Phosphodiesterases in development : on the regulation of cGMP signal transduction in the brain

Summary

The aim of the studies described in this thesis was to investigate nitric oxide (NO)-mediated guanosine 3',5'-cyclic monophosphate (cGMP) synthesis in the central nervous system (CNS), with respect to its relation to learning and memory. NO-cGMP signaling is found in virtually all regions of the CNS. Five protein families are involved in the regulation of the NO-cGMP signaling cascade. NO synthase (NOS) is responsible for the synthesis of NO, the endogenous activator of the NO-receptive guanylyl cyclase (GC), known as the soluble isoform of this enzyme (sGC). Binding of NO to sGC can lead to a hundredfold stimulation of cGMP synthesis. The produced cGMP plays an important role as a second messenger in cells and therefore cGMP levels need to be critically regulated. cGMP concentrations are for a large part regulated by the activity of cGMP-hydrolyzing enzymes, the 3',5'-cyclic nucleotide phosphodiesterases (PDEs). Nowadays, a total of 11 different PDE families have been identified, consisting of an estimated ninety-five different forms. Other proteins involved in NO-cGMP signaling are the cGMP-dependent protein kinases and the cGMP-regulated ion channels.

The involvement of a number of protein families in NO-cGMP signaling, expressed in various cell types and brain areas, points to the involvement of this cascade in numerous brain processes. In this thesis research was performed on the function of this signaling pathway in learning and memory. This was chosen for the reason that it had been found by our department and other external research groups that inhibition of NO synthesis had an adverse effect on learning and memory in a number of animal tasks, including the object recognition test. In contrast, application of a compound which can inhibit a PDE family of type 5 (PDE5) resulted in an improved performance in the object recognition task. The precise mechanism that occurs after application of a PDE5 inhibitor was unknown at the start of our investigations. Furthermore, it was not clear where PDE inhibitors are effective in the brain. Therefore, the localization of components involved in NO-cGMP signaling were studied in detail. Testing of novel PDE5 inhibitors was one of the approaches used in this research. Special emphasis was given to study the localization of PDE5 in the brain in comparison to other cGMP-hydrolyzing PDE families.

Chapter 1 gives a historical description of the discovery of NO-cGMP signaling and describes the different components involved in this cascade. The cellular targets of cGMP are discussed in detail with special emphasis on the description and biochemical characteristics of the superfamily of PDEs. Furthermore, studies on cGMP signaling in relation to learning and memory models are reviewed.

In **Chapter 2** the effects of the *in vivo* application of PDE5 inhibitors such as sildenafil and vardenafil on object recognition memory are described in relation to their administration *in vitro* using hippocampal slices. Both PDE5 inhibitors enhanced retention for object memory, with vardenafil being more potent than sildenafil. In agreement, *in vitro* analysis of both PDE5 inhibitors confirmed the higher potency of vardenafil. Both compounds induced local cGMP accumulation in neuronal fibers of the hippocampus. It is argued that the observed cGMP accumulation in the *in vitro* incubated hippocampus slice might be part of the underlying mechanism of memory improvement after *in vivo* administration of PDE5 inhibitors.

Since scarce information was available about the effects of selective inhibitors of PDE isoforms in the hippocampus, the properties of a number of PDE inhibitors on cGMP and cAMP accumulation in the hippocampus slices are described in greater detail in **Chapter 3**. Dose response curves showed a strong cGMP production after incubation of hippocampus slices with the non-selective PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX), the PDE2 inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and the mixed type inhibitor dipyridamole. Furthermore, it was observed using cGMP-immunocytochemistry, that these inhibitors stimulated cGMP accumulation throughout the hippocampal slices, whereas the PDE5 inhibitor zaprinast induced a more local cGMP production, similar as sildenafil or vardenafil (Chapter 2). Furthermore, cGMP accumulation in the hippocampus slices was quantified using a classical radioimmunoassay and by semi-quantitative measurements of cGMP-immunofuorescence intensity using an image analysis system. Both methods yielded essential identical results. However, the immunocytochemical approach has the additional advantage that the structures responding to the treatment are visible and can be characterized by double immunocytochemistry using structural and neurochemical markers.

