ELSEVIER

Brain Research 888 (2001) 275–286

BRAIN RESEARCH

www.elsevier.com/locate/bres

Research report

The effects of phosphodiesterase inhibition on cyclic GMP and cyclic AMP accumulation in the hippocampus of the rat

W.C.G. van Staveren*, M. Markerink-van Ittersum, H.W.M. Steinbusch, J. de Vente

Maastricht University, Department of Psychiatry and Neuropsychology, Universiteitssingel 50, POB 616, 6200 MD Maastricht, The Netherlands

Accepted 3 October 2000

Abstract

The effects of selective and non-selective 3′,5′-cyclic nucleotide phosphodiesterase (PDE) inhibitors on cGMP and cAMP accumulation were studied in rat hippocampal slices incubated in vitro. The following PDE inhibitors were used: vinpocetine and calmidazolium (PDE1 selective), erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA, PDE2 selective), SK&F 95654 (PDE3 selective), rolipram (PDE4 selective), SK&F 96231 (PDE5 selective), the mixed type inhibitors zaprinast and dipyridamole, and the non-selective inhibitors 3-isobutyl-1-metylxanthine (IBMX) and caffeine. cGMP levels were increased in the presence of different concentrations of IBMX, EHNA, dipyridamole, vinpocetine and rolipram. cGMP immunocytochemistry showed that incubation with different inhibitors in the presence and/or absence of sodium nitroprusside resulted in pronounced differences in the extent and regional localization of the cGMP response and indicate that PDE activity in the hippocampus is high and diverse in nature. The results suggest an interaction between cGMP and cAMP signalling pathways in astrocytes of the rat hippocampus.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Second messengers and phosphorylation

Keywords: Phosphodiesterase; cGMP; cAMP; Hippocampus; Nitric oxide; Inhibitor

1. Introduction

Nitric oxide synthase (NOS) has a widespread distribution in the central nervous system (CNS) [4,46]. In the hippocampus, two constitutive isoforms of NOS have been described, i.e. the neuronal NOS (nNOS) and the endothelial NOS (eNOS), also referred as NOS-I and NOS-III respectively [13]. In this brain area, both isoforms are activated through calcium/calmodulin dependent pathways, which are triggered by the *N*-methyl-D-aspartate (NMDA)-type glutamate receptors [21]. NO diffuses from the site of synthesis to the target structures which are neurons and astrocytes close to the site of the NO production [10,20,21,29,35]. The soluble isoform of guanylyl cyclase (sGC) presents an important target molecule for NO [5,28]. NO activates sGC by binding to the heme group of this enzyme which leads to an increased cGMP synthesis. cGMP and the other second messenger cAMP are inactivated by hydrolytic cleavage of their 3′-phosphoester bonds to form 5′-GMP and 5′-AMP by the superfamily of enzymes known as the cyclic nucleotide phosphodiesterases (PDEs). At present, the PDEs have been classified into 11 different families, i.e. PDE1 to PDE11, based on their substrate and inhibitor profiles together with their structural characteristics [1,6,14,43]. Within families multiple splice variants of those isozymes exist which makes the number of PDE isozymes more than 30 [1,23]. A number of these enzymes were shown to be localized regionally [2,19,24,25,27,31,36–39,47] and may be expressed to different degrees even within one cell type [1,25].

From previous studies it is known that the PDE activity in the hippocampus is high (e.g. [12]). We also found evidence for the presence of cGMP-hydrolyzing PDE activity in the hippocampus which is not or only partly inhibited by IBMX [12]. In addition, it was shown in brain slices that zaprinast, an inhibitor of cGMP-specific PDE activity (PDE5 and PDE9), increased NO-mediated cGMP

*Corresponding author. Tel.: +31-43-388-1168; fax: +31-43-367-1096.

E-mail address: w.vanstaveren@np.unimaas.nl (W.C.G. van Staveren).

0006-8993/01/$ – see front matter
PII: S0006-8993(00)03081-X

accumulation especially in the CA2/CA3 region and the stratum lacunosum moleculare of the hippocampus. In contrast, IBMX, a non-selective PDE inhibitor, increased cGMP levels in varicose fibers and astrocytes throughout the hippocampus [11]. Thus, the choice of the PDE inhibitor is a very important factor when cyclic nucleotide levels are studied in complex tissues. In this respect it is striking that in the canine proximal colon only a combination of zaprinast and IBMX was effective in inhibiting NO-mediated cGMP in smooth muscle cells [40].

In order to study the possibilities of locally increasing cyclic nucleotide levels using selective inhibitors of PDE activity [11], we measured the effects on cGMP and cAMP accumulation in hippocampal slices, after the incubation with a number of PDE inhibitors with different selectivity profiles. In addition, we visualized the effect of these inhibitors on the accumulation of NO-mediated cGMP synthesis using cGMP immunocytochemistry. A comparison was made between the determination of cGMP levels by the use of a radioimmunoassay and by the evaluation of cGMP immunofluorescence intensity using an image analysis system.

2. Materials and methods

2.1. Animals

Experiments were performed on hippocampal slices obtained from adult male Lewis rats (200–240 g). The effect of zaprinast on two different rat strains was studied in female Lewis and Wistar rats (obtained from Charles River). The animals were housed under standard conditions at the local animal facility. All experiments were approved by the committee on animal welfare according to Dutch governmental rules.

2.2. Tissue preparation

Animals were decapitated and their brains were immediately removed. Brains were transferred to ice-cold Krebs–Ringer bicarbonate buffer (Krebs incubation buffer) of the following composition: 121.1 mM NaCl, 1.87 mM KCl, 1.17 mM KH_2PO_4, 1.15 mM $MgSO_4.7H_2O$, 24.9 mM $NaHCO_3$, 2.0 mM $CaCl_2.2H_2O$ and 11.0 mM glucose.

Hippocampal slices (400 μm) were prepared as described previously [10]. Slices were incubated in Krebs incubation buffer at 36°C, under an atmosphere of 95% O_2 and 5% CO_2 at pH 7.4. The incubations lasted 40 min; when appropriate, the last 10 min in the presence of the NO donor sodium nitroprusside (SNP) in a concentration of 0.1 mM. The PDE inhibitors were present from the start of the incubation and were added in different concentrations to the slices. Vinpocetine, calmidazolium, EHNA, SK&F 96231, SK&F 95654, rolipram, dipyridamole and zaprinast had to be dissolved in dimethylsulfoxide (DMSO), therefore a final concentration of 1% DMSO was present in all experiments. In an earlier study it was found that 1% DMSO had no effect on cGMP levels in hippocampal slices (unpublished results).

2.2.1. Radioimmunoassay

cGMP and cAMP levels were determined in individual hippocampal slices, incubated as described above, using a radioimmunoassay as published previously [9]. Briefly, the incubations were terminated by placing the slices into a solution of 5% trichloroacetic acid. Subsequently, the samples were sonicated and centrifuged. The supernatant was used for the determination of the cGMP and cAMP content, measured by a radioimmunoassay according to Steiner and coworkers [44]. The pellet was used for the analysis of the protein content according to Lowry and coworkers [30].

Each condition was measured in two different slices per animal and for each PDE inhibitor three different rats were tested. In each slice, both cGMP and cAMP levels were measured in triplicate and the median was taken for each sample. In the radioimmunoassay, a level of 0.6 fmol cGMP and 5 fmol cAMP could be detected. cGMP and cAMP levels were corrected for the protein content of each slice.

2.3. Immunocytochemistry

After the incubation, slices were fixed with ice-cold fixative solution of 4% freshly prepared depolymerised paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at 4°C. The slices were then fixed for another 90 min with 4% paraformaldehyde containing 10% sucrose. After washing overnight at 4°C in 0.1 M phosphate buffer (pH 7.4) containing 10% sucrose, the slices were frozen in CO_2. Cryostat sections (10 μm) were cut, thawed onto chrome–alumn/gelatin coated slides and processed for immunocytochemistry.

