Anesthetic interactions with lysophosphatidate signaling

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Anesthetic interactions with
lysophosphatidate signaling

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Cover: author's impression of a *Xenopus laevis* oocyte under two-electrode voltage clamp. The cell is impaled by two electrodes. A micropipette releases lysophosphatidate onto the cell. The inset shows a lysophosphatidate molecule interacting with its receptor in the cell membrane.

Anesthetic interactions with lysophosphatidate signaling

PROEFSCHRIFT

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aan de Universiteit Maastricht,
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*to Marijke,
to my parents and children,
and to the people in my laboratory, without whom this never could have happened*

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Table of Abbreviations

ADP	Adenosine bisphosphate
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole propionate
ATP	Adenosine triphosphate
BK channel	Ca ²⁺ -activated K ⁺ channel
BSA	Bovine serum albumin
C terminus	Carboxy terminus
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
cLPA	Cyclic LPA
CMV	Cytomegalovirus
CNS	Central nervous system
cRNA	Complementary RNA
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
EC ₅₀	Half-maximal effect concentration
EGTA	Ethylene glycol tetraacetic acid
G protein	GTP-binding protein
GABA _A receptor	Gamma-amino butyric acid type A receptor
GCR	G protein-coupled receptor
GDP	Guanosine diphosphate
GDP β S	A non-phosphorylatable GDP analog
Gpp(NH)p	A non-hydrolyzable GTP analog
GTP	Guanosine triphosphate
GTP γ S	A non-hydrolyzable GTP analog
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethylsulfonic acid)
IC ₅₀	Half-maximal inhibitory concentration
IP ₃	Inositoltrisphosphate
K _{ACh} channel	Muscarinic-regulated K ⁺ channel
LPA	Lysophosphatidate (lysophosphatidic acid)
MAC	Minimal alveolar concentration
MAP kinase	Mitogen-activated protein kinase
mRNA	Messenger RNA
N terminus	Amino terminus
NMDA receptor	N-methyl-D-aspartate receptor
NR1, NR2	NMDA receptor subunit 1 and 2
PA	Phosphatidic acid
PAF	Platelet aggregating factor
PIP ₂	Phosphatidylinositolbisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
RNA	Ribonucleic acid
S1P	Sphingosine-1-phosphate
TXA ₂	Thromboxane A ₂

CHAPTER 1

Introduction and aims

Marcel E. Durieux

Introduction and aims

INTRODUCTION

After more than a century of investigation, it appears that a common site of action for volatile and most non-volatile anesthetics may have been identified. The GABA_A ligand-gated ion channel has for some time been recognized as the primary site of action for non-volatile drugs, such as barbiturates, benzodiazepines and steroid anesthetics. Propofol also appears to exert its primary action at this ionotropic receptor. Recent data indicate that volatile anesthetics, in clinically relevant concentrations, similarly enhance GABA_A signaling, and a defined molecular target on the GABA_A receptor has been localized. Thus, it appears that a unitary model of anesthesia may actually be the correct one. Ketamine, with its primary action at the NMDA glutamate receptor, appears one of the few exceptions to this model.

However, if the GABA_A receptor were the *only* site of action for anesthetics, their side effects would be expected to be the same. This is obviously not the case. Therefore, if most anesthetics indeed induce anesthesia through the single mechanism of GABA_A enhancement, their varying side effect profiles have to be explained through interactions with other systems. Particularly for volatile drugs this is not surprising, as these require very high (low millimolar) concentrations for clinical effect, and their lipophilic nature makes them likely to interact (possibly non-specifically) with a variety of lipophilic sites. In contrast, non-volatile anesthetics are usually therapeutically active in micromolar concentrations. They would be expected to interact with fewer sites, and likely in a specific manner.

Thus, one of the research challenges in the years to come will be to elucidate, at the molecular level, anesthetic sites of interaction in addition to the GABA_A receptor. These secondary actions of anesthetics will define their unique side effect profile and can aid in the development of drugs with fewer adverse actions. This thesis reports some of our research in this area.

Clearly, the number of potential secondary molecular targets for anesthetics is overwhelming. Some have been probed in significant detail, many others have not been studied at all. In an effort to narrow the field, we focused on the physicochemical parameter that has driven anesthetic mechanisms research since the beginning of the century: the Meyer-Overton hypothesis. Most anesthetics are lipophilic, therefore *lipophilic targets may be likely sites of secondary anesthetic interaction*. One clear example of such lipophilic targets are G protein-coupled receptors for lipophilic mediators. These include receptors for a variety of compounds relevant to anesthesiology and critical care, including the prostaglandin and leukotriene receptors, the thromboxane receptor and the platelet-activating factor receptor.

Study of interactions between anesthetics and G protein-coupled receptors requires an appropriate model: one where receptors can be expressed functionally and in isolation, where they can be compared with each other in a similar setting, where volatile and non-volatile anesthetics can be administered in a reproducible manner, and where anesthetic effects on intracellular signaling can be studied in detail. Such a model was not established at the time, and we therefore developed one using *Xenopus* oocytes, already used widely for the investigation of G protein-coupled receptors, and used previously to study anesthetic effects on ion channels.

As an initial test of the system, we decided to focus on a lipid mediator receptor which we had found to be already expressed in oocytes: a novel receptor for the phospholipid lysophosphatidate (LPA). Although the (patho) physiology of the compound is as yet poorly de-

INTRODUCTION

scribed, it is known to induce (among other actions) platelet aggregation, contraction of smooth muscle (including vascular smooth muscle), changes in cellular morphology and mitosis. We felt that it would form a useful model for the study of anesthetic interactions with lipid mediator signaling. In addition, our studies would enhance the understanding of the physiology of LPA signaling.

AIMS

We hypothesized that *different anesthetics interfere to different degrees with LPA signaling. These effects are localized at the membrane receptor rather than in the intracellular signaling pathways.* Specifically, the goals of the study were to:

1. Establish the *Xenopus* oocyte model for the study of anesthetic effects on G protein-coupled receptors for lipophilic mediators, by (a) developing software for the efficient study of G protein-coupled receptors in oocytes, and (b) determining the background presence of G protein-coupled receptors in the model.
2. Describe the physiology of LPA signaling, as well as signaling by the related compound sphingosine-1-phosphate, in *Xenopus* oocytes.
3. Study the effects of several classes of anesthetics (volatile, non-volatile, and local) on LPA-mediated Ca^{2+} signaling in *Xenopus* oocytes.

This thesis reports the results of investigations planned to accomplish these Aims. It is organized as follows:

- *Chapter 2* provides a review of our current understanding of anesthetic actions on membrane signaling molecules. The goal is not as much to be completely comprehensive, as well as to focus on those molecules where interactions appear to have a high likelihood of being relevant to anesthetic effects or side effects.
- *Chapter 3* reviews LPA signaling. The main cellular effects of the compound and the molecular mechanisms behind those effects are discussed, and potential (patho)physiological roles for the compound are described.
- *Chapter 4* is a review of the use of *Xenopus* oocytes for the study of G protein-coupled receptors. General properties of the oocyte are discussed, as well as the major ways in which the system can be used in experimental practice. This chapter will provide the reader with a framework to help interpret use of the model for the study of anesthetic interactions.
- *Chapter 5* (Am J Physiol 263:C896-C900, 1992) describes the characterization of the LPA receptor in *Xenopus* oocytes, and provides an introduction to the methodology used throughout the remainder of the thesis.
- *Chapter 6* (Am J Physiol 264:C1360-C1364, 1993) describes the characterization of a second lipid mediator G protein-coupled receptor in the oocyte: that for the ligand sphingosine-1-phosphate.

CHAPTER 1

- *Chapter 7* (Comp Meth Progr Biomed 41:101-105, 1993) reports the development of a software system specifically designed to facilitate G protein-coupled receptor signaling in *Xenopus* oocytes.
- *Chapter 8* (FEBS Lett 337:235-238, 1994) describes an endogenous response in the oocyte that potentially could confound experimental studies: a trypsin-induced Ca^{2+} -activated Cl^- current. In addition, the study is the first to use, in a practical investigation, the software described in Chapter 6.
- *Chapter 9* (Anesth Analg 83:1090-1096, 1996) is the first of three chapters reporting anesthetic interactions of various classes of anesthetics with LPA signaling. This chapter describes the interaction between propofol and LPA signaling.
- *Chapter 10* (Anesthesiology 86:660-669, 1997) reports the interactions of the volatile anesthetics halothane and isoflurane with LPA signaling.
- *Chapter 11* (Anesthesiology 86:1112-1119, 1997) describes the differential effects of the local anesthetics lidocaine and bupivacaine on LPA signaling.
- *Chapter 12* summarizes the reported results, and recommends further studies.

CHAPTER 2

Anesthetic actions on membrane signaling molecules

Marcel E. Durieux

Anesthetic Actions on Membrane Signaling Molecules

INTRODUCTION

Despite more than a century of research, anesthetics are one of the very few drug classes for which a mechanism of action has not been definitively established. Research into this area during the first half of the century was largely guided by the strong correlation between lipid solubility and anesthetic potency described by Meyer¹ and Overton.² This relationship suggested a lipid site of anesthetic action, and the most logical lipid site for anesthetics to act would appear to be the cell membrane. However, despite the development of several elegant hypotheses, such as changes in membrane fluidity³⁻⁵ or volume,⁶ no clear membrane mechanism for anesthetic action has emerged. In retrospect, this is not completely surprising, as at 1 MAC a concentration of approximately 70 mM of anesthetic should be present at the lipid site of action, which would amount to one anesthetic molecule for each 2,000 Å, or one anesthetic molecule for each 60 lipid molecules.⁷ This suggests that the anesthetic would act merely as a minor membrane contaminant. Although by itself this does not rule out the possibility of a significant effect, it makes it less likely. Consistent with this calculation, large amounts of anesthetics are usually required to induce measurable membrane effects. Effects that occur at clinically relevant anesthetic concentrations can usually be mimicked by very small changes in temperature (< 1 °C), which itself are without measurable anesthetic action.⁸

Whereas a large number of anesthetic molecules would be necessary to substantially change lipid membrane behavior, one anesthetic molecule could in theory modify the behavior of a single membrane protein significantly. For these and other reasons, interest has shifted over the last decades toward investigating anesthetic effects on protein molecules. The inhibition by anesthetics of firefly luciferase demonstrated the capability of anesthetics to interfere specifically with an isolated protein.⁹ Remarkably, this interaction takes place at concentrations similar to those relevant for anesthesia. In addition, some anesthetic actions were shown to be stereoselective, suggesting direct interactions with proteins.¹⁰ Subsequently, proteins have been shown to be common targets for anesthetics.⁸

Most relevant for anesthetic action are of course the membrane signaling proteins, and it is to them that this review will be restricted. From an investigational point of view, they suffer a disadvantage: their position in the membrane makes it almost impossible to determine whether anesthetic effects are in fact due to direct interactions with the protein, or due to modification of their lipid environment. This issue has not yet been resolved, although stereospecificity studies strongly suggest a direct protein interaction.

It should be realized that a protein site of anesthetic action does not negate the Meyer-Overton rule. In other words, even if anesthetics act primarily on a protein site, there is still a strong correlation with lipid solubility. Therefore, the protein site of interest is likely to be of a hydrophobic nature. For this reason, we hypothesized that the receptors for lipophilic ligands (which should have a lipophilic binding pocket) should be a likely target for anesthetic action.

In the following sections, our current understanding of anesthetic actions on the two major groups of cell signaling proteins will be discussed. Particular emphasis will be on interactions with volatile anesthetics, as their site of action is less clearly defined than is that of the injected drugs. References are largely to the more recent literature.

Figure 1. The GABA_A receptor. The GABA_A receptor-chloride channel protein complex is a hetero-oligomer composed of 5 subunits (I-V), including α , β , γ , δ and ρ polypeptides. Each subunit has 4 putative membrane-spanning domains (1-4, represented by cylinders), one or more of which contribute to the wall of the ion channel pore.

ION CHANNELS

As the major effect of anesthetics is on the central nervous system, and as neurons depend on ion fluxes to convey their electrical signals, ion channels have long attracted attention as potential sites of action for anesthetics.

Ionotropic receptors

The ionotropic receptors, or ligand-gated ion channels, comprise a superfamily of membrane molecules characterized by the presence of one or more ligand binding sites and an ion channel within the same macromolecular complex. Binding of ligand to the receptor induces a conformational change that leads to opening of the ion channel. Ionotropic receptors have in

common with many voltage-gated ion channels that they consist of a number of molecular subunits, allowing for a large variety of functional receptors, the distribution of which across the brain has by and large not yet been established. Thus, studies of these receptors always run the risk of investigating a subunit composition that is not particularly relevant to the neuronal substrate of anesthetic action. Ionotropic receptors for excitatory neurotransmitters (such as the nicotinic acetylcholine receptor and the glutamate receptors) become permeable to cations (particularly Ca^{2+} and Na^{+}) when activated; in contrast, receptors for inhibitory neurotransmitters (such as the GABA_A and glycine receptors) become permeable to Cl^{-} . In this section, anesthetic effects on GABA_A , NMDA glutamate, and nicotinic acetylcholine receptors will be reviewed.

GABA_A receptors

Of the ionotropic receptors, the GABA_A receptor has received most attention as a site of anesthetic action. It has been well established as the primary target for most non-volatile anesthetics, such as the barbiturates, benzodiazepines, steroid anesthetics and propofol. In addition, alcohol interacts with this receptor, and recent evidence indicates that it is an important site of action for volatile anesthetics as well.

The receptor is thought to form a pentameric structure, composed of members from the five subunit families (α , β , γ , δ , and ρ , see Figure 1).¹¹ The subunits are structurally related: each consists of a single polypeptide with four putative membrane-spanning domains. As there are multiple subtypes of the subunits, the potential number of different GABA_A receptors is staggering (up to 3000); in reality, however, it seems more likely that only approximately 500 types are expressed.¹¹ The second transmembrane domain of each subunit is thought to contribute to the ion channel. The extracellular N-terminus contains consensus sites for N-linked glycosylation, and in some units the intracellular domains contain consensus sites for phosphorylation by protein kinase A and C, as well as by tyrosine protein kinases. Other areas on the subunits form binding domains for a variety of compounds. The main GABA binding pocket is localized on the α subunit, as is the benzodiazepine binding site.

The evidence for effects of volatile anesthetics on GABA_A receptors is compelling. A number of models have been used to study these actions, and virtually without exception they have shown enhancement of GABA signaling by relevant concentrations of anesthetic compounds. Harris et al, for example, demonstrated that halothane, isoflurane and sevoflurane enhanced flunitrazepam binding to the benzodiazepine receptor site by increasing ligand affinity.¹² That these effects translate to functional changes in receptor behavior was shown by Lin et al,¹³ who expressed GABA_A receptors in *Xenopus* oocytes and measured the effects of enflurane on their signaling properties. The anesthetic enhanced GABA-activated currents in a manner dependent on the subunit composition of the expressed channels. Longoni et al. have demonstrated similar effects of halothane on GABA receptors in rat brain cortical slices,¹⁴ and Harrison et al. reported comparable findings with isoflurane.¹⁵ Recently, sevoflurane has also been reported to modulate GABA signaling.¹⁶

Importantly, the effects of volatile agents on this receptor complex have been shown to be stereoselective, strongly suggesting a specific (i.e. protein) site of action. Flunitrazepam binding to benzodiazepine receptors is enhanced more by S(+) isoflurane than by R(-) isoflurane.¹⁷ EC_{50} s were 0.44 and 0.88 mM, respectively. Potency of Cl^{-} to increase flunitrazepam binding was increased 3.2 and 1.5 fold, respectively. Similar results were reported by Hall et al, who studied GABA-induced Cl^{-} currents in cerebellar Purkinje cells.¹⁸ They observed a maximal

Figure 2. The NMDA receptor. Like the GABA_A receptor complex, the NMDA receptor is a macromolecule with multiple binding and modulatory sites, including those for glutamate (or NMDA), glycine, phencyclidines (PCP) and Mg²⁺. In addition, the complex contains a cation channel permeable to K⁺, Ca²⁺ and Na⁺. The ion is positively modulated by glutamate and glycine, and negatively modulated by PCP and Mg²⁺.

stereoselective effect (approximately 2 fold) in favor of S(+) isoflurane at the EC₅₀ concentration for general anesthesia (310 μ M). At lower anesthetic concentrations the stereoselectivity became less marked. Similar findings have been reported using rat brain synaptic microvesicles.¹⁹ The action of halothane on the GABA_A receptor is also stereoselective, and associated with genetic loci different from those that distinguish between isomers of isoflurane.²⁰ Thus, the anesthetics probably act through different sites.

In further support of a protein site of action are data demonstrating that the effect of anesthetics depends on the subunit composition of the GABA_A receptors. As mentioned, this was already noted in the functional model employed by Lin et al.¹³ Harris et al. showed that expression of the β 2 subunit reduced the efficacy of both halothane and isoflurane in enhancing flunitrazepam binding.²¹ Of particular relevance are their findings that non-volatile anesthetics acting on the GABA_A receptor (alphaxalone and pentobarbital) were affected differently, suggesting

that volatile anesthetics act on separate sites. The $\alpha 6$ subunit appeared promising as a potential site of volatile anesthetic action,²² but recently it was shown that knockout mice lacking the $\alpha 6$ subunit have normal responses to volatile anesthetics. Mihic et al.²³ reported recently that two specific amino acid residues in transmembrane domains 2 and 3 are critical for allosteric modulation of both GABA_A and glycine receptors by alcohols and enflurane.

In summary, the data currently available suggest that the ionotropic GABA_A receptor is a likely site of action of anesthetics. In fact, as all anesthetics tested appear to enhance signaling at clinically relevant concentrations, it may well be the primary site of action for most of them (with the prominent exception of ketamine). However, if it were the *only* site, one would anticipate the clinical effects and side effects of these anesthetics to be similar. This, they clearly are not, and therefore other sites of activity must exist. Likely potential targets will be discussed briefly in the following sections.

NMDA glutamate receptors

Whereas the GABA_A receptor is established as the primary site of action of most non-volatile anesthetics and appears a likely primary site of action of volatile drugs as well, there is one clear exception: ketamine acts primarily on the NMDA glutamate receptor. It inhibits functioning of this receptor in a non-competitive manner, thus inhibiting excitatory neurotransmitter signaling. The different mode of action most likely explains the unusual clinical anesthetic effects of ketamine. Recent data suggest that other anesthetics also interact with the NMDA receptor to various degrees, making it worthy of consideration.

Glutamate acts on several classes of ionotropic receptors, as well as on metabotropic receptors. Three main types of ionotropic glutamate receptors are known, designated by their selective ligands: the *N*-methyl-D-aspartate (NMDA) receptors (Figure 2), the α -amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, and the kainate receptors. Often the latter are grouped together as non-NMDA receptors. NMDA receptors are more sensitive to anesthetics than non-NMDA receptors²⁴ and have received most attention as anesthetic targets. Therefore, this discussion will be limited to NMDA receptors only.

NMDA receptors consist of two subunits, NR1 and NR2. Of the latter, 4 subtypes (A through D) are established. In addition, many alternative forms exist of both subunits, yielding a large variety of potential receptors.²⁵ The NMDA receptor exhibits unusual pharmacologic behavior. It is characterized by high Ca²⁺ permeability (high single channel conductance and slow gating), voltage-dependent Mg²⁺ block, and a requirement for glycine as a co-agonist for channel activation. All these actions take place at the NR1 subunit. Together, these properties allow the receptor to play a key role in memory processes.²⁶ However, the massive cellular influx of Ca²⁺ after glutamate excitation can have toxic effects on cells as well, and glutamate overstimulation (excitotoxicity), mainly mediated by NMDA receptors, is an established model of neuronal injury. Thus, inhibition of these receptors by anesthetics could not only account for part of clinical anesthesia, but be potentially neuroprotective as well.

As mentioned above, ketamine is well established as a non-competitive antagonist at the NMDA receptor.²⁷⁻³⁰ It is effective at clinical concentrations. In addition, it acts in a stereospecific manner, the S(+) form being approximately 2-3 times as potent as the R(-) isomer, strongly suggesting that its action is specific. More recently, volatile anesthetics have similarly been shown to interact with NMDA receptors. Enflurane and halothane (0.2-0.3 mM) inhibit glutamate-stimulated ⁴⁵Ca uptake in rat brain microvesicles by as much as 60-80%.³¹ Interestingly,

the anesthetics also diminish desensitization after prolonged exposure to NMDA. Both anesthetics, as well as methoxyflurane, chloroform and diethylether, have been shown to decrease glutamate-induced binding of the NMDA antagonist MK-801.³² Conversely, CNS administration of NMDA antagonists has modest anesthetic effects. For example, intrathecal application of NMDA antagonists reduces MAC of isoflurane by 10-20%.³³

In agreement with an inhibitory action of anesthetics on NMDA signaling, anesthetics have been shown to ameliorate glutamate-induced excitotoxicity. A protective action of ketamine is well established, and the traditional contraindication of its use in neurotrauma patients is being reconsidered. Volatile anesthetics have similarly been shown to limit excitotoxic neuronal injury.³⁴ In addition, much recent attention has focused on their ability to inhibit glutamate release,³⁵⁻⁴⁰ and enhance glutamate uptake.^{41, 42} Together, these findings offer exciting new insight into the neuroprotective actions of anesthetics.

In brief, ketamine has its primary site of action on the NMDA receptor, and the volatile anesthetics have inhibitory effects on this system as well. Interactions with NMDA signaling may be relevant both to anesthetic effects, as well as to neuroprotective actions of the anesthetics.

Nicotinic acetylcholine receptors

The nicotinic receptors can be divided broadly in two classes: the muscle types and the neuronal types. Although neuronal nicotinic receptors are present in the CNS in much lower quantities than the glutamate receptors, they still play important roles, as is e.g. borne out by the syndrome of nicotine tolerance. (Interestingly, in insects this situation is reversed: there nicotinic receptors form the main CNS excitatory receptor, whereas neuromuscular transmission employs amino acid receptors.^{42, 43}) The neuronal nicotinic receptor is a pentameric structure, constructed out a variety of possible subunits. Whereas the muscle type consists of two α units, and one each of β , δ and ϵ subunits (or $(\alpha)_2\beta\gamma\delta$ in fetal muscle), the neuronal receptors show a much greater variety of subtypes.⁴⁴ At present it is not clear how these are combined in vitro. Of those receptors that do not bind α -bungarotoxin, 6 α and 4 β subunits have been cloned, and of the α -bungarotoxin-binding receptors, several additional α subunits are known to exist. It is important to keep in mind that many combinations of two α subunits with or without other subunits provide functioning receptor/channel complexes. However, it is most times not clear if receptors with such a configuration actually exist in vivo.

From studies with the muscle-type receptor, it has become clear that the agonist acetylcholine binds to the α subunit, and that both α subunits require a bound acetylcholine molecule before the channel will open.

It has been well established that muscle nicotinic receptors are inhibited by anesthetics.⁴⁵ The closely packed receptors in *Torpedo* membranes have been used to map the binding sites for ¹⁴C halothane by photoaffinity labeling.⁴⁶ Within the α subunit, more than 90% of label was found within the four transmembrane regions. As was the case with studies on the GABA_A receptor, it appears that different anesthetics have different binding domains, as unlabeled halothane reduced labeling more than did isoflurane.

More recently, the neuronal subtypes have been shown to be even more sensitive to volatile anesthetics than muscle receptors, albeit in a complicated differential manner. Violet et al. recently expressed neuronal and muscle type nicotinic receptors in *Xenopus* oocytes and studied the effects of halothane, isoflurane, sevoflurane and propofol on their signaling.⁴⁷ Neuronal re-

CHAPTER 2

ceptors were significantly more sensitive than muscle receptors (IC_{50} concentrations 10-35 fold less). In fact, the receptors were found to be inhibited at concentrations below those required for general anesthesia. However, the sensitivity to anesthetic depends critically on subunit composition. For example, when expressed in *Xenopus* oocytes, $\alpha_4\beta_2$ neuronal receptors have been shown to be inhibited by isoflurane and propofol (IC_{50} 85 and 19 μM , respectively).⁴⁸ In contrast, a monomeric α_7 receptor was not affected by the anesthetics.

In view of the uncertainty about subunit compositions of these receptors in vivo, results from reconstitution experiments should be interpreted with caution. Nonetheless, it appears that at least some nicotinic receptors are inhibited by remarkably low concentrations of anesthetics, making them a likely target of anesthetic action.

Interestingly, activation of neuronal nicotinic receptors has been shown to convey significant analgesia.⁴⁹

Voltage-gated ion channels

The second major class of ion channels are those regulated by voltage rather than by ligand binding. In contrast to some of the ionotropic receptors, voltage-gated channels tend to be highly selective for specific ions, and they are therefore conveniently classified as to the permeant ion. The classes to be discussed here are the Ca^{2+} and K^+ channels, as these are channels which have been shown convincingly to be a target for anesthetics. Na^+ channels have generally been shown to be relatively resistant to anesthetic action (with the obvious exception of local anesthetics), although recent data indicates that neuronal Na^+ channels may be significantly more sensitive than the peripheral channels that are studied routinely. Cl^- channels play several important roles, but have not been shown to be a significant target for anesthetics.

Voltage-gated ion channels show a bewildering diversity of types. Many of these have been defined by pharmacological means, in particular by the use of specific channel blockers. Molecular biology methods, however, have shown that many more clones exist than can be differentiated by pharmacologic means. The study of anesthetic interactions with all these subtypes will undoubtedly occupy electrophysiologists for years to come.

Ca^{2+} channels

Historically, Ca^{2+} channels have been classified in 5 groups, based on their electrophysiologic and pharmacologic characteristics.⁵⁰ T-type channels (low-voltage activated channels) activate at negative potentials (-50 to -70 mV), whereas L, N, Q and P types (high-voltage activated) activate at more positive potentials (-30 mV and above). The four high-voltage activated types are differentiated on the basis of pharmacological criteria: L-type channels are blocked by dihydropyridines, N-type channels by *Conus geographicus* toxin ω -conotoxin GVIA, and P-type channels by *Agenelopsis aperta* toxins ω -agatoxin IVA and FTX. Thus far, no specific blockers are available for Q-type channels.

Structurally, Ca^{2+} channels consist of several subunits. The primary one of these is the α_1 subunit, which contains both the pore-forming region as well as the voltage sensor. This unit consists of four repeats of a structural motif of six transmembrane domains. This motif appears throughout the voltage-gated ion channel superfamily. Ancillary subunits (α_2 , β , γ and δ) modulate the behavior of the α_1 subunit. Cloning efforts have yielded cDNAs for α_1 subunits with properties that match the main types of channels as described above. Thus, α_{1A} is thought to en-

code P and/or Q type channels, α_{1B} encodes N-type channels, and α_{1C} and α_{1D} encode L-type channels. Considering the modulatory influence of the ancillary subunits and the fact that the α_1 subunits exist in several forms and splice variants, the functional variety of Ca^{2+} channels is likely to be significantly greater than that predicted by pharmacological and electrophysiological means alone.

Inhibitory effects of anesthetics on Ca^{2+} channels have been well described, although most studies have employed channels in native cells, and the exact subunit composition is therefore not known. Although these inhibitory effects are often modest, the essential role of voltage-gated Ca^{2+} channels in regulating neurotransmitter release at the synapse makes even moderate actions of anesthetics relevant. The change in membrane potential resulting from an action potential arriving at the synaptic terminal leads to opening of Ca^{2+} channels, and it is the resulting Ca^{2+} influx into the synaptic terminal that forms the trigger for exocytosis of vesicles containing neurotransmitters. Anesthetics have been shown to inhibit release of neurotransmitters, in particular glutamate, presumably by blocking Ca^{2+} channels and inhibiting Ca^{2+} entry.^{35,38}

Remarkably, it appears that Ca^{2+} channels are sensitive to virtually all anesthetics tested, which enhances the likelihood that this inhibition is part of anesthetic action *per se*, rather than a side effect of the compounds. Most studies have been performed on cardiac channels,⁵¹⁻⁵⁴ and neuronal channels have only been investigated recently. Adrenal chromaffin cells have been used as a popular model for the study of neuronal channels, and anesthetics have been shown to inhibit Ca^{2+} channels present in these cells. For example, halothane, methoxyflurane, etomidate and methohexital block these channels, primarily by reducing probability of channel opening and increase in inactivation.⁵⁵ Isoflurane inhibits calcium currents in neurons from sensorimotor cortex⁵⁶ as well as in pyramidal neurons from hippocampus.⁵⁷ Both high-voltage activated and low-voltage activated channels appear sensitive. In fact, a recent report suggests that isoflurane (but not halothane) may block T-type channels more potently than it blocks L or N-type channels.⁵⁸ These results suggest that anesthetic action is not dependent on the specific complement of ancillary subunits. In other words, anesthetics act probably on the α_1 subunit. Initial studies using recombinant expression of defined channels are in progress, and these should be able to confirm this, as well as yield additional insight into the molecular mechanisms of action on these channels.

K⁺ channels

K^+ channels, which have as their primary role the regulation of the resting membrane potential, have been shown to exist in an enormous variety of forms. In fact, it is far from clear why so many different types are necessary to perform a relatively straightforward electrophysiologic task. These channels are structurally some of the most interesting ones. The classic K^+ channel (*Shaker*, originally cloned from *Drosophila*) has as its primary structure a single domain with six putative transmembrane regions, i.e. the structural analog of one-fourth of a Ca^{2+} channel. However, variants exist, including one subtype that consists of a single transmembrane domain only.⁵⁹ Currently, the mammalian voltage-activated K^+ channels are divided into 6 subfamilies, with a total of approximately 20 main members. Of many of these, further subtypes have been cloned.

In addition to the voltage-activated K^+ channels, K^+ channels exist that are regulated by intracellular intermediates. Of particular interest are K_{ATP} channels that are controlled by intracellular ATP concentrations. These are thought to play an essential role in the phenomenon of

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myocardial preconditioning, and volatile anesthetics, in particular isoflurane, have been shown to activate these channels directly.⁶⁰⁻⁶² Anesthetics similarly inhibit Ca^{2+} -activated K^+ (BK) channels,⁶³ as well as those K^+ channels regulated by muscarinic stimulation in heart (K_{ACh}).⁶⁴

Anesthetic interactions with voltage-gated K^+ channels have been studied only fairly recently, and with particular emphasis on cardiac K^+ channels;⁶⁵ relatively little is known about their neuronal counterparts. Halothane and isoflurane decrease the open state probability of K^+ channels in cerebral arterial cells.⁶⁶ Ketamine and halothane have been shown to inhibit $\text{Kv}2.1$ K^+ channels from brain, expressed recombinantly in *Xenopus* oocytes.⁶⁷ Interestingly, this inhibition was voltage dependent for halothane, but not for ketamine. Deletion of the C-terminus resulted in decreased sensitivity to both anesthetics.

METABOTROPIC RECEPTORS

The metabotropic receptors form a very large group of signaling molecules. They can be divided in several broad classes, the protein tyrosine kinase receptors and the G protein-coupled receptors (GCRs). The protein tyrosine kinase receptors are large molecular complexes. Ligand binding induces activation of a phosphorylating enzyme activity in the intracellular segment of the molecule. As phosphorylation is one of the major mechanisms of cellular regulation, such receptors can have a variety of cellular effects. Many of these actions are relatively long-term, and possibly because of this reason anesthetic interactions with protein tyrosine kinase receptors have not been studied in significant detail. Therefore, this group will not be discussed further.

GCRs, in contrast, are much smaller. Ligand binding results in activation of an associated GTP-binding protein (G protein) that subsequently influences cellular processes. The number of GCRs is large, and still growing. Approximately a hundred defined receptors have been cloned, while in addition several dozens of clones exist that have the molecular characteristics of a GCR, but lack a known ligand (so-called orphan receptors). In addition, the olfactory epithelium expresses hundreds of GCRs, which are thought to mediate the sense of smell, and another large group, with unknown function, is expressed on sperm cells. Taken together, the superfamily has more than one thousand members.

