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cGMP immunocytochemistry in aorta, kidney, retina and brain tissues of the rat after perfusion with nitroprusside

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Summary. The distribution of cyclic guanosine 3′,5′ monophosphate (cGMP) producing cells in various organs of the rat were studied immunocytochemically using antibodies raised against formaldehyde-fixed cGMP. Sodium nitroprusside (SNP), a direct activator of guanylate cyclase and vasodilator, was used to enhance cGMP levels. In order to reach all organs optimally, whole body perfusion was performed using a modified Krebs-Ringer buffer at 37° C, aerated with 5% CO_2/95% O_2, also containing isobutyl methyl xanthine (IBMX); a phosphodiesterase inhibitor. After 15-min pre-perfusion, SNP was added to the perfusate, followed by fast fixation with ice-cold 4% paraformaldehyde-phosphate buffer. After vehicle perfusion, only the retina showed cGMP immunoreactivity in the photoreceptor and ganglion layer, while other organs lacked cGMP immunoreactivity. After 15-min perfusion with SNP (10 μM), enhanced cGMP immunostaining was seen in smooth muscles of the aorta, amacrine-like cells in the retina, glomeruli of the kidney cortex, blood vessels in the dura mater, as well as cells in the pineal and in the median eminence. The results indicate that the distribution and the reactivity of cGMP producing cells, situated outside the blood brain barrier, can be studied by immunocytochemistry after pharmacological manipulations of the intact tissue with a nitrovasodilator using whole body perfusion.

Introduction

Ever since its detection in mammalian tissues, cyclic guanosine 3′,5′ monophosphate (cGMP) was thought to have a second messenger function (Waldman and Murad 1987). However, contrary to cAMP, the second messenger role of cGMP has been established in only a few biological systems. cGMP has so far been found to mediate, as a second messenger, cellular signal transduction processes in vascular smooth muscle (Rapoport and Murad 1983; Waldman and Murad 1987) and in vertebrate retinal rods (Lamb 1986).

In most cases the exact cellular localization of the cGMP pool in various tissues, in reponse to hormonal stimulation, is unknown. Histochemical methods should enable the visualization of cGMP at the cellular level. An immunocytochemical method for cGMP localization using unfixed tissue was developed by Steiner et al. (1972), and further applied by Wedner et al. (1972), Steiner et al. (1976) and Spruill and Steiner (1979). A disadvantage of this method is the loss of free cyclic nucleotides during the procedure due to diffusion or metabolism in the unfixed tissue (Steiner et al. 1976; Cumming et al. 1980; Ortez et al. 1980). Furthermore, although these Steiner antibodies have been used on formaldehyde-fixed material (Ariano 1983; Chan-Palay and Palay 1979), they are only slightly reactive with cGMP fixed in gelatin model sections and do not react with cGMP fixed in protein on nitrocellulose (De Vente et al. 1989).

Recently, a new antibody was introduced for the visualization of cGMP in fixed tissue, using antibodies raised against formaldehyde-fixed cGMP (De Vente et al. 1987a). With this method the localization of cGMP was visualized in the hippocampus (De Vente et al. 1988) and in cells of the superior cervical ganglion (De Vente et al. 1987b; Steinbusch et al. 1988). It was demonstrated that the intensity changes of cGMP immunoreactivity, after pharmacological manipulations in these tissues in vitro, mirrored the changes in cGMP concentrations as determined by radioimmunoassay (De Vente et al. 1987a and c, 1988). This indicates the specificity of the new method.

The immunocytochemical procedure for cGMP requires fast fixation due to the rapid diffusion of the intracellular localized cGMP. This procedure has been used successfully on small tissue pieces or tissue slices. The tissues were pretreated in vitro in order to stimulate cGMP production and to prevent fast metabolic degradation. It would be interesting to study pharmacological effects in large intact tissues with respect to the cellular localization of cGMP. However, in this respect, it will be difficult to achieve the required speed of fixation. In an attempt to find a solution to this problem, we have studied cGMP immunostaining in various organs after whole body perfusion of rats with sodium nitroprusside, an activator of soluble guanylate cyclase (Waldman and Murad 1987). Perfusion of the intact rat leads to good penetration of these drugs and fast fixation of the tissues. The results of this study indicate that whole body perfusion combined with immunocytochemistry enables studies on cGMP responses at the cellular level of intact organs to be performed. These studies should contribute to a better view of the role of cGMP as a second messenger.