Frozen sections were dried for 20 min at room temperature, followed by three 5 min washes with Tris-buffered saline (TBS). Sections were incubated overnight at 4°C with sheep anti-formaldehyde fixed cGMP, diluted 1:4000 in TBS containing 0.3% Triton X-100 (TBS-T). The specificity of this antibody has been detailed elsewhere [10]. Washing consisted of 10 min of TBS, followed by TBS-T and another 10 min of washing with TBS at room temperature. The primary antibody was visualized by the incubation of sections for 1 h at room temperature with Fluorescein (FITC)–conjugated rabbit anti-sheep immunoglobulins (Jackson), diluted 1:30 in TBS-T. After having been washed, sections were mounted and studied with an Olympus AX-70 microscope, equipped with a narrow band MNIBA-type FITC filter, or a MNG filter for Cy3 fluorescence (Chroma Technology Corporation). These filters made it possible to photograph FITC or Cy3 fluorescence

without any bleeding of the other fluorescent marker through the filter [10].

Glial fibrillary acidic protein (GFAP) was stained with mouse anti-GFAP serum (Innogenetics), diluted 1:10 in TBS-T and visualised with donkey anti-mouse Cy3 (Jackson), diluted 1:800 in TBS-T.

2.4. Image analysis

For the semi-quantitative measurement of cGMP, sections were stained for cGMP as described above and the primary antibody was visualised by the incubation of sections for 1 h at room temperature with the Alexa 488 donkey anti-sheep IgG (H+L) conjugate (Molecular Probes), diluted 1:100 in TBS-T. Each condition was tested in hippocampal slices obtained from three different animals and from each slice, three different sections of the hippocampal area were studied per animal. All sections were stained and analysed at the same time under standard conditions. Pictures of the stratum lacunosum moleculare and the CA1 area were made at a magnification of 20× using a Sony Power HAD 3CCD Color Video Camera. All pictures were analysed with the computer program analySIS Vers. 3.0. For each image, a color separation of the green image was done and the mean grayvalue of each area was estimated as a measure for the cGMP content of the hippocampal area. All measurements were corrected for control sections which were incubated without the primary antibody.

2.5. Statistical analysis

To determine whether different concentrations of the PDE inhibitor tested, differed from its control, a Student Newman–Keuls test and a Student *t*-test were used.

2.6. Chemicals

IBMX was from Janssen Chimica; zaprinast, dipyridamole and EHNA from Sigma; rolipram and calmidazolium from RBI; vinpocetine from Tocris; SK&F 96231 and SK&F 95654 were kindly donated by SmithKline Beecham. L-NAME and SNP were obtained from Fluka.

3. Results

3.1. cGMP levels in hippocampal slices after incubation with different PDE inhibitors

The effect of different PDE inhibitors on cGMP levels in hippocampal slices measured by a radioimmunoassay is shown in Fig. 1. In the absence of a PDE inhibitor, the cGMP content of the hippocampal slice was 0.42±0.08 pmol/mg protein and rose till 1.77±0.17 pmol/mg protein in the presence of 0.1 mM SNP (mean±S.E.M.; significantly different from control (0 M) without SNP, Student *t*-test, $P<0.01$). As shown in Fig. 1, in the absence of SNP the cGMP content in hippocampal slices was increased by IBMX (1 mM), EHNA (100 μM), dipyridamole (100 μM) and vinpocetine (1 μM and higher) compared to its controls. No changes in cGMP levels were found when slices were incubated with rolipram or zaprinast.

Incubation of slices with 0.1 mM SNP in the presence of different concentrations of IBMX, EHNA or dipyridamole, resulted in a concentration dependent increase of cGMP levels. Rolipram increased cGMP levels significantly at 1 μM and 100 μM and the cGMP content was only raised in the presence of the highest dose of zaprinast (100 μM). On a molar basis EHNA and dipyridamole appeared to be the most potent inhibitors. In the presence of SNP we did not find a significant increase in cGMP accumulation by vinpocetine (Fig. 1) or calmidazolium (not shown).

3.2. cAMP levels in hippocampal slices after incubation with different PDE inhibitors

As shown in Fig. 2, in the absence of PDE inhibitors the basal cAMP content of hippocampal slices was 37.94±4.94 pmol/mg protein (determined in six animals). This is in the range of reported hippocampal levels [17]. Rolipram strongly increased cAMP levels while no effect on the cAMP content was found in the presence of zaprinast, dipyridamole or IBMX. There was no effect of SNP on cAMP levels regardless of the PDE inhibitors being present. Incubation of hippocampal slices with 10 μM forskolin (an activator of adenylyl cyclase) or 10 μM noradrenalin, both in the presence of IBMX, resulted in large increases of cAMP levels.

3.3. cGMP immunostaining after incubation with different PDE inhibitors

When hippocampal slices were incubated in vitro without PDE inhibitors, cGMP immunostaining was nearly absent (Fig. 3A). No effect on cGMP immunocytochemistry was observed when slices were treated with vinpocetine, calmidazolium, SK&F 95654, SK&F 96231 or zaprinast (data not shown). In the presence of 1 mM IBMX isolated fibers were observed distributed at random throughout the hippocampal slice, with a cluster of thin, punctate fibers in the stratum lacunosum moleculare (data not shown). The NOS inhibitor N^G-nitro-L-arginine (L-NAME) abolished the effect of IBMX (not shown). When slices were incubated with 0.1 mM EHNA (Fig. 3C) a similar staining pattern was seen as with IBMX incubation.

Rolipram (0.1 mM) increased cGMP in astrocytes weakly (Fig. 3E and 3F); this effect might be observed in any region of the hippocampus. Incubation of the slices in the presence of L-NAME did not have an effect on