All of these have similar molecular characteristics. They are generally several hundred to a thousand amino acids in length, and contain 7 stretches of 20 to 25 hydrophobic amino acids. These hydrophobic domains are thought to form α helices and traverse the membrane, thus anchoring the receptor to the cell (for this reason, the family is often referred to as the "seven transmembrane" family). The result of this arrangement is the creation of three intracellular and three extracellular loops. The amino terminus is located extracellularly, whereas the carboxyterminus is located intracellularly. The functional domains of the receptor have been elucidated in reasonable detail, despite the fact that no crystallographic detail is as yet available for the GCRs. It is thought that the 7 transmembrane domains arrange in a funnel-like structure, the inside of which forms the ligand binding domain. The intracellular domains, particularly the third intracellular loop and the C terminus, bind to the G protein. G protein specificity can be determined by a remarkably small number of amino acids in the third intracellular loop, as elegantly demonstrated by mutation studies with the muscarinic receptors.⁶⁸ In most of these receptors, the amino terminus carries one or more consensus sequences for glycosylation. In addition, phosphorylation sites are often present on the intracellular domains.

G proteins come in two classes: small (cytoplasmic) G proteins and heterotrimeric (membrane) G proteins. Common to all is their mechanism of function. In their resting state, they bind a molecule of GDP. When activated (by a GCR in the case of heterotrimeric G proteins, and by another messenger in the case of cytoplasmic G proteins) this GDP is exchanged for a GTP molecule. The activated G protein can now perform functions within the cell until it is inactivated when an intrinsic enzyme activity hydrolyzes the GTP to GDP. The critical point about this hydrolytic activity is that it is (molecularly speaking) very slow: on the order of seconds. As a result, brief (on the order of milliseconds) activation of a receptor can lead to more prolonged activation of the intracellular signaling machinery.

GCRs bind to heterotrimeric G proteins, so called because they consist of three subunits: α , β and γ . Of these, the β and γ subunits are associated so tightly that for practical purposes they can be viewed as a single unit, often termed the $\beta\gamma$ subunit. The α subunit contains both the GDP-GTP binding domain as well as the hydrolytic activity, and was classically thought to be the "business end" of the molecule, with the $\beta\gamma$ subunit roaming freely and inactively, serving as an anchor and a sink of free α subunits. This view turns out to be incorrect: the $\beta\gamma$ subunit has activating functions as well. For example, it interacts with phospholipase C β and the muscarinic K⁺ channel. G protein binding to the associated receptor enhances receptor affinity for agonist. This phenomenon, known as GTP shift, is used as presumptive evidence that a receptor is G protein coupled.

Several classes of heterotrimeric G proteins exist, indicated by subscripts. The classical types are G_s and G_i, which stimulate and inhibit, respectively, the enzyme adenylate cyclase, thereby leading to changes in cytoplasmic cAMP concentrations. G_q proteins (and G_o in brain) activate phospholipase C (PLC), and thereby induce the generation of inositoltrisphosphate (IP₃) and diacylglycerol (DAG) from phosphatidylinositolbisphosphate (PIP₂). IP₃ acts on its own receptor/channel complex on intracellular Ca²⁺ storage sites, and induces release of Ca²⁺ from these sites, thereby raising intracellular Ca²⁺ concentrations. DAG activates protein kinase C (PKC) leading to phosphorylation of a variety of targets (including the receptors that initiated the cascade). In recent years, cloning efforts have shown each of these classes of G proteins to consist of a number of members, but their functional differences are as yet poorly defined.

Despite the huge number of GCRs, only a few have received attention as potential anesthetic sites of action. Some of this lack of information is a result of the fact that these receptors are technically not easy to study: although the receptors are molecularly smaller than most channels and consist of a single subunit only, they are only one part of very complex signaling pathways. Therefore, it is not sufficient to determine the effects on the receptor itself: actions on intracellular signaling should be investigated as well.

The receptors for which most evidence exists as to interactions with anesthetics are the muscarinic acetylcholine receptors (Figure 3), and these findings will be discussed in some detail. In addition, we have studied effects of anesthetics on receptors for lipid mediators (LPA, thromboxane A₂⁶⁹ and prostaglandin E₂). Results from experiments with LPA receptors will be described in subsequent chapters. Although occasional reports have suggested effects of anesthetics on other GCRs (such as serotonin receptors⁷⁰), insufficient data exists to declare these systems anesthetic targets. Studies of anesthetic effects on intracellular signaling systems have yielded somewhat variable results, but it appears warranted to conclude that at clinical concentrations anesthetics affect neither intracellular Ca²⁺ nor cAMP signaling.⁸

Figure 3. Muscarinic acetylcholine signaling. Muscarinic signal transduction is mediated by members of the G protein-linked membrane receptor superfamily. m1, m3 and m5 receptors couple to members of the G_q family of G proteins. Their main functional role is to cause activation of phospholipase C, resulting in liberation of inositoltrisphosphate (IP₃) and diacylglycerol (DAG). IP₃ induces an increase in intracellular Ca²⁺ through release from intracellular stores, and, secondarily, activates Ca²⁺-dependent systems such as Ca²⁺/calmodulin-dependent kinases. DAG directly activates protein kinase C (PKC). m2 and m4 receptors couple to members of the G_i family of G proteins. Their main functions are to cause G_{iα}-mediated activation of K⁺ channels and to inhibit adenyl cyclase activity. Decreases in cAMP and cAMP-dependent protein kinase activity produce a state of decreased phosphorylation of effector systems.

Muscarinic acetylcholine receptors

Anesthetic effects on muscarinic receptors are well established. Aronstam and coworkers have published a series of papers reporting the effects of various anesthetics on muscarinic receptor binding. A summary of their findings has appeared in print as well.⁷¹ Several studies investigated the effect of halothane on agonist⁷² and antagonist⁷³ binding. The conclusions drawn from this work were that (1) the anesthetic enhanced antagonist binding by slowing the rate of ligand dissociation; and (2) the anesthetic inhibited agonist binding (by 48% with 10% halothane).

The site of action of these effects was studied in more detail by investigating the interactions of anesthetics with G protein functioning.^{72, 73} As would be anticipated, the investigators found that addition of the non-hydrolyzable GTP analog Gpp(NH)p to the binding mixture resulted in decreased binding of muscarinic receptor agonist, i.e. GTP shift. They then determined the effect of halothane on this Gpp(NH)p-induced shift in the binding curve, and observed that the Gpp(NH)p concentration-response relationship was shifted to the right. In other words, a higher concentration of the GTP analog was necessary to induce a similar decrease in agonist binding. In the absence and presence of 5% halothane, the IC₅₀ values for the inhibitory effect of Gpp(NH)p were 0.7 and 83 μ M, respectively - a hundred-fold difference. These findings were interpreted as an ability of halothane to stabilize high-affinity G protein-receptor complexes.

Hence, it appears that halothane affects both receptor binding as well as receptor-G protein interaction. In a subsequent study, the effect of the anesthetic on G protein functioning - its ability to hydrolyze bound GTP - was investigated. Halothane was found not to inhibit binding of radiolabeled GTP analog to G proteins. However, the anesthetic blocked completely the stimulation of G protein GTPase activity induced by acetylcholine, with a half-maximal effect at the clinically relevant halothane concentration of 0.3 mM. The site of this effect was not determined. Largely similar findings were obtained with other anesthetics.

The conclusion to be drawn from these studies is that anesthetics may interfere with several components of the muscarinic signaling pathway. However, it should be kept in mind that (1) most times a mixture of muscarinic receptor subtypes was studied, (2) the anesthetics were administered in relatively high, and clinically unequal, concentrations, and (3) anesthetic effects on functional properties of the receptor-G protein unit were not addressed specifically. Nonetheless, the results are in general agreement with those obtained later from functional studies.

Lin et al described the effects of enflurane on muscarinic acetylcholine receptors from extracted brain mRNA,⁷⁴ and we have reported effects of several anesthetics on expressed muscarinic receptor clones.^{75, 76} These studies were performed in *Xenopus* oocytes, a convenient model for such investigations. As will be described in more detail in Chapter 4, in the oocyte, activation of Ca²⁺-signaling GCRs leads to generation of IP₃ and release of intracellular Ca²⁺, which in turn opens an endogenous Ca²⁺-activated Cl⁻ current, easily assayed by 2-electrode voltage clamp. Lin et al. found that enflurane (2%) inhibited virtually completely currents induced by 100 nM acetylcholine, but not 10 μ M acetylcholine. This suggests competitive components to the interaction. Similar to these findings, we observed that halothane, in clinically relevant concentrations, inhibited m1 muscarinic signaling in this model. At a halothane concentration of approximately 0.3 mM, currents were depressed by 60%. Studies with other expressed receptors and microinjection of intracellular mediators indicate that this anesthetic effect takes place at either the receptor or coupled G protein.

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Studies with other anesthetics, however, have shown that these results can not necessarily be extrapolated. Isoflurane, at equipotent concentrations, was without effect on m1 signaling.⁷⁶ Recently, we have shown that desflurane shows a biphasic effect: at lower concentrations (5%) it enhances m1 signaling, and at higher concentrations (10%) it depresses it.⁷⁶ The mechanism for this action is unclear at present, although we have shown that the anesthetic - like the others - is without effect on IP₃ signaling. These findings are of significance, as the differences in anesthetic sensitivity imply that muscarinic inhibition is unlikely to underlie the anesthetic state *per se*. Instead, muscarinic inhibition is more likely to account for some of the specific side effects of clinically used anesthetics.

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CHAPTER 3

Lysophosphatidate signaling

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Lysophosphatidate signaling

This chapter will review our current state of understanding of signaling by the compound lysophosphatidate (LPA). LPA is a highly intriguing compound: it is generated in large amounts during a process as common as platelet aggregation, its receptor is present almost ubiquitously, its cellular effects are varied and pronounced. At the same time we are still at a loss as to the physiologic or pathophysiologic role of the compound, and we are not in the possession of adequate pharmacologic tools such as highly selective antagonists or a receptor clone (although both have been reported, they are not yet accepted by the scientific community). From the viewpoint of anesthetic interactions the compound is of interest as inhibition of several of its actions (e.g. platelet aggregation and vascular smooth muscle contraction) could potentially have detrimental results in the surgical patient. The presence of an LPA receptor in *Xenopus* oocytes provides a model system where effects on receptor as well as intracellular pathways can be probed in detail.

The contents of this chapter are modified and updated from a book on this topic published by the author in 1995.¹

STRUCTURAL PROPERTIES OF LPA

LPA (1-acyl-2-*sn*-glycero-3-phosphate) is the simplest phospholipid known (see Chapter 6, Figure 1). For many years, the compound had been considered only as a constituent of lipid bilayer membranes and an intermediate in phospholipid metabolism, where it acts as a precursor in the synthesis of phosphatidic acid.²

LPA consists of a glycerol backbone with an acyl chain substituted in the *sn*-1 position, a hydroxyl group in the *sn*-2 position and a phosphate group in the *sn*-3 position. As the length and saturation of the acyl chain can vary, it is technically not correct to speak of LPA *per se*. Rather, there is a family of lysophosphatidic acids, and the composition of the acyl chain determines partially the potency of the compound. In fact, recent findings appear to indicate that LPA is only one member of a family of lipid phosphoric acid mediators, possibly acting on related receptors. Other members of this group include sphingosine-1-phosphate, for which a signaling role is established, and amantadine-1-phosphate. Lynch et al. have proposed that phosphorylated N-acyl ethanolamines may occur naturally.³

The physicochemical properties of LPA have been studied in surprisingly little detail. Nonetheless, this information is of significance, as particularly the solubility properties of LPA are unusual for a signaling molecule and are relevant for defining the putative physiologic role of LPA. As an amphiphilic compound, LPA will form micelles in aqueous solutions at higher concentrations. The compound has a critical micellar concentration of approximately 1.3 mM in 0.1 M NaCl, 10 mM HEPES buffer (pH 7.4).⁴ Nonetheless, several studies have shown only modest influence of the macromolecular form of LPA on its functional activity.⁵ The compound binds Ca^{2+} avidly, in a 1:1 molar ratio, and aqueous LPA solutions become visibly turbid when Ca^{2+} is added.⁴ Ca^{2+} -induced turbidity can be prevented by the presence of bovine serum albumin (BSA), which also binds LPA, as discussed below.

It is of importance to mention that LPA, at concentrations that induce its pharmacologic effects, is not lytic to cells. This is in contrast to other phospholipids, in particular lysophosphatidylcholine, which has pronounced detergent properties. Jalink et al. showed conclusively that LPA concentrations greater than 1 mM did not induce leakage of indo-1 out of cells, whereas

lysophosphatidylcholine readily permeabilized cells at 10-fold lowered concentrations.⁴ Although this result may not be extrapolated to all models (e.g. *Dictyostelium discoideum* is lysed by 200 μM LPA⁶), 100 μM LPA should be safe for most cell types.

The structure-activity relationship of LPA is being actively investigated. It has been known for a considerable amount of time that activity of the compound is closely related to structure of the phosphate and hydroxyl moieties: virtually any modification of this structure inactivates the compound completely. For example, neither phosphatidic acid (with an *sn*-2 hydrocarbon chain) or lysophosphatidylcholine (with a choline group attached to the phosphate) are able to elicit Ca^{2+} transients in most cell types.⁴ The acyl tail also has an influence on activity, but structural changes are tolerated better. For example, optimal potency of LPA for increasing rat blood pressure was obtained with a chain length of 16 and 2 or 3 double bonds.⁷ Similar findings were obtained in smooth muscle contraction⁸ and platelet aggregation⁹ assays. Lynch et al. have reported the relationship between total backbone length and activity of LPA, and found optimal activity at a chain length of 24-25 atoms.³

SIGNALING PROPERTIES OF LPA

LPA exhibits a profound array of actions on a variety of cellular systems. Indeed, the ubiquity of responsiveness to LPA has hindered efforts to clone the receptor for the compound. In this section, a brief overview will be provided of the main cellular effects of LPA. It should be noted that the vast majority of these studies were performed in cultured, immortalized cell lines. It is possible that the immortalized state or other selection procedures inherent in cell culture (such as adhesiveness) may be related to the ubiquitous LPA response.

Smooth muscle

Many of the early studies of LPA signaling were performed in smooth muscle. One of the initial observations of a physiologic effect of LPA was reported in 1978, when Tokumura described a transient pressor response in rats after the intravenous injection of crude soybean lecithin.¹⁰ The active compound was isolated and found to be LPA.⁷ The effects were species dependent: in rats and guinea pigs the compound was hypertensive, but in cats and rats it was hypotensive. These differences were shown by Schumacher to result from a species-specific effect on platelet aggregation:¹¹ in species where LPA activates platelets the resulting aggregates are trapped in the pulmonary circulation, and the sudden increase in pulmonary vascular resistance induces circulatory collapse. In contrast, in those animals where LPA does not appreciably aggregate platelets, an increase in blood pressure is observed as a result of direct smooth muscle contractile effects of the compound.

A number of other smooth muscle preparations have been used to study the contractile actions of LPA. LPA was found to contract uterine smooth muscle,⁸ as well as intestinal smooth muscle.^{12, 13} In all these systems, potency depended on chain length and saturation in the manner described in the previous section. Interestingly, despite the fact that the original observations of LPA's action on smooth muscle derived from the vasculature, it was not until recently that direct observations of the cerebral circulation demonstrated a contractile action of LPA on the vasculature.¹⁴ In pial arterioles a modest contraction (up to 15%) was induced by the compound. We have shown recently that LPA has a more profound contractile action on larger cerebral arteries

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(unpublished observations). It is unfortunate that this area has been relatively neglected, as a role for LPA in the vasculature appears likely.

Mitogenic effects

In 1986, Moolenaar published a seminal paper reporting that phosphatidic acid (PA) acted as a growth factor for fibroblasts.¹⁵ Since that time it has been shown that many commercial PA sources contain significant amounts of LPA.^{16, 17} As LPA has been shown to be a more potent mitogen than PA¹⁸ it is likely that LPA was responsible for the growth promoting actions of PA in the original studies. In addition to being mitogenic, the compound induced proto-oncogene expression after 1 h of treatment. The cells in these experiments were serum-starved before being tested. Serum itself was approximately as potent a mitogen as the combination of PA and insulin. In view of later studies, it seems likely that this effect is due to the presence of LPA on serum albumin, as discussed below.

The mitogenic action of LPA has since been shown to be highly specific. Van Corven et al. tested a large variety of phospholipids and related compounds for mitogenic activity, and found only PA and LPA to be active.¹⁹ Specifically, they could demonstrate the importance of the LPA backbone for cellular activity. Changes in the phosphate group (e.g. lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylinositol), changes in the fatty acid chain linkage (e.g. lyso-platelet-activating factor) and changes in the *sn*-2 position of the glycerol backbone (e.g. platelet-activating factor) lead to inactive compounds. LPA-induced proliferation is sensitive to pertussis toxin, suggesting that a G_i protein is involved in the signal transduction pathway.¹⁹

Intracellular Ca²⁺ release

In the 1986 study by Moolenaar, PA (possibly contaminated with LPA) was also shown to induce release of Ca²⁺ from intracellular stores in A431 human carcinoma cells.¹⁵ This effect was specific for the compound and not mimicked by several structurally related molecules. The action of (L)PA was shown to be associated with increases in IP₃, suggesting that the compound induces phospholipase C activation. In a more detailed investigation, Jalink demonstrated that LPA itself also increases intracellular Ca²⁺ levels in an IP₃-dependent manner.⁴ This effect was shown not to be sensitive to pertussis toxin (and therefore most likely mediated by a G_q protein). However, the action could be blocked by phorbol ester treatment. The effect was again highly specific.

The ability of LPA to induce Ca²⁺ transients has been evaluated in a large variety of cell types, and has been found to be present in virtually every cell type tested. Of relevance to the present report, *Xenopus laevis* oocytes also responded to LPA with intracellular Ca²⁺ transients, which in this model induce a Ca²⁺-activated Cl⁻ current through endogenous channels. Only very few cell types have been found to be unresponsive. These unresponsive cells include fresh human neutrophils, mast cells, Jurkat T cells, monocytes, peripheral blood lymphocytes and K562 cells (although a few contradictory reports exist). Interestingly, all these cells are of hematopoietic lineage, and most of them are part of the immune system. Platelets, on the other hand, do respond to LPA with a Ca²⁺ transient.

Platelet aggregation and LPA generation by platelets

Schumacher et al. demonstrated in 1979 that a factor present in serum "aged" for 18 to 24 h induced platelet aggregation *in vivo*.¹¹ The compound was tentatively defined as LPA, and LPA was shown to have the same effects. The effect is concentration-dependent as well as species-dependent: rat, rabbit and guinea pig platelets do not respond to the compound, feline platelets aggregate at a threshold of approximately 0.1 $\mu\text{g/ml}$, and human platelets aggregate at approximately 6-10 $\mu\text{g/ml}$.

Later studies have shown this platelet-aggregating activity of LPA to be Ca^{2+} - and Mg^{2+} -dependent, to be optimal at a pH of 7.50 and to be enhanced by the presence of fibrinogen. LPA enhances ADP-induced platelet aggregation as well.^{16, 20} Its signaling pathway is separate from that of PAF.^{21, 22} Watson et al. have demonstrated that LPA acts at an extracellular site on the platelet.²³

Not only does LPA induce platelet aggregation, it is also generated by activated platelets.²⁴ This LPA, mostly of the palmitoyl, stearoyl or arachidonoyl variety,²⁵ appears to be released extracellularly.²⁶ Because of this release (and possibly because of generation by stimulated neutrophils [Durieux ME: unpublished observations]) LPA is present in micromolar concentrations in serum.²⁶ In serum, it is bound tightly to serum albumin²⁷ at a relative LPA:albumin ratio of 2:1.²⁸ In fact, many of the cellular effects of serum may be due to LPA. For example, Moolenaar, in his paper describing the mitogenic actions of (L)PA, noted already that serum induced similar actions. Serum-induced sensitization of cAMP stimulation in atrocytoma cells,^{29, 30} neurite retraction in nerve growth factor-stimulated PC12 cells,^{31, 32} and Sp2 shape changes³³ are examples of serum effects due to the presence of LPA. It is likely, therefore, that LPA is one of the main active factors in the serum routinely used to supplement cell culture. Some LPA is also present in plasma.³⁴

Cytoskeletal effects

As mentioned above, LPA has dramatic effects on the shapes of some cell types: neurite retraction is observed in nerve growth factor-stimulated PC12 cells,³² and even more rapid rounding has been observed in neuroblastoma cells.³⁵ Similar findings were reported in glioma cells.³⁶ In addition, LPA reverses cAMP-induced stellation in BHK21 cells³⁷ and spinal cord astrocytes,³⁸ and induces shape changes in a variety of other fibroblasts lines. Of particular interest may be a recent report of LPA-induced contraction in Dupuytren's fibroblasts.³⁹ Although the mechanisms underlying these effects are likely to be varied, one common pathway may be effects of LPA on the cytoskeleton. Neurite retraction in NG108-15 cells, for example, is blocked by cytochalasin B.⁴⁰ In fact, LPA has been shown to induce actin stress fiber assembly. In an effort to determine the factor(s) in serum responsible for maintenance of the actin stress fiber system, Ridley and Hall determined activities of a number of growth factors in serum, and found LPA to be the one most able to mimic the serum action.⁴¹ Similar findings have been reported by Ha and Exton.⁴²

In addition, LPA is known to affect several adhesion molecules. Both LPA and PA enhance the interaction between fibronectin and integrins,⁴³ although this effect could be observed in lipid vesicles only, not in platelet membranes. LPA enhancement of binding to integrins may be a common property, as similar findings have been reported in other systems. For example, Checovich and Mosher showed that binding of fibronectin to MG-63 osteosarcoma cells is en-

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hanced in a dose-dependent manner by LPA.⁴⁴ This appeared due to an increase in the number of fibronectin binding sites, rather than a change in affinity. Recent evidence suggests that Rho, which is activated by LPA, is involved in signaling downstream of integrins.⁴⁵

In view of LPA's action on adhesion, it is of interest that most of the cell types found to be unresponsive to LPA are part of the hematopoietic system, and therefore do not adhere under normal circumstances.

These cytoskeletal effects may explain in part the findings of modulation by LPA of tumor invasion, a process highly dependent on adhesion and cytoskeletal action. For example, LPA induces invasion of AH130 hepatoma cells (MM1 clone).⁴⁶

Non-mammals

All the effects described thus far were on mammalian cells. However, LPA responses are not limited to mammals. In *Xenopus laevis* oocytes LPA induces Ca^{2+} -activated Cl^- currents.^{28, 47, 48, 49} This will be described in more detail in the remainder of this thesis.

In addition, LPA has been shown to be a chemoattractant for *Dictyostelium discoideum* amoebae.⁶ The compound is approximately half as efficient an attractant as cAMP, the classic chemoattractant for this system. LPA-induced chemotaxis occurs over the concentration range of 0 to 2 μM . These findings suggest that LPA signaling is an evolutionary ancient system, spread widely through the animal kingdom. Thus, LPA signaling may have a high survival value.

LPA SIGNALING

The remarkable variety of cellular effects induced by LPA appears to suggest that the compound may act in a non-specific manner. However, data indicating that the compound is not lytic in cells,⁴ that it does not act when applied intracellularly,^{23, 47, 48} and that a large variety of related compounds are without effect indicate that LPA has a specific, extracellular site of action. Thus, one would expect to find evidence for a membrane receptor system. Unfortunately, ligand binding assays with LPA have thus far been found to be poorly reproducible,^{50, 51} probably because of excessive non-specific membrane binding of the lipophilic compound, as well as metabolism. The ubiquitous presence of LPA responses in cells commonly used for expression cloning and the absence of a dependable binding assay have greatly hampered efforts to clone the LPA receptor. Although several cDNA clones have been suggested to encode LPA receptors, none of them can at the time of writing be conclusively assigned that role. Thus, most evidence for the existence of an LPA receptor is relatively indirect. In contrast, the intracellular pathways influenced by LPA are much better defined. In this section, I will briefly review these data.

Evidence for an LPA receptor

Evidence for the existence of an LPA receptor system is as follows: (1) LPA acts extracellularly; (2) some responses to LPA are sensitive to pertussis toxin; (3) LPA signaling is affected by direct activators and inactivators of G proteins; and (4) a photoaffinity analog labels an LPA binding protein in the plasma membrane.

As mentioned before, intracellular application of LPA is without effect. Watson et al. generated LPA intracellularly in platelets from didecanoylglycerol, and found it not to induce

aggregation.²³ In *Xenopus* oocytes, we⁴⁸ and others⁴⁷ have shown that intracellular application of LPA does not induce Ca^{2+} -activated Cl^- currents. In addition, LPA microinjected into N1E-115 cells does not induce shape changes, whereas extracellularly applied LPA does.³⁵ La^{3+} , which interacts strongly with negatively charged phospholipids, completely blocks the LPA-induced Ca^{2+} signal in fibroblasts, even when added after initiation of the response.⁴ As La^{3+} is virtually impermeable in most cell types, the ability of the compound to terminate LPA actions must take place extracellularly. Similarly, bovine serum albumin, which binds LPA, is able to reverse LPA-induced platelet aggregation.²² Thus, the site of action of LPA is likely to be extracellular.

The mitogenic activity of LPA is inhibited by pertussis toxin,¹⁹ strongly suggesting a G protein-coupled pathway. LPA inhibition of adenylate cyclase is also inhibited by pertussis toxin.⁵² Additional evidence for a role for G_i in LPA signaling has been provided by Carr et al.⁵³

If LPA acts through a G protein-coupled receptor, one would anticipate sensitivity of the responses to GTP analogs. Such has indeed been shown. GTP γ S potentiates LPA-induced IP_3 generation and GDP β S inhibits it.^{19, 54} Thus, LPA signaling is likely to involve one or more G protein-coupled pathways.

Van der Bend et al.⁵⁵ reported studies using a radiolabeled, photoactivated LPA analog (^{32}P -diazirine-LPA). The compound was found to act as an agonist, and, when applied to N1E-115 cells, labeled a single, 38 kD band in the plasma membrane. The band was digested completely by trypsin, and in addition was inhibited competitively by unlabeled LPA, but not by other phospholipids. The labeled protein did not appear to contain a significant amount of N-linked oligosaccharides. When tested in tissues, significant labeling was observed in membranes from brain homogenates, but labeling was undetectable in liver and faint in lung.

Taken together, these data provide reasonably convincing evidence that LPA signals through one or more G protein-coupled receptors. In fact, several recent papers have reported cloning of LPA receptors. Thus far, however, none of these has passed the test of conveying LPA responsivity on a non-LPA responsive cell type. An et al.⁵⁶ cloned a cDNA homologous to that encoding sheep Edg2 from human lung. It encodes a 364 amino acid protein, most abundant in brain, with a hydrophilicity profile compatible with a G protein coupled receptor. Edg2-transfected fibroblasts showed enhanced responses to LPA, and overexpression in CHO cells correlated with enhanced LPA binding. The mouse Edg2 counterpart was similarly published as an LPA receptor.⁵⁷ Guo et al. reported cloning of a different cDNA from *Xenopus* oocytes.⁵⁸ Antisense constructs injected into oocytes inhibited LPA responses in these cells. At the moment it is unclear which, if any, of these receptors is "the" LPA receptor.

What appears to be clear is that multiple receptors for LPA exist. Using L-palmitoyl-serine and N-palmitoyl-tyrosine, two compounds that antagonize oocyte LPA responses, and a cyclic form of LPA, cLPA, Liliom et al. were able to differentiate high- and low-affinity receptors on *Xenopus* oocytes.^{59, 60}

Intracellular signaling pathways

LPA activates multiple G proteins intracellularly: as mentioned above, the mitogenic response to the compound is sensitive to pertussis toxin, whereas the Ca^{2+} response is not. Thus, coupling to at least two G proteins appears likely, one linked to mitogenic effects and sensitive to pertussis toxin, presumably G_i , and one coupled to Ca^{2+} release and insensitive to pertussis toxin, presumably G_q . This latter pathway is sensitive to phorbol ester. In addition, van Corven et al. have shown that LPA treatment of cells induces arachidonic acid release, which, based on con-

centration-response data, GTP γ S sensitivity and pertussis toxin and phorbol ester blockade, appears to be mediated by a third G protein class.¹⁹

LPA inhibits agonist-induced cAMP accumulation in several models, including fibroblasts,¹⁹ glioma cells,⁶¹ and hamster adipocytes.⁶² This effect is due to G protein-mediated inhibition of adenylate cyclase.⁵² In fibroblasts, half-maximal inhibition is obtained at an LPA concentration of approximately 0.1 μ M. Experiments with GTP analogs and pertussis toxin have demonstrated that this effect is mediated through a G protein, and Wong et al. have shown that the inhibitory effects of LPA on cAMP accumulation are mimicked completely by a constitutively active mutant of G_{i2}.⁶³

In addition to inhibiting cAMP generation in response to agonist, LPA sensitizes cells to agonist, so that agonist stimulation results in levels of cAMP higher than those found in the absence of LPA. This effect was observed first with serum,²⁹ and later the serum effect was shown to be due to LPA.³⁰ LPA-induced sensitization is mediated by G_i and protein kinase C, as shown by block of the effect with pertussis toxin and phorbol ester.

LPA activates members of the *ras* family of small cytoplasmic G proteins. This was initially suggested in a study by Yu et al.,⁶⁴ where it was shown that neutralizing antibody against *c-ras* was able to reverse phosphatidic acid-induced cell proliferation. Van Corven et al.⁶⁵ studied *ras* activation by LPA in more detail. LPA, as well as thrombin and epidermal growth factor, induced a rapid accumulation of GTP on p21^{ras} in Rat-1 cells. The EC₅₀ for LPA was approximately 20 to 30 nM. Pretreatment with pertussis toxin largely abolished LPA-induced p21^{ras} activation, but cholera toxin was without effect. The cell-permeant cAMP analog 8-Br-cAMP was also without effect, suggesting that p21^{ras} activation by LPA is independent of a G_i mediated decrease in cAMP. In contrast, tyrosine-specific phosphorylation does appear involved, as LPA strongly induces tyrosine phosphorylation in fibroblasts and the protein kinase inhibitor genistein inhibits both LPA-induced phosphorylation and p21^{ras} activation.

LPA also activates *rho*, another member of the *ras* family, and it is this effect that is responsible for LPA's profound effects on the cytoskeleton. The exoenzyme C3 transferase inactivates *rho* proteins by ADP-ribosylation, and also inhibits the effects of LPA on the cytoskeleton.⁴¹ *Rho* activity also appears to play a role in the stimulation by LPA of tumor cell invasion, as LPA-induced invasiveness of MM1 tumor cells could be inhibited by C3 treatment.⁴⁶ A *Rho* GDP/GTP exchange factor, RhoGEF, may link LPA receptor activation with *Rho* activity, inducing neurite retraction and cell rounding.⁶⁶

As indicated, LPA induces protein phosphorylation. At least two of the kinases involved are known: MAP kinase and p125^{FAK}. Kumagai et al. reported that LPA induced phosphorylation of p125^{FAK},⁶⁷ which may play a role in the LPA-mediated actions on focal adhesions and the cytoskeleton. In addition, the investigators demonstrated LPA-induced phosphorylation of MAP kinase, which is likely to be the link to the mitogenic actions of LPA. Similar findings were reported by Hordijk et al.,⁶⁸ and the effects on p125^{FAK} were confirmed by Seufferlein and Rozenfurt.⁶⁹ The action of LPA in these studies was rapid: maximal effects were generally observed after 2 to 5 minutes. Pertussis toxin is without effect on p125^{FAK} phosphorylation, but, in contrast, MAP kinase activation is inhibited completely. Staurosporine, an inhibitor of several serine/threonine and tyrosine-specific protein kinases also inhibits MAP kinase phosphorylation as well as p21^{ras} activation induced by LPA. These findings have been confirmed in a subsequent study.⁷⁰

In contrast to its effect on p21^{ras}, 8-Br-cAMP blocks LPA-induced MAP kinase activation.⁷¹ The mechanism of this effect appears to be rapid dephosphorylation of constitutively active MAP kinase.