Materials and methods

Perfusion. Six male Wistar rats (200–240 g), obtained from Harlan/CPB (Zeist, The Netherlands), were housed on a 12:12-h light/dark cycle. Experiments were performed in the first 6 h of the light period. The rats were anaesthetized with Nembutal (60 mg/kg) per-

fused via catheter into the aorta and bled via opening the right atria. Preperfusion was started for 15 min at 37° C with a modified Krebs-Ringer buffer of the following composition (m*M*): NaCl 121.1; KCl 1.87; KH_2PO_4 1.17; $MgSO_4$ 1.15; $NaHCO_3$ 24.9; $CaCl_2$ 1.2; Glucose 11.0; aerated with 5% CO_2/95% O_2 (pH 7.4). This pre-perfusion was performed under constant pressure (110–130 mm Hg), maintained by adapting the perfusate flow. The flowrate was in a range of 30–40 ml/min.

To prevent rapid degradation of cGMP, 1 m*M* IBMX (isobutyl methyl xanthine, Janssen Chimica Beerse, Belgium), a phosphodiesterase inhibitor, was added to the buffer for all experiments. Hereafter, the rats were perfused with the same buffer, to which 10 μ*M* sodium nitroprusside (Janssen Chimica) or vehicle was added for another 15 min. Nitroprusside was added to the buffer less then ten minutes before use. Subsequently, the buffer was replaced by freshly made ice-cold 4% paraformaldehyde solution in 0.1 *M* phosphate buffer (pH 7.4) for 12 min.

Tissue treatment. After perfusion fixation, the tissues of brain, eyes, pineal, kidney, dura mater, aorta and several blood vessels were dissected out and postfixed for 2 h at 0° C in the same fixative. The tissue pieces were then immersed for 1.5 h at 0° C in 5% sucrose dissolved in 0.1 *M Tris* buffered saline (TBS) at pH 7.4. The tissue pieces were then frozen with CO_2 gas, 20-μm cryostat sections were cut (Reichert-Jung frigocut 2800) and thawed on to chrome-alum coated glass slides. The dura mater pieces were kept free-floating and processed as such.

Immunocytochemistry. Tissue sections and dura mater pieces were incubated overnight at 4° C with rabbit antiserum against formaldehyde-conjugated cGMP (Code cGMP 73-5; De Vente et al. 1989, diluted 1:600 in TBS containing 0.5% Triton X-100 (TBS-T). The sections were then incubated at room temperature with goat anti-rabbit immuno globulin G (Nordic, Tilburg, The Netherlands) diluted 1:60 and allowed to soak for 2 h. Finally, the sections were incubated for 1 h at room temperature with PAP (Dakopatts Copenhagen, Denmark) diluted 1:600 in TBS-T. The precipitation product was then nickel-intensified according to the procedure of Hancock (1982) as follows: 12-min processing with diaminobenzidine (DAB) (Sigma St. Louis, USA), 0.2 g/l in *Tris* at pH 7.6; to which 6.0 g/L nickel ammonium sulphate had been added. Between each incubation step, 3 washing steps were performed with TBS-T, TBS and TBS-T. During the DAB processing the 3 washing steps only contained *Tris*-HCl buffer (pH 7.6).

The sections were dehydrated, a coverslip was placed over the sections and mounted with Entellan (Merck, Darmstadt, FRG). Dura mater tissue was collected on to microscope glass slides and air dried, after which it was dehydrated and covered as above. Photomicrographs were taken on an Olympus BH-2 microscope with Ilford Pan F Film (50 ASA).

Results

Aorta

In transverse sections of the aorta after vehicle administration, smooth muscle cell layers were lightly stained (Fig. 1a). The elastic membranes in the media did not show cGMP immunoreactivity. After perfusion with nitroprusside, the smooth muscle cells show an increase in cGMP immunostaining (Fig. 1b). The elastic membranes in the media still lack cGMP immunoreactivity, whereas the effect of nitroprusside is evident in all smooth muscle cell layers.

In blood vessels, such as carotid artery and vena cava, similar increases of cGMP immunoreactivity have been observed after nitroprusside stimulation.

Retina

After administration of vehicle, most of the layers of the retina are clearly distinguishable (Fig. 2a). The photoreceptor cell layer shows clear cGMP immunoreactivity and staining is also present in the ganglion cell layer. Other layers of the retina are not intensely stained.

When nitroprusside is added to the perfusate (Fig. 2b), the cGMP immunoreactivity is greatly enhanced in cell bodies of the inner nuclear and outer plexiform layer with fibrous projection into the inner plexiform layer. In addition, the cGMP immunoreactivity in the photoreceptor cell layer and the ganglion cell layer is also increased after nitroprusside stimulation (Fig. 2b).

However, this is not a consistent observation. In almost all experiments the intensity of cGMP immunoreactivity in the photoreceptor cell layer is high after administration of vehicle only. It does not increase after nitroprusside stimulation, whereas the increased staining in inner nuclear and outer plexiform layer remains.

Kidney

Transverse and tangential sections through the kidney give an overview of all the cell types present in the medulla and cortex of this organ. After administration of vehicle only, there is only a slight staininig intensity in blood vessel structures. When nitroprusside is added, the blood vessel structures show an increase in cGMP immunoreactivity similar to the increase seen in aorta. In addition, cGMP immunoreactivity is increased in glomeruli in the cortex, probably present in the visceral layer and in particular, in the podocytes.

Dura mater

The dura mater, the outer meningeal layer covering the whole brain is a vascularized membrane. It shows cGMP immunoreactivity after vehicle administration, especially in the vessel walls (Fig. 4a). After stimulation of guanylate cyclase by nitroprusside, vascular structures show elevated levels of cGMP immunoreactivity. This seems to be confined to the regions of the vessel walls, and an increase of cGMP immunoreactivity also occurs in the underlying cellular layer (Fig. 4b).

Brain structures

Several brain regions were examined for cGMP immunoreactivity. No increased cGMP immunoreactivity was detected after nitroprusside perfusion compared to vehicle in the cortex, septum, thalamus, hippocampus, cerebellum and brain stem. However, the vascular elements in all these structures show enhanced immunoreactivity after nitroprusside infusion. Furthermore, in the median eminence, a larger increase of cGMP immunoreactivity was observed after nitroprusside treatment compared to vehicle only conditions (Fig. 5).

In the median eminence, this increase was seen in the parts of the structure that also bear fibres of neurosecretory cells projecting into the pituitary gland (Fig. 5). In the pineal gland, a few immunostained elements were found after perfusion with vehicle only. After stimulation with nitroprusside, the immunostaining is greatly enhanced throughout the pineal gland (Fig. 6).

Discussion

The biological role of cGMP in the regulation of vascular smooth muscle tone is reasonably well understood (Ignarro

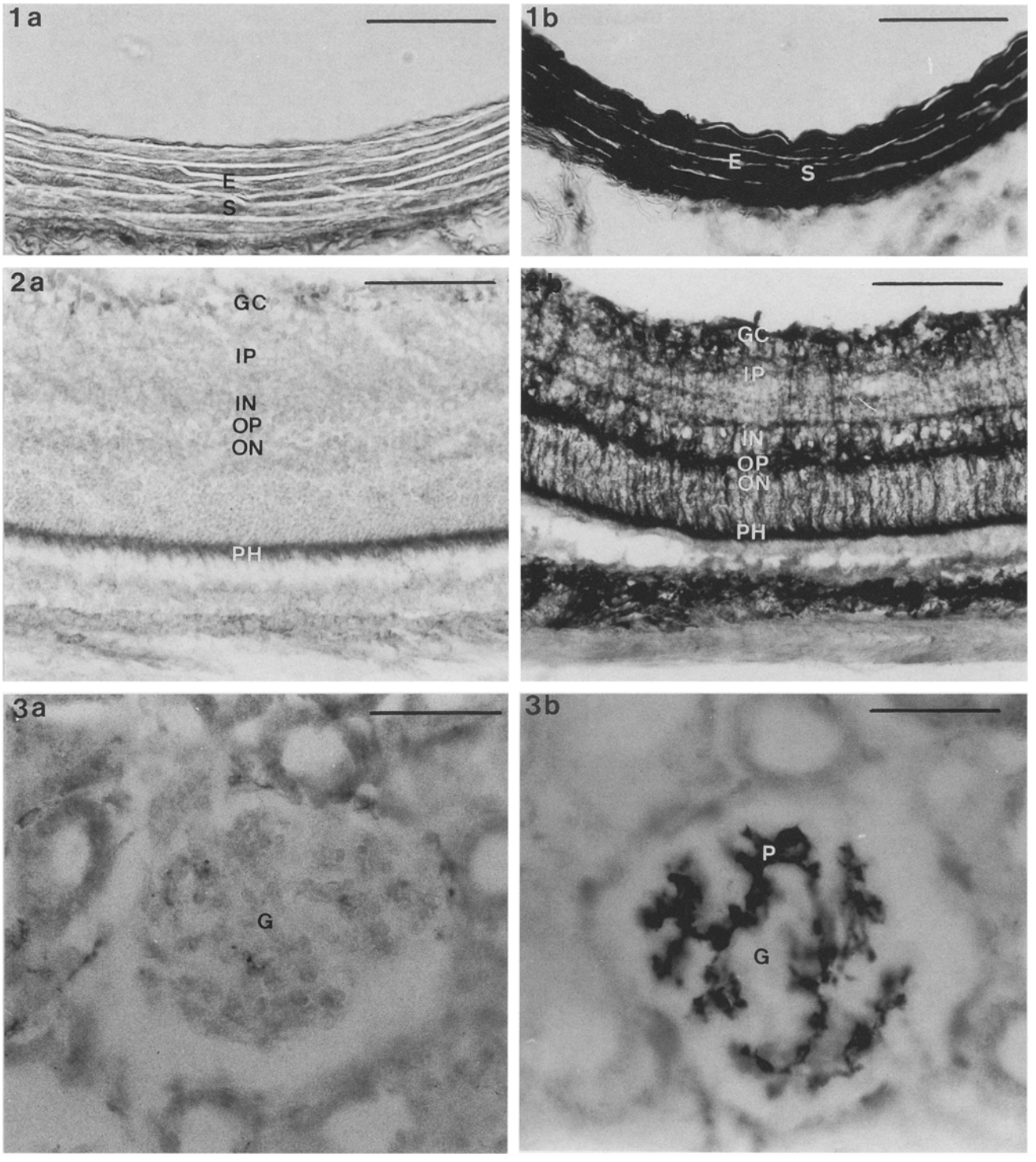
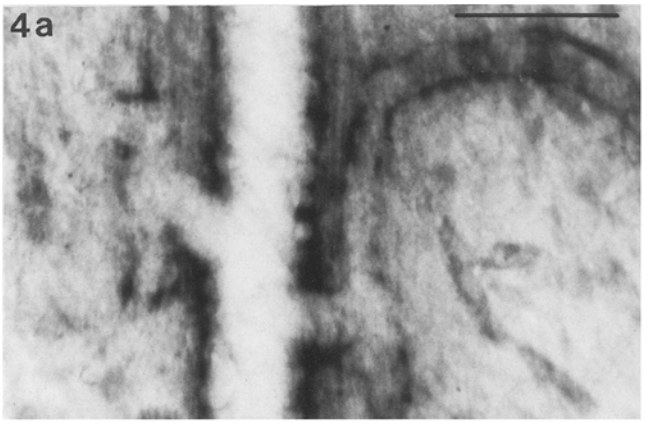


Fig. 1a and b. Photomicrograph of cGMP immunostaining in rat transected aorta after whole body perfusion with (**a**) Krebs-Ringer buffer or (**b**) Krebs-Ringer buffer containing 10 μ*M* sodium nitroprusside. *E*=elastic membranes, *S*=smooth muscle. *Bar*: 100 μm

Fig. 2a and b. Photomicrograph of cGMP immunostaining in rat transected retina after whole body perfusion with (**a**) Krebs-Ringer buffer or (**b**) Krebs-Ringer buffer containing 10 μM sodium nitroprusside. *PH*=photoreceptor cell layer; *ON*=outer nuclear, *OP*=outer plexiform, *IN*=inner nuclear, *IP*=inner plexiform, *GC*=gangion cell layer. *Bar*: 100 μm

Fig. 3a and b. Photomicrograph of cGMP immunostaining in rat kidney glomerulus after whole body perfusion with (**a**) Krebs-Ringer buffer or (**b**) Krebs-Ringer buffer containing 10 μ*M* sodium nitroprusside. *G*=glomerulus. *P*=podocytes. *Bar*: 100 μm

and Kadowitz 1985). Nitrovasodilators, such as sodium nitroprusside, are known to relax smooth muscles through activation of guanylate cyclase. This results in elevated levels of intracellular cGMP (Ignarro and Kadowitz 1985; Ra-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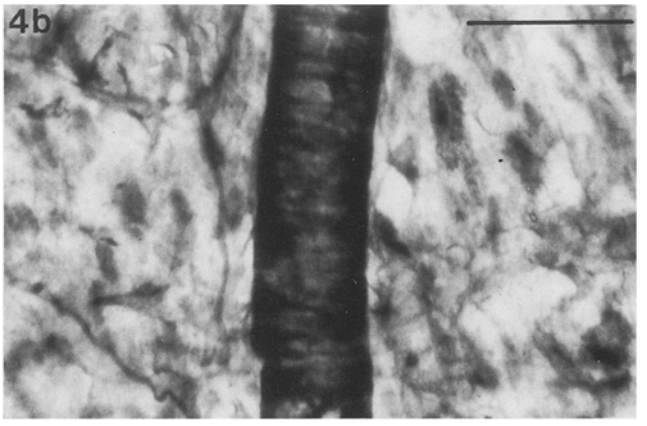
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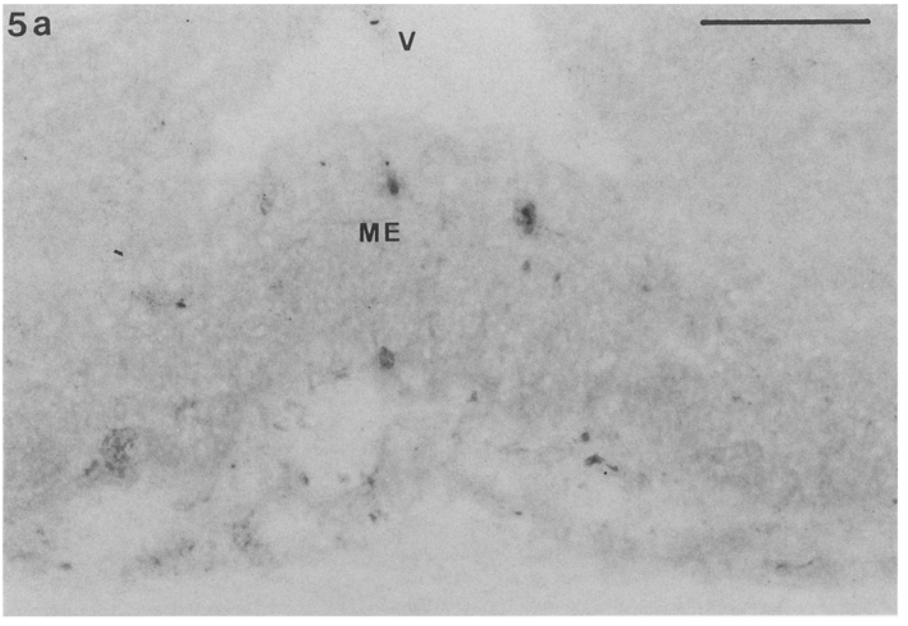
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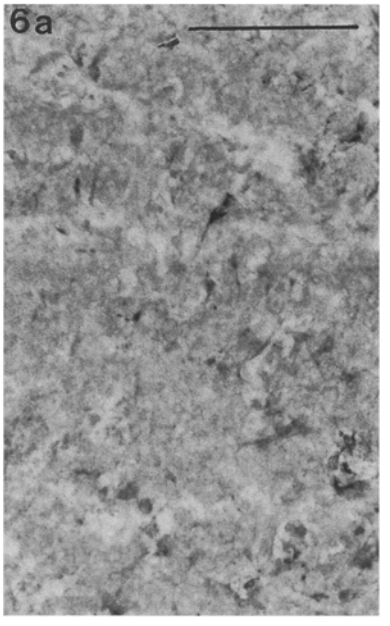
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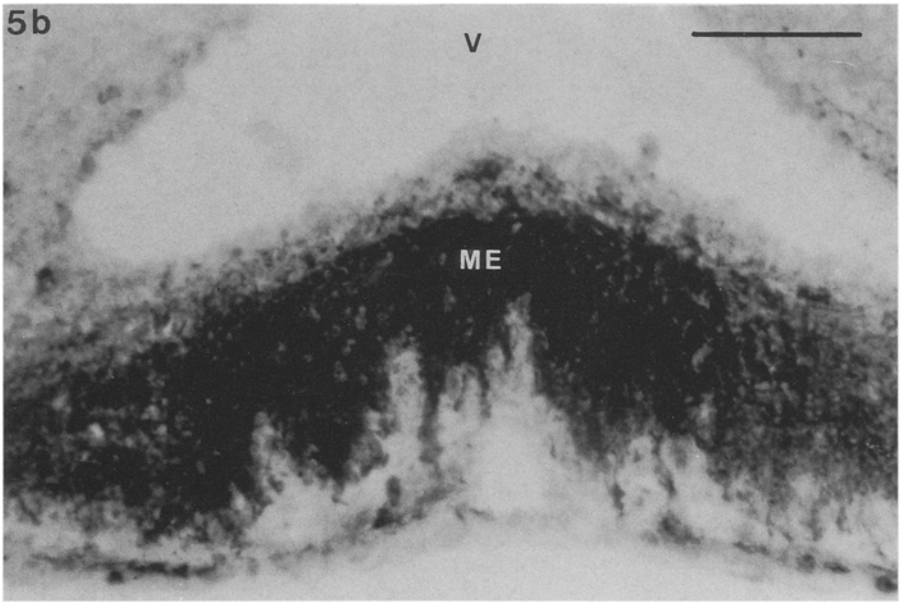
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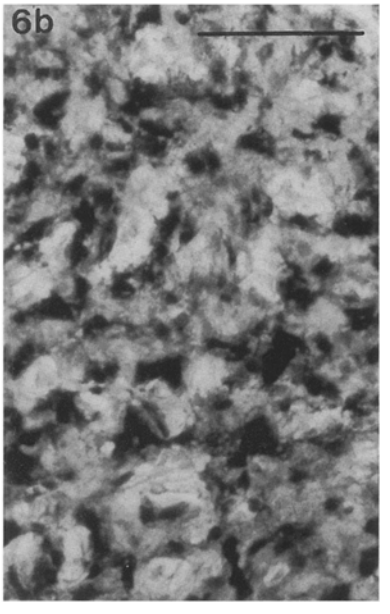
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poport and Murad 1983; Waldman and Murad 1987). We have observed an increase of cGMP immunoreactivity in aorta smooth muscle after infusion with nitroprusside (Fig. 1). Nitroprusside also induces an increase in cGMP immunoreactivity in other vascular structures, such as the dura mater, the vasculature of the kidney, the carotid artery and the vena cava. These observations are in agreement with other reports that nitroprusside induces cGMP responses in every vascular element, with no tissue specificity (Katsuki et al. 1977).

In addition to the effects in vascular tissues, nitroprusside increases cGMP levels in many other tissues (Waldman and Murad 1987). In the retina, we have observed a large increase of cGMP immunoreactivity after nitroprusside stimulation, specifically in the inner nuclear, inner plexiform and outer plexiform layers. Ferendelli and de Vries (1983) studied cGMP and guanylate cyclase concentrations in microdissected regions of the retina. They found high levels of cGMP in the photoreceptor layer. This corresponds to the cGMP immunostaining we have found after vehicle perfusion.

Furthermore, the above authors found high levels of guanylate cyclase present in inner plexiform, inner nuclear and outer plexiform layer, whereas cGMP levels were low in these regions. Nitroprusside stimulates guanylate cyclase to produce more cGMP. Therefore, our observations on the distribution of cGMP producing cells in the retina are in agreement with the distribution of guanylate cyclase described by Ferendelli and de Vries (1983).

Our studies show that the guanylate cyclase present in the inner nuclear and outer plexiform layers is predominantly situated in amacrine-like cells, whereas the guanylate cyclase present in the inner plexiform layer resides mainly in fibrous structures.

The presence of cGMP immunoreactivity in the photoreceptor cell layer seems to be in agreement with the role of cGMP in photoperception (Kaupp and Koch 1986; Gold and Nakamura 1987; Lamb 1986). The observation that nitroprusside has little effect on cGMP immunostaining in the photoreceptor cell layer might be explained by the high level of guanylate cyclase activation under basal conditions. This generates high levels of cGMP in the photoreceptor cell layer, as indicated also by immunocytochemical staining (Fig. 2). Further enhancement is not detectable by the present methodology or does not occur.

In the kidney, nitroprusside induces an elevation in cGMP immunoreactivity in the glomeruli. This appears most predominantly in podocytes (Fig. 3). Dousa et al. (1980) also described an elevation of cGMP immunoreactivity in the kidney after nitroprusside treatment, especially in intercapillary (probably mesangial) areas. Biochemical evidence exists that basal cGMP levels in suspensions of glomeruli and tubules of rat renal cortex increased two-fold after incubation with IBMX (0.5 m*M*), whereas, after addition of nitroprusside (1.0 m*M*), a ten-fold higher level was measured in glomeruli compared to tubules (Dousa et al. 1980). Our observations are in line with these data (Fig. 3).

In spite of pronounced effects on cGMP levels after stimulation with nitroprusside in varous peripheral organs, the effects on cGMP immunostaining in the central nervous system (CNS) are scarce. Structures in the CNS, outside the blood-brain barrier, revealed a cGMP stimulation after nitroprusside stimulation. Both the pineal gland and the median eminence showed an increase in cGMP immunoreactivity after nitroprusside perfusion. The fact that, in other brain structures, no cGMP immunoreactivity could be detected seems to be in contrast with reports that cGMP and guanylate cyclase are present in several brain structures (Bartfai 1980). However, in vitro studies with hippocampus slices revealed an increase in cGMP immunoreactivity after nitroprusside incubation. Furthermore, these studies show a good correlation between elevation of cGMP concentration, as measured biochemically, and increase of cGMP immunoreactivity after nitroprusside or atrial natriuretic factor stimulation (De Vente et al. 1988). Sodium nitroprusside is a hydrophilic compound which is not likely to cross the blood-brain barrier. Therefore, the above findings are fully in line with a very limited penetration of the blood-brain barrier by sodium nitroprusside.

Infusion of nitroprusside led to an increase of cGMP immunoreactivity in several tissues. In blood vessels structures, these increases in cGMP levels result in vasodilation, which is the actual observed therapeutic effect of nitroprusside treatment.

The question arises: What are the functional implications of enhancement of cGMP levels in other structures? An increase in cGMP levels may indicate additional pharmacological effects of sodium nitroprusside. A logical suggestion would be that the elevation of cGMP in retina possibly affects visual processes. However, no side effects on vision are described during clinical use of nitroprusside.

In the kidney, glomerular filtration is not affected by sodium nitroprusside (Scriabine 1980), while enhanced cGMP immunoreactivity is observed after nitroprusside infusion.

It is possible that, normally, the cGMP detected in glomeruli is functional in glomerular filtration, but that further elevation of these levels does not induce a greater effect because the concentration of cGMP is probably not rate-determining for glomerular filtration.

The experiments performed indicate that systemic perfusion of nitroprusside leads to increased cGMP immunoreactivity in several tissues. This method lends itself to pharmacological manipulations, as shown by our nitroprusside experiments. Still, one has to realize that whole body perfusion with Krebs-Ringer buffer creates an artificial state, different from the in vivo situation, since the oxygen concentration in Krebs-Ringer buffer is much lower than in blood. However, the problems of penetration of the tissue and the required fast fixation were circumvented by distributing the compound and fixative throughout the capillary system of the animal. Conclusively, using this method, it

Fig. 4a and b. Photomicrograph of cGMP immunostaining in blood vessel in rat dura mater after whole body perfusion with (**a**) Krebs-Ringer buffer or (**b**) Krebs-Ringer buffer containing 10 μ*M* sodium nitroprusside. *Bar*: 100 μm

Fig. 5a and b. Photomicrograph of cGMP immunostaining in rat transected median eminence after whole body perfusion with (**a**) Krebs-Ringer buffer or (**b**) Krebs-Ringer buffer containing 10 μ*M* sodium nitroprusside. *V*=third vehicle, *ME*=median eminence, *Bar*: 100 μm

Fig. 6a and b. Photomicrograph of cGMP immunostaining in rat transected pineal gland after whole body perfusion with (**a**) Krebs-Ringer buffer or (**b**) Krebs-Ringer buffer containing 10 μ*M* sodium nitroprusside. *Bar*: 100 μm

is possible to study the cellular localization of guanylate-cyclase containing elements. Similar experiments can be performed with other agents that stimulate cGMP production, such as e.g. atrial natriuretic factor (ANF) and acetylcholine (Waldman and Murad 1987). In these cases, the cellular localization of functionally active ANF or acetylcholine receptors could be studied in peripheral organs.

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