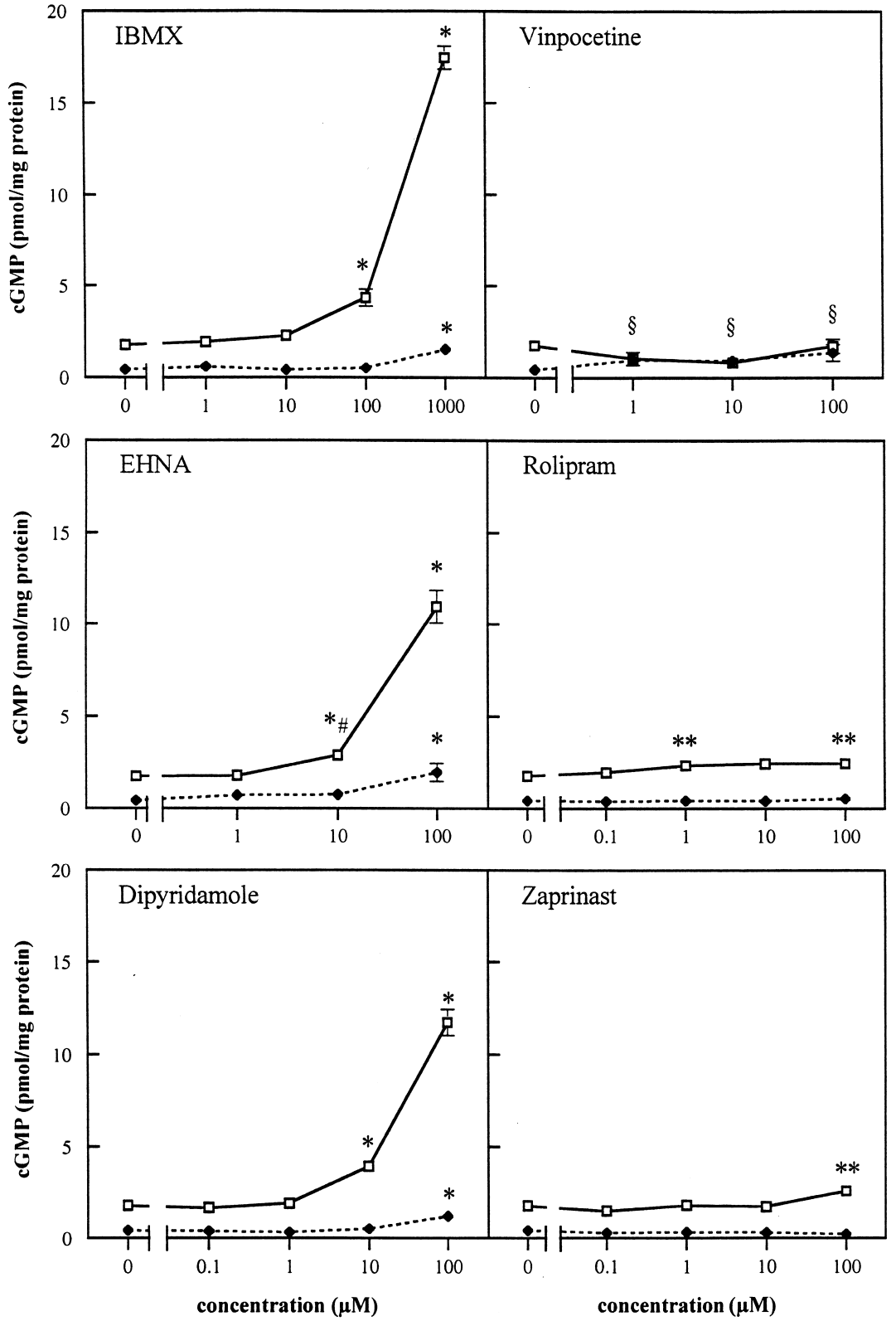

Fig. 1. Effect of PDE inhibitors on cyclic GMP levels in hippocampal slices of the rat, in the absence (♦- - -♦) or presence (□——□) of 0.1 mM SNP, measured by a radioimmunoassay. Each value is the mean (±S.E.M.) of three animals. Each concentration was performed in duplicate per animal and assayed in triplicate in a radioimmunoassay. Values which were different from control (0 M) (Student Newman–Keuls test; $P<0.01$) were marked with an asterisk (*). Values which were different from control tested with a Student *t*-test were marked as **($P<0.05$) and *#($P<0.01$). §, different from control without SNP ($P<0.05$).

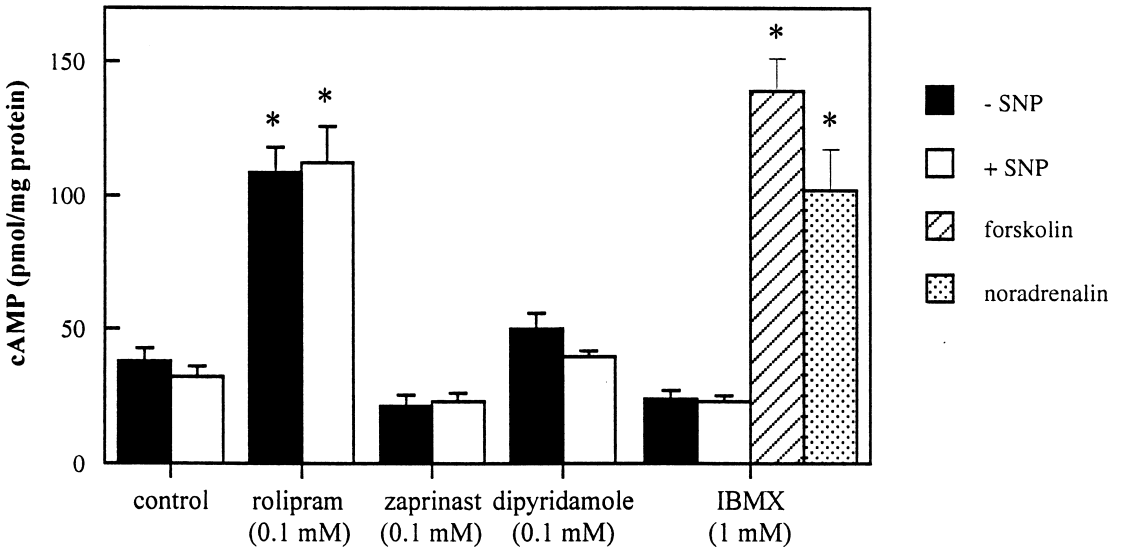

Fig. 2. Effect of PDE inhibitors on cAMP levels in hippocampal slices of the rat, in the presence or absence of 0.1 mM SNP, measured by a radioimmunoassay. Forskolin and noradrenalin were both used in a concentration of 10 μM. Each value is the mean (+S.E.M.) of three animals. Each concentration was performed in duplicate per animal and assayed in triplicate in a radioimmunoassay. Values which were different from control (Student *t*-test; $P<0.01$) were marked with an asterisk.

rolipram induced cGMP immunostaining (data not shown). Dipyridamole (0.1 mM) strongly increased cGMP in the smooth muscle layer of what appeared to be the larger blood vessels of the hippocampus (Fig. 3D).

3.4. cGMP accumulation after stimulation of sGC by SNP in the presence of PDE inhibitors

Incubation of hippocampal slices with 0.1 mM SNP in the absence of PDE inhibitors, showed a few intensely stained fibers which, taking into account all experiments (>10), might be observed in any region of the hippocampus (not shown). cGMP immunocytochemistry after in vitro incubation of the slices in the presence of IBMX and SNP has been described previously (e.g. [11]). This combination of drugs resulted in cGMP accumulation in a multitude of varicose fibers throughout the hippocampus (Fig. 3B). Similar staining patterns were observed when EHNA or dipyridamole were used (Fig. 4B and 4G). In contrast, the combination of SNP with the inhibitors zaprinast (Fig. 4E) or SK&F 96231 (Fig. 4F) increased cGMP immunostaining in neuronal fibers, especially in the CA2/CA3 region and the stratum lacunosum moleculare. Astrocytes and a few neuronal cells were found to be cGMP immunopositive after incubation of the slices with IBMX, EHNA, zaprinast or dipyridamole in the presence of SNP. The effects of combinations of calmidazolium (data not shown), vinpocetine (Fig. 4A), SK&F 95654 (Fig. 4C) with SNP were not different from the effect of SNP alone.

When different concentrations of EHNA or dipyridamole in combination with SNP were tested, cGMP immunostaining was still visible at low concentrations (Fig. 5). In contrast, in the radioimmunoassay the effect of 1 μM of these inhibitors on the cGMP content was not significantly different from controls (Fig. 1).

Incubation of slices with rolipram and SNP resulted in cGMP immunostaining in astrocyte-like cells and a few isolated varicose fibers scattered throughout the hippocampus (Fig. 4D). Using double immunostaining of cGMP in combination with GFAP, these cells could indeed be identified as astrocytes (see also Fig. 3E and 3F). When slices were incubated with rolipram combined with IBMX and SNP, cGMP staining was observed similar as in the case of IBMX and SNP, although the cGMP immunostaining in the astrocytes was more pronounced (Fig. 6A). Strikingly, only a subpopulation of astrocytes showed cGMP immunoreactivity (Fig. 6B).

3.5. Image analysis of cGMP immunostaining after treatment with IBMX or zaprinast

Hippocampal slices were incubated with different concentrations of IBMX or zaprinast (1 mM to 0.1 μM) and the effect on cGMP immunostaining in the stratum lacunosum moleculare and the CA1 area was measured by a semi-quantitative image analysis (Fig. 7). In the absence of SNP, the cGMP content in the stratum lacunosum moleculare was increased at the highest concentration of IBMX (1 mM) compared to control (0 M) (Student Newman–Keuls test, $P<0.01$), while no effect was measured of this dose in the CA1 area. Incubation of slices with different concentrations IBMX in the presence of SNP, resulted in a concentration dependent increase in cGMP immunostaining in the stratum lacunosum molecu-

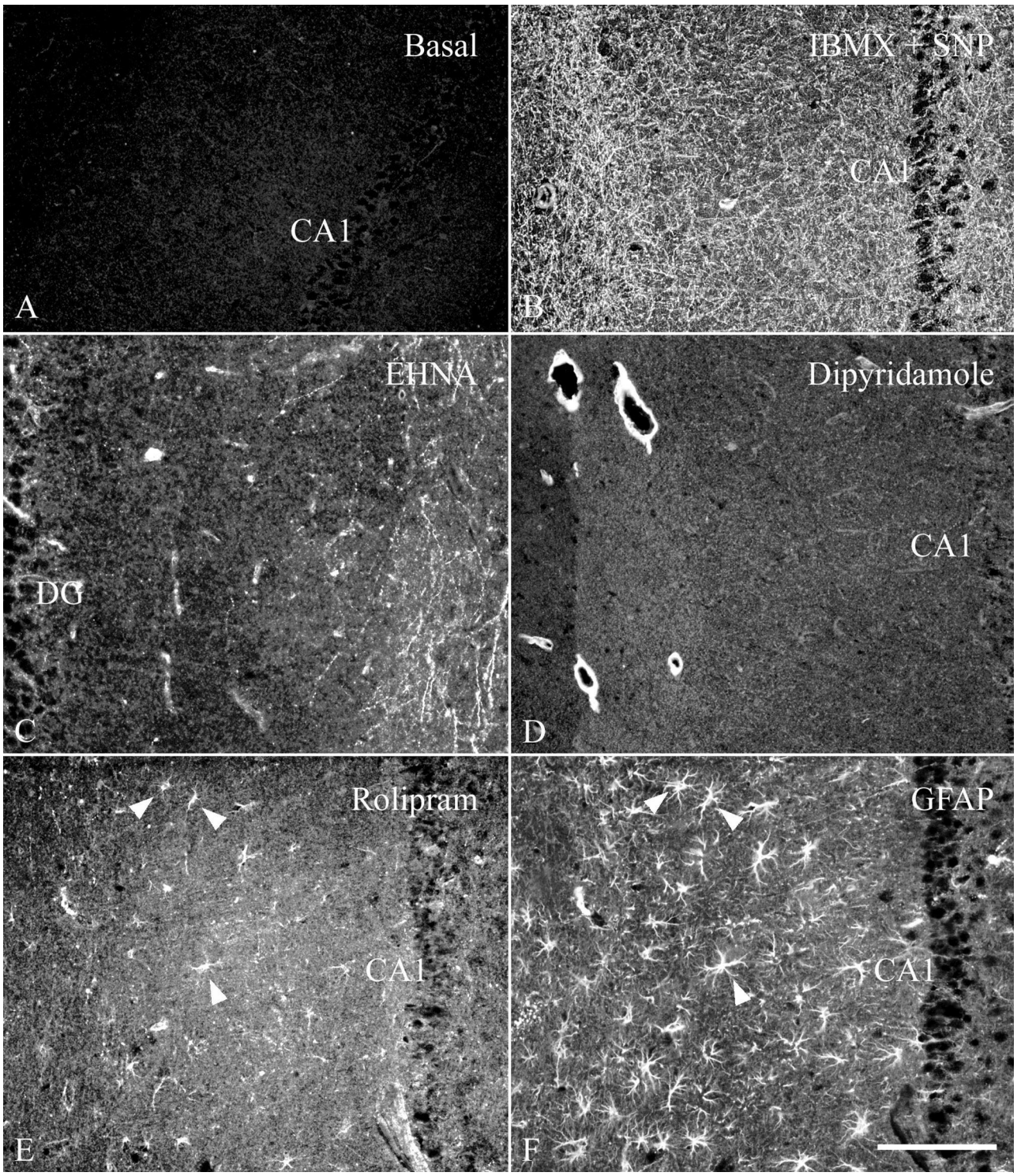

Fig. 3. Localization of cGMP immunoreactivity in hippocampal slices incubated in vitro: (A) in the presence of 1 mM IBMX; (B) combination of 1 mM IBMX and 0.1 mM SNP; (C) 0.1 mM EHNA; (D) 0.1 mM dipyridamole; (E) 0.1 mM rolipram. The immunostaining in (F) with GFAP shows the same field as depicted in (E) indicating that rolipram increases cGMP in astrocytes (arrowheads). DG, dentate gyrus. Bar represents 100 μm for all pictures.

lare. In the CA1 area, a raised cGMP level was only found at the highest dose of IBMX used (1 mM).

In the absence of SNP, no effect of zaprinast could be detected in the stratum lacunosum moleculare or the CA1 area. When slices were treated with SNP and different concentrations of zaprinast, a significant increase in the intensity of the cGMP immunostaining was detected in both areas at the highest dose tested (1 mM).

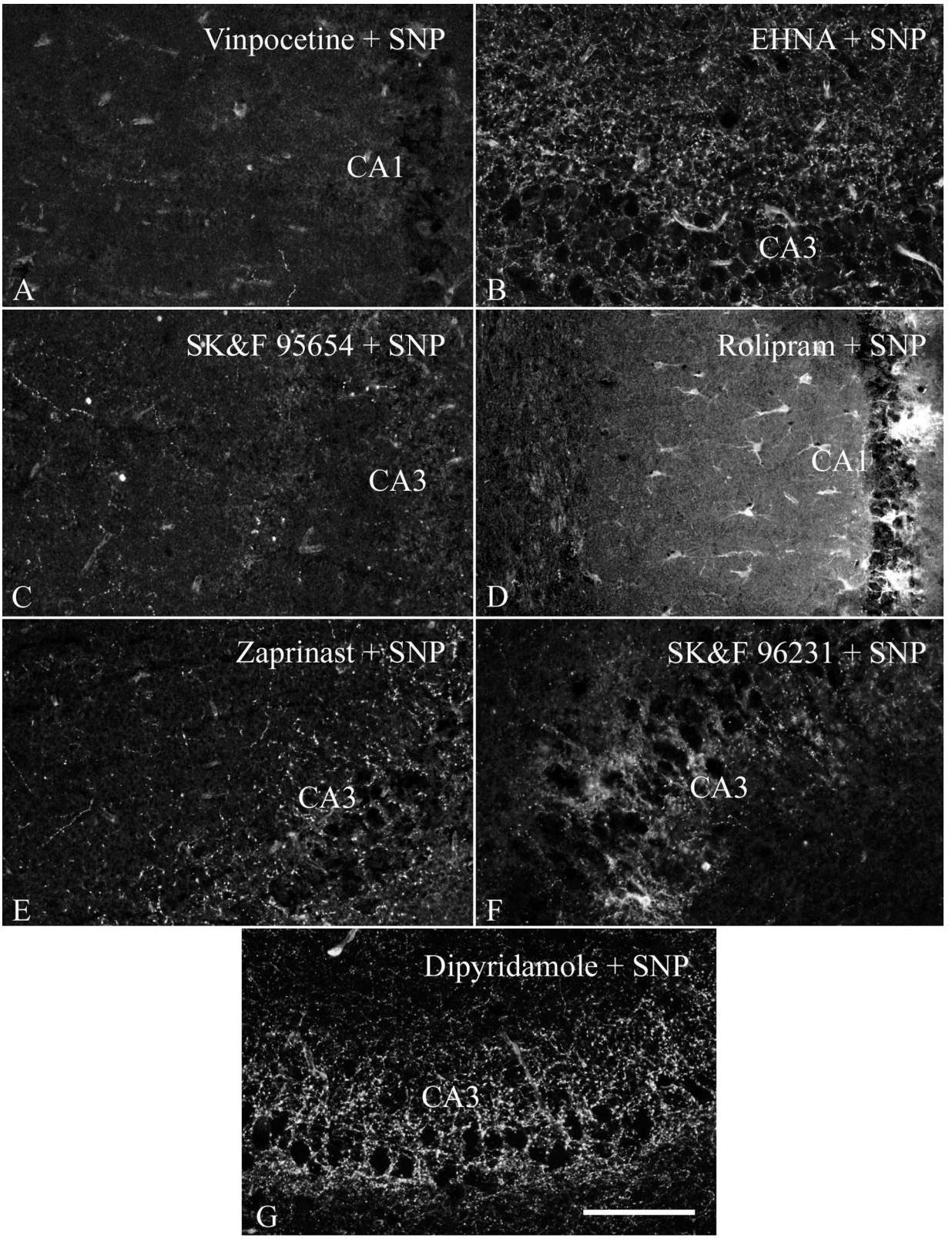

Fig. 4. Localization of cGMP immunoreactivity in hippocampal slices incubated in the presence of 0.1 mM SNP and: (A) 0.1 mM vinpocetine; (B) 0.1 mM EHNA; (C) 0.1 mM SKF 95654; (D) 0.1 mM rolipram; (E) 0.1 mM zaprinast; (F) 0.1 mM SKF 96231; (G) 0.1 mM dipyridamole. Bar represents 100 μm for all pictures.

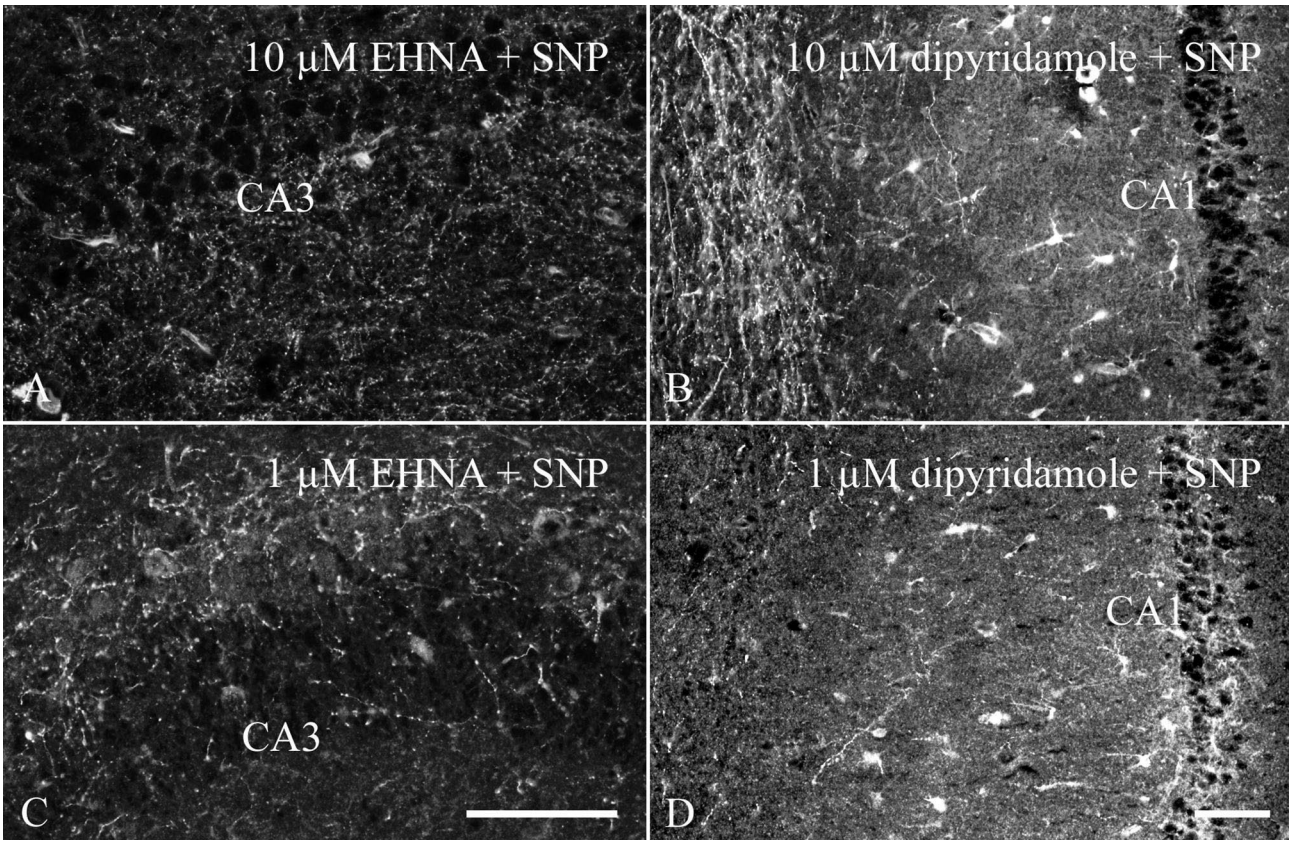

Fig. 5. Localization of cGMP immunoreactivity in the hippocampal slice incubated in the presence of 0.1 mM SNP and 1–10 μM of EHNA or dipyridamole. Bar (c) represents 100 μm for all pictures.

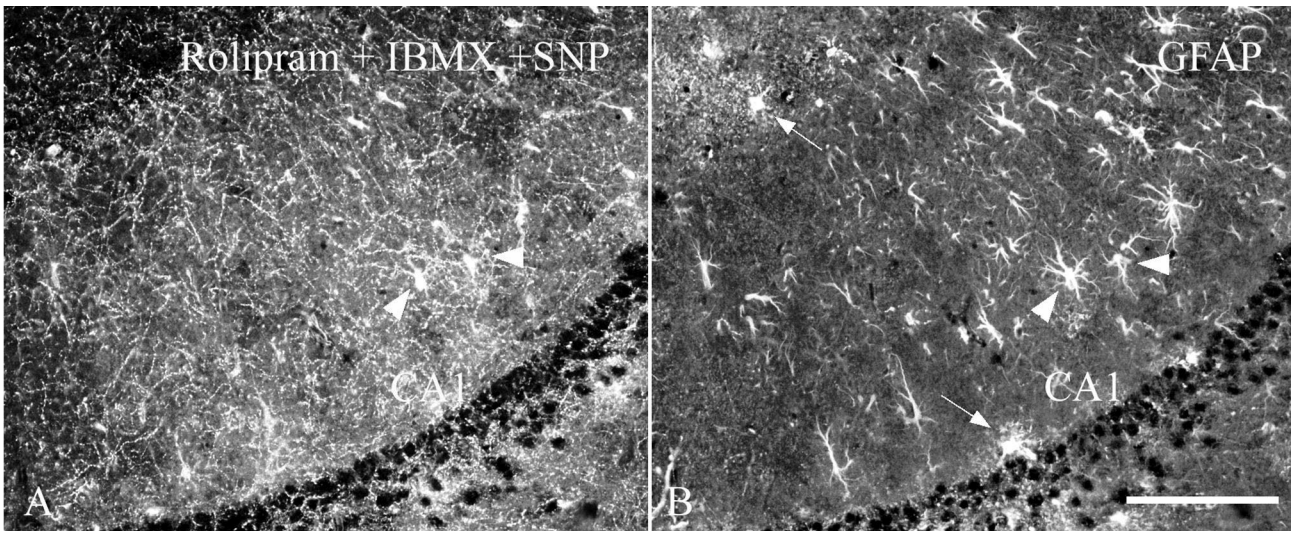

Fig. 6. Localization of cGMP immunoreactivity (A) in the hippocampal slice incubated in the presence of 0.1 mM SNP and 0.1 mM rolipram in combination with 1 mM IBMX. GFAP immunostaining in (B) shows the same field as in (A) indicating the cGMP accumulation in a subpopulation of astrocytes. Arrowheads present colocalization of cGMP and GFAP in astrocytes, arrows indicate astrocytes not stained for cGMP. Bar represents 100 μm for both pictures.

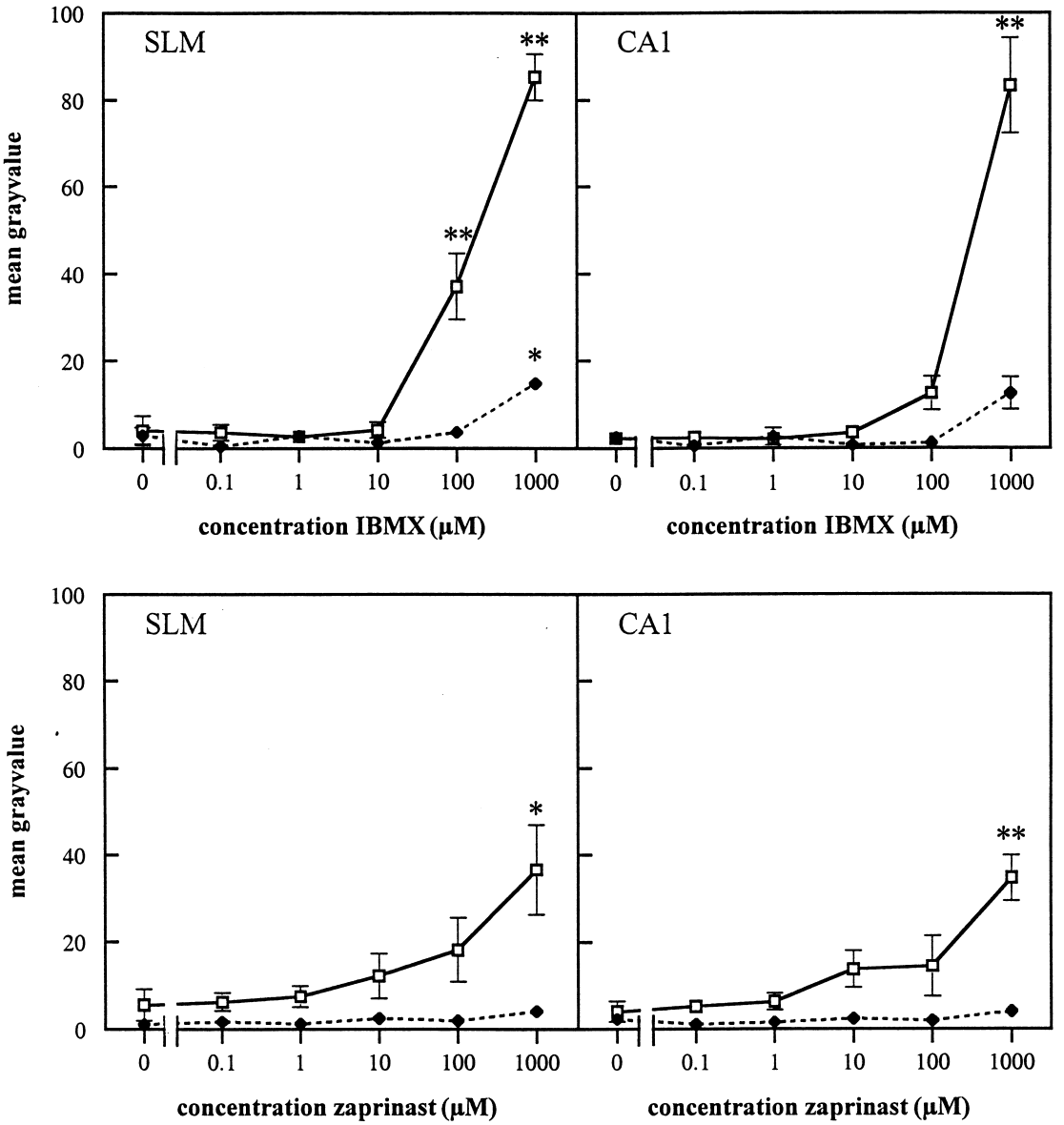

Fig. 7. The effect of IBMX and zaprinast on the fluorescence intensity of the cGMP immunostaining in the stratum lacunosum moleculare (SLM) and the CA1 area of hippocampal slices of the rat, in the absence (♦- - -♦) or presence (□——□) of 0.1 mM SNP, measured by image analysis. Each value is the mean (±S.E.M.) of three animals. For each concentration three different sections were analysed per animal. Values which were different from control (0 M) (Student Newman–Keuls test) were marked as *($P<0.05$) and **($P<0.01$).

4. Discussion

In this study, the effects of different PDE inhibitors on cGMP and cAMP levels in the hippocampus were investigated. In addition, we used cGMP immunocytochemistry to localize the structures synthesising cGMP in the presence of PDE inhibitors and an NO donor. cGMP production was quantified by two different methods, i.e. by a radioimmunoassay and by taking the immunofluorescence intensity as a measure for the cGMP content. Both methods showed a similar pattern in the dose response curves when IBMX and zaprinast were analysed. It was found that the radioimmunoassay was more sensitive than the image analysis. At low concentrations, a significant effect could be detected by the radioimmunoassay but not by the use of image analysis. However, the use of cGMP immunocytochemistry has the advantage that cGMP increases can be tracked which could otherwise escape detection. This is exemplified by Fig. 5 where it is shown that low concentrations of EHNA and dipyridamole indeed increased cGMP in varicose fibers and (probably) astrocytes, whereas in the radioimmunoassay no significant increase in cGMP was measured. Thus we decided to localize the structures which accumulate cGMP in the presence of highly selective PDE inhibitors in combination with an NO donor.

At present, several PDE types have been localized in the rat hippocampus. It has been shown with in situ hybridisation or immunocytochemistry that PDE1, PDE2, PDE3 and PDE4 are present in this brain area of the rat [19,31,37,38,45]. In an attempt to gain more insight in the localization of PDEs with different selectivity profiles, several PDE type inhibitors were used in this study. The PDE inhibitors studied include the highly selective inhibitors vinpocetine, calmidazolium (both PDE1 inhibitors), EHNA (PDE2) [34] and rolipram (PDE4). In addition, we used zaprinast and dipyridamole, which were until recently, considered to be highly selective inhibitors of PDE5 [7,32]. Recent evidence showed that zaprinast inhibited PDE9 also [16] and dipyridamole has affinity for PDE7 [22], PDE8 [15,41], PDE10 [18,42] and the recently cloned PDE11 [14]. In this study IBMX was used as a non-specific PDE inhibitor.

Selective inhibitors have a high affinity for a particular PDE. In our study, the effects in the radioimmunoassay are found at a relative high concentration of most inhibitors which could point to unspecific inhibition. However, if all the effects found would be due to unspecific reactions then it was expected that all the staining patterns were the same. Boulton and coworkers [3] reported that 2 μM zaprinast increased the cGMP content in the hippocampus. As shown in Fig. 1 we did not measure an effect of zaprinast at such low concentrations. To investigate if strain or sex differences might cause this difference we repeated the experiments using female Wistar rats, as used by Boulton and coworkers, and compared them with female Lewis rats. As shown in Table 1, we were not able to reproduce the effects that were found with zaprinast.

In literature, it has been shown that the potency of a PDE inhibitor might be higher on purified enzymes than found in cells or tissues. Coste and Grondin [7] reported differences in the potency of the PDE5 inhibitor 1,3-dimethyl-6-(2-propoxy - 5 - methanesulfonylamidophenyl) - pyrazolo[3,4d]-pyrimidin-4-(5H)-one (DMPPO) when an enzymatic PDE5 assay was compared to a cell model. A lower potency for inhibitors in a cell model or slices might be explained by cell permeability or the intracellular metabolism. These factors are not involved when the purified enzyme is used. Furthermore, it is not known whether more than one PDE type is present in one cell. Therefore, it is possible that if a certain PDE is inhibited this could have an effect on another PDE type in this cell as well, which in turn can influence the cyclic nucleotide levels also.

Using immunocytochemistry for cGMP, it was found that the largest part of the NO-mediated cGMP immunostaining was present in varicose fibers. As shown previously [11,12], inhibition of the PDE activity using non-specific PDE inhibitors as methylxanthines like IBMX (caffeine and theophylline not shown) in combination with an NO donor resulted in cGMP-accumulation in varicose fibers throughout the hippocampus. As a somewhat varying response between experiments, cGMP immunostaining was also found in astrocytes and a few interneurons under these conditions. In some experiments a very low level of cGMP immunoreactivity was possibly observed in pyramidal cells in unstimulated slices. However, in the stimulated slice the cGMP staining in these cells could not normally be observed, probably due to the intensity of the cGMP immunofluorescence in the surrounding fibers (Fig. 3B), although a redistribution of cGMP through an as yet unknown mechanism might also be involved. If cGMP immunoreactivity is to be present in cell somata in the pyramidal cell layer, as has been suggested [3], the concentration must be very low.

The cGMP immunoreactive fibers in the hippocampus have not yet been characterized in terms of the major neurotransmitters and it is known neither from which projections area(s) these fibers originate. cGMP immunostaining has been observed only in an occasional NOS immunoreactive hippocampal interneuron. The hippocampal cGMP-positive fibers in our study do not correspond in any aspect to the localization and direction of pyramidal dendritic or axonal fibers as visualized using eNOS antibodies or the modified NADPH–diaphorase stain [13].

It must be noted that EHNA, dipyridamole and IBMX exert different actions in adenosine pharmacology. EHNA is a potent inhibitor of adenosine deaminase whereas

Table 1
The effects of zaprinast and IBMX on cGMP levels (pmol/mg protein) in the absence or presence of 0.1 mM SNP in hippocampal slices from female Lewis ($n=3$) and Wistar ($n=3$) rats (means±S.E.M.)[a]

	Without SNP (pmol/mg protein)		In the presence of SNP (pmol/mg protein)	
	Lewis	Wistar	Lewis	Wistar
Control	0.03±0.16	0.96±1.19	1.25±0.54	1.33±0.51
1 μM zaprinast	N.D.	0.27±0.46	1.08±0.19	0.98±0.63
10 μM zaprinast	N.D.	0.06±0.26	1.51±0.28	1.56±0.61
100 μM zaprinast	N.D.	N.D.	2.54±0.20*	2.36±0.83
1 mM zaprinast	0.13±0.26	0.17±0.32	5.66±0.30*	5.40±1.63*
1 mM IBMX	2.69±0.32**	1.62±0.24	20.33±3.39**	15.98±3.75*

[a] All conditions were tested in duplicate per animal and assayed in triplicate in a radioimmunoassay. Values which were different from control (Student Newman–Keuls test or a Student *t*-test) were marked with *($P<0.05$) or **($P<0.01$). N.D., not detectable.

dipyridamole is an inhibitor of adenosine uptake, and IBMX is an adenosine receptor antagonist. If adenosine was involved in the cGMP response, we would expect EHNA and dipyridamole to have similar effects, because both compounds increase the adenosine concentration outside the cell (see e.g. [8]). Indeed, the cGMP immunostaining is rather similar when EHNA or dipyridamole is used in the presence of SNP. IBMX is an adenosine antagonist and, following the above reasoning, in the presence of IBMX it might be expected that the cGMP staining would be less than the immunoreactivity obtained in the presence of EHNA or dipyridamole. However, in the presence of IBMX, cGMP immunoreactivity was found in the hippocampus and was strongly increased in the presence of SNP. Furthermore, we did not observe any effect with a non-selective adenosine receptor agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) in a concentration of 0.1 mM, in the presence or absence of different PDE inhibitors on cGMP immunostaining in the hippocampus slice. Therefore, we conclude tentatively that adenosine in not involved in the cGMP-response in the hippocampus slice.

Mayer and coworkers (1992) [33] reported that especially the calcium dependent PDE1 activity is responsible for cGMP breakdown in the rat brain. Although we did observe a small increase in cGMP levels in the presence of vinpocetine alone, no effect was measured in the presence of SNP and vinpocetine. In addition, we did not find any effect of calmidazolium on cGMP levels in the hippocampus, the frontal brain, or the cerebellum of the rat. Under the conditions used, our results indicate that the inhibition of PDE1 does not have a major effect on cGMP breakdown in the hippocampus. In addition, no effect was found of the inhibition of PDE3 by SK&F 95654 in our study also.

The results obtained with rolipram were unexpected. It was found that rolipram influenced both cGMP and cAMP levels. Rolipram alone increased the cAMP concentration in the hippocampus slice till a very high level, whereas a small raise in the cGMP content was detected also. When the effect of rolipram was studied by immunocytochemistry, cGMP positive astrocytes were found. These data suggest an interaction between cGMP and cAMP signalling pathways in the hippocampus, resulting in a massive increase in cAMP and a more restricted increase in cGMP in a subpopulation of astrocytes. This effect of rolipram on cGMP levels does not appear to be a general effect in all brain tissue as we found little, if any, cGMP immunostaining in frontal cortical areas after incubation of slices in the presence of rolipram and SNP (not shown). Nevertheless, this observation is not completely without precedent, because recently it was reported that rolipram increased cGMP levels in endothelial cells through an NO-dependent mechanism [26]. A difference between our results and the observations of Kessler and Lugnier [26] is that the effect of rolipram on cGMP levels in the hippocampus was unaffected by the presence of L-NAME, suggesting that NO was not involved.

Our results permit the following conclusions. First, our results indicate that several PDE isoforms are present in the rat hippocampus and function as part in the NO–cGMP signal transduction pathway. Nevertheless, the non-selective component in the pharmacological profile of the inhibitors used in the present study, makes it unlikely that the approach of cGMP immunocytochemistry can be used for further characterization of the localization of PDE isoforms. Secondly, the results obtained with rolipram strongly suggest that PDE4 is present in a subpopulation of astrocytes in the hippocampus.

References

[1] J.A. Beavo, Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms, Physiol. Rev. 75 (1995) 725–748.

[2] M.L. Billingsley, J.W. Polli, C.D. Balaban, R.L. Kincaid, Developmental expression of calmodulin-dependent cyclic nucleotide phosphodiesterase in rat brain, Dev. Brain Res. 53 (1990) 253–263.

[3] C.L. Boulton, A.J. Irving, E. Southam, B. Potier, J. Garthwaite, G.L. Collingridge, The nitric oxide-cyclic GMP pathway and synaptic depression in rat hippocampal slices, Eur. J. Neurosci. 6 (1994) 1528–1535.

[4] D.S. Bredt, P.M. Hwang, S.H. Snyder, Localization of nitric oxide synthase indicating a neural role for nitric oxide, Nature 347 (1990) 768–770.

[5] D.S. Bredt, S.H. Snyder, Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum, Proc. Natl. Acad. Sci. USA 86 (1989) 9030–9033.

[6] M. Conti, S.L.C. Jin, The molecular biology of cyclic nucleotide phosphodiesterases, Prog. Nucl. Acid Res. Mol. Biol. 63 (1999) 1–38.

[7] H. Coste, P. Grondin, Characterization of a novel potent and specific inhibitor of type V phosphodiesterase, Biochem. Pharmacol. 50 (1995) 1577–1585.

[8] R.A. Cunha, A.M. Sebastiao, J.A. Ribeiro, Inhibition by ATP of hippocampal synaptic transmission requires localized extracellular catabolism by ecto-nucleotidases into adenosine and channeling to adenosine A1 receptors, J. Neurosci. 18 (1998) 1987–1995.

[9] J. De Vente, J.G.J.M. Bol, L. Hudson, J. Schipper, H.W.M. Steinbusch, Atrial natriuretic factor-responding and cyclic guanosine monophosphate (cGMP)-producing cells in the rat hippocampus: a combined micropharmacological and immunocytochemical approach, Brain Res. 446 (1988) 387–395.

[10] J. De Vente, D.A. Hopkins, M. Markerink-Van Ittersum, P.C. Emson, H.H.H.W. Schmidt, H.W.M. Steinbusch, Distribution of nitric oxide synthase and nitric oxide-receptive, cyclic GMP-producing structures in the rat brain, Neuroscience 87 (1998) 207–241.

[11] J. De Vente, D.A. Hopkins, M. Markerink-van Ittersum, H.W.M. Steinbusch, Effects of the 3′,5′-phosphodiesterase inhibitors isobutylmethylxanthine and zaprinast on NO-mediated cGMP accumulation in the hippocampus slice preparation: an immunocytochemical study, J. Chem. Neuroanat. 10 (1996) 241–248.

[12] J. De Vente, H.W.M. Steinbusch, On the stimulation of soluble and particulate guanylate cyclase in the rat brain and the involvement of nitric oxide as studied by cGMP immunocytochemistry, Acta Histochem. 92 (1992) 13–38.

[13] J.L. Dinerman, T.M. Dawson, M.J. Schell, A. Snowman, S.H. Snyder, Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity, Proc. Natl. Acad. Sci. USA 91 (1994) 4214–4218.

[14] L. Fawcett, R. Baxendale, P. Stacey, C. McGrouther, I. Harrow, S. Soderling, J. Hetman, J.A. Beavo, S.C. Phillips, Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A, Proc. Natl. Acad. Sci. USA 97 (2000) 3702–3707.
[15] D.A. Fisher, J.F. Smith, J.S. Pillar, S.H. St Denis, J.B. Cheng, Isolation and characterization of PDE8A, a novel human cAMP-specific phosphodiesterase, Biochem. Biophys. Res. Commun. 246 (1998) 570–577.
[16] D.A. Fisher, J.F. Smith, J.S. Pillar, S.H. St Denis, J.B. Cheng, Isolation and characterization of PDE9A, a novel human cGMP-specific phosphodiesterase, J. Biol. Chem. 273 (1998) 15559–155564.
[17] J.C. Fowler, J.M. O'Donnell, Antagonism of the responses to isoproterenol in the rat hippocampal slice with subtype-selective antagonists, Eur. J. Pharmacol. 153 (1988) 105–110.
[18] K. Fujishige, J. Kotera, H. Michibata, K. Yuasa, S. Takebayashi, K. Okumura, K. Omori, Cloning and characterization of a novel human phosphodiesterase that hydrolyses both cAMP and cGMP (PDE10A), J. Biol. Chem. 274 (1999) 18438–18445.
[19] T. Furuyama, Y. Iwahashi, Y. Tano, H. Takagi, S. Inagaki, Localization of 63-kDa calmodulin-stimulated phosphodiesterase mRNA in the rat brain by in situ hybridization histochemistry, Mol. Brain Res. 26 (1994) 331–336.
[20] J. Garthwaite, Glutamate, nitric oxide and cell–cell signalling in the nervous system, Trends Neurosci. 14 (1991) 60–67.
[21] J. Garthwaite, C.L. Boulton, Nitric oxide signaling in the central nervous system, Annu. Rev. Physiol. 57 (1995) 683–706.
[22] J.M. Hetman, S.H. Soderling, N.A. Glavas, J.A. Beavo, Cloning and characterization of PDE7B, a cAMP-specific phosphodiesterase, Proc. Natl. Acad. Sci. USA 97 (2000) 472–476.
[23] M.D. Houslay, The N-terminal alternately spliced regions of PDE4A cAMP-specific phosphodiesterases determine intracellular targeting and regulation of catalytic activity, Biochem. Soc. Trans. 24 (1996) 980–986.
[24] Y. Iwahashi, T. Furuyama, Y. Tano, I. Ishimoto, Y. Shimomura, S. Inagaki, Differential distribution of mRNA encoding cAMP-specific phosphodiesterase isoforms in the rat brain, Mol. Brain Res. 38 (1996) 14–24.
[25] D.M. Juilfs, H.J. Fulle, A.Z. Zhao, M.D. Houslay, D.L. Garbers, J.A. Beavo, A subset of olfactory neurons that selectively express cGMP-stimulated phosphodiesterase (PDE2) and guanylyl cyclase-D define a unique olfactory signal transduction pathway, Proc. Natl. Acad. Sci. USA 94 (1997) 3388–3395.
[26] T. Kessler, C. Lugnier, Rolipram increases cyclic GMP content in L-arginine-treated cultured bovine aortic endothelial cells, Eur. J. Pharmacol. 290 (1995) 163–167.
[27] R.L. Kincaid, C.D. Balaban, M.L. Billingsley, Differential localization of calmodulin-dependent enzymes in rat brain: evidence for selective expression of cyclic nucleotide phosphodiesterase in specific neurons, Proc. Natl. Acad.Sci. USA 84 (1987) 1118–1122.
[28] R.G. Knowles, M. Palacios, R.M. Palmer, S. Moncada, Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase, Proc. Natl. Acad. Sci. USA 86 (1989) 5159–5162.
[29] V. Lev-Ram, T. Jiang, J. Wood, D.S. Lawrence, R.Y. Tsien, Synergies and coincidence requirements between NO, cGMP, and Ca^{2+} in the induction of cerebellar long-term depression, Neuron 18 (1997) 1025–1038.
[30] O.H. Lowry, N.J. Rosebrough, A.L. Farr, F.J. Randall, Protein measurements with the Folin reagent, J. Biol. Chem. 193 (1951) 265–275.
[31] N. Ludvig, V. Burmeister, P.C. Jobe, R.L. Kincaid, Electron microscopic immunocytochemical evidence that the calmodulin-dependent cyclic nucleotide phosphodiesterase is localized predominantly at postsynaptic sites in the rat brain, Neuroscience 44 (1991) 491–500.
[32] C. Lugnier, P. Schoeffter, A. Le Bec, E. Strouthou, J.C. Stoclet, Selective inhibition of cyclic nucleotide phosphodiesterases of human, bovine and rat aorta, Biochem. Pharmacol. 35 (1986) 1743–1751.
[33] B. Mayer, P. Klatt, E. Bohme, K. Schmidt, Regulation of neuronal nitric oxide and cyclic GMP formation by Ca^{2+}, J. Neurochem. 59 (1992) 2024–2029.
[34] A.M. Michie, M. Lobban, T. Muller, M.M. Harnett, M.D. Houslay, Rapid regulation of PDE-2 and PDE-4 cyclic AMP phosphodiesterase activity following ligation of the T cell antigen receptor on thymocytes: analysis using the selective inhibitors erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) and rolipram, Cell. Signal. 8 (1996) 97–110.
[35] T.J. O'Dell, R.D. Hawkins, E.R. Kandel, O. Arancio, Tests of the roles of two diffusible substances in long-term potentiation: evidence for nitric oxide as a possible early retrograde messenger, Proc. Natl. Acad. Sci. USA 88 (1991) 11285–11289.
[36] J.W. Polli, R.L. Kincaid, Expression of a calmodulin-dependent phosphodiesterase isoform (PDE1B1) correlates with brain regions having extensive dopaminergic innervation, J. Neurosci. 14 (1994) 1251–1261.
[37] R.R. Reinhardt, C.A. Bondy, Differential cellular pattern of gene expression for two distinct cGMP-inhibited cyclic nucleotide phosphodiesterases in developing and mature rat brain, Neuroscience 72 (1996) 567–578.
[38] D.R. Repaske, J.G. Corbin, M. Conti, M.F. Goy, A cyclic GMP-stimulated cyclic nucleotide phosphodiesterase gene is highly expressed in the limbic system of the rat brain, Neuroscience 53 (1993) 673–686.
[39] H. Sakagami, Y. Sawamura, H. Kondo, Synchronous patchy pattern of gene expression for adenylyl cyclase and phosphodiesterase but discrete expression for G-protein in developing rat striatum, Mol. Brain Res. 33 (1995) 185–191.
[40] C.W. Shuttleworth, C. Xue, S.M. Ward, J. De Vente, K.M. Sanders, Immunohistochemical localization of 3′,5′-cyclic guanosine monophosphate in the canine proximal colon: responses to nitric oxide and electrical stimulation of enteric inhibitory neurons, Neuroscience 56 (1993) 513–522.
[41] S.H. Soderling, S.J. Bayuga, J.A. Beavo, Cloning and characterization of a cAMP-specific cyclic nucleotide phosphodiesterase, Proc. Natl. Acad. Sci. USA 95 (1998) 8991–8996.
[42] S.H. Soderling, S.J. Bayuga, J.A. Beavo, Isolation and characterization of a dual-substrate phosphodiesterase gene family: PDE10A, Proc. Natl. Acad. Sci. USA 96 (1999) 7071–7076.
[43] S.H. Soderling, J.A. Beavo, Regulation of cAMP and cGMP signaling: new phosphodiesterases and new functions, Curr. Opin. Cell Biol. 12 (2000) 174–179.
[44] A.L. Steiner, C.W. Parker, D.M. Kipnis, Radioimmunoassay for cyclic nucleotides. I. Preparation of antibodies and iodinated cyclic nucleotides, J. Biol. Chem. 247 (1972) 1106–1113.
[45] S. Suda, M. Nibuya, T. Ishiguro, H. Suda, Transcriptional and translational regulation of phosphodiesterase type IV isozymes in rat brain by electroconvulsive seizure and antidepressant drug treatment, J. Neurochem. 71 (1998) 1554–1563.
[46] S.R. Vincent, H. Kimura, Histochemical mapping of nitric oxide synthase in the rat brain, Neuroscience 46 (1992) 755–784.
[47] C. Yan, J.K. Bentley, W.K. Sonnenburg, J.A. Beavo, Differential expression of the 61 kDa and 63 kDa calmodulin-dependent phosphodiesterases in the mouse brain, J. Neurosci. 14 (1994) 973–984.