The links between the LPA receptor(s) and MAP kinase are not yet clearly established. G_i activation by LPA induces tyrosine kinase activity and recruitment of Grb2, which causes GDP-GTP exchange on *ras*. Recently, a tyrosine-phosphorylated protein (p100) was reported that binds to Grb2 in a G_i dependent manner.⁷² The p100-Grb2 complex may thus form the link between the G protein and MAP kinase.

ROLE FOR LPA

Despite the insights gained in recent years into the cellular effects of LPA and the molecular mechanisms underlying these actions, it is still unclear what the physiologic or pathophysiologic role of the compound is. Most of the research has been performed at the cellular or tissue level; very few studies have investigated effects in organs or animals. However, some patterns have emerged that indicate potential roles for the compound.

At present, the main source of LPA in the body appears to be activated platelets, which would suggest a site of action in the vasculature. In addition, injured fibroblasts have been shown to release LPA.⁷³ As LPA constricts vascular smooth muscle, activates platelets, and induces fibroblast and vascular smooth muscle⁷⁴ proliferation, this suggests a potential role in the response to vascular injury. It is of interest that blood cells that are not involved in repair are some of the very few cells known to be unresponsive to the compound. As a lipid, LPA would be unlikely to migrate far in the hydrophilic environment of the vasculature, which would prevent undesired actions far removed from the injury site. In addition to a repair function in the vasculature, LPA could play a beneficial role in wound healing. We have shown LPA levels in acute wounds to be increased (Durieux ME: unpublished observations), and the possibility of enhancing wound healing by external LPA application is currently under investigation.

If LPA indeed plays a role in the maintenance of vascular integrity, the possibility exists that it also plays a role in one or more pathophysiologic states. One possibility would be an etiologic role in the development of cerebral vasospasm seen after subarachnoid hemorrhage, when it could be released in large amounts after clotting of subarachnoid blood. In fact, LPA levels have been shown to increase after experimental subarachnoid hemorrhage in piglets,¹⁴ and we have shown LPA levels to be increased after subarachnoid hemorrhage in humans, as well as in an experimental rabbit model.⁷⁵ In addition, LPA, in the concentrations present after subarachnoid hemorrhage, constricts cerebral blood vessels: the effect on pial arterioles is modest,¹⁴ but basilar artery is constricted approximately 50%, both in vitro and in vivo, by 1 μ M LPA (Durieux, unpublished observations). Interestingly, LPA has several other detrimental effects on brain. It increases permeability of tight junctions in cultured brain endothelial cells,⁷⁶ and it decreases glutamate and glucose uptake by astrocytes.⁷⁷

Thus, despite the fact that a clear role for LPA in physiology or pathophysiology remains to be established, an investigation of anesthetic effects on LPA signaling seems warranted. In addition to providing information on the interactions between anesthetics and a novel signaling molecule with a variety of remarkable actions, it will serve as a paradigm for the investigation of anesthetic interactions with G protein coupled receptors in general, and in particular those for lipophilic ligands.

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CHAPTER 4

RECEPTOR EXPRESSION IN OOCYTES

Marcel E. Durieux

Receptor expression in oocytes

The potential of the *Xenopus laevis* oocyte as an expression and cloning system was first realized in 1971. In that year Gordon et al. for the first time expressed foreign protein in the oocyte by injection of messenger RNA.¹ During the intervening 25 years oocyte expression has developed into a highly versatile method for the study of recombinant proteins, and in particular for membrane signaling proteins such as G protein-coupled receptors (GCRs) and ion channels. Although the expression principles first laid down in Gordon's paper are to a large extent still valid, a variety of novel methodologies has been devised over the years, which have helped to make oocyte expression more flexible and powerful. Examples are the measurement of Ca^{2+} -induced Cl^- currents and of $^{45}\text{Ca}^{2+}$ efflux as convenient methods for the functional study of Ca^{2+} signaling receptors, and the development of cDNA injection into the germinal vesicle to bypass the need for mRNA preparation. More rarely used, but very elegant variations on the basic theme are the use of aequorin, both as a reporter of intracellular Ca^{2+} release and a control of adequate mRNA expression, and the combined expression of GCRs and G protein-regulated ion channels functioning as reporter molecules.

During the past ten years, the oocyte system has been used primarily as a cloning system. However, the system is also a powerful tool for the functional study of receptors. Chimeric or otherwise mutated receptors can be expressed easily and their structure/function relationships studied. Similarly, the functional properties of potential agonists and antagonists can be tested easily, in order to supplement binding studies. These uses of the system are likely to increase over the years to come.

In this chapter I will give an overview of use of the *Xenopus* oocyte expression system as a functional assay for the study of receptor properties. This section is based on a book chapter by the author.²

THE *XENOPUS* OOCYTE

The African Clawed Toad, *Xenopus laevis*, is an aquatic species of toad found primarily in southern Africa (despite the fact that the animal biologically is a toad, it has historically been called a frog, and I will concede to this established terminology). The animal has long been used for laboratory purposes, as it is remarkably easy to maintain and quite resistant to disease. In the past, injecting female frogs with a urine sample and watching for release of eggs was a routine method of pregnancy testing. Being aquatic, the animals can be kept in tanks without the need for a dry surface. In addition, the presence of several compounds with antibiotic activity in *Xenopus* skin makes infection at the site of operation remarkably rare. They easily adapt to commercial food and have few special temperature, water and light requirements.

Female *Xenopus* frogs carry a large supply of oocytes. Although most of these are immature cells, removal of mature oocytes will cause some of the immature cells to develop into mature cells. Thus, oocytes can be retrieved multiple times from a single frog. Mature oocytes have to be separated from the - usually much more numerous - immature ones. Dumont distinguished 6 stages of oocyte maturation,³ of which only stage 5 and 6 are suitable for expression purposes. These are easily recognized by being the largest oocytes, showing a clear division in dark and light poles, and uniform coloration of each pole. Oocytes with a white spot in the dark half should be avoided; these are undergoing spontaneous maturation (so-called germinal vesicle breakdown). The isolated oocytes obtained can be maintained for more than a week.

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As obtained from the ovary, the oocytes are surrounded by a thin layer of follicle cells, recognizable only by the presence of blood vessels and a shiny appearance. The combination of oocyte and follicular layer is at times termed a follicle. For most purposes, the follicle cells need to be removed, as they impede access of agonists to the oocyte, and in addition - and in contrast to the oocyte proper - contain a large array of endogenous receptors and channels. As the follicle cells connect to the oocyte by gap junctions, activation of signaling systems in the follicle cells can result in transmission of electrical or chemical signals to the oocyte,⁴ which can confound experimental results. Hence, the oocytes need to be defolliculated. Two common methods are in use. There seems to be little difference between them in the (minimal) amount of damage to membrane proteins. In manual defolliculation, the follicular layer is removed from each oocyte individually, using microforceps and a dissection microscope. Although requiring a significant amount of skill and time, this method probably provides the best assurance that complete defolliculation has been achieved, as each oocyte is inspected individually. Alternatively, oocytes can be defolliculated by gentle shaking in collagenase for several hours.

After defolliculation, the oocyte plasma membrane is still covered with an additional layer, the (acellular) vitelline membrane. This layer gives significant strength to the oocyte. Its removal is not necessary for most receptor studies. However, oocyte patch clamping requires removal of at least parts of the vitelline membrane, which can be accomplished manually after shrinking the cell by immersion in hypertonic solution.

Defolliculated Dumont stage 5 or 6 *Xenopus* oocytes are approximately 1.2 to 1.5 mm in diameter, and consist of well defined dark brown and light brown hemispheres. The dark brown color is caused by melanin accumulation; albino oocytes are white all over. If the oocyte were to develop into an egg and be fertilized, the dark hemisphere, termed the animal pole, would develop into the rostral segment of the frog, whereas the light hemisphere, termed the vegetal pole, would develop into the caudal segment. The distinction between animal and vegetal pole is relevant for several reasons. First, the germinal vesicle (the technical term for the oocyte nucleus) is located in the center of the animal pole, and approximately one third the oocyte diameter in size. Although not visible, its fixed location allows microinjection of cDNA. Second, the distribution of receptors and channels (endogenous as well as recombinantly expressed) over the oocyte surface is not homogeneous. Although exceptions exist, expression is generally considered to be better over the animal pole,⁵ and agonist application is therefore better performed over that segment of the oocyte membrane.

The internal volume of the oocyte, approximately 0.5 to 1 μ l, consists largely of yolk. In fact, it is this remarkable yolk production that makes the oocyte such an effective protein factory. In order to produce these large amounts of protein, the cell duplicates part of its genome encoding the building blocks for ribosomes, and as a result it has a complement of protein synthesizing machinery far exceeding that of other cells. The other large component of the interior is, as stated, the germinal vesicle. It can be visualized easily by carefully pulling open the oocyte membrane: it will appear as a small clear sphere within the white yolk protein.

Endogenous G protein-coupled receptors

One of the major advantages of the *Xenopus* oocyte for use as an expression system for GCRs is its remarkable paucity of endogenous receptors. In fact, only a single receptor type is consistently present, that for the phospholipid agonist lysophosphatidate (see Chapter 5).⁶ Whether this receptor also mediates responses to sphingosine-1-phosphate, or whether a separate

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sphingosine-1-phosphate receptor is present is still an unresolved question (see Chapter 6).⁷ In fact, as discussed in Chapter 3, multiple subtypes of LPA receptor appear to exist in the oocyte. The presence of the lysophosphatidate receptor can be used to test for functional oocyte signaling.

A number of studies report the presence of other receptors endogenously expressed in *Xenopus* oocytes. However, most times these receptors are located in the follicular cell layer, and their activation leads to oocyte responses only because of the gap junctions present between oocyte and follicle cells. If oocytes are meticulously defolliculated, such responses almost invariably disappear or become so small to be practically irrelevant. Examples of such receptors endogenously present in follicles are muscarinic⁸⁻¹⁰ and angiotensin^{4, 10, 11} receptors. Oocytes from occasional frogs (so-called "variant donors") may exhibit consistently large responses to acetylcholine.¹² Despite the fact that these have received considerable attention, they are rare, and the fact that many investigators have successfully studied recombinantly expressed angiotensin and muscarinic receptors in *Xenopus* oocytes indicates that such occasional intrinsic oocyte responses form no hindrance to the study of exogenous receptors. We have expressed both muscarinic and angiotensin receptors, and never observed endogenous responses in defolliculated oocytes.^{13, 14} In contrast, folliculated oocytes will show Ca^{2+} transients in response to angiotensin,⁴ acetylcholine,^{12, 15} follicle-stimulating hormone,¹⁶ adenosine,¹⁶ β -adrenergic agonists,¹⁶ and other compounds.

The oocyte shows responses to extracellularly applied trypsin that are indistinguishable from those observed after GCR activation (see Chapter 8).¹⁷ These have recently been shown to be mediated by G proteins.¹⁸ Whether or not a receptor for trypsin or a related enzyme (analogous, maybe, to the thrombin receptor?) is present in the oocyte membrane is still an open question; however, if material used to stimulate oocytes (e.g. cell extracts) contains trypsin, responses may be anticipated.

Endogenous signaling systems

Despite its paucity of endogenous receptors, the oocyte contains the appropriate machinery to link activation of exogenously expressed receptors to intracellular responses. For the purpose of GCR expression, the most important intracellular signaling cascade is G_q/G_{12} -linked Ca^{2+} release, which can be quantified conveniently through an induced Cl^- current. Other G proteins are present, however, and able to link receptor activation to opening of recombinantly expressed channels. Finally, a number of other endogenous channels are present in the oocyte, and may be activated - whether intended or not - by manipulation of the oocyte system.

G_o/G_q activation: Ca^{2+} release and Ca^{2+} -activated Cl^- current

Activation of GCRs, expressed in the oocyte membrane, that couple to G_o or G_q proteins endogenously present leads to activation of the oocyte phospholipase C (PLC) and release of inositoltrisphosphate (IP_3) from phosphatidylinositolbisphosphate (PIP_2). The oocyte has been shown to contain only a single subtype of PLC linking GCR stimulation to Ca^{2+} release, namely PLC- β .¹⁹ The IP_3 generated by PLC activation releases Ca^{2+} from intracellular stores. Intracellular Ca^{2+} signaling in the oocyte has received a significant amount of attention over the past years, as it has been shown to be a complicated and even beautiful process. When confocal microscopy is used to study albino oocytes loaded with Ca^{2+} -sensitive dye, it can be shown that Ca^{2+} is not

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released as a single gradient across the oocyte, but rather as complex spirals that emerge from several points, move through the oocyte volume, and annihilate on contact.^{20, 21} These Ca^{2+} waves arise because of an interplay between IP_3 -stimulated release and Ca^{2+} -activated Ca^{2+} release, and are probably responsible for the fluctuations observed in the Ca^{2+} -activated Cl^- currents described next.

Whereas confocal microscopy provides detailed insight in the kinetics of Ca^{2+} release, for most purposes it is sufficient to have a quantifiable measure of total release. This can be conveniently obtained by measuring Ca^{2+} -activated Cl^- current ($I_{\text{Cl}(\text{Ca})}$). The oocyte contains endogenous Cl^- -selective ion channels that are gated by the intracellular Ca^{2+} concentration and probably play a role in maintaining oocyte polarity and segregation of intracellular contents. Hence, if membrane potential and ionic gradients across the cell membrane are chosen appropriately, intracellular Ca^{2+} release will result in an inward Cl^- current that is measured easily by two-electrode voltage clamp. Either peak current (in μA) or total charge movement (the integral of the current trace, in μC) have been used as a measure of Ca^{2+} release. As charge transfer provides us the closest correlation when plotted against the logarithm of agonist concentration, we routinely use charge movement as a measure of intracellular Ca^{2+} release. A data acquisition program is available that automates the integration step (see Chapter 7).²²

Other G proteins: cAMP and channel activation

The ease with which intracellular Ca^{2+} release is studied in the oocyte has made the system a favorite for the investigation of Ca^{2+} -linked GCRs, and it has been used much less often for the study of receptors activating other G proteins. However, it is certainly possible to use the system to study such receptors.

Activation of expressed receptors coupling to G_s or G_i proteins leads to the expected changes in cAMP concentration. Thus, these G proteins are present and able to couple appropriately to exogenously expressed receptors. Unfortunately, G_i -linked receptors are still difficult to study, as raising cAMP levels sufficiently to observe decreases after receptor activation remains problematic. This appears to be due to relative insensitivity of the oocyte adenylyl cyclase to the stimulating agent forskolin.¹⁶ The oocyte G_i is sensitive to pertussis toxin, although in general much higher incubation concentrations and times are used (e.g. 1-2 $\mu\text{g}/\text{ml}$ for 12 h) than employed for cells with a smaller volume.

A more promising method of studying G_i -coupled receptors is by coexpression of a G protein regulated ion channel, such as a K channel.

Endogenous channels

For the investigator interested in using the oocyte system for expression of GCRs, the Ca^{2+} -activated Cl^- channel described above is no doubt the most important endogenous ion channel present. However, a number of other channels are expressed, and researchers should be aware of these, as their unplanned activation might lead to confounding results.

The presence of voltage-activated Ca^{2+} channels is well established.²³ They are activated by depolarization of the oocyte membrane from between -100 and -60 mV to more than -30 mV, and have a decay constant of approximately 0.5 s. Because Ca^{2+} is normally present in physiological solutions bathing the oocyte, activation of these channels will lead to Ca^{2+} influx and subse-

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quent activation of $I_{Cl(Ca)}$, which could confound experimental results. Therefore, the holding potential of the oocyte should be maintained constant during experiments with GCRs.

Other currents described to be present in the oocyte include a hyperpolarization-induced Cl^- current, a depolarization-induced slow K^+ current, and a depolarization-induced slow Na^+ current. None of these responses should interfere with the study of GCRs, as long as membrane potential is maintained constant during experimentation. For a more in depth discussion of these endogenous channels, the reader is referred to a thorough review article.²⁴

TECHNIQUES OF OOCYTE EXPRESSION

This section will discuss the two main methods by which GCRs can be expressed in *Xenopus* oocytes: by microinjection of RNA into the cytoplasm, or by microinjection of DNA into the germinal vesicle.

Despite meticulous technique and consistency in method, variation in expression levels will still be occasionally observed. It is not certain where this variation derives from. Clearly, diseases or changes in the animals' environment (temperature, water quality, food) may play a role, but even if these are ruled out, some degree of variation may persist. A seasonal variation has been suggested, but in our experience there is no consistent distribution over the year. Despite the fact that often no specific problem can be found and corrected, suboptimal expression tends to be temporary. Often, simply waiting for a few weeks will resolve the problem. Alternatively, some new frogs can be acquired and used.

RNA injection

The original, and still the most commonly used, method of expressing receptors in *Xenopus* oocytes is by intracellular microinjection of RNA encoding the protein to be expressed. The RNA can either be extracted from tissue (mRNA), or be prepared in vitro from a DNA template (cRNA). RNA injection into oocytes is performed into the vegetal pole, in order not to injure the germinal vesicle.

cDNA injection

Although used less commonly, receptor expression by injection of cDNA into the oocyte germinal vesicle has the advantage that smaller amounts of nucleic acid are needed, and that the step of cRNA preparation can be skipped. There is, however, some additional complexity to the technique. The cDNA is injected as the uncut vector construct. In order to allow the oocyte RNA polymerase to transcribe the cDNA, an appropriate promoter should be incorporated in the vector. Several specifically designed oocyte promoters have been developed, but the presence of a CMV promoter upstream of the cDNA to be expressed has been shown to be effective as well.

Although it was known that the germinal vesicle is localized in the animal pole, it was for some time considered necessary to centrifuge the oocytes at low speed, with the animal pole facing outward, in order for the germinal vesicle to move against the cell membrane, where it was visible as a whitish spot and could be injected easily. However, this manipulation is time consuming, as oocytes need to be aligned individually, and in addition it tends to damage the cells, decreasing their survival rate. It has become clear by now that the position of the germinal

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vesicle is sufficiently consistent that it can be injected blindly with a high success rate. Expression by germinal vesicle injection often requires more culture time than expression by cytoplasmic injection.

THE STUDY OF GCRs IN OOCYTES

Once a GCR of interest is expressed in the oocyte membrane, an assay system is needed to determine its presence and function. As the questions that can be addressed using this system (from cloning to the effect of mutations in the receptor to the study of changes in agonist structure) range wide, a number of techniques have been developed to study GCRs expressed in oocytes. In this section, some of these will be discussed, and their advantages and disadvantages will be compared. In the context of the present thesis, only techniques used to study Ca^{2+} -signaling receptors ($\text{I}_{\text{Cl}(\text{Ca})}$, $^{45}\text{Ca}^{2+}$, and aequorin assays) will be discussed. Descriptions of methods to study receptors signaling through other intracellular mediators (co-expression with channels, cAMP measurements) are available elsewhere.² The technique for applying anesthetics to oocytes will be described in subsequent chapters.

Ca^{2+} -induced Cl^- currents

The most widely used oocyte assay for the study of GCRs is measurement of $\text{I}_{\text{Cl}(\text{Ca})}$ in response to intracellularly released Ca^{2+} . The technique is relatively straightforward, rapid, and provides consistent quantitative results. The main practical difficulties with the technique derive from the difference in response size and kinetics as compared with "standard" electrophysiologic data; therefore, many hardware and software systems have difficulty dealing with them. For example, the membrane capacitance of the oocyte is enormous when compared with that of other cells, and as a result, the capacitance transient occurring after a membrane potential step easily saturates standard voltage clamp amplifiers. Similarly, many electrophysiology software packages have difficulty dealing with currents that develop over many seconds or even minutes, rather than milliseconds to seconds. Thus, specific hardware and software has been developed for this task (see Chapter 7).

In general, only a single response should be elicited from each oocyte, as receptor desensitization may occur. However, if required by the experimental protocol (e.g. studies investigating reversibility of inhibition), multiple responses can be obtained from most expressed receptors by allowing the oocyte to rest 10-30 min between agonist applications. In our laboratory, we reuse, at times, oocytes after one or more days of rest.

A number of variations on routine $\text{I}_{\text{Cl}(\text{Ca})}$ measurement can be used to study effects of compounds on intracellular signaling pathways. For example, localization of site of action of drugs known to interfere with GCR signaling can be easily accomplished using the oocyte. Microinjection of the compound into the cell can determine if its site of action is intracellular or extracellular. Determining the effect of the compound on $\text{I}_{\text{Cl}(\text{Ca})}$ induced by microinjection of intracellular mediators such as IP_3 or Ca^{2+} can determine its exact site of action within intracellular signaling pathways.

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$^{45}\text{Ca}^{2+}$ efflux

A different method to assess intracellular Ca^{2+} release indirectly is by the measurement of efflux of Ca^{2+} from the oocyte. The procedure is based on the presence in the oocyte membrane of endogenous voltage-gated Ca^{2+} channels. Cells are first loaded with $^{45}\text{Ca}^{2+}$. Agonist-induced intracellular Ca^{2+} release will induce $I_{\text{Cl}(\text{Ca})}$, as described above. In a non-voltage clamped cell, this inward current will depolarize the membrane, inducing opening of endogenous voltage-gated channels. $^{45}\text{Ca}^{2+}$ will flow through these channels and appear in the medium. This can then be assayed quantitatively by scintillation counting.

Although the method allows testing of multiple oocytes at the same time, it lacks some of the advantages of the electrophysiologic technique. The sensitivity and time resolution of the $^{45}\text{Ca}^{2+}$ assay is less than that obtained with direct $I_{\text{Cl}(\text{Ca})}$ measurements, and the intracellular manipulations possible with the electrophysiology technique are not as easily performed. In addition, the method has the inherent disadvantage of requiring the use of a radioactive compound (however, $^{45}\text{Ca}^{2+}$ spills are cleaned easily with EGTA).

Aequorin

The use of aequorin is another approach to circumvent the necessity of individual oocyte testing. In addition, it provides a built-in control for adequacy of protein expression.

Aequorin is a protein obtained from jellyfish that emits light when it binds Ca^{2+} . Its cDNA has been cloned, and either the protein (50 nl of 5 mg/ml²⁵) or its mRNA can be injected into oocytes for expression.²⁶ Aequorin luminescence in response to intracellular Ca^{2+} release can be used as a reporter for receptor activation. Although the technique can be performed in regular oocytes,²⁵ light emission may be measured more conveniently in albino oocytes. The advantage of injection of mRNA (or cDNA) encoding aequorin over injection of the protein itself is in the control it offers for adequate expression. In those oocytes that express a functional receptor, intracellular Ca^{2+} will be released in response to agonist, bind to aequorin, and result in light emission, which is detected by the luminometer. Alternatively, for more detailed study, a single oocyte can be observed under a microscope with attached video imaging system. If desired, electrophysiological measurements can be performed at the same time.²⁵

To ascertain that the injected nucleic acid is adequately expressed and that the cellular signaling system is functional, the cells can be exposed to lysophosphatidate. Light emission in response to this agonist confirms that the oocyte signaling system is intact, and that at least the aequorin is adequately expressed.

CONCLUDING REMARKS

Within the past few years, creative strategies have been employed to enhance the value of *Xenopus* oocytes for expression cloning and functional characterizations of GCRs. In particular, the expression of ion channels that act as reporters of GCR-activated signaling pathways promises to be an important advance in the study of GCRs that do not activate the endogenous Ca^{2+} -activated Cl^- current. As nearly all of the GCRs that have been characterized by pharmacologic criteria have been identified by molecular cloning, the use of the oocyte as an expression cloning system is likely to decline. The increasing number of orphan GCRs combined with the decreas-

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ing number of known endogenous ligands may prompt the development of strategies to screen orphan GCRs for responsiveness to various bioactive compounds. As novel uses and techniques continue to be developed, *Xenopus* oocytes are likely to remain an important tool in future studies of GCR structure and function.

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CHAPTER 5

**Lysophosphatidic acid induces a pertussis toxin-sensitive
Ca²⁺-activated Cl⁻ current in *Xenopus laevis* oocytes**

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Lysophosphatidic acid induces a pertussis toxin-sensitive Ca^{2+} -activated Cl^- current in *Xenopus laevis* oocytes

ABSTRACT

Lysophosphatidic acid (LPA) induces a Ca^{2+} -activated Cl^- current in defolliculated *Xenopus laevis* oocytes. The response appears mediated by a specific membrane receptor, because no current is induced when related compounds [phosphatidic acid (PA), lysophosphatidylcholine (LPC), and lysophosphatidylserine (LPS)] are applied extracellularly or when LPA is injected intracellularly. Incubation in pertussis toxin prevents the response. The response is mediated by a Ca^{2+} -activated Cl^- current because 1) it is abolished by intracellular ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA; 5 mM) but not affected by changes in extracellular Ca^{2+} concentration and 2) the reversal potential becomes more positive at lower Cl^- concentrations. Suramin (2 mM) blocks the LPA-induced current, but PA, LPS, LPC, and the platelet-activating factor antagonist WEB-2086 do not. The response is dose dependent for LPA concentrations from 10^{-8} to 10^{-3} M. Incubation of oocytes in LPA does not induce germinal vesicle breakdown. These findings suggest that this novel oocyte response to LPA is mediated by a specific membrane receptor linked to a pertussis toxin-sensitive G protein.

Key words: G protein-coupled receptors; chloride channels; suramin; platelet-activating factor

INTRODUCTION

The polar lipid L- α -lysophosphatidic acid (monoacylglycerol $\Delta 3$ -phosphate; LPA) has attracted attention as a potential intercellular messenger because it causes species-specific blood pressure changes when injected intravenously,¹⁴ aggregates platelets,¹² and contracts uterine¹⁵ and colonic¹⁶ smooth muscle. LPA is also a mitogen in Rat-1 cells and human foreskin fibroblasts.¹ These two cell types respond to LPA application with phosphoinositide hydrolysis and a transient elevation of intracellular Ca^{2+} as measured by [^3H]inositol phosphate accumulation and Indo-1 fluorescence.¹ In these cell lines, this effect was not prevented by pertussis toxin (PT) pretreatment. In the present study, we report that defolliculated *Xenopus laevis* oocytes respond to LPA with a Ca^{2+} -activated Cl^- current and that, in contrast to the findings in mammalian cells, this response is blocked by incubation in PT. The presence of this response offers a new experimental system for study of the putative LPA receptor and should be considered when expressing membrane receptors in the *X. laevis* oocyte.

MATERIALS AND METHODS

Materials

LPA (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate) and phosphatidic acid (PA; 1,2-dipalmitoyl-*sn*-glycero-3-phosphate) were obtained from Avanti Polar Lipids (Alabaster, AL) and were dissolved in a 1% solution of bovine serum albumin (BSA; ICN Biomedicals,

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Cleveland, OH). PT was a kind gift of Dr. E. Hewlett at the University of Virginia. The platelet-activating factor (PAF) antagonist WEB-2086 was kindly provided by Dr. T. Y. Shen at the University of Virginia. Molecular biology reagents were obtained from Promega (Madison, WI). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Oocyte harvesting, incubation, and injection

Female *X. laevis* were obtained from Xenopus I (Ann Arbor, MI), housed in an established frog colony, and fed regular frog brittle twice weekly. For removal of oocytes, a frog was anesthetized by immersion in ice until unresponsive. A 1-cm incision was made in a lower abdominal quadrant, and a lobule of ovarian tissue, containing 200-300 oocytes, was removed and placed in modified Barth's solution [MBS; in mM: 88 NaCl, 1 KCl, 2.4 NaHCO₃, 0.41 CaCl₂, 0.82 MgSO₄, 0.3 CaNO₃, 0.1 gentamicin, and 15 N¹-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.6]. The wound was closed in two layers, the animal was allowed to recover from anesthesia, kept in a separate tank overnight, and returned to the colony the following day. The ovarian tissue was washed immediately and copiously in MBS and dissected into small clusters of 20-50 oocytes. Dumont stage V and VI oocytes were isolated manually and cultured in MBS at 18-19°C. Before each experiment, oocytes were defolliculated manually. For injection of mRNA, folliculated oocytes in MBS were placed on ice and injected with 50 nl of mRNA in 100 mM KCl, using a manual microinjector (Microdispenser; Drummond Scientific, Broomall, PA). The cells were cultured for 3 days and defolliculated manually before the experiment.

Electrophysiological recording

A single defolliculated oocyte was placed in a recording chamber (3 ml vol), perfused at 3-4 ml/min with a solution containing (in mM) 150 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 dextrose, and 10 HEPES (pH 7.4). Microelectrodes were pulled in one stage from capillary glass (type 6010; AM-1 Systems, Everett, CA) on a Flaming/Brown micropipette puller (model P-86; Sutter Instrument, Novato, CA) and beveled to sharpness on an electrode grinder (Narishige KG-6, Tokyo, Japan). They were filled with 3 M KCl, and tip resistances were usually 1-3 MΩ. The cell was voltage clamped using a conventional two-microelectrode voltage clamp amplifier (Oocyte Clamp OC725; Warner, New Haven, CT) connected to commercially available data acquisition and analysis software (Axon Instruments, Foster City, CA) and an IBM-compatible personal computer. Membrane current was sampled at 16.7 Hz and recorded for ~10 s before and 50 s after application of the test compounds. Before drug application the bath perfusion was stopped, and compounds were delivered in 30-μl aliquots over 1-2 s using a handheld micropipette positioned ~5 mm from the oocyte. Responses were quantified by integrating the current trace by quadrature and are reported as microcoulombs (mean ± SE). All experiments were performed at room temperature.

To determine the reversal potentials of the induced current, responses to 10 μM LPA were determined at several holding potentials. This was repeated at several extracellular Cl⁻ concentrations. Low Cl⁻ solutions were prepared by reducing the amount of NaCl and replacing it with an isosmolar amount of N-methyl-D-glucamine and by adjusting the pH with methanesulfonic acid. Each oocyte was tested at one potential in one bath solution. Reversal potentials were estimated from the x-intercept of a plot of charge movement vs. holding potential.

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Intracellular injections

For the intracellular injections of LPA and ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), a third micropipette was used, connected to an automated nitrogen-driven microinjector (Picospritzer, General Valve, Fairfield, NJ). The micropipette was calibrated volumetrically by counting the number of injections needed to expel a known volume of solution. It was then introduced in the cell, and a 50-nl volume was administered. The adequacy of injection was verified by observing the slight increase in cell size on injection.

RNA transcription

The rat M1 muscarinic acetylcholine receptor cDNA clone, a 2.8-kilobase pair *Xho* I fragment in the pGEM1 vector (Promega, Madison, WI), was a gift of Dr. T. I. Bonner (National Institute of Mental Health, Bethesda, MD). It was linearized with *Hind* III, transcribed *in vitro* in the presence of a capping analogue (⁷mGpppG), and the resulting mRNA was diluted in 100 mM KCl to a concentration of 1 mg/ml.

RESULTS

LPA induces a current in X. laevis oocytes

Application of LPA (10^{-7} to 10^{-3} M) to oocytes at a holding potential of -70 mV resulted in a transient inward current (Figure 1A). Typically, $1 \mu\text{M}$ LPA induced a 2- to $6\text{-}\mu\text{C}$ response. Vehicle (BSA 1%) alone did not induce this response. The current developed after a latency of ≥ 1 s and consisted of a fast inward component, followed by a relaxation over several seconds, on which small fluctuations were superimposed. The oocyte response to LPA desensitized only partially. If the recording chamber was adequately washed with LPA-free solution (150 ml over 30 min), multiple responses could be obtained from a single oocyte (3 oocytes, 1 frog; Figure 1B).

Response to LPA is specific

Because LPA is a lipophilic compound, the currents observed might have been caused by nonspecific interactions with the oocyte membrane. To differentiate nonspecific effects from specific interactions, we performed the following experiments.

Structurally related compounds do not induce the response. We tested the LPA analogues PA (8 oocytes, 3 frogs), lysophosphatidylcholine (78 oocytes, 6 frogs), and lysophosphatidylserine (LPS; 16 oocytes, 3 frogs) for activity and for their ability to block the LPA response. Except for PA, none of these compounds induced currents. PA ($1 \mu\text{M}$) induced occasional small responses. As a group, responses to PA ($0.3 \pm 0.2 \mu\text{C}$) were not significantly different from responses to application of vehicle. None of these compounds blocked the oocyte response to LPA (data not shown).

LPA is related structurally to PAF, which mediates its actions through a G protein-mediated pathway. The PAF receptor can be blocked by several structurally unrelated com-

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Figure 1. LPA-induced currents in *Xenopus* oocytes. A: Inward current induced by 100 μ M lyso-phosphatidic acid (LPA) in defolliculated *X. laevis* oocyte at holding potential of -70 mV. Application of 1% bovine serum albumin (BSA) alone does not induce response. Peak current of LPA response, 1,100 nA; total charge movement, 6.3 μ C. B: LPA-induced current does not significantly desensitize. LPA (100 μ M) was applied repeatedly to single oocyte at holding potential of -70 mV and washed with ~ 150 ml over 30 min between applications. Charge movements: 2.0, 4.4, 2.7 and 2.8 μ C. Holding currents at beginning of each recording: -146 , -181 , -171 and -156 nA, respectively.

pounds.¹¹ We tested the PAF antagonist WEB-2086 (a synthetic benzodiazepine; 5 oocytes, 1 frog) for agonist and antagonist activity. It neither induced currents nor blocked the LPA-induced current in oocytes (data not shown).

The anthelmintic polyanionic drug suramin, which inhibits the mitogenic responses to LPA in fibroblasts,² decreased the currents induced in oocytes by LPA. At a concentration of 2 mM, the response to 10 μ M LPA was decreased from 4.6 ± 0.4 to 1.4 ± 0.1 μ C (16 oocytes, 3 frogs), a decrease of 70%. This decrease could not be reversed by washing the bath (150 ml over 30 min). Combinations of 100 μ M LPA-2mM suramin or 10 μ M LPA-0.5 mM suramin also resulted in an irreversible decrease in the LPA-induced current (data not shown).

Intracellular injection of LPA does not induce a current. A volume of 50 nl LPA (10^{-5} M) injected into the oocyte did not result in a current response, whereas LPA applied extracellu-

Figure 2. LPA response is PT sensitive. *A*: LPA (1 μ M) applied to defolliculated oocyte incubated over night in 2 μ g/ml pertussis toxin (PT; charge movement 0.3 μ C) and to control oocyte from same frog incubated in modified Barth's solution (charge movement 5.7 μ C). Holding potential, -70 mV. LPA response is PT sensitive. *B*: Methylcholine (MeCh: 3 μ M) applied to defolliculated oocytes 3 days after injection with 50 ng M_1 muscarinic acetylcholine receptor mRNA, one incubated overnight in 2 μ g/ml PT (charge movement 8.7 μ C), and one control oocyte from same frog (charge movement 10.6 μ C). Holding potential, -70 mV. Methylcholine response is not PT sensitive.

larly to the same oocyte did produce the usual response (5 oocytes, 2 frogs; see Figure 3A). This finding suggests that LPA acts through an extracellular site of action and not through nonspecific membrane interactions.

LPA response is PT sensitive

Overnight incubation in 2 μ g/ml PT, a dose shown to inhibit PT-sensitive G proteins in the *X. laevis* oocyte,^{8,9} decreased the oocyte response to 10^{-4} M LPA from 8.8 ± 1.6 ($n = 48$) to 0.9 ± 0.3 ($n = 52$) μ C, a decrease of 90% (Figure 2A). This finding was reproduced in oocytes from 9 of 10 frogs. In oocytes injected with mRNA encoding the M_1 muscarinic acetylcholine receptor, incubation in PT did not decrease the response to 1 μ M methylcholine (Figure 2B),

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Figure 3. *A:* LPA (50 nl, 10 μ M) injected into defolliculated oocyte at holding potential of -70 mV does not induce a current. Small deflection seen is attributed to motion artifact induced by injection. LPA applied extracellularly to same oocyte afterwards induces typical current (19.3 μ C). This suggests an extracellular site of action for LPA. *B:* Intracellular injection of EGTA (50 nl, 100 nM) abolishes response to LPA (100 μ M) in defolliculated oocyte at holding potential of -70 mV. This suggests that LPA response is mediated by intracellular Ca^{2+} . *C:* LPA (10 μ M)-induced currents in defolliculated oocytes in 0 and 2 mM extracellular Ca^{2+} . Charge movements are 3.5 and 2.8 μ C, respectively.

suggesting that PT did not have a direct effect on intracellular Ca^{2+} release or on opening of Ca^{2+} -dependent Cl^- channels. Thus the LPA effect appears to be mediated through a G protein-linked pathway.

Oocyte LPA response is a Ca^{2+} -dependent Cl^- current

The *X. laevis* oocyte membrane contains Ca^{2+} -dependent Cl^- channels.³ To test the hypothesis that the current seen on LPA application was dependent on intracellular Ca^{2+} , we tested oocytes for LPA responses before and after intracellular injection of a 50- to 100-nl volume of 100 mM EGTA (estimated intracellular concentration 5-10 mM). We were unable to elicit the

Figure 4. Reversal potential determinations of LPA-induced current in *X. laevis* oocytes. Plot shows charge movement as function of holding potential at 3 Cl^- concentrations. Each point consists of at least 10 measurements. Error bars indicate SE. Reversal potential: 158 mM Cl^- , -15 mV; 16 mM Cl^- , +10 mV; and 3 mM Cl^- , +45 mV. This indicates that current is carried mainly by Cl^- .

LPA effect after EGTA injection (13 oocytes, 2 frogs; Figure 3B), suggesting that intracellular Ca^{2+} is a mediator of the current. Injection of 50 nl KCl (150 mM) did not block the response to LPA (data not shown). To determine whether influx of Ca^{2+} across the cell membrane is important for the response, we recorded LPA-induced currents in oocytes while perfusing the recording chamber with nominally Ca^{2+} -free medium (0 mM Ca^{2+} , no EGTA; 21 oocytes, 3 frogs; Figure 3C) as well as with high (6 mM) Ca^{2+} medium (3 oocytes, 1 frog). In neither case was the magnitude of the induced current affected, suggesting that the response is not dependent on extracellular Ca^{2+} .

To establish the ionic nature of the LPA-induced current, we measured its reversal potential in solutions with different Cl^- concentrations (Figure 4). In 158 mM Cl^- the reversal potential was approximately -15 mV, in 16 mM Cl^- it was +10 mV, and in 3 mM Cl^- it was +45 mV. These findings indicate that the current is mainly carried by Cl^- .

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Figure 5. Oocyte LPA response is dose dependent. *A*: currents induced by 3 concentrations of LPA in defolliculated oocyte at holding potential of -70 mV . Charge movements are 1.37, 6.18, and $9.61 \mu C$, respectively. LPA response is dose dependent. *B*: variability of oocyte response to LPA is demonstrated in this box plot. At least 15 oocytes were tested at each concentration (total ~ 130 oocytes). Each box includes 50% of measurements; vertical line encompasses 75%. Horizontal line within each box indicates median.

Oocyte LPA response is dose dependent

The oocyte response to LPA is dependent on the dose applied. Higher concentrations of LPA induced larger currents in individual oocytes (Figure 5A). There was, however, much variability between oocytes, and, even when >15 cells were used to determine the average charge movement at each concentration of LPA, we could not demonstrate a sigmoidal dose-response relationship (Figure 5B). This variation was not eliminated by constructing a cumulative dose-response curve (data not shown) nor by testing all doses in a single oocyte, even with copious washes between applications. However, a half-maximal effect was obtained at $\sim 1 \mu M$ LPA. At concentrations above the critical micellar concentration [$\sim 1.3 \text{ mM}$ (7)], we observed a progressive decrease in membrane resistance, possibly due to nonspecific interactions of LPA with the oocyte membrane.

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LPA does not induce oocyte maturation

Because LPA induces mitosis in fibroblast lines,² we tested its capability to induce germinal vesicle breakdown (GVBD), the precursor to mitosis in oocytes. Twenty folliculated cells were incubated overnight in 10^{-4} M LPA and were observed under a microscope the next day for the presence of a white spot at the animal pole, an indicator of GVBD. Control oocytes were incubated with progesterone (5 μ M), which is the physiological inducer of GVBD. The experiment was performed in two sets of oocytes from different frogs. In both cases, 50-60% of the cells incubated in progesterone showed GVBD, whereas none of the LPA-incubated cells showed this response.

DISCUSSION

We report that application of LPA to defolliculated *X. laevis* oocytes induces a Ca^{2+} -activated Cl^- current. This response is specific for LPA, is dose dependent, and is sensitive to PT. These results suggest that the *X. laevis* oocyte has a specific receptor for LPA that is linked to a PT-sensitive G protein. Its function is at present unknown but is not related to inducing GVBD. Because the response is present in defolliculated oocytes, this putative receptor is localized on the oocyte itself and not only on the follicle cells. The fraction of the current response blocked by PT incubation puts an upper limit on the amount of LPA effect that can be ascribed to other nonspecific interactions and is <15% in our study. During the course of our studies, we learned that others also had observed an inward current in *X. laevis* oocytes induced by LPA application.⁶

Our results have potential limitations. First, PA also induced occasional small currents. These, however, could be due to contamination with LPA, which has been shown to be present in commercial PA sources.⁷ Alternately, PA is less water soluble than LPA, so the effective concentration might have been lower. Second, it is conceivable that the absence of responses after intracellular LPA application were not a result of the inability of LPA to reach an extracellular site of action but were due to the high intracellular protein concentration that precluded the LPA from ever reaching the membrane and exerting a nonspecific effect.

Nonetheless, our findings are highly suggestive that the LPA response is mediated by an intrinsic oocyte receptor, probably coupled to a PT-sensitive G protein. This receptor would be different from the receptor described on mammalian cells in several ways. In mammalian fibroblasts, the Ca^{2+} response (but not the mitogenic response) to LPA has been shown to be PT insensitive,¹ so we postulate that either the LPA receptor is linked to a different G protein in *X. laevis* oocytes than in mammalian cells, or amphibian G proteins show a different PT sensitivity. Furthermore, Jalink et al.⁷ demonstrated that the LPA response in fibroblasts is subject to homologous, but not heterologous, desensitization, whereas we found that the oocyte LPA response does not desensitize. However, only after prolonged bath perfusion could full responses again be obtained, possibly indicating that metabolism of LPA at its site of action plays a role in the reappearance of the response. This difference in pharmacological behavior between two species makes the putative LPA receptor an interesting subject for structure-function relationship studies.

The LPA response in *X. laevis* oocytes shows marked variability, and we were unable to establish that the dose dependency of the response falls along a sigmoid curve. We tried multiple approaches using many cells, obtained from several frogs, but obtained similar results in each

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case. The variability could be explained by the many amplification and modulation steps that take place between activation of a receptor and Cl^- moving across the cell membrane. More likely, however, is the possibility that the delivery of LPA to its site of action is variable, as a result of its binding affinity for the plasma membrane, micelle formation, and binding to the BSA vehicle. Also, rapid metabolism of LPA might decrease the effective concentration of the drug at its site of action. The concentration at the receptor might therefore be different from the applied concentration and vary from measurement to measurement.

Our findings are of importance for investigators involved in expressing putative membrane receptors in *X. laevis* oocytes. Membrane receptors can activate several phospholipases, not only C but also D and A2,^{4,5,10,13} and it is therefore conceivable that activation of an expressed receptor could generate LPA in sufficient quantities to activate the oocyte LPA receptor, generating a Ca^{2+} transient. In that case, an observed current would not be a direct effect of the expressed receptor.

In summary, we report the presence of a novel endogenous response in *X. laevis* oocytes, a Ca^{2+} -activated Cl^- current induced by LPA. The response appears mediated by a specific membrane receptor linked to a PT-sensitive G protein.

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CHAPTER 6

**Responses to sphingosine-1-phosphate in *X. laevis* oocytes:
Similarities with lysophosphatidic acid signaling**

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Responses to sphingosine-1-phosphate in *X. laevis* oocytes: Similarities with lysophosphatidic acid signaling

ABSTRACT

Sphingosine-1-phosphate (S1P, 50 μ M) induces inward currents in *Xenopus laevis* oocytes voltage clamped at -70 mV. The currents are Ca^{2+} -activated Cl^- currents, as shown by a reversal potential of -20 mV and absence of the response after intracellular injection of ethylene glycol-bis(β -aminoethyl ether) -N,N,N',N'-tetraacetic acid (EGTA; 10 mM). The response is nearly indistinguishable from that to the related compound lysophosphatidic acid (LPA), and complete cross-desensitization occurs between LPA and S1P responses. Both the LPA and S1P responses are inhibited by suramin (2 mM) and dithiothreitol (5 mM). These responses appear mediated by a specific membrane receptor, since intracellular injection of S1P (5 μ M) does not induce currents, and sphingosine and the related compounds sphingosylphosphorylcholine and N,N-dimethylsphingosine, all at 100 μ M, neither induce currents nor block the response to S1P. HEK-293 and COS-1 cells respond with intracellular Ca^{2+} release to both 50 μ M S1P and 10 μ M LPA; K-562 cells do not. No cross-desensitization was noted in the responsive cells. Our findings indicate that S1P and LPA might act through the same mechanism, probably a membrane receptor.

Key words: G protein-coupled receptors; suramin; dithiothreitol; HEK-293; K-562; COS-1

INTRODUCTION

Over the past years several classes of molecules have been shown, unexpectedly, to act as intercellular messengers. Naturally occurring cellular metabolites, because of their ubiquity, were only reluctantly considered as intercellular mediators. However, the demonstration that adenosine,²¹ glutamate,²² and ATP¹⁷ signal through specific G protein-coupled receptors (GCR) has proven this view wrong. There were similar doubts whether highly lipophilic fatty acid derivatives could act through specific membrane receptors. Many investigators favored a direct action on the plasma membrane to explain their effects. Again, however, examples of such compounds acting on receptors or enzymes have now been documented: the prostaglandins,¹² platelet-activating factor,¹⁸ the cannabinoids,¹⁵ and diacylglycerol.¹¹

Lysophosphatidic acid (1-acyl-*sn*-glycerol-3-phosphate, LPA; see Figure 1A) is a phospholipid with a range of cellular actions, including Ca^{2+} accumulation, platelet aggregation, and cytoskeletal effects.^{2,19,24,25} It is mitogenic for fibroblasts¹³ but inhibits tumor cell growth.⁶ LPA elicits Ca^{2+} -activated Cl^- currents in *Xenopus* oocytes^{5,7,8} and is probably the factor inducing oscillatory currents in these cells on exposure to serum.²³ Being a natural, albeit minor, membrane constituent and an intermediate in phospholipid biosynthesis, the concept of LPA as a signaling molecule met resistance on both issues mentioned when it was postulated to mediate its varied effects through a specific receptor. However, there is now compelling biochemical evidence that LPA acts through a specific membrane receptor coupled to several G proteins.^{1,5,24}

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Recently, there has been increased interest in another phospholipid, sphingosine-1-phosphate (S1P; see Figure 1A) that also induces intracellular Ca^{2+} transients,⁹ has mitogenic effects in certain cells,²⁷ but inhibits growth of tumor lines.²⁰ Many researchers expect S1P to have an intracellular site of action, and the possibility of a membrane receptor for this compound has not received much attention. Prompted by the resemblance in structure and effects between LPA and S1P, we studied the relationship between responses to these compounds. In this report, we show the presence, in *Xenopus laevis* oocytes and several mammalian cell lines, of an endogenous S1P response that shows many similarities with LPA signaling. The S1P response, like the LPA response, might be mediated through a membrane receptor.

MATERIALS AND METHODS

Materials

S1P was prepared using the method of van Veldhoven et al.²⁶ Briefly, sphingosylphosphorylcholine (Matreya, Pleasant Gap, PA) was treated with phospholipase D (EC 3. 1. 4. 4) from *Streptomyces chromofuscus* (Sigma, St. Louis, MO), and the product was isolated by selective precipitation in water, acetone, and methanol-chloroform. Purity of the compound was verified by thin-layer chromatography. When developed in *n*-butanol-acetic acid-water (6:2:2 vol/vol) and sprayed with ninhydrin, a single spot at the expected retardation factor (R_f 0.45) was obtained. In addition, proton nuclear magnetic resonance of the product confirmed the absence of a choline group (data not shown). The compound was dissolved by sonication in 1% fatty acid-free bovine serum albumin (BSA; ICN Biochemicals, Cleveland, OH) in water to a concentration of ~5 mM (assuming a yield of 70%, as reported for this method). Sphingosine and N,N-dimethylsphingosine were obtained from Matreya and lysophosphatidic acid from Avanti Polar Lipids (Alabaster, AL), and they were dissolved in 1% BSA. Suramin was obtained from FBA Pharmaceuticals (West Haven, CT). Pertussis toxin was a kind gift of Dr. E. Hewlett at the University of Virginia and obtained from List Biological Laboratories (Campbell, CA). All other chemicals were from Sigma.

Oocyte culture and testing

Our technique for *X laevis* oocyte harvesting and culture has been described previously.⁵ Before each experiment, oocytes were defolliculated manually. A single oocyte was placed in a recording chamber filled with 3 ml of a solution containing (in mM) 150 NaCl, 5 KCl, 1 MgCl_2 , 2 CaCl_2 , 10 dextrose, and 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.4). Microelectrodes were pulled in one stage from 1.5-mm capillary glass (BBL with fiber; World Precision Instruments, Sarasota, FL) on a micropipette puller (model 700C; David Kopf Instruments, Tujunga, CA), and the tips were broken to a diameter of ~10 μm . They were filled with 3 M KCl, and tip resistances were usually 1-3 M Ω . The cell was voltage clamped using a two-microelectrode voltage clamp amplifier (oocyte clamp OC725A; Warner Corporation, New Haven, CT), connected to a data acquisition system (DAS-8; Keithly Metrabyte, Taunton, MA) running on an IBM-compatible personal computer. Membrane current was sampled at 125 Hz and recorded for 5 s before and 55 s after application of the test compounds. Compounds were delivered in 30- μl aliquots over 1-2 s using a hand-held micropipettor positioned ~5 mm from

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the oocyte. Responses were quantified by integrating the current trace by quadrature and are reported as microcoulombs (means $\pm$ SE), since this better reflects Cl^- flux than peak current does.^{5,8} All experiments were performed at room temperature. For intracellular injections a third micropipette was used, connected to an automated nitrogen-driven microinjector (PLI-100; Medical Systems, Greenvale, NY). The micropipette was calibrated volumetrically by counting the number of injections needed to expel a known volume of solution. It was then introduced into the cell, and a 50-nl volume was administered. The adequacy of injection was verified by observing the slight increase in cell size on injection.

Cell culture and testing

HEK-293 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in 1:1 Dulbecco's modified Eagle's medium (DMEM) and F-12 medium containing 10% fetal bovine serum (FBS). COS-1 cells were obtained from Derk Bergsma, SmithKline Beecham Pharmaceuticals (Swedeland, PA), and grown in DMEM containing 5% FBS. K-562 cells were a gift from Dr. Craig L. Slingsluff at the University of Virginia and were grown in RPMI medium containing 10% FBS. All were maintained at 37°C in an atmosphere of 5% CO_2 . Changes in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in cultured cell lines were measured using the fluorescent Ca^{2+} indicator indo-1. Confluent cell layers were removed from the dishes with 6 ml of a 0.01% trypsin-0.02% EDTA solution, immediately transferred to HEPES-Krebs-Ringer bicarbonate solution (HKRB; in mM: 20 HEPES, 103 NaCl, 4.77 KCl, 0.5 CaCl_2 , 1.2 MgSO_4 , 1.2 KH_2PO_4 , 25 NaHCO_3 , and 15 glucose, as well as 0.25% BSA, pH 7.40), and centrifuged for 4 min at 200 g. Cells were resuspended in HKRB and incubated for 40 min to 1 h with 4 μM of the acetoxymethyl ester of indo-1 in 100% dimethyl sulfoxide at 37°C. Cells were centrifuged, resuspended to a density of 1.5×10^6 cells/ml HKRB, and held at 37°C before use. Fluorescence was measured in a temperature-controlled fluorometer (SLM 8000C; SLM Instruments, Urbana, IL) at an excitation wavelength of 332 nm (slit width 2 nm) and emission wavelengths of 400 and 485 nm (slit widths 16 nm). $[\text{Ca}^{2+}]_i$ was calculated from the fluorescence intensity using the method of Lückhoff.¹⁴ Maximum and minimum fluorescence ratios were calculated by the sequential addition of 75 μM digitonin and 5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), respectively.

RESULTS

*S1P induces $I_{\text{Cl}(\text{Ca})}$ in *X. laevis* oocytes*

S1P (50 μM) induced transient inward currents when applied to oocytes at a holding potential of -70 mV (Figure 1B). Average charge movement was $-3.9 \pm 0.64 \mu\text{C}$, with some variability between oocytes from different frogs (Figure 2A). The responses developed after a latency of at least 5 s and occasionally up to 20 s and consisted of a fast inward component, followed by a recovery over several seconds with superimposed fluctuations. Because this shape is typical for Ca^{2+} -activated Cl^- currents in oocytes, we performed experiments to demonstrate the involvement of Ca^{2+} and Cl^- . Intracellular injection of the Ca^{2+} chelator EGTA (50 nl of 100 mM, intracellular concentration ~ 10 mM) inhibited the current completely (5 oocytes from 1 animal; Figure 1C), suggesting that the response is mediated by a change in $[\text{Ca}^{2+}]_i$. This is in

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Figure 1. S1P responses in oocytes. *A:* Molecular structures of sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA), indicating their structural similarity. *B:* Large inward current induced by 50 μM S1P in defolliculated *X. laevis* oocyte voltage clamped at -70 mV. Peak current of S1P response is 1,700 nA; total charge movement is -12 μC. Bovine serum albumin vehicle did not induce responses. *C:* Intracellular injection of 50 nl 100 mM EGTA (intracellular concentration ~10 mM) inhibits responses to 50 μM S1P in defolliculated oocyte at holding potential of -70 mV. This indicates that free intracellular Ca^{2+} is involved in response. *D:* Reversal potential determination of S1P-induced responses in *X. laevis* oocytes. Plot shows charge movement as function of holding potential in solution containing 158 mM Cl^- . Error bars indicate SEM. Reversal potential is -20 mV, indicating that the current is carried mainly by Cl^- .

accordance with previous reports of S1P causing intracellular Ca^{2+} release (9). The measured reversal potential of -20 mV (Figure 1D) indicates that the current is carried mainly by Cl^- , presumably through Ca^{2+} -activated Cl^- channels known to exist in the oocyte membrane (3). During the first minutes after a S1P response, no further currents could be induced by S1P. However, if the recording chamber was washed with copious amounts of S1P-free solution (150 ml over 30 min) multiple responses could be obtained from a single oocyte (3 oocytes from 1 animal; data not shown), indicating that tachyphylaxis did not occur.

Figure 2. S1P response is specific. *A*: Responses to 50 μM S1P in defolliculated oocytes voltage clamped at -70 mV, with (+) and without (-) prior application of sphingosine (SPH), sphingosylphosphorylcholine (SPC), or *N,N*-dimethylsphingosine (DMS). S1P response is not affected by these analogues. Note variation in S1P response among batches of oocytes. *B*: Responses to S1P or LPA in defolliculated oocytes voltage clamped at -70 mV, with (+) or without (-) prior application of LPA or S1P, respectively. There is crossdesensitization between compounds. *C*: S1P (50 nl, 50 μM) injected into defolliculated oocyte at holding potential of -70 mV does not induce inward response, but extracellular application to the same oocyte afterward induces typical Ca^{2+} -activated Cl^- current. This suggests extracellular site of action for S1P.

Related sphingolipids do not induce responses

We tested the S1P analogues sphingosine (11 oocytes from 3 animals), sphingosylphosphorylcholine (lysosphingomyelin; 5 oocytes from 2 animals) and *N,N*-dimethylsphingosine (6 oocytes from 2 animals) for agonist and antagonist activity. When applied at 100 μM , none of these compounds induced currents in oocytes nor did they block the response to subsequent administration of 50 μM S1P (Figure 2A). This lack of response to structurally similar compounds makes nonspecific membrane effects of S1P unlikely.

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Figure 3. S1P responses in mammalian cells. *A:* Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) measurement by indo-1 fluorescence in HEK-293 cells. Repeated application of 50 μM S1P (S) desensitizes response but does not inhibit subsequent responses to 10 μM LPA (L). ATP (10 μM ; A) is used to confirm intact signaling mechanism. *B:* No changes in $[\text{Ca}^{2+}]_i$ are seen when 50 μM S1P or 10 μM LPA are applied to K562 cells. ATP (10 μM) application confirms that $[\text{Ca}^{2+}]_i$ signaling pathway is intact in these cells.

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Responses to S1P and LPA cross-desensitize

We found that a response to the related compound LPA (Figure 1A) consistently inhibited responses to S1P applied subsequently (Figure 2B). Conversely, S1P blocked responses to LPA (Figure 2B). Responses to agonists of other GCRs (muscarinic acetylcholine M_1 and angiotensin II receptors) expressed in oocytes were not inhibited by prior application of LPA or S1P (data not shown). Because the cross-desensitization suggested that LPA and S1P act through a similar pathway, we studied the concordance between the properties of these compounds more closely.

S1P responses appear mediated through a membrane receptor

In view of the similarities between LPA and S1P responses, we investigated whether the polyanionic compound suramin, which blocks the putative LPA receptor,^{2,5} also inhibited oocyte responsiveness to S1P. At a suramin concentration of 2 mM, the response to S1P was decreased by 89%, from 1.58 ± 0.39 to 0.18 ± 0.13 μC (25 oocytes from 4 animals), similar to its effect on the LPA response.^{2,5} Dithiothreitol (DTT), a disulfide-reducing agent, has been shown to reduce ligand-receptor interaction on several GCR, including the angiotensin II receptor,¹⁰ presumably by disrupting disulfide bonds in the receptor. When oocytes were incubated for 15 min in 5 mM DTT, responses to both S1P (11 oocytes from 2 frogs) and LPA (4 oocytes from 1 frog) were eliminated completely. As a control experiment, we expressed the M_1 muscarinic receptor in oocytes and found that the Ca^{2+} -activated Cl^- currents induced by 1 μM methylcholine was not sensitive to DTT incubation. These data suggest that a membrane receptor with at least one extracellular disulfide bond might be part of the S1P and LPA signaling pathway. Previously, we reported consistent sensitivity of the oocyte LPA response to pertussis toxin (PT; 2 mg/ml overnight).⁵ In our present experiments, we found highly variable responses to PT between batches of oocytes, with responses to 10 μM LPA consistently inhibited (>50% of control) in only 5 of 15 batches of oocytes. S1P responses were inhibited also in these groups but not in the others. Thus there was complete consistency between LPA and S1P PT sensitivity. To determine further whether S1P acts on an extracellular or intracellular site, we tested the ability of injected S1P to induce Ca^{2+} -activated Cl^- currents. A volume of 50 nl S1P injected into the oocyte did not induce a current, whereas S1P applied extracellularly to the same oocyte resulted in a charge movement of -1.68 ± 0.86 μC (8 oocytes from 2 animals; Figure 2C). Similar results have been obtained with LPA.^{5,8} This finding indicates that S1P acts on an extracellular site, rather than on an intracellular target or through nonspecific membrane interactions, and again underscores the similarities between S1P and LPA responses.

*The S1P response in mammalian cell lines is different from that in *X. laevis* oocytes*

To study the concordance between S1P and LPA signaling further, we tested several immortalized cell lines for responsiveness to S1P and LPA. COS-1 (5 batches) and HEK-293 (10 batches) cells showed transient intracellular Ca^{2+} accumulation on stimulation with 50 μM S1P or 10 μM LPA (Figure 3A). When we tested the ability of S1P to block responses to subsequent LPA, no inhibition was noted, even after the S1P response was desensitized by several applications of S1P (Figure 3A). Similarly, treatment with LPA did not inhibit S1P responses. Overnight incubation in 5 mM DTT did, but 2 mg/ml PT did not, reduce responses to LPA or S1P.

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K-562 cells (7 batches) consistently showed no $[Ca^{2+}]_i$ changes in response to either S1P or LPA (Figure 3B) but were responsive to ATP and thrombin. In preliminary experiments, HeLa, C₆ glioma, and bovine aortic endothelial cells have been responsive to both LPA and S1P, whereas Jurkat T and EL-4 cells were unresponsive. Therefore we have found complete concordance between responses to LPA and S1P.

DISCUSSION

We report that application of the phospholipid S1P to *Xenopus* oocytes results in a Ca^{2+} -activated Cl^- current. The S1P response appears mediated through a specific pathway, possibly involving a membrane receptor, and is nearly indistinguishable from the LPA response. In several mammalian cell lines, S1P also induces intracellular Ca^{2+} accumulation, but through a mechanism that shows several differences with that in oocytes. Our hypothesis that these findings can be explained by LPA and S1P acting through a similar or even identical pathway is difficult to prove conclusively in the absence of binding studies. Nonetheless, our data are consistent with the hypothesis and suggest that a specific membrane receptor might be part of the signaling mechanism. As the lysophospholipid literature bears out, most research groups have focused exclusively on either the glycerol-based or the sphingosine-based family, and the functional and structural similarities between S1P and LPA appear to have gone unnoticed. Our findings suggest that at least some reports of LPA and S1P effects might be addressing the same phenomena.

One potential difficulty with these studies is that the binding to BSA and insolubility of LPA and S1P affect the amount of drug available for interaction with the signaling system, and the concentration applied is probably different from that present at the site of action. There is therefore no inconsistency between the similar magnitude of the responses to LPA and S1P and the differences in concentrations used. Dose-response studies in oocytes with LPA have been unsuccessful,^{5,7} probably due to solubility problems, and therefore we have not attempted establishing a dose-response relationship with the even less soluble S1P.

We were surprised to find significant variability of the oocyte LPA response to PT, whereas in a previous study⁵ we found consistent inhibition. At present, we have no explanation for this result; one possible reason could be seasonal variation in the availability of NAD in the oocyte. The important result, however, is the concordance in sensitivity between S1P and LPA.

The only difference we found between LPA- and S1P-induced currents was a several second longer delay after application of S1P. We cannot determine from our observations whether this longer delay is due to metabolism of S1P to another compound or simply a result of the extreme insolubility of S1P and different dissociation kinetics from the BSA vehicle. It has been shown that, in mammalian cells, S1P induces the formation of phosphatidic acid,⁴ but the time course is at least an order of magnitude too slow to account for the S1P responses we observed.

It is not possible to conclude from the oocyte studies that S1P and LPA act through a membrane receptor, but the cross-desensitization, sensitivity to suramin and DTT, and absence of response after intracellular injection are consistent with that hypothesis. Unfortunately, definitive confirmation of the existence of a S1P receptor and of the identity of the putative LPA and S1P receptors is difficult to obtain. Competition binding studies are virtually impossible due to the heavy membrane partitioning of these lipophilic compounds, resulting in unacceptable

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levels of nonspecific binding. Also, there is a lack, at present, of specific antagonists for either LPA or S1P. Molecular cloning of the LPA/S1P receptor(s) would be very helpful to settle this issue.

If there is a single LPA/S1P receptor in the *Xenopus* oocyte, the question arises whether LPA or S1P is the natural ligand for this receptor. This again is not easy to study. It is conceivable, although to our knowledge no precedent exists, that two separate pathways, one resulting in the formation of LPA, the other of S1P, might act through a single receptor as a final common pathway. In mammalian cells, the lack of cross-desensitization is suggestive of the existence of separate LPA and S1P receptors. Again, molecular cloning of the receptors would be helpful in explaining these findings.

In summary, we have demonstrated that S1P induces Ca^{2+} -activated Cl^- currents in *Xenopus* oocytes. This novel endogenous oocyte response shows a high degree of similarity to, and cross-desensitization with, the response to LPA. In mammalian cells cross-desensitization was not seen, but in the cell types tested to date, there is complete concordance between the Ca^{2+} transients induced by S1P and those elicited by LPA. Our findings indicate that S1P and LPA act through similar, or even identical, pathways, probably through specific membrane receptors.

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CHAPTER 7

**OoClamp: an IBM-compatible software system for
electrophysiologic receptor studies in *Xenopus* oocytes**

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OoClamp: an IBM-compatible software system for electrophysiologic receptor studies in *Xenopus* oocytes

ABSTRACT

A software system for IBM-compatible microcomputers running MS-DOS or Microsoft Windows 3.1 is described which facilitates the acquisition, analysis and storage of data from electrophysiologic studies of receptors expressed in *Xenopus laevis* oocytes. The system is designed to provide standardization of test conditions, automation of all routine functions, rapid, on-line analysis of data, and self-documentation and compact storage of data files. All system settings are optimized for use with the *Xenopus* expression system, but can be adapted to other large cells. An example application, expression of muscarinic acetylcholine receptors in *Xenopus* oocytes, is described.

Key words: Electrophysiology; *Xenopus laevis*; Oocytes; Voltage clamp; Receptors

INTRODUCTION

The oocytes of the African clawed toad *Xenopus laevis* play an increasingly important role in the study of many membrane ion channels and receptors. Foreign messenger RNA, when injected into these unusually large cells, is translated into protein and inserted into the cell membrane after the appropriate post-translational modifications. The cells can then be studied easily with electrophysiologic or other methods. One particularly helpful feature of the *Xenopus* oocyte is the presence of Ca^{2+} -activated Cl^- channels in the membrane.¹ These channels pass a Cl^- current $I_{\text{Cl}(\text{Ca})}$ proportional to the Ca^{2+} concentration inside the cell.

As many receptors, when stimulated, induce increases in intracellular Ca^{2+} , the function of these receptors can be studied by electrophysiologically measuring $I_{\text{Cl}(\text{Ca})}$.² The same assay system also offers a useful approach for cloning the genetic material for such receptors.³

This novel concept, electrophysiologic study of membrane receptors, offers significant challenges to existing electrophysiologic hardware and software, as most systems are designed primarily for the study of ion channels in small cells. *Xenopus* oocytes are several orders of magnitude larger than most cell types, resulting in very high membrane capacitance and necessitating voltage clamp amplifiers with the ability to pass large currents. Several of such 'oocyte amplifiers' are commercially available, but the software needs have not been addressed. Specifically, two problem areas exist. First, $I_{\text{Cl}(\text{Ca})}$ resulting from receptor stimulation has a much longer time scale (seconds to minutes) than current through most voltage or ligand-gated ion channels (milliseconds to seconds), and available software is not designed to handle these long acquisitions suitably. For example, some electrophysiologic data acquisition programs do not display captured data during acquisition, nor is it possible to abort trials. For experiments on a millisecond time scale this offers no difficulties, but for trials of minutes in duration these are serious disadvantages: a separate oscilloscope is necessary to monitor progress of the experiment, and trials that will yield no useful data (e.g. when clamp oscillation occurs on test compound addi-

tion) cannot be interrupted. Scale labeling in some packages demonstrates how the software was developed for small cells and short time courses: a typical *Xenopus* experiment might last an unwieldy '120 000 ms' (rather than 2 min), and yield a current of '5000 nA' (rather than 5 μ A). A second problem relates to the need to perform experiments on many oocytes in the most efficient manner possible. Particularly during molecular cloning many hundreds of oocytes need to be tested rapidly, and software optimized for standardization of test conditions, automation of routine functions, rapid analysis, and self-documentation and compact storage of data files will ease the effort considerably. Again, present systems are not designed for such experiments. For example, $I_{Cl(Ca)}$ is analyzed most appropriately by integration of the current trace,^{4,5} and most programs do not allow this integration on-line during acquisition.

OoClamp was designed to provide, in combination with an appropriate voltage clamp amplifier and acquisition hardware, an optimized system for acquisition, analysis and storage of $I_{Cl(Ca)}$ in *Xenopus* oocytes. Many of the program's features make it useful for the study of other slow currents in large cells. To illustrate the functionality of the program, it was used for acquisition and analysis of $I_{Cl(Ca)}$ induced by acetyl- β -methylcholine in *Xenopus* oocytes expressing the M_1 muscarinic acetylcholine receptor. Another application has been published elsewhere.⁶

PROGRAM DESCRIPTION

The OoClamp system consists of three programs: (i) OoClamp, which performs all data acquisition and analysis functions and requires the presence of acquisition hardware, (ii) OoCalc, a stand-alone version of the OoClamp analysis module, used to analyze OoClamp data on computers without acquisition hardware, and (iii) OoConv, a utility to convert the compact OoClamp data files to a format that can be imported easily into graphics and spreadsheet programs.

OoClamp runs in 640 x 480 color graphics mode, with a 500 x 350 pixel screen area reserved for data display. To maximize speed in using the program, all features were designed to be accessed by single-keystroke choices from menus. The program runs in one of three modes: oscilloscope, acquisition, or analysis.

Oscilloscope mode

This mode shows a real-time trace of the voltage amplifier current output. Under most conditions, this obviates the need for a separate oscilloscope during impalement and stabilization of the oocyte. From this mode, a number of program parameters, optimized for receptor studies in oocytes, can be adjusted, including trial and sweep time (10 s to 2 min) and upper and lower screen limits (-5 μ A to +5 μ A). Tick marks can be set for ease of screen reading. An 'auto save' feature controls automatic saving of data files after acquisition and after integration. All these settings are retained when the system is shut down. Acquisition and analysis mode are entered from oscilloscope mode.

Acquisition mode

Once this mode is entered, the program samples data for the predetermined period, plotting the acquired data in real time. As standardized application of test compounds to the oocyte is desirable, a bell can be set to sound at a preset time into acquisition. Also, a real time clock indi-

tion) cannot be interrupted. Scale labeling in some packages demonstrates how the software was developed for small cells and short time courses: a typical *Xenopus* experiment might last an unwieldy '120 000 ms' (rather than 2 min), and yield a current of '5000 nA' (rather than 5 μ A). A second problem relates to the need to perform experiments on many oocytes in the most efficient manner possible. Particularly during molecular cloning many hundreds of oocytes need to be tested rapidly, and software optimized for standardization of test conditions, automation of routine functions, rapid analysis, and self-documentation and compact storage of data files will ease the effort considerably. Again, present systems are not designed for such experiments. For example, $I_{Cl(Ca)}$ is analyzed most appropriately by integration of the current trace,^{4,5} and most programs do not allow this integration on-line during acquisition.

OoClamp was designed to provide, in combination with an appropriate voltage clamp amplifier and acquisition hardware, an optimized system for acquisition, analysis and storage of $I_{Cl(Ca)}$ in *Xenopus* oocytes. Many of the program's features make it useful for the study of other slow currents in large cells. To illustrate the functionality of the program, it was used for acquisition and analysis of $I_{Cl(Ca)}$ induced by acetyl- β -methylcholine in *Xenopus* oocytes expressing the M_1 muscarinic acetylcholine receptor. Another application has been published elsewhere.⁶

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Acquisition mode

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ates time elapsed since the beginning of the trial, a feature useful for standardization of multiple interventions during a single acquisition. If desired, the acquisition can be aborted at any moment. For maximum efficiency, the system automatically switches to analysis mode at the end of the acquisition, and, if 'auto save' has been activated in oscilloscope mode, stores the acquired data at this point.

OoClamp samples 12-bit data at a fixed rate of 125 Hz. This rate is sufficient both to prevent aliasing of 60 or 50 Hz noise, the primary noise source for oocyte voltage clamp systems, and to sample adequately the relatively slow changes in current resulting from receptor activation. Sampling is in background (interrupt driven), and is therefore not interrupted or delayed by CPU activity, a feature particularly important when OoClamp is run under Microsoft Windows. A fixed number of 500 data points is used to record each trial, and OoClamp determines the number of A/D conversions averaged to calculate each point as

$$n = \text{int}\left(\frac{125 * t}{500}\right)$$

where t is the total acquisition time. Therefore, for a 10-s trial each data point will be the average of 2 conversions; for a 120-s trial each point will be the average of 30 conversions.

Analysis mode

This mode can be entered manually from oscilloscope mode, or automatically after completion of a trial. The primary use of analysis mode is for rapid integration of the current trace. A segment of interest can be highlighted using the cursor keys, and a horizontal cursor is positioned to indicate base line (see Figure 1). With a single keystroke the area delimited is then integrated, using the quadrature method.⁷ If 'auto save' has been activated in oscilloscope mode, the system will also store the data automatically on disk, together with the integration result and the positions of the highlighted segment and base line cursor. If necessary, Y-axis scaling and tick settings can be retroactively adjusted to ensure that traces fit on the display, and for ease of reading. The horizontal cursor can also be used to measure current directly, as a digital readout of its position is available (see Figure 1).

From analysis mode files can be saved, and saved files can be loaded for analysis. To maximize speed in using the system, OoClamp generates its own data file names, based on date and a sequential numbering for experiments on each day, resulting in intuitive file names. For example, 'OC060393.14' contains data of the 14th trial run on June 3, 1993. This file naming system is not affected by exiting and restarting the system. Similarly, save and load paths, which can be specified separately, are retained when the system is shut down.

Several features are included to increase speed when reviewing large numbers of data files. The date of acquisition only needs to be entered once. Further references to files acquired on that day are simply by number. Also, single-keystroke commands are available to load the next or previous file written on the specified day.

OoClamp data files are designed to use minimal disk space, and to be self-documenting. To minimize the size of data files, only the 500 averaged data points are stored. The time scale is reconstructed during loading from the trial duration, which is stored in the file header. Additional information stored (see Table 1) allows the system to reconstruct completely the screen settings in use as the data were saved.

Table 1
File header information stored with OoClamp data files

Name	Use
Max Y value	To reconstruct Y-axis scale
Min Y value	To reconstruct Y-axis scale
X tick setting	To reconstruct X-axis scale
Y tick setting	To reconstruct Y-axis scale
Acquisition time	To reconstruct X-axis scale
Horizontal cursor position	To reconstruct position during integration
Left delimiter	To reconstruct delimited trace segment
Right delimiter	To reconstruct delimited trace segment
Integration result	To display

OoConv

Due to the compact data storage and the presence of a header with additional information (see Table 1), importing OoClamp data files into other programs is not straightforward. For this reason, a utility program, OoConv, is included. OoConv, using the header information, expands OoCalc data files to ASCII files that can be read easily by graphics and spreadsheet systems.

Hardware and software specifications

OoClamp is designed to work with a Keithley-Metrabyte DAS-8 data acquisition and control board.⁸ No additional software is necessary, as all drivers are included in the OoClamp system. All OoClamp data acquisition functions are performed using standard DAS-8 function calls. A VGA graphics board with either a color or monochrome monitor are also required. The system needs approximately 70 k conventional memory, and will work well in an environment with 128 k left available. OoClamp is written in Microsoft QuickBasic, version 4.5, and has been tested under MS-DOS 5.0 and as a non-Windows application under Microsoft Windows 3.1.

AVAILABILITY

Further information concerning the distribution of OoClamp can be requested from the author.

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CHAPTER 8

Trypsin induces Ca^{2+} -activated Cl^- currents in *X. laevis* oocytes

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Trypsin induces Ca^{2+} -activated Cl^- currents in *X. laevis* oocytes

ABSTRACT

The protease trypsin induces Ca^{2+} -activated Cl^- currents when applied in concentrations as low as 0.1 mg/ml to defolliculated, voltage clamped *X. laevis* oocytes. The response is dose-dependent and specific, as other proteases (chymotrypsin, Lys-C and Arg-C), or trypsin pretreated with soybean trypsin inhibitor, did not induce currents. Intracellular trypsin injection did not induce responses. The current does not appear to result from proteolytic activation of the endogenous receptor for lysophosphatidic acid, the only known Ca^{2+} -mobilizing receptor consistently present in oocytes. These results suggest the presence on the oocyte membrane of a specific receptor for trypsin.

Key words: Oocyte; *Xenopus laevis*; Trypsin; Voltage clamp; Membrane receptor; Lysophosphatidic acid

INTRODUCTION

The oocytes of the African clawed toad *Xenopus laevis* are a tool used commonly for the expression and cloning of membrane receptors of the G protein-coupled receptor superfamily.¹ One of several advantages of this system is the relative absence of endogenous Ca^{2+} release-activating receptors. Although other agonist-induced responses have been described, particularly to muscarinic agonists,^{2,3} the only responses present consistently in native oocytes are to the phospholipids lysophosphatidic acid (LPA)⁴⁻⁷ and sphingosine-1-phosphate (SIP)⁸, which may well signal through the same receptor. To prevent confounding results when expressing receptors in oocytes, it is important for investigators to be aware of the presence of any endogenous signaling systems in these cells. Here we describe the presence of a novel endogenous oocyte response, a Ca^{2+} -activated Cl^- current induced by the protease trypsin. This response, which is dose-dependent and specific, appears to result from activation of a membrane receptor system.

MATERIALS AND METHODS

Materials

LPA (1-oleyl-2-hydroxy-*sn*-glycero-3-phosphate) was obtained from Avanti Polar Lipids (Alabaster, AL) and was dissolved in a 1% solution of bovine serum albumin (BSA; ICN Biochemicals, Cleveland, OH). The endoproteases Arg-C and Lys-C were obtained from Boehringer-Mannheim (Indianapolis, IN). All other chemicals were obtained from Sigma (St. Louis, MO).

Oocyte culture and testing

Our technique for *Xenopus laevis* oocyte harvesting and culture has been described previously.⁴ Prior to each experiment, oocytes were defolliculated manually. A single oocyte was

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placed in a recording chamber filled with 3 ml of a solution containing (mM): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, dextrose 10, HEPES 10 (pH 7.4). Microelectrodes were pulled in one stage from 1.5 mm capillary glass (BBL with fiber, World Precision Instruments, Sarasota, FL) on a micropipette puller (model 700C, David Kopf Instruments, Tujunga, CA), and the tips were broken to a diameter of approximately 10 μm . They were filled with 3 M KCl, and tip resistances were usually 1-3 M Ω . The cell was voltage clamped (at -70 mV, unless otherwise noted) using a two-microelectrode voltage clamp amplifier (Oocyte Clamp OC725A, Warner Corp., New Haven, CT), connected to an IBM-compatible personal computer with data acquisition hardware (DAS-8, Keithly Metrabyte, Taunton, MA) and software (OoClamp⁹). Membrane current was sampled at 125 Hz, and recorded for 5 s before, and 55 s after, application of the test compounds. Compounds were delivered in 30 μl aliquots over 1-2 s using a hand-held micropipettor positioned approximately 5 mm from the oocyte. Responses were quantified by integrating the current trace by quadrature and are reported as μC (mean $\pm$ S.E.M.). All experiments were performed at room temperature and, unless otherwise noted, at least 5 oocytes were used for each datapoint. For intracellular injections a third micropipette was used, connected to an automated, nitrogen-driven microinjector (PLI-100, Medical Systems Corp., Greenvale, NY). The micropipette was calibrated volumetrically by counting the number of injections needed to expel a known volume of solution. It was then introduced into the cell, and a 50 nl volume was administered. The adequacy of injection was verified by observing the slight increase in cell size on injection.

RESULTS

Application of trypsin to defolliculated oocytes voltage clamped at a holding potential of -70 mV induced transient inward currents (see Figure 1A). The responses developed after a latency of several seconds, and consisted of a fast inward component, followed by a fluctuating return to baseline over a period of 5-10 s. Typically, 1 mg/ml trypsin induced charge transfers of -2 μC , but responses could be elicited with concentrations as low as 0.1 mg/ml. The response was dose-dependent within the dose range tested (see Figure 1B). Vehicle alone (BSA 1 %) did not induce responses. Even if after trypsin application the oocyte was washed for 30 min with trypsin-free solution, no subsequent trypsin responses could be elicited. Therefore, the trypsin response desensitizes completely.

In shape and kinetics these responses are very similar to Ca^{2+} -activated Cl^- currents ($I_{Cl(Ca)}$) induced by activation of endogenous or expressed G protein-coupled receptors in *Xenopus* oocytes.⁵ For this reason we tested the hypothesis that the responses induced by trypsin are $I_{Cl(Ca)}$. Intracellular injection of the Ca^{2+} chelator EGTA (50 nl of a 100 mM solution, resulting in an approximate intracellular concentration of 5 mM) completely abolished the response to subsequent application of trypsin (Figure 2A). Therefore, an increase in intracellular Ca^{2+} level appears to mediate the response. To establish the nature of the ionic current, we elicited responses to trypsin at various holding potentials, and determined the reversal potential of the current (Figure 2B). The reversal potential of -15 mV at an extracellular Cl^- concentration of 158 mM indicates that the current is carried mainly by Cl^- ions.

Figure 1. Trypsin-induced currents in *Xenopus* oocytes. *A:* When applied (at arrow) to a defolliculated oocyte, voltage clamped at -70 mV, trypsin (2 mg/ml) induces an inward current. Charge transfer is -9.4 μC . *B:* Dose-dependency of the oocyte response to trypsin.

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Figure 2: The trypsin response in *Xenopus* oocytes is a $I_{Cl(Ca)}$. *A: Left:* control oocyte, injected with 50 nl water, and subsequently exposed (at arrow) to trypsin (1 mg/ml). Charge transfer is $-14.3 \mu C$. *Right:* oocyte, injected with 50 nl EGTA (100 mM), and subsequently exposed (at arrow) to trypsin (1 mg/ml). Except for application artifact, no response is seen, indicating an increase in intracellular Ca^{2+} concentration mediates the response. Time between injections and applications is approximately 1 minute. *B:* Plot of charge transfer versus holding potential. Each point contains data of at least 5 oocytes. A trypsin concentration of 1 mg/ml was used. The reversal potential at -15 mV indicates the current is carried mainly by Cl^- ions.

Next, we studied the mechanism of activation of $I_{Cl(Ca)}$ by trypsin. To ensure that the responses were due to trypsin, and not to a contaminant, we treated the trypsin solution (1.6 mg/ml) with soybean trypsin inhibitor before application, and found that oocyte responses were virtually eliminated ($-0.4 \pm 0.2 \mu C$). Trypsin inhibitor alone did not induce responses (data not shown). To determine whether the action of the protease was intracellular or extracellular, we injected trypsin (50 nl, 10 mg/ml) into oocytes voltage clamped at -70 mV. No responses were observed, yet viability of the oocytes could be confirmed by responses to 1 mg/ml trypsin applied subsequently to the outside of the same oocytes, either after 1 minute ($-9.0 \pm 2.1 \mu C$; see Figure 3A) or after 5 min ($-7.5 \pm 1.6 \mu C$; $n = 4$). These responses were similar to those obtained in oocytes injected with 50 nl water ($-11.2 \pm 2.7 \mu C$; $n = 3$). Therefore, an extracellular site of action appears likely. To ascertain that the current did not result from nonspecific proteolysis of membrane constituents, we tested several other proteases for their ability to induce currents. Application of chymotrypsin at a concentration of 1 mg/ml resulted in small application artifacts only. Similarly, the proteases Arg-C and Lys-C, which cleave a subset of the trypsin cleavage sites, did not induce currents when applied at these concentrations. In addition, responses to trypsin were not reduced by exclusion of Ca^{2+} from, and addition of, 10 mM EGTA to the bath solution, eliminating trypsin-induced Ca^{2+} leakage into the oocyte as an explanation of the $I_{Cl(Ca)}$. Finally, we did not note increases in holding potential after responses to trypsin, indicating the absence of a non-specific increase in membrane leakiness. Thus, the action of trypsin is specific. Another mechanism explaining trypsin-induced $I_{Cl(Ca)}$, would be proteolytic activation of an endogenous G protein-coupled receptor in the oocyte membrane. The only receptor consistently present in native oocytes is that for LPA.^{4,7} When activated by LPA this receptor does not desensitize.^{4,5} However, if trypsin activated the LPA receptor by proteolysis one would expect subsequent unresponsiveness to LPA. Therefore, we applied trypsin (1 mg/ml) to defolliculated oocytes, washed the oocytes in trypsin-free solution for 30 min, and then applied LPA (10^{-5} mM). Responses of $-8.7 \pm 2.4 \mu C$ were obtained, indicating that proteolytic digestion of the LPA receptor had not occurred (Figure 3B).

DISCUSSION

We report the presence of an endogenous response to trypsin in defolliculated *X. laevis* oocytes. The response is a $I_{Cl(Ca)}$, appears to have an extracellular site of action, and does not seem to involve activation of the endogenous G protein-coupled receptor for LPA. Knowledge of this response is relevant for researchers using oocytes for expression of Ca^{2+} -mobilizing receptors.

Based on the data presented, it is not possible to determine the signaling mechanism that links the extracellular action of trypsin to the release of intracellular Ca^{2+} . The specificity of the effect makes general destruction of membrane proteins an unlikely explanation. The presence of response in the absence of extracellular Ca^{2+} eliminates the possibility of trypsin-induced Ca^{2+} leak. The LPA receptor, the only G protein-coupled receptor known to be present consistently in oocytes, does not appear proteolytically activated. A direct action on the Cl_{Ca} channel cannot be ruled out, but appears unlikely, as: (1) the time delay and response kinetics mimic closely those seen during receptor activation, and (2) a (presumably permanent) change in the channel would be unlikely to result in the transient currents observed. Therefore, the most likely explanation for the trypsin responses appears to be the presence of a specific trypsin receptor in these cells.

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Figure 3. The trypsin response in *Xenopus* oocytes is mediated through an extracellular system different from the LPA receptor. *A:* Trypsin (10 mg/ml, 50 nl) injected into the oocyte ('in') does not elicit responses whereas trypsin (1 mg/ml) applied subsequently to the outside of the same oocyte ('on') results in a charge transfer of $-12.5 \mu\text{C}$. Therefore, the site of action is probably extracellular. *B: Left:* LPA (10^{-5}M) applied to a control oocyte elicits a charge transfer of $-11.1 \mu\text{C}$. *Right:* after treatment of oocyte with trypsin (1 mg/ml) and 30 min wash in trypsin-free solution, LPA (10^{-5}M) still elicits a current (charge transfer $-9.2 \mu\text{C}$). Therefore, trypsin has not degraded the LPA receptor, making trypsin signaling through this system unlikely.

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Based on the response morphology, this receptor might belong to the G protein-coupled receptor superfamily. In analogy to the thrombin receptor, another G protein-coupled receptor activated by a protease, this putative trypsin receptor might well be activated through a tethered ligand mechanism.^{10,11} This would explain the complete desensitization of the trypsin response. However, the trypsin doses needed to induce currents are higher than those activating other G protein-coupled receptors, and an alternative explanation of the responses would be trypsin binding, with low affinity, to a receptor in the oocyte membrane for another ligand, as yet unreported. Final determination of the mechanism of action will need additional investigation.

Unresolved at this time is the function of the endogenous trypsin response in oocytes. As $I_{Cl(Ca)}$ in these cells is presumably a fertilization signal,¹² the responses to trypsin, LPA or sphingosine-1-phosphate might be involved in signaling that sperm penetration has occurred. Interestingly, in several studies lysophospholipids and trypsin have been shown to have similar effects on components of the reproductive system. Both compounds are able to induce the acrosome reaction and capacitate bovine spermatozoa,¹⁴ allowing penetration of the zone pellucida of oocytes. Also, a cyclic nucleotide phosphodiesterase has been isolated from *Xenopus* oocytes that can be activated by trypsin or lysophospholipids, as well as by Ca^{2+} -calmodulin.¹⁴ Several studies have demonstrated the presence of trypsin-related proteases in the ejaculate of mice¹⁵ and humans.¹⁶ The functional relevance of these findings, as well as the relationship, if any, to our finding of an endogenous oocyte $I_{Cl(Ca)}$ in response to lysophospholipids and trypsin, remains to be evaluated.

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CHAPTER 9

**Interactions between propofol and lipid mediator receptors:
Inhibition of lysophosphatidate signaling**

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Interactions between propofol and lipid mediator receptors: Inhibition of lysophosphatidate signaling

ABSTRACT

As a highly lipophilic drug, propofol may interact with lipophilic domains in addition to its likely primary site of action on the γ -aminobutyrate_A (GABA_A) receptor. Likely candidates for such interaction are the G protein-coupled membrane receptors for lipid intercellular mediators. The phospholipid lysophosphatidate (LP) has attracted attention as such a signaling molecule. It has a variety of biological actions, including vasoconstriction. We therefore studied the interaction between propofol and the LP receptor. Intracellular Ca^{2+} release in response to LP was assessed by measuring Cl^- flux through Ca^{2+} -activated Cl^- channels in *Xenopus* oocytes. The average charge movement in response to $\text{LP } 10^{-7} \text{ M}$ was 2.0 ± 0.2 microCoulombs. Propofol in Intralipid® (0.01%) dose-dependently inhibited LP signaling (50% inhibitory concentration [IC_{50}] 5.38 μM). Propofol 28 μM inhibited LP signaling by 81%. Intralipid® (0.01%) was without effect. To ascertain that intracellular signaling pathways and the Ca^{2+} -activated Cl^- channel were not affected by propofol, we tested the effects of propofol (5.6 μM) on currents induced by methylcholine (10^{-7} M) in oocytes expressing the ml muscarinic acetylcholine receptor. No inhibition was observed. As both receptors share the same intracellular signaling pathway, we conclude that clinically relevant concentrations of propofol most likely inhibit the LP receptor or its G protein. Inhibition of LP signaling may explain some of propofol's vasodilating actions.

Key words: General anesthetics: propofol; *Xenopus laevis*; Receptors: lysophosphatidate, muscarinic acetylcholine

INTRODUCTION

Propofol (2,6-diisopropylphenol) has rapidly established itself as one of the most commonly used intravenous anesthetics. Like the benzodiazepines, the barbiturates, and etomidate, its likely primary site of action appears to be the γ -aminobutyrate_A (GABA_A) receptor-channel complex.¹⁻³ However, propofol is significantly more lipophilic than these other GABAergic anesthetics. Therefore, it might be expected to interact with additional sites in the central nervous system and the periphery; such interactions might explain some of the compound's specific secondary effects, such as peripheral and coronary vasodilation.⁴

Of particular interest as sites of propofol action are the receptors for lipophilic mediators. Whereas until recently it was considered unlikely that hydrophobic molecules could act as intercellular mediators, several classes of such lipid-derived compounds have now been well described. The prostaglandins and leukotrienes are prominent examples, and a novel group of phosphate-containing compounds, including lysophosphatidate^{5,6} and sphingosine-1-phosphate,⁷ has recently attracted much attention. An intracellular analog of such lipid signaling compounds is diacylglycerol, the second messenger that activates protein kinase C.

Lysophosphatidate (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate; LP) signaling has been described in considerable detail over the past years.^{5,6,8} The compound has a variety of actions in

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various preparations: it contracts smooth muscle, aggregates platelets, is mitogenic in fibroblasts, and induces morphologic changes in neurons and other cells. In many cell types it releases intracellular Ca^{2+} and decreases cyclic adenosine monophosphate levels. These effects are mediated through a G protein-coupled membrane receptor. The compound is a simple phospholipid (Figure 1A) and is quite hydrophobic. Since the binding site on the LP receptor is presumably hydrophobic as well, we hypothesized that propofol might interact with this LP receptor. Such interaction could explain some of propofol's vasodilating properties.

We therefore studied the effects of clinical concentrations of propofol on LP signaling. As a model system we used *Xenopus laevis* oocytes. These cells have an endogenous, well described LP signaling system linked to intracellular Ca^{2+} release, which is quantified conveniently by measuring current through Ca^{2+} -activated Cl^- channels ($I_{\text{Cl}(\text{Ca})}$, Figure 1B).^{7,9} In addition, the size of the cells makes them very accessible and easily studied. To localize the site of action of propofol, we compared its effect on LP signaling with that on recombinantly expressed ml muscarinic acetylcholine receptors, which signal through the same intracellular pathway. Specifically, we attempted to answer the following questions: 1) Does propofol, in clinically relevant concentrations, affect signaling through LP receptors? 2) If so, in which segment of the signaling pathway is this effect localized? 3) Does the propofol vehicle, Intralipid®, have a role in this effect?

METHODS

The study protocol was approved by the Animal Research Committee at the University of Virginia.

Oocyte harvesting

Female *X. laevis* toads were obtained from Xenopus I (Ann Arbor, MI), housed in an established frog colony, and fed regular frog brittle twice weekly. For removal of oocytes, a frog was immersed in 7.65 mM solution of tricaine until unresponsive to a painful stimulus (toe pinching). A 1-cm long incision was made in a lower abdominal quadrant and a lobule of ovarian tissue, containing approximately 200 oocytes, was removed and placed in modified Barth's solution (containing in mM: NaCl 88, KCl 1, NaHCO_3 2.4, CaCl_2 0.41, MgSO_4 0.82, $\text{Ca}_2(\text{NO}_3)_2$ 0.3, gentamicin 0.1, HEPES 15; pH adjusted to 7.6). The wound was closed in two layers, and the animal was allowed to recover in a separate tank overnight.

The ovarian tissue was washed immediately with modified Barth's solution and dissected into small clusters of 20-50 oocytes. Mature oocytes (Dumont Stage V and VI) were isolated and the follicular layer was removed manually from each oocyte using microforceps.

Receptor expression

LP receptors are expressed endogenously in *Xenopus* oocytes. The rat ml muscarinic acetylcholine receptor was expressed recombinantly. Its complementary DNA (cDNA) consists of a 2.8-kilobasepair fragment in a commercial vector (pGEM1; Promega, Madison, WI). The construct was linearized by digestion with the nuclease *Hind* III, and complementary RNA (cRNA) was transcribed *in vitro* using the bacteriophage RNA polymerase T7. A capping ana-

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$\log(^m\text{GpppG})$ was included in the reaction to generate capped transcripts, as these are translated more efficiently in the oocyte. The resulting mRNA was quantified by spectroscopy and diluted in water to a concentration of 0.1 mg/mL. Oocytes were each injected with 3.5–5 ng of ml muscarinic receptor mRNA using an automated microinjector (Nanoject; Drummond Scientific, Broomall, PA). Adequacy of injection was confirmed by noting the slight increase in cell size during injection. The cells were then cultured in modified Barth's solution at 18°C for 3 days before study.

Electrophysiology

A single defolliculated oocyte was placed in a perfusable recording chamber (3 mL volume) filled with Tyrode's solution (containing in mM: NaCl 150, KCl 5, MgCl_2 1, CaCl_2 2, dextrose 10, HEPES 10; pH adjusted to 7.4). Microelectrodes were pulled in one stage from capillary glass (BBL with fiber; World Precision Instruments, Sarasota, FL) on a micropipette puller (model 700C; David Kopf Instruments, Tujunga, CA). Tips were broken to a diameter of approximately 10 μm , providing a resistance of 1–3 M Ω . Electrodes were filled with 3 M KCl.

The cell was voltage clamped using a two microelectrode oocyte voltage clamp amplifier (OC725C; Warner Corporation, New Haven, CT) connected to a data acquisition and analysis system running on an IBM-compatible personal computer. The acquisition system consisted of a DAS-8 A/D conversion board (Keithley-Metrabyte, Taunton, MA) and analysis was performed with OoClamp software.¹⁰ Holding potential was -70 mV. Cells that did not show a stable holding current of less than 1 μA during a 1-min equilibration period (less than 5% of cells tested) were excluded from analysis. Membrane current was sampled at 125 Hz and recorded for 5 s before and 55 s after application of the test compounds.

Drug application

LP was dissolved in 1% bovine serum albumin (fatty acid free). To ensure that the hydrophobic LP was in solution, it was vortexed vigorously and sonicated (Benson 250 Sonifier, Danbury, CT) at 5% output in two 5-s intervals each day before use.

Propofol was added to the bath as a 30- μL volume of 100-fold concentrated stock in 1% Intralipid[®] (an intravenous fat emulsion) in Tyrode's solution (final Intralipid[®] concentration 0.01% for all propofol concentrations), and 1 min was allowed for equilibration. The agonist (methylcholine 10^{-7} M for ml muscarinic-injected oocytes; LP 10^{-7} M for uninjected oocytes) was then delivered in a 30- μL aliquot over 1–2 s using a micropipettor positioned approximately 5 mm from the oocyte.

Intralipid[®] was added to the bath as a 30- μL volume of 100-fold concentrated 1% stock (final concentration 0.01%), and 1 min was allowed for equilibration. LP was then delivered to the oocyte in the same manner as in the propofol experiments. All experiments were performed at room temperature.

Data analysis

$I_{\text{Cl}(\text{Ca})}$ was used as a measure of agonist-induced Ca^{2+} -release. Responses were quantified by integrating the current trace by quadrature and are reported as charge movements in units of microCoulombs, as this reflects Ca^{2+} release better than peak current does.⁹

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Responses are reported as mean $\pm$ SEM. As variability between batches of oocytes is common, responses were normalized to controls from the same batch to allow inclusion of data from multiple frogs into the same comparison. Oocytes from at least two frogs were used for each treatment. Differences between treatment groups were analyzed using Student's unpaired t-test. $P < 0.05$ was considered significant.

Materials

The rat ml muscarinic acetylcholine receptor cDNA was obtained from Dr. T. I. Bonner (National Institute of Mental Health, Bethesda, MD). LP was obtained from Avanti Polar Lipids (Alabaster, AL). Bovine serum albumin was obtained from ICN Biomedicals (Cleveland, OH). Propofol was obtained from Zeneca Pharmaceuticals (Wilmington, DE). Intralipid[®] was obtained from KabiVitrum Inc. (Alameda, CA). Molecular biology reagents were obtained from Promega (Madison, WI). All other chemicals were obtained from Sigma (St. Louis, MO).

RESULTS

LP signaling

Uninjected oocytes responded to lysophosphatidate LP 10^{-7} M with a transient inward current (Figure 1C). The current developed after a latency of ≥ 1 s and consisted of a fast inward component followed by a relaxation over several seconds, on which small fluctuations were superimposed. This is the typical response pattern to LP in these cells.⁹ Average charge movement in response to LP 10^{-7} M was 2.0 ± 0.2 μ C ($n = 65$). We have previously shown that this response to LP is specific and represents a Cl⁻ flux through Ca²⁺-activated Cl⁻ channels.⁹

Effects of propofol on LP signaling

We then tested the ability of propofol to interfere with LP signaling. Propofol in clinically relevant concentrations (2.8–28 μ M) did not elicit responses in uninjected oocytes (data not shown), but depressed LP-induced currents in a dose-dependent manner. Sample traces showing typical responses to LP 10^{-7} M in the presence of various concentrations of propofol are shown in Figure 2A, whereas Figure 2B represents the concentration-response relationship. Curve fitting to the Hill equation yielded a 50% inhibitory concentration (IC₅₀) of 5.38 μ M and a slope of 5.74. Propofol in a concentration of 28 μ M depressed the LP response by 81%. This incomplete inhibition by large propofol concentrations suggests that the effect of propofol on LP signaling may not be exclusively competitive antagonism.

To exclude effects on LP signaling of the Intralipid[®] solvent, its ability to interfere with LP signaling was also tested. Intralipid[®] (0.01%) did not elicit responses in uninjected oocytes (data not shown), nor did it inhibit responses to LP. The average LP response in the presence of Intralipid[®] was 2.1 ± 0.4 μ C, similar to the control response (1.8 ± 0.5 μ C) (Figure 2C).

Figure 1. Lysophosphatidate (LP) structure and signaling in *Xenopus* oocytes. *A:* Structure of LP. The circled "P" represents a phosphate group. The striped rectangle represents an acyl chain (18:1 in the present study). *B:* Signaling pathway linking stimulation of the endogenous LP receptor in *Xenopus* oocytes to Cl⁻ channel opening. The double line represents the cell membrane. The agonist LP binds to its receptor and the resulting signal is transduced via the coupled G protein (G) to phospholipase C (PLC). PLC produces the second messenger inositoltriphosphate (IP₃) from phosphatidylinositolbiphosphate (PIP₂). Calcium (Ca²⁺) released from intracellular stores by IP₃ then activates calcium-dependent chloride channels in the cell membrane. The resulting Cl⁻ flux (*I*_{Cl(Ca)}) is a measure of intracellular Ca²⁺ release. *C:* Example of inward Cl⁻ current induced by 10⁻⁷ M LP in a defolliculated oocyte. Holding potential was -70 mV. Charge movement was 3.6 μC.

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Figure 2. Propofol inhibits lysophosphatidate (LP) signaling in oocytes. All oocytes are voltage clamped at a holding potential of -70 mV. *A*: Examples of $I_{\text{Cl(Ca)}}$ induced by LP alone ($3.1 \mu\text{C}$) and in the presence of 5.6 and $28 \mu\text{M}$ propofol (1.7 and $0 \mu\text{C}$, respectively). *B*: Propofol has a dose-dependent inhibitory effect on $I_{\text{Cl(Ca)}}$ induced by 10^{-7} M LP in uninjected oocytes. The number of oocytes used for each data point is indicated. The 50% inhibitory concentration is $5.38 \mu\text{M}$; maximum depression is 81%. *C*: Responses to 10^{-7} M LP in control oocytes, and those exposed to 0.01% Intralipid®. Intralipid® does not affect LP signaling.

Figure 3. Propofol (5.6 μ M) does not inhibit m1 muscarinic signaling in oocytes. All oocytes are voltage clamped at a holding potential of -70 mV. *A*: Methylcholine (MCh; 10^{-7} M) applied to defolliculated oocytes 3 days after injection with 3.5 ng ml receptor mRNA, one incubated for 1 min in 5.6 μ M propofol, and one control oocyte from the same frog (each response is 3.2 μ C). *B*: Responses to 10^{-7} M MCh in control oocytes and those exposed to 5.6 μ M propofol. n = number oocytes in each group.

Effects of propofol on ml signaling

To ascertain that the effect of propofol on LP signaling was not due to interference with intracellular pathways or the Cl^- channel, we expressed the ml muscarinic acetylcholine receptor in *Xenopus* oocytes. Like the LP receptor, the ml receptor is a member of the G protein-coupled receptor superfamily. When expressed in oocytes it also induces $I_{\text{Cl}(\text{Ca})}$ through a mechanism that, apart from receptor and possibly G protein, is considered to be the same as that for LP signaling. Lack of propofol inhibition of muscarinic signaling would therefore make an effect on the signaling pathway downstream of the LP receptor unlikely.

Methylcholine 10^{-7} M, a nonhydrolyzable agonist at the ml muscarinic acetylcholine receptor, was without effect on uninjected oocytes (data not shown), but induced $I_{\text{Cl}(\text{Ca})}$ in oocytes expressing the ml receptor. These currents had a somewhat shorter latency and larger peaks than did LP-induced currents, but were otherwise similar (Figure 3A). Average charge movement was $5.8 \pm 0.7 \mu\text{C}$ ($n = 52$). Presence of $5.6 \mu\text{M}$ propofol (close to the IC_{50} for LP inhibition, $5.4 \mu\text{M}$) did not interfere with muscarinic signaling (Figure 3B). Therefore, inhibition of LP signaling by this concentration of propofol is unlikely to be explained by effects on intracellular pathways or the Ca^{2+} -activated Cl^- channel of the oocyte.

DISCUSSION

The main finding of our study is that propofol, in clinical concentrations, profoundly inhibits LP-induced $I_{\text{Cl}(\text{Ca})}$ in *Xenopus* oocytes. The vehicle Intralipid® was without effect. Propofol concentrations that depressed LP signaling by 50% were without effect on $I_{\text{Cl}(\text{Ca})}$ induced by methylcholine in oocytes expressing the ml muscarinic receptor.

This latter finding suggests strongly that propofol's site of action on LP signaling is the LP receptor and not a site downstream in the signaling pathway. In the *Xenopus* oocyte only a single isoform of phospholipase C couples receptor activation to Ca^{2+} release.¹¹ Therefore, both LP and ml receptors couple to this isoform, and the signaling pathway downstream from this point (including the intracellular Ca^{2+} release mechanism and the Ca^{2+} -activated Cl^- channel) will be the same for both receptors. The comparable kinetics of the responses supports this (the slightly slower responses to LP presumably being due to its poor water solubility and its binding to albumin). As $5.6 \mu\text{M}$ propofol was without effect on ml signaling, it is unlikely that its inhibitory action on LP signaling takes place downstream of the LP receptor or the associated G protein. One issue that deserves further study is the interaction between propofol and LP bound to the physiologic carrier protein, albumin.

Several potential limitations of our model system should be considered. First, we performed our experiments at room temperature, and interactions between propofol and LP receptors might be different in homeothermic animals at higher temperatures. In addition, lack of propofol effect on muscarinic signaling might in theory be due to disturbed functioning of the (rat) receptor clone at low temperatures. However, this is unlikely as we have shown previously that halothane¹² and ketamine¹³ inhibit muscarinic signaling in this model in a manner comparable with that found in other systems. Thus, although it cannot be ruled out that temperature might affect protein functioning to some degree, we thought it more important to maintain the cell membrane in its normal state of fluidity. A second potential issue with our choice of model is that the *Xenopus* LP receptor might be of a different type, potentially with a different response to

propofol, than its mammalian homolog. As the LP receptor has not yet been cloned, no molecular biological evidence exists for multiple receptor subtypes. Similarly, no published pharmacological or functional studies suggest the existence of different receptor subtypes. Indeed, LP signaling in oocytes has been shown to be quite comparable to that in other cell types. Therefore, the flexibility of the oocyte model far outweighs the remote possibility of a different propofol effect in another model. Thirdly, endogenous muscarinic receptors have been reported in *Xenopus* oocytes.¹⁴ However, we have not observed these in this or our previous studies;^{9,12,13} apparently many frogs do not express such receptors.

Although it is well established that propofol's primary site of action is on the GABA_A receptor/Cl⁻ channel,¹⁻³ this does not rule out other interactions. In fact, several other activities of the compound have been described. Propofol affects another ligand-gated ion channel, the nicotinic acetylcholine receptor. In a concentration of 15 μ M it decreased channel open time without affecting single channel conductance.¹⁵ Effects on voltage-activated channels have also been reported. For example, propofol at concentrations between 30 and 150 μ M reduced sodium channel fractional open time and interfered with voltage-dependent steady state activation.¹⁶ Similarly, K⁺ currents in PC12 cells were inhibited by propofol (50 μ M to 1 mM),¹⁷ and 300 μ M propofol inhibited Ca²⁺ currents in chick sensory neurons.¹⁸ However, the compound also has effects on second messenger-mediated systems. In concentrations ranging from 0.17 to 280 μ M it induced release of intracellular Ca²⁺ in embryonic rat brain cells (but propofol did not induce I_{Cl(Ca)} in our model) and reorganization of the actin cytoskeleton in human astrocytes.¹⁹ It also activated protein kinase C with an 50% effective concentration of 240 μ M.²⁰ The latter finding is of particular interest in the current context, as protein kinase C is physiologically activated by a lipid agonist, diacylglycerol.

One issue of importance in interpreting such studies is the concentration of propofol used. It is quite difficult to determine the relevant concentration at the site of action for a drug as lipophilic as propofol, that in addition is very highly protein-bound (>95%). The minimally effective propofol plasma concentration for general anesthesia is considered to be around 1 μ g/mL, or approximately 5 μ M.²¹ Free concentrations would of course be considerably smaller, but on the other hand enrichment might take place at the (lipophilic) site of action. We think that studies demonstrating effects of propofol at concentrations larger than 50 μ M should be interpreted cautiously. The IC₅₀ observed in the present study, 5.4 μ M, is certainly within the clinically relevant range.

Although outside the scope of the study, it is interesting to speculate whether inhibition of LP signaling might explain some of propofol's side effects. LP has a wide variety of actions in several systems. Best described are its mitogenic,^{22,23} platelet aggregating,²⁴ and smooth muscle contractile effects.²⁵ In addition, it induces actin stress fiber rearrangement and resulting cellular shape changes.²⁶ Its physiologic or pathophysiologic role is unknown, although a function in the vasculature seems likely. Supporting this hypothesis are findings that activated platelets form the primary source of LP,²⁷ that LP is a vasoconstrictor,²⁸ and that the compound induces mitosis in vascular smooth muscle cells.²³ If LP does play a role in the maintenance of vascular tone, inhibition of LP signaling might explain some of the vasodilatory actions of propofol. However, this hypothesis will require additional study.

In summary, we have demonstrated that clinically relevant concentrations of propofol significantly inhibit LP signaling, an effect most likely taking place at the LP receptor or its associated G protein. Our findings imply that, at the concentrations used during general anes-

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sia, propofol has more than a single site of action. Therefore, study of interactions between propofol and other lipid mediator receptors (such as those for the prostaglandins) are indicated.

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CHAPTER 10

**Differential inhibition of lysophosphatidate signaling
by volatile anesthetics**

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Differential Inhibition of Lysophosphatidate Signaling by Volatile Anesthetics

ABSTRACT

Background: Volatile anesthetics have been found to interfere with the functioning of several G protein-coupled receptors, effects that may be relevant to the mechanism of anesthetic action. Lysophosphatidate (1-acyl-2-*sn*-glycero-3-phosphate; LP) is the simplest natural phospholipid. It has pronounced biological effects and signals through a specific G protein-coupled receptor. Because of its lipophilicity, the LP receptor is a feasible site of anesthetic interaction. Therefore, the authors investigated the effects of halothane and isoflurane on LP signaling using *Xenopus* oocytes.

Methods: Mature oocytes were harvested from *Xenopus* frogs, isolated, and defolliculated manually. Lysophosphatidate receptors are endogenously present in these cells. Angiotensin receptors were expressed recombinantly to study anesthetic effects on intracellular signaling. Oocytes were studied individually with a two-electrode voltage clamp at room temperature. Integrated Ca^{2+} -activated Cl^- currents ($\text{I}_{\text{Cl}(\text{Ca})}$) were used to evaluate the effects of anesthetics on changes in intracellular Ca^{2+} concentration in response to receptor agonists (10^{-7} M LP or 10^{-7} M angiotensin II) or intracellular inositoltrisphosphate (IP_3) injection.

Results: Halothane depressed LP signaling in a concentration-dependent manner, with half-maximal inhibition at 0.23 mM and virtually complete inhibition at 0.34 mM. Responses could be recovered after an anesthetic-free wash. Oocyte injection with heparin, an IP_3 receptor antagonist, completely blocked LP and angiotensin signaling, indicating similar IP_3 -dependent pathways. However, $\text{I}_{\text{Cl}(\text{Ca})}$ induced by angiotensin receptor activation or intracellular IP_3 injection were not inhibited by halothane. Isoflurane, at comparable concentrations, did not depress LP responses in oocytes significantly.

Conclusions: Lipid mediator signaling can be affected profoundly by volatile anesthetics. At clinically relevant concentrations, halothane and isoflurane have different effects on LP signaling. The inhibitory effects of halothane on the LP signaling pathway occur before the IP_3 receptor.

Key words: Anesthetics, volatile: halothane; isoflurane. Receptors: angiotensin; G protein-coupled; lysophosphatidate. *Xenopus laevis* oocytes.

INTRODUCTION

Volatile anesthetics interact with various cellular systems, although the relative importance of these interactions to clinical anesthesia is largely unknown. Much recent research has focused on anesthetic-protein interactions. Effects on N-methyl-D-aspartate,¹⁻³ and gamma amino-butyric acid receptor^{2,4,5} signaling seem to offer the most promise for explaining the mechanism of general anesthesia. However, interactions with other systems may also be important, either in modulatory roles or by inducing anesthetic side effects.

Although anesthetic interactions with ion channel function have been studied in some detail, effects on second messenger-linked receptors have been investigated to a limited extent only. Nonetheless, studies have shown pronounced inhibition of, for example, muscarinic acetyl-

choline⁶⁻⁹ and serotonin⁹⁻¹¹ receptor functioning, making the interactions between anesthetics and G protein-coupled receptors a worthwhile field of investigation. Of specific relevance are receptors for lipid mediators, because a hydrophobic ligand-binding domain might be a likely site of anesthetic action. The field of lipid mediators has attracted much attention recently and includes important messengers such as the prostaglandins and platelet-activating factor.

Lysophosphatidate (1-acyl-2-hydroxy-*sn*-glycero-3-phosphate; LP) is a novel lipid mediator,^{12,13} originally known only as a precursor in *de novo* lipid synthesis.¹⁴ However, LP also has several pronounced biological effects, including the induction of smooth muscle contraction,¹⁵⁻¹⁷ cellular proliferation,¹⁸⁻²⁰ and platelet aggregation.²¹⁻²³ Many of these effects may be due to LP's pronounced ability to induce actin stress fiber reorganization, and they are mediated through a specific G protein-coupled receptor for the compound.²⁴ Although some volatile anesthetics exhibit effects opposite to those of LP signaling in various cell types, suggesting an interaction, and although the lipophilicity of LP makes its receptor a feasible site for such interaction, the direct anesthetic effects on the LP signaling system have not been studied.

We hypothesized that LP signaling can be inhibited by volatile anesthetics. To test this hypothesis, we studied the effects of halothane and isoflurane on LP signaling in *Xenopus laevis* oocytes. *Xenopus* oocytes form a good model to study the compound because these cells express a well-described endogenous LP signaling system²⁵⁻²⁸ coupled to intracellular Ca^{2+} release (Figure 1A). In addition, the presence of Ca^{2+} -activated Cl^- channels allows convenient measurement of this Ca^{2+} release by electrophysiologic means.²⁶⁻²⁹ In this study, we tried to answer the following questions:

1. Do volatile anesthetics, at clinically relevant concentrations, affect LP signaling in oocytes?
2. If so, are there differences in effect between halothane and isoflurane, two anesthetics used frequently in clinical practice?
3. Can these effects be localized to a specific segment of the signaling pathway?

MATERIALS AND METHODS

Animals

The study protocol was approved by the Animal Research Committee at the University of Virginia. Female *Xenopus laevis* frogs were obtained from Xenopus I (Ann Arbor, MI), housed in an established frog colony, and fed regular frog brittle twice per week. To remove the oocytes, each frog was anesthetized by immersion in 0.2% methanesulfonate salt solution until unresponsive to a painful stimulus (toe pinching). A 1-cm-long incision was made in a lower abdominal quadrant and a lobule of ovarian tissue, containing approximately 200 oocytes, was removed and placed in modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 0.41 mM CaCl_2 , 0.82 mM MgSO_4 , 0.3 mM Ca_2NO_3 , 15 mM HEPES, and 0.1 mg/ml gentamicin, pH adjusted to 7.4). The wound was closed in two layers and the frog was allowed to recover from anesthesia, kept in a separate tank overnight, and returned to the colony the next day.

The ovarian tissue was immediately and copiously washed in modified Barth's solution and dissected into small clusters of 20 to 50 oocytes. Mature oocytes (Dumont stages V and VI) were isolated, defolliculated manually with microforceps, and maintained in modified Barth's solution.

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Materials

Lysophosphatidate (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate) stock was obtained from Avanti Polar Lipids (Alabaster, AL) and diluted in a 1% solution of fatty acid-free bovine serum albumin (BSA; ICN Pharmaceuticals, Costa Mesa, CA) to appropriate concentrations. Halothane and isoflurane were obtained from Halocarbon Laboratories (River Edge, NJ) and Ohmeda PPD (Liberty Corner, NJ), respectively. All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Electrophysiologic recording

A single defolliculated oocyte was placed in a perfusable recording chamber (3-ml volume) filled with Tyrode's solution (150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.2 mM CaCl₂, 10 mM dextrose, 10 mM HEPES, pH adjusted to 7.4). Microelectrodes were pulled in one stage from capillary glass (BBL with fiber; World Precision Instruments, Sarasota, FL) on a micropipette puller (model 700C; David Kopf Instruments, Tujunga, CA). Electrode tips were broken to a diameter of approximately 10 μ m, providing a resistance of 1 to 3 M Ω , and filled with 3 M KCl. The cell was voltage clamped using a two-microelectrode oocyte voltage-clamp amplifier (OC725A; Warner Corp., New Haven, CT) connected to a data acquisition and analysis system running on an IBM-compatible personal computer. The acquisition system consisted of a DAS-8 A/D conversion board (Keithley-Metabyte, Taunton, MA), and analysis was performed with OoClamp software.³⁰ Holding potential was -70 mV, and only cells exhibiting stable holding currents less than 1 μ A during a 1-min equilibration period were included in analysis. Membrane current was sampled at 125 Hz and recorded for 5 s before and 55 s after application of the appropriate receptor agonist. Agonists were delivered in 30- μ l aliquots for a period of 1 to 2 s using a hand-held micropipettor positioned approximately 5 mm from the oocyte. Unless otherwise indicated, washout did not occur during the time scale of traces presented. To prevent desensitization of the receptor or signaling pathways from influencing the results, each oocyte received only a single agonist application. Responses were quantified by integrating the current trace by quadrature and are reported in microcoulombs (μ C). All experiments were performed at room temperature.

Anesthetic administration

To determine the effects of halothane and isoflurane on LP-induced currents, output from the appropriate vaporizer was bubbled through a reservoir filled with 30 ml Tyrode's solution and allowed to equilibrate for at least 10 min. Air at a flow rate of 500 ml/min was used as the carrier gas. After equilibration, the solution from the reservoir was perfused through the recording chamber at a rate of approximately 10 ml/min, and measurements were obtained after ten bath volumes had been exchanged (3 min). The concentrations of the anesthetics in the recording chamber were quantified by gas chromatography: Triplicate samples from the recording chamber were equilibrated with air and analyzed in a gas chromatograph (Aerograph 940; Varian Analytical Instruments, Walnut Creek, CA) calibrated with the appropriate standard. Results were converted to concentrations in liquid using Kreb's solution/air partition coefficients at 25°C³¹ and to

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corresponding partial pressure (vol%) at that temperature.³² Each oocyte was exposed to a single concentration of anesthetic only.

Intracellular injection

To study inositoltrisphosphate (IP₃)-induced Ca²⁺-activated Cl⁻ currents (I_{Cl(Ca)}) a third microelectrode, connected to an automated microinjector (Nanoject; Drummond Scientific, Broomall, PA), was inserted into the oocyte under voltage clamp. Oocytes were injected with 50 nl IP₃ (final concentration 100 µM) and adequacy of injection was confirmed by noting a small increase in cell size. Membrane currents were recorded for 5 s before and 55 s after IP₃ injection. Microinjection pipettes were examined for obstruction before and after recording. Only experiments in which tips were fully functional before and after injection were included in the analysis.

For intracellular injection of heparin, an automated microinjector (Nanoject) was used. Oocytes were injected with 60 nl heparin (final concentration 120 ng/µl) at least 30 min before agonist application. Adequacy of injection was confirmed by observing the small increase in cell size on injection.

cRNA synthesis and injection

The rat AT_{1A} angiotensin II receptor clone was obtained from Dr. K. R. Lynch (University of Virginia, Charlottesville, VA) as a cDNA of 1.2 kilobasepair in the CDM8 vector (Invitrogen, San Diego, CA). The construct was linearized with the nuclease *Xho* I and transcribed in the presence of a capping analog by T7 polymerase.

Oocytes were injected with 50 nl cRNA (3-5 ng) in water using an automated microinjector (Nanoject). The adequacy of injection was confirmed by noting the slight increase in cell size during injection. The cells were cultured in modified Barth's solution for 3 days before study.

Data analysis

Responses are reported as means ± SEM. Differences among treatment groups were analyzed using Student's *t* test. If multiple comparisons were made, data were analyzed using analysis of variance followed by a *t* test corrected for multiple comparisons. Probability values less than 0.05 were considered significant. Concentration-response curves were fit to the following logistic function, derived from the Hill equation:

$$y = y_{\min} + (y_{\max} - y_{\min}) \left(1 - \frac{x^n}{x_{50}^n + x^n} \right)$$

where $y_{\max}$ and $y_{\min}$ are the maximum and minimum obtained response, n is the Hill coefficient, and x_{50} is the concentration at which the half-maximal response occurs.

Figure 1. Endogenous lysophosphatidate (LP) signaling in *Xenopus* oocytes. *A*: The signaling pathway linking LP receptor activation to chloride (Cl⁻) channel opening. The double line represents the cell membrane. The agonist LP binds to its receptor, which transduces the signal via the coupled G protein (G) to phospholipase C (PLC), which produces the second messenger inositoltrisphosphate (IP₃) from phosphatidylinositolbisphosphate (PIP₂). Calcium (Ca²⁺), released from IP₃-sensitive intracellular stores, then activates Ca²⁺-dependent Cl⁻ channels in the cell membrane. The resulting Cl⁻ current (I_{Cl(Ca)}) is integrated and expressed in microcoulombs (μC; charge movement). *B*: Inward Cl⁻ current induced by 10⁻⁷ μM LP (second trace) but not by 1% bovine serum albumin (first trace) in the same defolliculated oocyte. The charge movement in response to LP is 2.9 μC.

RESULTS

Lysophosphatidate induces an inward current in oocytes

Whereas application of 1% BSA had no effect on oocytes at a holding potential of -70 mV (Figure 1B), application of 10⁻⁷ M LP (approximately the median effective concentration in this model³³), diluted in 1% BSA, resulted in transient inward currents of several microamperes. The current developed after a short latency period (~5 s) and consisted of a fast inward component followed by a relaxation over several seconds with superimposed small fluctuations. We and others have shown previously that the oocyte responses to LP are due to release of Ca²⁺ from

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Figure 2. Halothane inhibits lysophosphatidate (LP) signaling in oocytes. *A*: Sample traces of LP-induced $I_{C(Ca)}$ in the absence and presence of halothane. Charge movements are 2.1 μC , 0.7 μC , and 0.2 μC , respectively. Halothane concentrations in oocyte bath are indicated above current traces. *B*: Halothane depresses LP (10^{-7} M)-induced $I_{C(Ca)}$ in a concentration-dependent manner. The half-maximal inhibitory concentration is 0.23 ± 0.02 mM (0.43%). Each oocyte was exposed to a single halothane concentration only. The number of oocytes used per treatment group is indicated above each data point. *C*: Halothane inhibition is reversible. Lysophosphatidate (10^{-7} M) induced $I_{C(Ca)}$, three different groups: in the absence of halothane (first bar), after exposure to 0.34 mM (0.64%) halothane (middle bar) and after 0.34 mM halothane exposure followed by a 20-min wash of Tyrode's solution (last bar). The number of oocytes used per group is indicated.

intracellular stores and subsequent activation of $I_{Cl(Ca)}$.^{25,26,28} The shape of the response is indistinguishable from responses induced by activation of other G protein-coupled receptors.

Halothane reversibly inhibits lysophosphatidate responses in oocytes

We tested the ability of halothane to interfere with LP signaling. Halothane at clinically relevant concentrations depressed LP-induced $I_{Cl(Ca)}$ in a concentration-dependent manner (Figures 2A and B). In the absence of halothane, average charge movement induced by 10^{-7} M LP was 1.6 ± 0.2 μ C. In the presence of 0.34 mM (0.64%) halothane, responses to LP were inhibited completely. Curve fitting using the Hill equation revealed a half-maximal inhibitory concentration of 0.23 ± 0.02 mM (0.43%) and a Hill coefficient of 6.3 ± 2.5 .

To determine if the effect of halothane was reversible, LP-induced currents were tested under three conditions: in the absence and presence of halothane and after exposure to halothane followed by a wash with anesthetic-free solution. Three separate treatment groups were used to prevent multiple agonist applications from desensitizing the LP receptor or intracellular signaling pathways. A high concentration of halothane was used because it was considered more likely to cause irreversible effects. Although LP-induced responses were depressed more than 95% in the presence of anesthetic, $I_{Cl(Ca)}$ obtained in response to LP after exposure to 0.34 mM (0.64%) halothane and a wash with anesthetic-free solution were similar in size to control responses (Figure 2C).

Halothane does not inhibit angiotensin responses in oocytes

In a previous study, we showed that 0.34 mM (0.64%) halothane does not affect responses to 10^{-7} M angiotensin II (ATII) in oocytes expressing the AT_{1A} angiotensin receptor.⁶ Apart from the receptor and possibly the G protein, LP and AT_{1A} receptors are presumed to signal through the same intracellular pathway, because they both induce Ca^{2+} release and Ca^{2+} -activated Cl^- channel activation and because the oocyte contains only a single molecular species of phospholipase C coupling receptor activation to intracellular Ca^{2+} release.³⁴ Therefore, as halothane had no inhibitory effect on angiotensin signaling, halothane's site of action is unlikely to be downstream of the LP receptor and G protein. To confirm our previous data, we expressed AT_{1A} receptors in oocytes and tested the ability of 0.34 mM (0.64%) halothane to inhibit $I_{Cl(Ca)}$ induced by 10^{-7} M ATII (as for LP, this concentration is approximately half-maximal in this model³⁵). As in our previous investigation, halothane at this concentration had no effect (Figure 3). We proceeded to confirm that LP and AT receptors signal through the same transduction pathway and that halothane has no effect on this common signaling pathway.

Lysophosphatidate and angiotensin receptors signal through inositoltrisphosphate

Both LP^{36,37} and angiotensin signaling³⁸ result in IP_3 release. To verify that these receptors indeed signal through this mechanism in our model, we injected heparin (MW = 3,000; 60 nM; final concentration, 120 ng/ μ l) into oocytes and tested their ability to respond to agonist application 30 min later. Heparin antagonizes the effect of IP_3 on its receptor and inhibits IP_3 -mediated Ca^{2+} responses in oocytes.³⁸ To ensure that the intracellular injection process did not impair the signaling ability of the cells, some oocytes were injected with 60 nl water, which

Figure 3. Halothane does not inhibit angiotensin signaling. *A*: Example traces of 10^{-7} M ATII-induced currents in oocytes expressing AT_{1A} angiotensin receptors. The arrow indicates the time of agonist application. Charge movements in the absence and presence of 0.34 mM halothane are 3.8 and 3.4 μC , respectively. *B*: Halothane does not significantly inhibit 10^{-7} M ATII-induced currents. The number of oocytes used per treatment group is indicated.

Figure 4. Lysophosphatidate (LP) and angiotensin receptors signal through inositoltrisphosphate (IP_3). *A:* Water (60 nl) preinjected into oocytes does not interfere with LP signaling (first trace) or angiotensin (AT) signaling (second trace, in oocyte injected with AT_{1A} cRNA 3 days previously). Charge movements are 4.6 μC and 2.9 μC , respectively. The arrow indicates the time of agonist application. *B:* Sample traces of oocytes preinjected with heparin (60 nl, 120 ng), an IP_3 receptor blocker, show inhibition of both LP and AT signaling. *C:* De-N-sulfated heparin (60 nl, 120 ng) preinjected into oocytes does not significantly affect LP or AT signaling. Charge movements are 2.8 μC and 1.7 μC , respectively. *D:* Heparin significantly blocks LP and AT signaling. Agonist (LP or ATII)-induced $I_{Cl(Ca)}$ in oocytes (non-RNA injected oocytes for LP, and AT_{1A} cRNA-injected oocytes for AT). + and - refer to oocytes injected and not injected with 120 ng heparin. The number of oocytes per treatment group is indicated * $P < 0.05$. ** $P < 0.01$. Pathway downstream of IP_3 generation is unaffected by the anesthetic.

was found to have no effect on LP or ATII-induced $I_{Cl(Ca)}$ (Figure 4A). However, responses to LP and ATII were blocked significantly by previous heparin injection (Figures 4B and D). In contrast, the de-N-sulfated form of heparin, which is a less potent IP_3 antagonist,³⁸ did not affect agonist-induced responses significantly (Figure 4C).

Halothane does not affect inositoltrisphosphate responses in oocytes

Because lack of effect on AT signaling suggests that halothane does not affect the signaling pathway downstream of receptor or G protein, we wanted to confirm that the anesthetic does not affect the intracellular Ca^{2+} release system or the Ca^{2+} -activated Cl^- channel. For this purpose we injected IP_3 into oocytes and determined the effect of halothane on $I_{Cl(Ca)}$ induced by the compound. Inositol trisphosphate (50 nl; final concentration, 100 μ M) induced currents similar in kinetics to those observed with LP or AT_{1A} signaling (Figure 5A). In preliminary experiments, we established that this concentration of injected IP_3 resulted in response sizes similar to those observed with 10^{-7} M LP or ATII. To ensure that the observed IP_3 -induced currents were not a result of the injection process, membrane currents were recorded after injections of 50 nl water. Intracellular injection of water did not induce any currents (first trace; Figure 5A). The average IP_3 -induced response was 4.2 ± 0.5 (n = 22). Halothane (0.34 mM; 0.64%) had no effect on these responses (Figures 5A and B; average charge movement, 4.2 ± 0.6 μ C; n = 20), indicating that the pathway downstream of IP_3 generation is unaffected by the anesthetic.

Isoflurane does not depress lysophosphatidate responses in oocytes

We also investigated the effects of isoflurane on LP-induced Cl^- currents. In contrast to halothane, isoflurane caused only modest depression of LP-induced currents in oocytes (Figure 6). The calculated half-maximal concentration was 0.22 ± 0.07 mM (0.46%), with a Hill coefficient of 3.4 ± 2.9 . Maximal inhibition was 45%, which was not statistically different from control (2.7 ± 0.3 μ C). Thus the effects of these two anesthetics on the LP response in oocytes are quite different.

DISCUSSION

In this study we show that halothane at clinically relevant concentrations completely inhibits LP, but not AT, responses in *Xenopus* oocytes. In contrast, equivalent concentrations of isoflurane induced no significant inhibition of LP signaling.

Effects and site of action of lysophosphatidate

Lysophosphatidate, the simplest natural phospholipid, has many biological effects. Examples include contraction of vascular³⁹ and other¹⁵ smooth muscle; aggregation of human and feline platelets;²¹ rapid neurite retraction in several neuronal cell lines;^{25,40,41, a} mitogenesis in

* Jalink K: Lysophosphatidate as an extracellular messenger; a study of early cellular effects and mechanism of action. Doctoral thesis, Jalink K. Leiden, the Netherlands, 1993; pp 81-7.

Figure 5. Halothane does not affect inositoltrisphosphate (IP_3) induced currents. *A*: Injected water (50 nl) does not elicit a current (first trace), but 100 μM IP_3 (50 nl, middle trace) induces $I_{Cl(Ca)}$ (charge movement is 3.2 μC). Halothane (0.34 mM) does not significantly affect IP_3 -induced currents (last trace, charge movement is 2.7 μC). *B*: Inositoltrisphosphate-induced responses in control oocytes and in those exposed to 0.34 mM halothane are similar. The number of oocytes used per data set is indicated.

fibroblasts^{18,19} through a mechanism involving p21^{ras} and the mitogen-activated protein (MAP) kinase pathway^{41,42}; and inhibition of proliferation in myeloma cells.⁴³ Many of these diverse effects presumably can be reduced to a primary action on the intracellular actin stress fiber system through an effect on the guanine triphosphate (GTP)-binding protein Rho.^{24,44} In addition, the compound inhibits cyclic adenosine monophosphate generation and induces Ca^{2+} release from intracellular stores through IP_3 generation. In *Xenopus laevis* oocytes, we and other investigators found that LP induces $\text{I}_{\text{Cl}}(\text{Ca})$ through this mechanism.²⁵⁻²⁸ The compound is released into serum by activated platelets,^{45,46} and its importance is demonstrated by the fact that many of the studies we refer to have shown that well-known serum effects such as neurite retraction and actin reorganization are, in fact, due to LP bound to serum albumin.

Although it has not been cloned yet, many studies document the existence of an LP receptor that couples to a G protein signaling cascade. When microinjected into cells, LP has no effect, implying an extracellular site of action.^{25,26,36,47} Additional evidence for a membrane receptor comes from work done by van der Bend *et al.*,⁴⁸ who, using a photoreactive, radiolabeled, cross-linking LP analog, identified a putative LP membrane receptor of 38-40 kD. In addition, responses to LP are blocked by suramin, an inhibitor of many ligand-receptor interactions. Several studies support a G protein-coupled signaling system for LP. Transient increases in Ca^{2+} levels in fibroblasts^{18,36} and oocytes^{25,26} induced by LP have kinetics typical of those of G protein-coupled receptor responses. In addition, some LP-induced responses are sensitive to pertussis toxin,^{18,26} and GTP γ S, a nonhydrolyzable GTP analog, potentiates the effects of LP.^{18,37} Lysophosphatidate action is highly specific. Virtually no structurally related compounds induce changes in Ca^{2+} levels in human fibroblasts or oocytes. Prostaglandins and platelet-activating factor have been shown clearly to signal through other receptor classes.^{49,50}

Site of anesthetic inhibition

The inhibition of LP responses by halothane observed in the present study is likely to occur at the receptor or G protein level. Suggestive evidence for this derives from our finding that angiotensin signaling is not inhibited by halothane concentrations that virtually abolish LP responses. The two agonists are presumed to signal through the same intracellular pathway and have similar potencies in our model. A single phospholipase C type has been shown to be involved in receptor-mediated Ca^{2+} signaling in oocytes,³⁴ making Ca^{2+} release from similar pools and activation of the same Cl^- channel very likely. The indistinguishable response kinetics in these cells supports this. Our heparin microinjection experiments show that both LP and $\text{AT}_{1\text{A}}$ receptors signal through IP_3 -sensitive Ca^{2+} stores. Furthermore, no other intracellular calcium release channels, such as ryanodine receptors, have been found in oocytes.³⁸ Therefore, apart from the receptors, and possibly the coupled G proteins, AT and LP signaling pathways can be considered identical, and lack of halothane on one, but not the other, pathway suggests an effect at the receptor level.

Additional evidence supporting lack of anesthetic interference with intracellular signaling mechanisms derives from our finding that IP_3 -induced currents are not affected by halothane. This indicates that the anesthetic does not affect the Ca^{2+} release mechanism or the Ca^{2+} -activated Cl^- channel. Our findings correspond with other studies in which low concentrations of halothane were shown not to affect IP_3 -linked Ca^{2+} signaling. For example, Smart *et al.*⁵¹ studied the effect of halothane (0.5-1%) on basal and carbachol-stimulated IP_3 production in SH-SY5Y human neuroblastoma cells and found no inhibition but rather a modest enhancement of IP_3 for-

mation in the presence of anesthetic. Hossain and Evers⁵² and Stern et al.⁵³ observed that halothane (0.5 mM) had almost no effect on thyrotropin-releasing hormone-induced, IP₃-mediated intracellular Ca²⁺ release.

Our data cannot rule out an effect of halothane on the LP molecule itself or on LP binding to albumin. However, such interactions are unlikely to explain our results, because volatile anesthetics also inhibit signaling through G protein-coupled receptors with agonists that are less hydrophobic and do not require albumin for solubilization. We have shown that halothane significantly inhibits signaling through muscarinic acetylcholine receptors.⁶ Lin et al.⁹ reported findings similar to ours, showing that although enflurane (1.8 mM) inhibited muscarinic and serotonin signaling, it did not affect the signaling cascade downstream of phospholipase C. A differential effect of isoflurane and halothane on LP binding to albumin is also unlikely. Although volatile anesthetics have been shown to compete with fatty acids for binding sites on albumin, halothane and isoflurane have similar binding constants: K_d was 1.4 mM for isoflurane and 1.3 mM for halothane.^{54,55} Therefore, our data are most compatible with an effect of halothane on the LP receptor or its associated G protein.

Anesthetic effects suggestive of lysophosphatidate inhibition

Besides being a useful model for investigating anesthetic - protein interactions, LP is an important compound to study because it is a likely regulator of cell proliferation, particularly in tumor cells. The lack of selective LP antagonists limits our understanding of this growth modulator, although recent reports,^{33,56} if confirmed, suggest that this problem may be resolved. Suramin inhibits LP responses, but it is nonspecific because it blocks many other growth factor actions. Many studies have shown volatile anesthetics to exhibit effects opposite of those of LP signaling, although some reports are conflicting. As noted earlier, LP is a mitogen and induces stress fiber assembly. Halothane inhibits growth of neuroblastoma cells in a dose-dependent manner by disrupting actin-like microfilaments of the mitotic apparatus.⁵⁷ Similar results were found in other cell types. Halothane also affects the distribution pattern of actin in fibroblasts and neuroblastoma cells⁵⁸ and prevents cell cleavage in sea urchin eggs,⁵⁹ another actin-dependent process. Both enflurane and halothane at equivalent minimum alveolar concentrations interfere with cell division in *Tetrahymena pyriformis*.⁶⁰ As previously noted, LP is a potent platelet aggregator and decreases cyclic adenosine monophosphate levels in many cell types. In contrast, halothane has been found to decrease aggregation in human platelets^{61,62} accompanied by increased cyclic adenosine monophosphate levels.⁶¹ Thus the LP antagonist-like properties and some side effects of volatile anesthetics may be due to interference with LP signaling.

CONCLUSIONS

Our findings show that lipid mediator signaling can be affected profoundly by anesthetics. It should be determined if prostaglandins and platelet-activating factor are similarly inhibited by volatile anesthetics. It is not possible to study direct interactions between anesthetics and the LP receptor, because a binding assay for the LP receptor has not been established. If, however, anesthetic interference with signaling occurs at the LP binding site, the simple molecular structure of many anesthetics might allow elucidation of the molecular determinants that define an LP antagonist. Finally, because the LP receptor is G protein linked, our findings provide additional

support for anesthetic interference with G protein-coupled receptors.

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CHAPTER 11

**Inhibition of lysophosphatidate signaling by
lidocaine and bupivacaine**

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Inhibition of Lysophosphatidate Signaling by Lidocaine and Bupivacaine

ABSTRACT

Background. Lidocaine and bupivacaine impair wound healing, but the mechanism of this side effect has not been determined. The phospholipid messenger lysophosphatidate is released from activated platelets and induces fibroblast and smooth muscle proliferation. Because it may play a role in wound healing, the authors studied the effects of local anesthetics on lysophosphatidate signaling in *Xenopus* oocytes.

Methods: Defolliculated *Xenopus* oocytes expressing endogenous G protein-coupled lysophosphatidate receptors were voltage clamped and studied in the presence or absence of lidocaine or bupivacaine. Lysophosphatidate-induced Ca^{2+} -activated Cl^- currents ($\text{I}_{\text{Cl}(\text{Ca})}$) were measured. To determine the site of action of the local anesthetics on the signaling pathway, the authors studied 1) the effects of local anesthetics on signaling induced by intracellular injection of the second messenger inositoltrisphosphate, and 2) the effects of local anesthetics on functioning of recombinantly expressed angiotensin II receptors signaling through the same pathways as the lysophosphatidate receptor.

Results: Lysophosphatidate signaling was inhibited in the presence of local anesthetics. The half maximal inhibitory concentrations (IC_{50}s) for lidocaine and bupivacaine were 29.6 mM and 4.7 mM, respectively. Neither responses induced by inositoltrisphosphate injection nor angiotensin signaling were influenced by local anesthetics.

Conclusions: Lysophosphatidate signaling is inhibited by the extracellular application of lidocaine or bupivacaine. In contrast, inositoltrisphosphate or angiotensin signaling was not affected by local anesthetics. Therefore, local anesthetics have a specific, effect on lysophosphatidate receptor functioning. As the local anesthetic concentrations used were similar to those observed after injection around surgical wounds, lysophosphatidate inhibition may play a role in the observed detrimental effects of local anesthetics on wound healing.

Key words: Local anesthetics: Lidocaine; bupivacaine. Lysophosphatidate signaling.

INTRODUCTION

Local anesthetics impair wound healing in various models. For example, the tensile strength of wounds is decreased when lidocaine is injected in wounds to reduce pain after operation.¹ Lidocaine, with or without epinephrine, also caused a 13-day delay in surgical wound healing in rats.² Although long-acting local anesthetics are becoming ever more important to provide pain relief after outpatient surgery, their effects on wound healing have not been reported.

The mechanisms responsible for the adverse effects of local anesthetics on wound healing are unclear. Suppression of mucopolysaccharide synthesis has been postulated.¹ In tissue cultures of fibroblasts, slices of newborn rat skin or *in vivo* granuloma tissue induced by subcutaneous implantation of steel cylinders in rats, application of lidocaine or bupivacaine inhibited synthesis of collagen to a greater extent than that of noncollagenous proteins. Glycosaminoglycan

synthesis was found defective as well.³ Nevertheless, the specific site of local anesthetic interference in the wound healing process is not known.

Lysophosphatidate is a phospholipid released by activated platelets and injured fibroblasts.^{4,5} The compound activates a specific membrane receptor and, through several coupled G proteins, activates a number of intracellular signaling cascades.⁶⁻⁹ Its effects include platelet activation, vasoconstriction, and induction of mitogenesis, particularly in fibroblasts.^{6,10,11} Thus the compound may be released after injury and play a role in wound healing. Therefore, we hypothesized that interference of local anesthetics with the lysophosphatidate signaling pathway might explain this phenomenon. We investigated the effects on lysophosphatidate signaling of lidocaine and bupivacaine, two local anesthetics of different lipid solubility and duration of action.

As a model, we used oocytes of the African clawed toad, *Xenopus laevis*. These cells express lysophosphatidate-specific G protein-coupled membrane receptors, the activation of which results in a Cl⁻ current induced by intracellular Ca²⁺ release (Figure 1A).⁷ To localize the site of local anesthetic action, we expressed the angiotensin receptor, which responds to a structurally different agonist but couples to the same intracellular signaling pathway as does the lysophosphatidate receptor. In addition, we determined the effects of the local anesthetics on signaling induced by microinjection of inositoltrisphosphate (IP₃). Specifically, we tried to answer the following questions:

1. Does lidocaine or bupivacaine interfere with lysophosphatidate-induced Ca²⁺-activated Cl⁻ currents; if so, are the calculated half maximal inhibitory concentration (IC₅₀s) comparable to concentrations reached after subcutaneous injection?
2. Are these effects reversible?
3. Where in the signaling pathway are these effects localized?

MATERIALS AND METHODS

Oocytes

The study protocol was approved by the Animal Research Committee of the University of Virginia. Adult female *Xenopus laevis* frogs were obtained from Xenopus I (Ann Arbor, MI) and housed in an established frog colony. To obtain oocytes, frogs were anesthetized with 1% tricaine (3-aminobenzoic acid ethyl ester) until they became unresponsive, and they were operated on ice. Oocytes were harvested through a 5-mm incision in the lower lateral abdominal wall and immediately placed in modified Barth's solution (containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 0.3 mM Ca(NO₃)₂, 15 mM HEPES, and 0.1 mM mg/ml gentamicin, with pH adjusted to 7.6). Frogs were returned to the main tank after recovering for 2 days in isolation. Oocytes (Dumont's stage 5 and 6) were defolliculated manually using microforceps and incubated in modified Barth's solution at 18°C for 24 h because this results in more consistent lysophosphatidate responses.

Receptor expression

Endogenous lysophosphatidate receptors were used to evaluate the influence of local anesthetics on lysophosphatidate signaling. The angiotensin receptor was expressed recombinantly. A 1.2-kilobase pair cDNA in the CDM8 vector (Invitrogen, San Diego, CA), encoding the rat AT_{1A} angiotensin II receptor, was a gift from Dr. K. R. Lynch at the University of Virginia. The construct was linearized with the nuclease *Xho* I and transcribed *in vitro* by T7 RNA polymerase in the presence of a capping analog. Oocytes were injected with 50 nl cRNA (5 ng) in water, using an automated microinjector (Nanoject; Drummond Scientific, Broomall, PA). Slight ballooning of the oocyte confirmed the adequacy of injection. The cells were cultured for 72 h at 18°C in modified Barth's solution before study.

Electrophysiologic studies

Lysophosphatidate or angiotensin receptor activation in the oocyte leads to G protein-mediated phospholipase C activity and release of IP₃ from phosphoinositolbisphosphate (Figure 1A). Inositoltrisphosphate then binds to its specific receptor on intracellular Ca²⁺ stores to release Ca²⁺, which in turn opens an endogenous Ca²⁺-activated Cl⁻ channel. The resulting current can be measured using a voltage clamp, and this Ca²⁺-activated Cl⁻ current (I_{Cl(Ca)}), integrated and expressed in microcoulombs (μC), is a measure of intracellular Ca²⁺ release.^{8,12-14} All experiments were performed at room temperature (approximately 22°C).

Microelectrodes were pulled in one stage from capillary glass (BBL with fiber; World Precision Instruments, Sarasota, FL) on a micropipette puller (model 700C; David Kopf Instruments, Tujunga, CA). Tips were broken to a diameter of approximately 10 μm, providing a resistance of 1-3 MΩ, and filled with 3 M KCl. A single oocyte was placed in a perfusable bath containing 3 ml Tyrode's solution (consisting of 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM dextrose, and 10 mM HEPES, with the pH adjusted to 6.4 with NaOH) with or without local anesthetic. Two microelectrodes were inserted into the oocyte, and a holding potential of -70 mV was applied to the membrane. The voltage clamp amplifier (OC725A; Warner Corp., New Haven, CT) was connected to a data acquisition and analysis system running on an IBM-compatible personal computer. The acquisition system was a DAS-8 A/D conversion board (Keithly-Metrabyte, Taunton, MA), and analysis was performed with OoClamp software.¹⁵ Occasional cells that did not show a stable holding current less than 1 μA during a 1-min equilibration period were excluded from analysis. Membrane current was sampled at 125 Hz and recorded for 5 s before and 55 s after application of the agonist (lysophosphatidate or angiotensin, 10⁻⁷ M). Agonists were delivered in 30-μl aliquots during 1 or 2 s using a micropipettor positioned approximately 1 mm from the oocyte. Responses were quantified by integrating the current trace by quadrature and are reported as microcoulombs because this reflects intracellular Ca²⁺ release better than peak current does.¹⁶ Oocytes were preincubated with local anesthetics at least 2 min before agonists were applied. In pilot experiments we found that inhibition develops within 60 s after exposure to the local anesthetic.

For intracellular injections of IP₃, a third microelectrode connected to an automated microinjector (Nanoject) was inserted into the oocyte. Cells were injected under voltage clamp, and the adequacy of injection was verified by observing the small increase in cell size on injection.

Figure 1. Endogenous lysophosphatidate signaling in *Xenopus laevis* oocytes. *A*: Signaling pathway linking activation of the lysophosphatidate receptor to chloride (Cl⁻) channel opening. The double line represents the cell membrane. The agonist lysophosphatidate binds to a G protein-coupled receptor, and the G protein activates phospholipase C. Phospholipase C releases inositoltrisphosphate (IP₃) from cell membrane-bound phosphatidylinositolbiphosphate. Calcium (Ca²⁺) is released from IP₃-sensitive intracellular stores and activates a Ca²⁺-dependent Cl⁻ channel in the cell membrane. *B*: Example of inward Ca²⁺-activated Cl⁻ current ($I_{Cl(Ca)}$) induced by 10⁻⁷ M lysophosphatidate in a defolliculated oocyte. The vehicle, 1% bovine serum albumin, has no effect. The current is integrated and expressed as charge movement (2.9 μ C).

Analysis

Results are reported as means $\pm$ standard error of the mean. Because variability among batches of oocytes is common, responses were at times normalized to controls for each batch. Differences between treatment groups were analyzed using analysis of variance and Student's unpaired t test. Probability values less than 0.05 were considered significant.

Materials

Molecular biology reagents were obtained from Promega (Madison, WI), and other chemicals were obtained from Sigma Chemical Company (St. Louis, MO). Lidocaine HCl and

Figure 2. Local anesthetics inhibit lysophosphatidate signaling. *A:* The Ca^{2+} -activated Cl^- current ($I_{\text{Cl}(\text{Ca})}$) induced by 10^{-7} M lysophosphatidate in control oocyte (4.6 μC). Below is an example of lysophosphatidate signaling inhibition by 42.7 mM lidocaine (1.5 μC) and 15.5 mM bupivacaine (1.1 μC). *B:* Lidocaine depresses lysophosphatidate-induced $I_{\text{Cl}(\text{Ca})}$ in a dose-dependent manner. The half maximal inhibitory concentration (IC_{50}) is 29.6 ± 8.2 mM. *C:* Bupivacaine depresses lysophosphatidate-induced $I_{\text{Cl}(\text{Ca})}$ in a dose-dependent manner. The IC_{50} is 4.5 ± 1.2 mM.

bupivacaine HCl (Sigma Chemical Co.) were diluted in Tyrode solution. Because of bupivacaine's low solubility at pH values greater than 6.4, all solutions were adjusted to pH of 6.4, and pilot experiments showed that lysophosphatidate and angiotensin signaling were not influenced by this lower value. Consecutive dilutions for dose-response calculations were made with pH-adjusted Tyrode's solution. Lysophosphatidate (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate [sodium salt]; Avanti Polar Lipids, Alabaster, AL) was prepared as a 10^{-4} M stock solution in 1% delipidated bovine serum albumin (ICN Biomedicals, Aurora, OH). Consecutive dilutions to 10^{-7} M were made in Tyrode's solution. Before experiments, lysophosphatidate solutions were sonicated for 5 s using a Branson Sonifier 250 (Misonix, Farmingdale, NY) to ensure that the compound was fully in solution. All other chemicals were obtained from Sigma Chemical Company.

Figure 3. Reversibility of local anesthetic inhibition of lysophosphatidate signaling. *A:* With 15.5 mM bupivacaine. *B:* With 42.7 mM lidocaine. Agonist was lysophosphatidate 10^{-7} M throughout. The first bar shows control responses to lysophosphatidate; the second bar shows inhibition of lysophosphatidate signaling by the local anesthetic. Recovery experiments as shown in the third bar of the graphs were conducted by placing the oocyte in local anesthetic solution for 5 min and then washing for 5 min before lysophosphatidate treatment (average control response, 3.1 μ C; control group, $n = 25$; other groups, $n = 5$).

RESULTS

Lysophosphatidate responses in Xenopus oocytes

Oocytes responded to $30 \mu\text{l } 10^{-7} \text{ M}$ lysophosphatidate with a transient inward current (Figure 1B). The vehicle (bovine serum albumin) had no effect. The current developed after 1-3 s and consisted of a fast component and a gradual return to baseline in 10 to 20 s, which is the typical response to G protein-coupled receptor stimulation in *Xenopus* oocytes. The average control response was $2.4 \pm 0.2 \mu\text{C}$.

Local anesthetic effects on lysophosphatidate signaling

Lysophosphatidate signaling was inhibited when oocytes were exposed to lidocaine or bupivacaine at concentrations used clinically for injection around wounds (Figure 2A). The inhibitory effect was concentration dependent; the IC_{50} was $29.9 \pm 8.2 \text{ mM}$ for lidocaine (Figure 2B) and $4.5 \pm 1.2 \text{ mM}$ for bupivacaine (Figure 2C). Hill coefficients were 1.5 and 1.6, respectively. At high local anesthetic concentrations, virtually complete inhibition of lysophosphatidate signaling was observed. In the presence of local anesthetics, a modest but consistent slowing of the response was noted, which was not investigated further.

Reversibility of local anesthetic effects on lysophosphatidate signaling

The reversibility of the local anesthetic inhibitory effect on lysophosphatidate signaling was investigated in three separate groups of oocytes because we were concerned that multiple lysophosphatidate applications might desensitize the lysophosphatidate receptor (Figure 3). In the first group, responses to 10^{-7} M lysophosphatidate were recorded in Tyrode's solution. In the second group, responses were determined in the presence of 42.8 mM lidocaine (1%) or 15.4 mM bupivacaine (0.5%); inhibitions to $26 \pm 7\%$ and $11 \pm 3\%$, respectively, of control values were seen. In the third group, oocytes were first placed in 42.8 mM lidocaine or 15.4 mM bupivacaine for 5 min, which was then completely washed out with Tyrode's solution. After 5 min of washing, responses to lysophosphatidate were recorded. Responses after bupivacaine washout were $115 \pm 17\%$ of control, and thus inhibition by bupivacaine was completely reversible. Responses after lidocaine washout were $58 \pm 11\%$ of control. Although this was not significantly different from control values, it suggests a prolonged and possible irreversible effect of lidocaine.

Site of action: effects on angiotensin_{1A} signaling

To determine that the effect of lidocaine and bupivacaine on lysophosphatidate signaling was specific and not due to nonspecific membrane interactions or inhibition of the Cl^- channel, we expressed the angiotensin_{1A} receptor, which, like the lysophosphatidate receptor, is a member of the G protein-coupled receptor superfamily. The angiotensin_{1A} receptor, when expressed in oocytes, also induces $\text{I}_{\text{Cl}(\text{Ca})}$ through a mechanism that, apart from receptor and possibly G protein, is considered to be the same as that for lysophosphatidate signaling. Lack of local anesthetic inhibition on angiotensin signaling would make unlikely a nonspecific membrane effect or

Figure 4. Angiotensin signaling is not inhibited by local anesthetics. *A:* Control response to 10^{-7} M angiotensin after injection of angiotensin_{1A} cRNA into oocyte 72 h before ($2.2 \mu\text{C}$). *B,C:* Lidocaine 42.7 mM and bupivacaine 15.4 mM have no effect on angiotensin signaling. Responses were 2.8 and 3.1 μC , respectively. *(D)* Lidocaine 42.7 mM and bupivacaine 15.4 mM have no significant effect on angiotensin signaling (all groups $n = 10$).

an effect on the signaling pathway downstream of the lysophosphatidate receptor (including the Cl^- channel). Angiotensin II (10^{-7} M) induced no responses in uninjected oocytes (data not shown) but induced currents in angiotensin_{1A}-injected oocytes that were indistinguishable from those induced by lysophosphatidate (Figure 4A; the average control response was 1.6 μC). Lidocaine and bupivacaine were without effect on angiotensin signaling. $I_{\text{Ca}(\text{Cl})}$ in response to 10^{-7} M angiotensin were $111.1 \pm 22\%$ of control in the presence of lidocaine (42.7 mM) and $92.1 \pm 38.1\%$ of control in the presence of bupivacaine (15.5 mM) (Figure 4). Therefore bupivacaine and lidocaine do not appear to affect the oocyte membrane, intracellular pathways, or the Ca^{2+} -activated Cl^- channel.

Figure 5. :Local anesthetics have no effect on inositoltrishosphate (IP₃) signaling. *A*: A 50-nl volume of IP₃ (100 mM) generates an I_{Cl(Ca)} similar to those induced by lysophosphatidate or angiotensin. *B*: No significant inhibition of IP₃-induced I_{Cl(Ca)} is observed when 42.7 mM lidocaine or 15.4 mM bupivacaine are added to the bath solution (the number of cells tested is shown in parentheses).

Site of action: intracellular inositoltrisphosphate injection

To confirm that local anesthetics do not interfere with intracellular Ca^{2+} release or the Ca^{2+} -activated Cl^- channel, we determined the effects of lidocaine and bupivacaine on $I_{\text{Cl}(\text{Ca})}$ induced by microinjection of IP_3 into the oocyte. Previously we showed that heparin, an IP_3 receptor antagonist, inhibits lysophosphatidate-induced $I_{\text{Cl}(\text{Ca})}$ completely, indicating that lysophosphatidate-induced Ca^{2+} release is mediated solely by IP_3 .^{6,12-14}

Intracellular injection of 50 nl of 100 mM IP_3 generated $I_{\text{Cl}(\text{Ca})}$ in the oocyte indistinguishable from those induced by extracellular lysophosphatidate (Figure 5A). However, in contrast to currents induced by lysophosphatidate application, size and kinetics of these responses were not affected by the presence of extracellular local anesthetic. Average responses to IP_3 were $3.5 \pm 0.9 \mu\text{C}$ in the control group, $3.3 \pm 0.8 \mu\text{C}$ in the presence of 42.7 mM lidocaine, and $3.0 \pm 0.6 \mu\text{C}$ in the presence of 15.5 mM bupivacaine (Figure 5B). These findings confirm that lysophosphatidate signaling inhibition by local anesthetics occurs early in the signaling pathway.

DISCUSSION

Our data show that the local anesthetics lidocaine and bupivacaine inhibit lysophosphatidate signaling in a concentration-dependent manner, with appreciable inhibition occurring at concentrations similar to those observed after injection around wounds. Lack of effect on signaling by angiotensin or microinjected IP_3 suggests that the lysophosphatidate receptor environment (receptor molecule, its lipid environment, or coupled G protein) is the site of action. These findings indicate that local anesthetics can interact significantly with lipid mediator receptors. More specifically, they may explain some observed effects of local anesthetics on the wound healing process. As bupivacaine is, and ropivacaine likely will be, used commonly to provide pain relief after surgery, the interactions of these long-acting local anesthetics with wound healing deserve to be studied in detail.

Several potential limitations of our model should be considered. First, we have only studied a single form of lysophosphatidate signaling (Cl^- currents induced by intracellular Ca^{2+} release), whereas several intracellular signaling cascades are activated by lysophosphatidate: decreases in cAMP, activation of Rho, prostaglandin synthesis, and *ras* activation, to mention just a few. No data suggest that these effects are transduced through different subtypes of receptor. Instead they are transduced through several G proteins, presumably coupling to a single, as yet uncloned, receptor subtype.⁶ Given the importance of intracellular Ca^{2+} signaling, we chose phospholipase C activation and IP_3 release as a pathway to investigate. Second, we used *Xenopus* oocytes to study this pathway, and consequently we performed our experiments at room temperature. This raises the question if the *Xenopus* lysophosphatidate receptor might behave differently from its mammalian counterpart. However, there is no molecular biological or pharmacologic evidence to suggest significant functional differences between lysophosphatidate receptors in various tissues. We have shown that the oocyte model closely replicates findings in other systems.^{11,14,16} The angiotensin_{1A} receptor derives from rat and therefore was expressed at a lower temperature than it normally functions in. However, this has not been shown to affect its signaling properties appreciably. Temperature differences in oocytes primarily affect latency of response of expressed receptors.¹⁷ Therefore we believe that the flexibility of the oocyte model outweighs these minor disadvantages.

The role of lysophosphatidate in wound healing is not known. However, because the compound is released primarily from activated platelets¹⁸ and injured fibroblasts,⁵ its presence in appreciable quantities around wounds seems likely. We have found in preliminary experiments that lysophosphatidate is absent in healthy skin but released in significant quantities after injury. Because its most prominent effects are fibroblast^{9,19} and smooth muscle cell²⁰ proliferation, vasoconstriction,²¹ platelet activation,^{22,23} and induction of changes in cell motility,²⁴⁻²⁶ it seems unlikely that its presence in wounds would have no effect on the wound healing process. Therefore we postulate that lysophosphatidate plays a physiologic role in wound healing, and that interference of local anesthetics with lysophosphatidate signaling can adversely affect the healing process.

Adverse effects of local anesthetics on wound healing have been well described,^{1-3,27} but the mechanisms remain unclear. Although local anesthetics are present in the wound for a short time only, their effects are noticeable for longer periods. For example, fibroblast proliferation and collagen deposition are impaired after application of local anesthetics³ and might explain subsequent decreases in wound breaking strength.¹ Although collagen deposition does not occur until 3-5 days after wounding,²⁸ a time when the local anesthetic is unlikely to be present still in appreciable concentrations, local anesthetic-induced delays in fibroblast proliferation could explain this. Transient inhibition of lysophosphatidate signaling in the early stages of wound healing may disturb the subsequent wound healing cascade. Alternatively, an irreversible component of lidocaine block of lysophosphatidate signaling, as suggested by our findings, might play a role. Clinically, signs of inflammation and occasional necrosis have been observed after local anesthetics were administered around wounds.^{1,27,29} Inhibition of proliferation and motility of immune system cells by local anesthetic block of lysophosphatidate signaling might explain some of these findings. On the other hand, inhibition of lysophosphatidate signaling by local anesthetics might have beneficial effects as well. Lysophosphatidate-induced vasoconstriction and platelet aggregation may be important immediately after wounding but will decrease blood flow in the regenerating wound bed. Local anesthetics might reverse this and thereby provide a better healing environment. Indeed, local anesthetics have been shown to inhibit platelet aggregation,³⁰⁻³² and reverse vasospasm,³³⁻³⁶ by mechanisms that have not been identified yet.

The exact site of interaction between the local anesthetics and the lysophosphatidate signaling pathway is important. Although the main site of action of local anesthetics is the Na⁺ channel,³⁷ secondary activities exist. For example, local anesthetics inhibit Ca²⁺ entry through voltage-gated channels, by direct interference with the channel molecule.³⁸ In our study, we could rule out an effect of the anesthetics on the Ca²⁺-activated Cl⁻ channel. As G protein-coupled pathways signal over many more intermediates than channels do, localization of the site of anesthetic action is relevant. The lack of effect after intracellular microinjection of IP₃ suggests that the site of action is proximal in the signaling pathway. This is confirmed by the lack of local anesthetic effect on angiotensin signaling. The latter finding, in addition, makes a nonspecific membrane effect unlikely. Therefore, the most likely site of action is the lysophosphatidate receptor itself. The lipid moiety of the local anesthetic molecule might interact with the presumed hydrophobic agonist binding site on the receptor. It is important that the more lipophilic local anesthetic bupivacaine (octanol-buffer partition coefficient 3420³⁹) inhibited lysophosphatidate signaling at significantly lower concentrations than did lidocaine (partition coefficient 366³⁹). (The agonist binding site might allow other anesthetic interactions, as we have shown recently that the lipophilic anesthetic propofol similarly interferes with lysophosphatidate signaling.⁴⁰) Although the sigmoidal shape of the concentration-inhibition relation is compatible

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with a receptor site of action, the variability associated with high lysophosphatidate concentrations^{7,14} makes it difficult to show competitive interactions conclusively. One other potential site of action deserves further study, namely a direct interaction with the lysophosphatidate molecule, or with lysophosphatidate binding to albumin, its physiologic carrier.

Our data indicate that commonly used local anesthetics can interfere significantly with lipid mediator signaling. Various lipid mediators (platelet-activating factor, prostaglandins, leukotrienes) are relevant in the perioperative context, making it important to understand their interactions with commonly used local anesthetics. This is of particular relevance to the newer, long-acting compounds.

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CHAPTER 12

SUMMARY AND CONCLUSIONS / SAMENVATTING EN CONCLUSIES

Marcel E. Durieux

Summary and conclusions

This thesis reports the development of a system for the study of molecular interactions between G protein-coupled receptors and anesthetics. Although the main site of action for most intravenous and inhaled anesthetics appears to be the GABA_A receptor (ketamine's action on the NMDA glutamate receptor is an exception), the different effect and side effect profiles of these drugs is a result of their interactions with other systems. As discussed in Chapter 2, ion channels - both voltage-regulated and ligand-gated - have received most attention as sites of anesthetic action. Indeed, many channel types have been shown to be affected. The nicotinic acetylcholine receptor is an example of an ionotropic receptor significantly inhibited by low concentrations of anesthetics. Voltage-gated Ca and K channels are similarly inhibited by inhaled anesthetics. In contrast, the large superfamily of G protein-coupled receptors (GCRs) has received relatively little attention. Although scattered reports exist of anesthetic interactions with GCRs, only the effects on muscarinic acetylcholine signaling have been investigated in some detail. Part of this lack of investigative attention is due to technical difficulties in studying GCRs in isolation. Therefore, we adapted the *Xenopus* oocyte model, long used for the expression and cloning of GCRs, to the study of anesthetic interactions with these signaling molecules. Of particular interest would be GCRs for lipid mediators, as these would be likely targets for (lipophilic) anesthetics.

As reviewed in Chapter 4, the *Xenopus* oocyte has a number of compelling advantages for use in such studies. The frogs are easily maintained and oocytes are obtained with a simple procedure. In addition, once oocytes are adequately defolliculated, they are remarkably devoid of endogenous GCRs, with the exception of those described in the present thesis. GCRs can be expressed either by microinjection of RNA (extracted from tissue or prepared in vitro) or cDNA. Particularly Ca²⁺-signaling GCRs are easily studied, as the oocyte expresses an endogenous Ca²⁺-dependent Cl⁻ channel, which is routinely used as a reporter for intracellular Ca²⁺ release. The results of the investigations in this thesis demonstrate that anesthetics of various classes can be applied without significantly modifying the behavior of the oocyte.

Chapters 5 and 6 describe our observations of two endogenous GCRs which are expressed consistently in oocytes: those for the phospholipid messengers lysophosphatide (LPA) and sphingosine-1-phosphate (S1P). These two compounds are closely related structurally and functionally. As described in Chapter 3, LPA signaling has received much investigative attention recently. The compound has long been known to be an essential intermediate in phospholipid metabolism, but only in recent years has it become clear that it also acts as a signaling molecule. Its cellular effects are varied and dramatic. The compound acts as a contractile agent on various types of smooth muscle, including vascular smooth muscle. It is a potent mitogen for many types of fibroblasts. It activates platelets and - in turn - is released into the blood stream from activated platelets. It induces shape changes in a number of cell types, including neuronal cells. Its relevance to biological systems is shown by the fact that LPA acts as a chemoattractant in a species as far removed from mammals as *Dictyostelium discoideum*. A variety of studies over the past ten years have provided detailed insight into the cellular mechanism of action of LPA. Although a membrane receptor has not yet been cloned convincingly, biochemical evidence strongly supports its existence. LPA binding to this receptor leads to intracellular activation of several G proteins, inducing IP₃ and DAG generation, as well as decreases in cAMP levels. The mitogenic and cytoskeletal actions of the compound are mediated by small cytoplasmic G proteins and the MAP kinase cascade. Thus, LPA is a molecule of significant interest. Some of its actions may be

relevant in the perioperative period, and anesthetic effects on LPA signaling are therefore worthy of investigation. As in addition the compound forms a good model for lipid mediator signaling in general, and the receptor is endogenously expressed in oocytes, we decided to study the effects of several classes of anesthetics on LPA signaling.

Chapter 5 reports our finding of the endogenous LPA response in *Xenopus* oocytes. LPA induced transient dose-dependent currents in uninjected cells. The response was found to be highly specific, as related compounds (phosphatidic acid, lysophosphatidylcholine, lysophosphatidylserine) were without effect. Intracellular LPA application did not induce intracellular Ca^{2+} release, strongly suggesting an extracellular site of action. In addition, the response to LPA could be inhibited by incubation in pertussis toxin, indicating G protein involvement. We could establish that the induced currents were Ca^{2+} -activated Cl^- currents, as they were abolished by intracellular injection of EGTA, and the reversal potential became more positive at lower extracellular Cl^- concentrations. Suramin, which had been shown to inhibit LPA responses in other systems, blocked the current. Hence, we concluded that this novel oocyte response to LPA was mediated by a specific membrane receptor linked to a pertussis toxin-sensitive G protein.

When searching for related responses in oocytes, we tested S1P, and found it also to induce currents. As detailed in Chapter 6, these currents were similarly Ca^{2+} -activated Cl^- currents, virtually indistinguishable from those induced by LPA. Intracellular injection of S1P was without effect, as was extracellular application of several compounds structurally related to S1P (sphingosine, sphingosylphosphorylcholine and N,N-dimethylsphingosine). In order to investigate if S1P and LPA might signal through the same receptor, we employed cross-desensitization, and found that LPA and S1P cross-desensitized completely. Both responses were inhibited by suramin and dithiothreitol. Thus, this suggests the existence of a single receptor for the two compounds. We were able to elicit responses to both LPA and S1P in HEK293 fibroblasts, but to neither of the compounds in K562 cells. However, no cross-desensitization between the compounds was observed in HEK293 cells.

As standard electrophysiology systems are not designed to function effectively with the long time courses and large currents induced by GCR signaling in oocytes, we developed a software system specifically geared to this function, as described in Chapter 7. OoClamp, as it is called, is a MS-DOS based package for the acquisition, analysis and storage of data from GCR studies in *Xenopus* oocytes. The system is designed to provide standardization of test conditions, rapid, on-line analysis of data, and self-documentation and compact storage of data files. All system settings are optimized for use with the *Xenopus* expression system. This system has been used for data acquisition in all subsequent studies reported in this thesis.

In the course of these investigations, we discovered an additional endogenous oocyte response: a current induced by trypsin. As described in Chapter 8, trypsin induces Ca^{2+} -activated Cl^- currents when applied in concentrations as low as 0.1 mg/ml to defolliculated, uninjected oocytes. The response is dose-dependent and specific, as other proteases (chymotrypsin, Lys-C and Arg-C) or trypsin pretreated with soybean trypsin inhibitor, were without effect. The response was due to an extracellular site of action, as microinjected trypsin did not induce currents. We also tested if the trypsin response could be due to proteolytic activation of LPA signaling, but found this not to be the case.

After these initial studies of oocyte physiology, we studied the effects of three classes of anesthetics on LPA signaling in oocytes. Chapter 9 reports the effects of propofol, a highly lipophilic intravenous anesthetic known to have its primary site of action on the GABA_A receptor. Propofol in Intralipid® dose-dependently inhibited LPA signaling (IC_{50} 5.38 μM). Propofol 28

μM inhibited LPA by more than 80%. Intralipid® (0.01%) was without effect. To ascertain that intracellular signaling pathways and the Ca^{2+} -activated Cl^- channel were not affected by propofol, we tested the effects of propofol on currents induced by methylcholine in oocytes expressing m1 muscarinic acetylcholine receptors. No inhibition was observed. As both receptors share the same intracellular signaling pathway, we concluded that clinically relevant concentrations of propofol inhibit LPA signaling, acting most likely on the receptor or the associated G protein. Thus, lipophilic anesthetics indeed interfere significantly with signaling through lipid mediator receptors.

Chapter 10 describes the effects of two volatile anesthetics, halothane and isoflurane, on LPA signaling. We found halothane to inhibit LPA signaling in a dose-dependent manner (IC_{50} 0.23 mM). Halothane 0.34 mM inhibited responses to LPA virtually completely. This effect was fully reversible. As we had shown previously that halothane inhibits functioning of muscarinic receptors, we used instead the $\text{AT}_{1\text{A}}$ angiotensin receptor to test for anesthetic effects on the shared intracellular signaling pathway. Halothane, even at the highest concentrations tested, was without effect on angiotensin signaling. To assure that both receptors signaled through the same pathway, we microinjected oocytes with heparin, an IP_3 receptor blocker. Responses to both LPA and angiotensin were inhibited by this treatment, confirming that both receptors signal through IP_3 . Currents elicited by microinjection of IP_3 into oocytes were not affected by halothane. Together, these findings confirm that the effect of halothane is proximal in the signaling pathway, most likely at the LPA receptor or G protein. In contrast to halothane, isoflurane was without effect on LPA signaling. Thus, volatile anesthetics can interfere with lipid mediator signaling, but the action is specific.

Finally, we studied the effect of two local anesthetics, lidocaine and bupivacaine, on LPA signaling, as described in Chapter 11. This study was of particular relevance as a role for LPA in wound healing seems likely, and local anesthetics have been shown to impair wound healing. Both compounds inhibited LPA signaling (IC_{50} 29.9 mM for lidocaine and 4.5 mM for bupivacaine - concentrations reached after local anesthetic injection around wounds). At high local anesthetic concentrations, virtually complete inhibition of LPA signaling was observed. The effect of bupivacaine was completely reversible, whereas lidocaine appeared to have a partially irreversible effect. Both compounds were without effect on angiotensin or IP_3 signaling.

Taken together, these studies show that the *Xenopus* oocytes is a flexible model for the study of anesthetic actions on GCR signaling, and that selected members of the three main classes of anesthetics interfere with at least one lipid mediator receptor.

Further studies suggest themselves. First, more detailed localization of the site of action is useful. We have now been able to induce Ca^{2+} -activated Cl^- currents in oocytes by microinjection of the non-hydrolyzable GTP analog GTP γS , and have found that halothane is without effect on these currents. Thus, the site of action of halothane is more proximal than the G protein effector domain, and therefore either involves the receptor, the receptor-G protein interface or early steps in G protein signaling (such as GDP-GTP exchange). We have obtained similar results have been obtained with local anesthetics. We have shown in addition that charged local anesthetics inhibit the LPA receptor at an intracellular site, using the non-permeable, permanently charged lidocaine analog QX314. Permanently uncharged compounds, exemplified by benzocaine, can also block LPA signaling, and as they interact with QX314 in a synergistic manner, the presence of two binding sites is suggested. It would be of great interest to test stereoisomers of some of the anesthetics, as a stereoselective effect would suggest a protein (receptor) site of action. Such studies are now in progress with the isomers of ropivacaine.

In addition, it is important to extend these results to other lipid mediator receptors. We have chosen the thromboxane A₂ (TXA₂) receptor because of its relevance in vasoconstriction and platelet aggregation, effects similar to those of LPA. We found both general anesthetics and local anesthetics inhibit TXA₂ signaling. Both isoflurane and halothane inhibit signaling, but competition studies suggest different site of action. This is in agreement with data obtained at other receptors, as discussed in Chapter 3. The effect of local anesthetics is profoundly time-dependent. After several hours incubation, concentrations of lidocaine and bupivacaine achieved in plasma during epidural anesthesia are found to inhibit TXA₂ signaling significantly. The mechanism of this delayed effect is under study.

It is also important to confirm findings obtained in a highly isolated model such as the oocyte to receptors in a more native environment. Therefore, studies of the effects of anesthetics on, e.g. LPA-induced platelet aggregation should be performed.

The number of lipid mediator receptors is large, and many of them play important roles during injury and repair. Thus, their functioning is of great importance during the perioperative period. Our data suggest that they may be targets for anesthetic action, and that anesthetics of several classes can significantly limit their functioning. This may have consequences for the bodily response during and after surgery, and a full understanding of these anesthetic-receptor interactions and their consequences is therefore warranted.

SAMENVATTING EN CONCLUSIES

Deze dissertatie beschrijft de ontwikkeling van een systeem voor de studie van moleculaire interacties tussen G-eiwit gekoppelde receptoren en anesthetica. Hoewel het belangrijkste effect voor de meeste intraveneus en per inhalatie toegediende anesthetica de GABA_A receptor schijnt te zijn (het effect van ketamine op de NMDA glutamaat receptor is een uitzondering), zijn de verschillende werkings- en bijwerkingsprofielen van deze geneesmiddelen een gevolg van interacties met andere systemen. Als beschreven in Hoofdstuk 2, hebben ionenkanalen - zowel potentiaal-gestuurd als ligand-gestuurd - de meeste aandacht gekregen als werkingsplaats van anesthetica. Van veel soorten kanalen is inderdaad aangetoond dat ze beïnvloed worden. De nicotinische acetylcholine receptor is een voorbeeld van een ionotropische receptor die in aanzienlijke mate geremd wordt door lage concentraties inhalatieanesthetica. Potentiaal-gestuurde Ca en K kanalen worden op gelijke wijze geremd. De grote superfamilie van G eiwit-gekoppelde receptoren (GCRs) heeft in tegenstelling relatief weinig aandacht gekregen. Hoewel verspreide rapporten bestaan over interacties tussen anesthetica en GCRs zijn alleen de effecten op muscarinische acetylcholine signalering in enig detail bestudeerd. Een deel van dit gebrek aan onderzoeksaandacht is het gevolg van de technische moeilijkheden in het bestuderen van GCRs in isolatie. Om die reden hebben wij het *Xenopus* oocyt model, reeds lang in gebruik voor expressie en clonering van GCRs, aangepast voor de studie van anesthetica interacties met deze signaalmoleculen. Van speciaal belang zouden zijn de GCRs voor lipiedmediatoren, aangezien deze waarschijnlijke doelen zouden vormen voor (lipofiele) anesthetica.

Als beschreven in Hoofdstuk 4, hebben *Xenopus* oocyten een aantal overtuigende voordelen voor het gebruik in dergelijke studies. De kikkers zijn makkelijk in onderhoud en oocyten zijn te verkrijgen met een eenvoudige ingreep. Bovendien zijn oocyten, eenmaal voldoende gedefolliculeerd, opmerkelijk vrij van endogene GCRs, met als uitzondering degenen beschreven in deze dissertatie. GCRs kunnen worden geëxprimeerd hetzij door microinjectie van RNA (ge-

extraheerd uit weefsel of in vitro geprepareerd) of cDNA. Met name GCRs die signaleren via Ca^{2+} zijn makkelijk te bestuderen, omdat de oocyt een endogeen Ca^{2+} -afhankelijk Cl^- kanaal bezit dat routinematig gebruikt wordt als een reporter voor intracellulair vrijgemaakt Ca^{2+} . De resultaten van de onderzoeken in deze dissertatie tonen aan dat anesthetica van verschillende klassen kunnen worden toegediend zonder de eigenschappen van de oocyt noemenswaardig te beïnvloeden.

Hoofdstuk 5 en 6 beschrijven onze waarnemingen betreffende twee endogene GCRs die consistent in oocyten geëxprimeerd zijn: voor de fosfolipide signaalmoleculen lysofosfatidaat (LPA) en sfingosine-1-fosfaat (S1P). Deze twee substanties zijn elkaar sterk verwant, zowel structureel als functioneel. Als beschreven in Hoofdstuk 3 is LPA signalering de laatste tijd onderwerp van veel onderzoeksinteresse. Het molecuul is lang bekend als een essentiële tussenstap in fosfolipied metabolisme, maar slechts in de afgelopen jaren is duidelijk geworden dat het ook functioneert als een signaal molecuul. Het werkt als contractiel agonist op verschillende soorten glad spierweefsel, inclusief vaatspierweefsel. Het is een krachtig mitogeen voor vele typen fibroblast. Het activeert trombocyten en - anderzijds - wordt door geactiveerde trombocyten in de bloedbaan vrijgemaakt. Het induceert vormveranderingen in een aantal celtypen, waaronder zenuwcellen. Het belang voor biologische systemen wordt duidelijk uit het feit dat LPA als chemoottractant functioneert in een soort zo ver verwijderd van zoogdieren als *Dictyostelium discoideum*. Een groot aantal studies over de afgelopen tien jaar heeft gedetailleerd inzicht opgeleverd in de cellulaire werkingsmechanismen van LPA. Hoewel een membraanreceptor nog niet overtuigend is gekloneerd wordt zijn bestaan krachtig gestaafd door biochemische bevindingen. Binding van LPA met zijn receptor leidt tot intracellulaire activering van verschillende G eiwitten, waardoor IP_3 en DAG gegenereerd worden, en cAMP niveaus dalen. De mitogene en cytoskelet effecten van LPA worden gemedieerd door kleine cytoplasmatische G eiwitten en de MAP kinase keten. LPA is derhalve een molecule met aanmerkelijke interesse. Een aantal van zijn effecten zijn mogelijk relevant in de perioperatieve periode, en het is daarom relevant om effecten van anesthetica op LPA signalering te bestuderen. Aangezien de substantie bovendien een goed model vormt voor lipied mediator signalering in het algemeen, en aangezien de receptor endogeen geëxprimeerd is in oocyten, besloten wij de effecten van verschillende klassen anesthetica op LPA signalering te bestuderen.

Hoofdstuk 5 rapporteert onze bevindingen omtrent de endogene LPA respons in *Xenopus* oocyten. LPA veroorzaakt tijdelijke, dosis-afhankelijke ionenstromen in niet-geïnjecteerde cellen. De response was uiterst specifiek, aangezien verwante stoffen (fosfatidaatzuur, lysofosfatidylcholine, lysofosfatidylserine) zonder effect waren. Intracellulaire toediening van LPA maakte geen intracellulair Ca^{2+} vrij, hetgeen sterk wijst op een extracellulaire werkingsplaats. De respons op LPA kon bovendien worden onderdrukt door incubatie in pertussis toxine, wijzend op een rol voor G eiwitten. Wij konden aantonen dat de geïnduceerde stromen Ca^{2+} -geactiveerde Cl^- stromen waren, aangezien ze onderdrukt werden door intracellulaire injectie van EGTA, en aangezien de omkeringspotentiaal meer positief werd met lagere extracellulaire Cl^- concentraties. Suramine, hetgeen LPA effecten in andere systemen onderdrukt, verminderde de stromen. Wij concludeerden dat deze nieuwe oocyt respons op LPA gemedieerd wordt door een specifieke membraan receptor gekoppeld met een pertussis toxine-gevoelig G eiwit.

Op zoek naar vergelijkbare reacties in oocyten testten wij S1P, en vonden dat het eveneens stromen induceerde. Als beschreven in Hoofdstuk 6 waren deze stromen eveneens Ca^{2+} -geïnduceerde Cl^- stromen, vrijwel niet te onderscheiden van degenen geïnduceerd door LPA. Intracellulaire injectie van S1P was zonder effect, evenals extracellulaire toediening van verschil-

lende structureel aan S1P verwante stoffen (singosine, sfingosylfosforylcholine en N,N-dimethylsfingosine). Om te onderzoeken of S1P en LPA via dezelfde receptor signaleren testten we kruisdesensitisatie, en vonden dat LPA en S1P volledig kruisdesensitiseren. Beide responsen werden onderdrukt door suramine en dithiothreitol. Dit suggereert derhalve het bestaan van een enkele receptor voor beide substanties. We waren in staat reacties op LPA en S1P te verkrijgen in HEK293 fibroblasten, maar op geen van beide stoffen in K562 cellen. Er werd evenwel geen kruisdesensitisatie waargenomen in HEK293 cellen.

Aangezien de gebruikelijke electrofysiologie systemen niet ontworpen zijn om effectief te functioneren met de lange tijdsduren en grote stromen die door GCRs in oocyten geïnduceerd worden, ontwikkelden we een software systeem specifiek ontworpen voor dit doel, als beschreven in Hoofdstuk 7. OoClamp, zoals het genoemd is, is een MS-DOS-gebaseerd pakket voor opname, analyse en opslag van gegevens verkregen met GCR studies in *Xenopus* oocyten. Het systeem is ontworpen om standaardisatie van test condities, snelle, on-line data analyse, en zelf-documentatie en compacte gegevensopslag te verschaffen. Alle systeem parameters zijn geoptimaliseerd voor gebruik met het oocyt expressie systeem. Dit systeem is gebruikt voor data opname in alle volgende studies beschreven in deze dissertatie.

In de loop van deze onderzoeken ontdekten we nog een endogene respons van de oocyt: een stroom geïnduceerd door trypsine. Als beschreven in Hoofdstuk 8 induceert trypsine, wanneer toegediend op gedefolliculeerde, ongeïnjecteerde oocyten in concentraties zo laag als 0.1 mg/ml, een Ca^{2+} -geactiveerde Cl^- stroom. De respons is dosis-afhankelijk en specifiek, aangezien andere proteasen (chymotrypsine, Lys-C en Arg-C), zowel als trypsine voorbehandeld met soja trypsine inhibitor, zonder effect waren. De respons was het gevolg van een extracellulaire werkingsplaats, aangezien gemicroïnjecteerd trypsine geen stromen induceerde. We testten ook of de trypsine respons een gevolg kon zijn van proteolytische activering van LPA signalering, maar dit bleek niet het geval.

Na deze voorbereidende studies van oocyt fysiologie bestudeerden we de effecten van drie klassen anesthetica op LPA signalering in oocyten. Hoofdstuk 9 rapporteert de effecten van propofol, een zeer lipofiel intraveneus anestheticum met zijn primaire werkingsplaats op de GABA_A receptor. Propofol in Intralipid[®] onderdrukte LPA signalering op dosis-afhankelijke wijze (IC_{50} 5.38 μM). Propofol 28 μM onderdrukte LPA signalering meer dan 80%. Intralipid[®] (0.01%) alleen was zonder effect. Om zeker te zijn dat intracellulaire signaalwegen en het Ca^{2+} -geactiveerde Cl^- kanaal niet beïnvloed waren door propofol testten we het effect van propofol op stromen geïnduceerd door methylcholine in oocyten die de m1 muscarinische acetylcholine receptor exprimeerden. Er werd geen inhibitie waargenomen. Aangezien beide receptoren dezelfde intracellulaire signaalketen delen concludeerden wij dat klinisch relevante concentraties propofol LPA signalering onderdrukken, waarschijnlijk werkend op de receptor of het geassocieerde G eiwit. Lipofiele anesthetica interfereren derhalve inderdaad aanzienlijk met receptoren voor lipied mediators.

Hoofdstuk 10 beschrijft de effecten van twee inhalatieanesthetica, halothaan en isofluraan, op LPA signalering. We vonden dat halothaan LPA signalering onderdrukte op dosis-afhankelijke wijze (IC_{50} 0.23 mM). Halothaan 0.34 mM onderdrukte LPA responsen vrijwel compleet. Dit effect was volledig reversibel. Aangezien we eerder aangetoond hadden dat halothaan de functie van muscarinische receptoren onderdrukt, gebruikten we de AT_{1A} angiotensine receptor om effecten van het anestheticum op de intracellulaire signaalketen te testen. Halothaan was zelfs in de hoogste concentraties zonder effect op angiotensine signalering. Om er zeker van te zijn dat beide receptoren over dezelfde keten signaleren microïnjecteerden we oocyten met

heparine, een IP_3 receptor antagonist. Responsen op zowel LPA als angiotensine werden door deze behandeling onderdrukt, aantonend dat beide receptoren via IP_3 signaleren. Stomen geïnduceerd door microinjectie van IP_3 in oocyten werden niet beïnvloed door halothaan. Tezamen duiden deze bevindingen er op dat het effect van halothaan proximaal in de signaalketen plaatsvindt, meest waarschijnlijk op de LPA receptor of G eiwit. Isofluraan, in tegenstelling tot halothaan, was zonder effect op LPA signalering. Volatile anesthetica kunnen derhalve interfereren met lipied mediator signalering, maar hun effecten zijn specifiek.

Tenslotte onderzochten we de effecten van twee lokaalanesthetica, lidocaine en bupivacaine, op LPA signalering, als beschreven in Hoofdstuk 11. Deze studie was van bijzonder belang aangezien een rol voor LPA in wondheling waarschijnlijk lijkt, en aangezien is aangetoond dat lokaalanesthetica wondheling nadelig beïnvloeden. Beide anesthetica onderdrukten LPA signalering (IC_{50} 29.9 mM voor lidocaine en 4.5 mM voor bupivacaine - concentraties die bereikt worden na injectie van lokaalanesthetica rond wonden). Met hoge concentraties lokaalanestheticum werd vrijwel volledige onderdrukking van LPA signalering waargenomen. Het effect van bupivacaine was volledig reversibel, maar lidocaine had een deels irreversibel effect. Beide stoffen waren zonder effect op angiotensine of IP_3 signalering.

Tezamen tonen deze studies aan dat *Xenopus* oocyten een flexibel model vormen voor de studie van de effecten van anesthetica op GCR signalering, en dat sommige leden van de drie grote klassen van anesthetica interfereren met op zijn minst één lipied mediator receptor.

Verdere studies zijn mogelijk. In de eerste plaats zou meer gedetailleerde localisatie van de werkingsplaats nuttig zijn. Wij zijn er nu in geslaagd om Ca^{2+} -geactiveerde Cl^- stromen te induceren in oocyten door microinjectie van het niet-hydrolyseerbare GTP analogon GTPyS, en we hebben gevonden dat halothane zonder effect is op deze stromen. De werkingsplaats van halothaan is derhalve stroomopwaarts van het G eiwit, en omvat dus de receptor of de receptor-G protein interface. We verkregen vergelijkbare resultaten met lokaalanesthetica. Bovendien hebben we aangetoond, gebruikmakend van het niet-permeabele, permanent geladen lidocaine analogon QX314, dat positief geladen lokaalanesthetica de LPA receptor intracellulair beïnvloeden. Permanent ongeladen lokaalanesthetica, bijvoorbeeld benzocaine, blokkeren LPA signalering eveneens, en aangezien ze synergistisch werken met QX314, is de aanwezigheid van twee bindingsplaatsen mogelijk. Het zou zeer interessant zijn om stereoisomeren van enkele van deze anesthetica te testen, aangezien een stereoselectief effect een eiwit (receptor) werkingsplaats zou suggereren. Dergelijke studies zijn nu in uitvoering met de stereoisomeren van ropivacaine.

Het is bovendien van belang deze resultaten uit te breiden tot andere lipid mediator receptoren. Wij hebben de thromboxaan A_2 (TXA_2) receptor gekozen vanwege zijn relevantie in vasoconstrictie en trombocytenaggregatie, effecten vergelijkbaar met die van LPA. We vonden dat algehele anesthetica zowel als lokaalanesthetica TXA_2 signalering onderdrukten. Zowel halothaan als isofluraan onderdrukten signalering, maar competitie studies suggereren verschillende werkingsplaatsen. Dit is in overeenstemming met gegevens van andere receptoren, als besproken in Hoofdstuk 3. Het effect van lokaalanesthetica is buitengewoon tijdfafhankelijk. Na enkele uren incubatie onderdrukken concentraties van lidocaine en bupivacaine als aanwezig in plasma tijdens epiduraalanesthesie TXA_2 signalering aanzienlijk. Het mechanisme voor dit vertraagde effect is onder studie.

Het is ook van belang om de gegevens verkregen in een zeer geïsoleerd model zoals de oocyt te bevestigen in een meer fysiologisch model. Studies van het effect van anesthetica op bv. LPA-geïnduceerde trombocytenaggregatie zijn daarom aangewezen.

Het aantal lipied mediator receptoren is groot, en vele spelen een belangrijke rol tijdens weefselbeschadiging en reparatie. Hun functie is dus van groot belang tijdens de perioperatieve periode. Onze gegevens suggereren dat zij een doel vormen voor anesthetica, en dat anesthetica van verschillende klassen hun functie aanzienlijk kunnen inperken. Dit kan gevolgen hebben voor de reacties van het lichaam tijdens en na operatieve ingrepen, en een volledig begrip van deze anesthetica-receptor interacties en hun consequenties is derhalve van belang.

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Curriculum Vitae

The author of this thesis was born on November 11, 1959, in Krommenie (The Netherlands). He attended secondary school at the Murmellius Gymnasium in Alkmaar.

After spending one year as an exchange student at Washington & Lee University, Lexington, VA, he commenced his studies in medicine at the University of Leiden (The Netherlands). He graduated *cum laude* in 1986.

After a year as Research Associate in the laboratory of David Longnecker at the University of Virginia, he completed his internship at Roanoke Memorial Hospitals, Roanoke, VA, and his residency training in Anesthesiology at the University of Virginia.

From 1991 through 1993 he worked in the laboratory of Kevin Lynch (Pharmacology) at the University of Virginia, as a Research Fellow.

In 1994 he took his position as Assistant Professor in the Department of Anesthesiology at the University of Virginia. In 1998 he was promoted to Associate Professor. He holds secondary appointments as Associate Professor in the Department of Neurological Surgery, and as Assistant Professor in the Department of Pharmacology.