Carbachol inhibits Na⁺-K⁺-ATPase activity in choroid plexus via stimulation of the NO/cGMP pathway

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Ellis, Dorette Z., James A. Nathanson, and Kathleen J. Sweedner. Carbachol inhibits Na⁺-K⁺-ATPase activity in choroid plexus via stimulation of the NO/cGMP pathway. Am J Physiol Cell Physiol 279: C1685–C1693, 2000.—Secretion of cerebrospinal fluid by the choroid plexus can be inhibited by its cholinergic innervation. We demonstrated that carbachol inhibits the Na⁺-K⁺-ATPase in bovine choroid tissue slices and investigated the mechanism. