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Natriuretic peptides, but not nitric oxide donors, elevate levels of cytosolic guanosine 3′,5′-cyclic monophosphate in ependymal cells ex vivo

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Abstract

Atrial natriuretic peptide-(1–28) (ANP), brain natriuretic peptide-(1–32) (BNP) and C-Type natriuretic polypeptide (CNP) occur in the brain, are concentrated in the anteroventral area of the third cerebral ventricle and participate in the regulation of body fluid homeostasis. The ventricles of the mammalian brain are lined by a continuous monolayered epithelium of polyciliated ependymal cells. In the adult rat, the ependymocytes continue to express the intermediate filament vimentin, but do not contain glial fibrillary acidic protein. Ependymal functions are poorly understood, but may extend to osmoregulation and volume sensing. Ependymal cells possess receptors for the natriuretic peptides, and in cell culture respond to them with an increase in their cyclic GMP content. In this study, a cyclic GMP-specific antibody was employed together with an ex vivo brain slice system to assess the ependymal response to ANP, BNP and CNP under close to life-like conditions. While ANP in concentrations of 0.1 nM and 1 nM had no effect, at concentrations of 10 nM and 100 nM it increased ependymal cyclic GMP levels in a concentration-dependent manner. The other natriuretic peptides BNP, and CNP, also increased the cyclic GMP content of ependymocytes, while nitric oxide (NO) donors had no effect. However, in contrast to the natriuretic peptides, the NO donors elevated the level of cyclic GMP in the brain parenchyma below the ependymal layer.

Keywords: Cyclic GMP; ANP; BNP; CNP; Nitric oxide; Ventricles; Brain slices

The natriuretic peptides ANP(1–28), BNP and CNP(1–32) function in the mammalian brain to regulate salt and water homeostasis via their receptors NPR-A and NPR-B (for review, see [12]). Most of the brain ANP appears to originate from the ANP-containing cells concentrated in the area of the anteroventral third ventricle (AV3V), which includes the organum vasculosum of the lamina terminalis (OVLT), a member of the circumventricular organs [10,11]. The AV3V area has been known for decades as essential for the regulation of body fluid homeostasis [2]. The ventricular system of the mammalian brain including the OVTL is covered by ependymal cells, a specialized type of glial cells, most of which carry kinocilia at their apical cell pole. The ependyma is suspected to take part in various aspects of brain function, probably including osmoregulation, as indicated by their critical positioning at the interface between the brain parenchyma and the cerebrospinal fluid (CSF), and by their expression of aquaporin 4 [18]. The CSF contains ANP [17], the levels of which respond to intracranial pressure [7] and are elevated in obstructive hydrocephalus [6]. The highest density of ANP binding sites in the brain has been found on the choroid plexus, a capillary convolute covered by specialized ependymal cells and responsible for CSF production [13,30]. A concomitant increase in the amount of ANP binding to the choroid plexus and in CSF production of growing rats may be taken as circumstantial evidence for the regulation of CSF production by ANP [14]. In addition, the peptide influences the morphology of choroid plexus epithelial cells [20]. The ventricular ependymal cells are anatomically situated to be exposed to natriuretic peptides released from the AV3V area. While they are known to affect the level of cyclic GMP in cultured ependymal cells [31], the present study aims at correlating the in vitro findings with an ex vivo paradigm.

Rat ANP, BNP and CNP were from Phoenix Pharmaceuticals, Belmont, CA, USA. Tissue-Tek Optimal Cutting Temperature (OCT) embedding medium for cryosectioning was from Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands.

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(Z)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA NONOate) was from Alexis/Kordia, Leiden, The Netherlands. Donkey anti-sheep IgG Alexa Fluor 488 conjugate, rabbit anti-mouse IgG Alexa Fluor 568 conjugate and Goat anti-rabbit IgG Alexa Fluor 568 conjugate were from Molecular Probes, now Invitrogen, Karlsruhe, Germany. Rabbit anti-GFAP, mouse anti-β-tubulin and mouse anti-vimentin antibodies were from Jackson ImmunoResearch (West Grove, PA, USA; purchased from Dianova, Hamburg, Germany). All other chemicals were from Sigma, Steinheim, Germany. Details of the generation and tests for specificity of the employed anti-cyclic GMP serum are given elsewhere [1,4,5]. Control rat brain slices, incubated for immunocytochemistry, but in the absence of primary cyclic GMP antiserum, showed no immunoreactivity. Preincubation of the antiserum with cyclic GMP (10 mM, 2 h, room temperature) completely prevented immunofluorescence on frozen sections, whereas preincubation with cyclic AMP or 5′-GMP (100 μM) did not affect immunoreactivity.

The superfusions were done with Krebs buffer (KB, 121.1 mM NaCl, 1.9 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25 mM $NaHCO_3$, 2.0 mM $CaCl_2 \cdot 2H_2O$, 11 mM glucose) or with KB supplemented with 222 mg/l IBMX, 1.7 mg/l PMSF and 40 mg/l bacitracin (KBI). The superfusion buffer was aerated with carbogen (5% (v/v) CO_2, 95% (v/v) N_2).

To prepare brain slices, rats were killed by decapitation, their brains removed immediately and put in a container with KB at 4 °C. After removal of the cerebellum, the brains were cut between Bregma −0.8 and −0.9 with a scalpel, glued to the stage of a Vibroslicer® (Camden Instruments, Loughborough, UK) with commercial cyanacrylate glue and cut into slices of 400 μm thickness. The slides were then partitioned in ice-cold KBI. For experimental incubation, a slice was warmed to room temperature under continuous carbogen aeration over 15 min and then mounted in the superfusion system. The tissue was superfused at 37 °C with aerated KBI for 30 min, before the compound of interest was added. The time allowed for the stimulation was 10 min, after which the slice was immediately fixed by immersion in 4% paraformaldehyde in 100 mM KPi, pH 7.3 (15 min, 4 °C). After the addition of 10% (w/v) sucrose, fixation was allowed to continue for a further 90 min. Superfluous fixative was washed out with 10% (w/v) sucrose in 100 mM KPi, pH 7.3 for 15 min. The fixed tissue was embedded in OCT for mounting and cutting into 10 μm sections in a cryostat. For cyclic GMP immunohistochemistry, the sections were thawed at room temperature for 15–20 min, rinsed three times with Tris-buffered saline (TBS) and then incubated overnight with antibody diluted in TBS/0.3% Triton X-100 (TBS-T). The dilutions were sheep anti-cyclic GMP, 1:4000; rabbit anti-GFAP, 1:500; mouse anti-vimentin, 1:500; mouse anti-β-tubulin, 1:500. After three washes (twice TBS, once TBS-T, 15 min each), the sections were incubated with the appropriate secondary antibody diluted 1:100 for 90 min. They were then washed again three times (twice TBS, once TBS-T, 15 min each) and covered with 80% glycerol (v/v in TBS, with 4′,6-diamidino-2-phenylindole (DAPI)) for coverslip attachment. Animal handling was compliant with the University of Maastricht Animal Ethics Committee and all pertinent laws.

The polyciliated ependymal cells of the ventricular wall do not contain GFAP, which is enriched in the subependymal layer (Fig. 1A). Instead, the kinocilia-bearing ependymocytes contain vimentin as intermediary filament protein, which is absent from the remaining normal brain parenchyma (Fig. 1B). Vimentin immunostaining reveals occasional ependymal processes of up to 50 μm length occurring in the striatal regions of the 3rd ventricle. These cells are distinct from tanycytes, which are also known to have basal processes. The kinocilia of the ventricular ependymal cells can be visualized by immunostaining against tubulin (Fig. 1C). The pattern of DAPI-stained ependymal cell nuclei is distinct from the pattern of nuclei in the parenchyma. Thus, a combination of immunostaining with a specific anti-cyclic GMP antibody and nuclear staining with DAPI would be sufficient to distinguish between cyclic GMP immunoreactivity in the ependyma and in the remaining brain parenchyma. To test the potency of ANP to elicit an increase in the intracellular concentration of cyclic GMP, the peptide was applied to living brain slices in concentrations of 0.1 nM, 1 nM, 10 nM and 100 nM. ANP increased the cyclic GMP level in the polyciliated ependymal cells of the slices in a concentration-dependent manner. The lowest concentration to yield discernable cyclic GMP immunoreactivity against the background was 10 nM (Fig. 1D–F). The strength of the signal was increased after the application of the agonist in 10-fold higher concentration (Fig. 1G). As opposed to ANP, the nitric oxide (NO) donor SNP did not increase ependymal cyclic GMP levels above the detection limit. Instead, the parenchyma exhibited higher cyclic GMP levels than after ANP stimulation (Fig. 1H). In addition to ANP, the other natriuretic peptides BNF and CNF were also able to elicit an increase in ependymal cyclic GMP concentrations (Fig. 2A and B). Like SNP, the NO donor DETA NONOate was also ineffective in elevating ependymal cyclic GMP (Fig. 2C) and produced no stronger ependymal signal than present in the background control (Fig. 2D). In choroid plexus, ANP and SNP acted on different structures. The natriuretic peptide elevated cyclic GMP in the plexus ependyma (Fig. 2E), while the NO donor increased the cyclic GMP concentration in the vascular endothelial cells below the ependymal layer (Fig. 2F).

The pattern of ependymal vimentin expression is species-dependent. While in humans, GFAP is only expressed during ependymal maturation and vimentin immunoreactivity disappears from the brain within the first postnatal weeks [26], bovine ependymocytes continue to express vimentin and to some extent GFAP into adulthood [25]. In adult rats, ependymocytes remain GFAP-negative (Fig. 1A) but vimentin-positive (Fig. 1B). During cryostat sectioning, the ependymal layer is frequently displaced from the ventricular surface, creating a source of error in ependymal research if denudated ventricle wall is mistaken for intact tissue. A staining for vimentin and visualization of the ependymal cilia may be used to guarantee ependymal integrity during an investigation. The characteristic pattern of densely aligned ependymal cell nuclei represents another valuable diagnostic feature for ependymal preservation.