In **Chapter 4** we studied sGC activation using NO donors and the novel NO-independent sGC stimulators YC-1 and BAY 41-2272. The objective was to compare the cellular structures synthesizing cGMP in three different brain areas with NO donors of different classes, varying in the mechanism of NO release. It was observed that although the NO donors differed to some extent in their potency to stimulate cGMP production, the cGMP-responsive cellular structures were similar, i.e. mainly in varicose fibers. In the cortex, cGMP-immunoreactivity was predominantly present in cholinergic fibers after incubation of the slices with an NO donor or YC-1 or BAY 41-2272 alone. In contrast, co-administration of an NO donor with YC-1 or BAY 41-2272 extended cGMP staining to GABAergic and glutamatergic neurons. It was concluded that sGC in GABAergic and glutamaterigic cells appears to be unresponsive to NO without the presence of a NO-independent activator of sGC. Another explanation might be that sGC is desensitized rapidly

and/or that the rate of cGMP breakdown in these cells greatly exceeds the rate of cGMP synthesis induced by NO only.

In **Chapter 5**, the rat cGMP-specific PDE type 9 (PDE9) was investigated in closer detail. Structural analysis of the cloned rat PDE9 revealed that this PDE is highly homologous to the mouse and human PDE9 and contains potential domains for regulation by protein kinases, including cGMP- and cAMP-dependent protein kinases. Furthermore, the cellular mRNA distribution patterns of PDE9 were investigated in the brain. It was found that PDE9 is highly expressed mainly in neurons throughout the rat and mouse brain, including strong expression in cerebellum and hippocampus.

Chapter 6 describes a comparison of the mRNA localization patterns of three cGMP-hydrolyzing PDE families, i.e. PDE2, PDE5 and PDE9, during development of the rat brain. PDE9 was the most abundantly expressed PDE throughout the brain, followed by PDE2 and PDE5. The expression patterns of each of the PDE families was maintained during the development of the brain, although in some cell types PDE expression was absent in early stages. For the first time it was demonstrated that PDE5 is expressed in other brain areas besides the cerebellum, as had been described previously, and was present in areas such as cortex and hippocampus. The results indicate that some cell types, such as the hippocampal pyramidal cells and the Purkinje cells in the cerebellum co-express the mRNAs of all three cGMP-degrading PDE families.

In **Chapter 7** a comparison was made between the localization of NO-mediated cGMP signaling in the mouse and rat hippocampus. It was found that the localization patterns of cGMP-immunoreactivity differ between the two species. In mouse hippocampus, cGMP staining is predominantly present in astrocytes, whereas cGMP is present mainly in varicose fibers in rat hippocampal slices. To investigate if this difference was due to a differential expression of components of cGMP signaling, the localization of the beta1 subunit of sGC and of PDE2, PDE5 and PDE9, three cGMP-hydrolyzing PDEs, was studied in rat and mouse hippocampus. It was concluded that although cGMP-staining patterns differ between rat and mouse hippocampus, localization patterns of sGC and PDE2, PDE5 and PDE9 mRNAs are similar in both species. These data point to that care should be taken when extrapolating results of experiments of NO-cGMP signaling in different species.

Final conclusions

Administration of PDE5 inhibitors to rats leads to an enhanced performance in an object recognition task. To obtain more insight in the mechanism of action of PDE5 inhibitors, these inhibitors in combination with inhibitors of other PDE families were investigated using *in vitro* incubation of brain slices. The method of *in vitro* incubation of slices is an appropriate method to investigate whether a compound of interest can stimulate cyclic nucleotide (cGMP and cAMP) levels in the brain and in which cellular structures. However, it can be concluded that this method does not provide a straightforward answer to the question whether the observed memory improvement after *in vivo* application of a PDE5 inhibitor is due to an increase in cGMP levels through PDE5 inhibition. This is because an effect on cGMP levels was observed at relative high concentrations of the PDE5 inhibitor. At these high inhibitor concentrations, other proteins such as other members of the PDE family will possibly be inhibited in addition to PDE5. Since the final concentration of a PDE5 inhibitor in the brain, or intracellular is not known after *in vivo* administration, a direct correlation between the *in vitro* and *in vivo* data is difficult. This situation is even more complex as we also have to take into account the expression of multiple members of various PDE families in one cell type, and/or cellular compartment, and the differential regulation of each PDE family by events such as phosphorylation, and regulation by cGMP and/or cAMP. Nevertheless, the observed memory enhancing effect by PDE5 inhibitors holds promise for future therapeutic use as cognition enhancers and, it should therefore be investigated whether these compounds can also improve cognitive performance in humans.

Samenvatting

Het doel van de studies beschreven in dit proefschrift was een onderzoek te verrichten aan de stikstof monoxide (NO)-gestimuleerde guanosine 3',5'-cyclic monophosphate (cGMP) vorming in de hersenen, in relatie tot leren en geheugen. NO-geïnduceerde cGMP productie wordt aangetroffen door het gehele brein. Een vijftal families van eiwitten speelt een belangrijke rol bij de regulatie van het NO-cGMP signaaloverdracht systeem. NO synthase (NOS) zorgt voor de vorming van NO, dat vervolgens het enzym genaamd oplosbaar guanylyl cyclase (sGC) kan activeren tot de vorming van cGMP. Binding van NO aan het sGC kan leiden tot een honderdvoudige stimulatie van de cGMP productie. Het gevormde cGMP speelt een belangrijke rol als een boodschapper molecuul in cellen en daarom moeten cGMP niveaus intracellulair sterk gereguleerd worden. cGMP gehalten worden voor een groot gedeelte bepaald door de activiteit van cGMP-afbrekende enzymen, de zogeheten 3',5'-cyclic nucleotide fosfodiesterases (PDEs). Op dit moment zijn elf verschillende PDE families geïdentificeerd, waarbij het totale aantal verschillende vormen binnen deze familie geschat wordt op vijfennegentig. Daarnaast zijn bij de cGMP signaaloverdracht nog andere families betrokken, waaronder de cGMP-afhankelijke proteïne kinasen en de cGMP-gereguleerde ionkanalen.

Het feit dat verschillende eiwitfamilies betrokken zijn bij NO-cGMP signaaloverdracht die tot expressie gebracht worden in verschillende celtypen en hersengebieden, wijst erop dat dit signaaloverdracht systeem betrokken is bij een groot aantal hersenfuncties. In dit proefschrift werd onderzoek gedaan naar de rol van NO-cGMP signaalroute in leren en geheugen. De reden hiervoor was dat eerdere studies door de vakgroep en externe onderzoeksgroepen hadden aangetoond dat remming van de NO vorming een negatief effect heeft op leren en geheugen taken in een aantal diersoorten. In tegenstelling, toediening van een stof die het type 5 van de PDE familie (PDE5) kan remmen, een PDE5 remmer, resulteerde in een verbetering van de prestatie in een geheugentaak bij ratten, namelijk in de object herkenningstaak. Het werkingsmechanisme dat optreedt na toediening van een PDE5 remmer was onbekend tijdens de start van het in dit proefschrift beschreven onderzoeksproject. Ook was het onduidelijk waar PDE remmers in de hersenen hun effect uitoefenen. Daarom werd de lokalisatie van componenten betrokken bij NO-cGMP signaaloverdracht nader bestudeerd. Hiervoor werd ondermeer gebruik gemaakt van het testen van nieuw ontwikkelde PDE5 remmers. Speciale aandacht werd gegeven aan het bestuderen van de lokalisatie van PDE5 in combinatie met verscheidene andere cGMP-afbrekende PDE families.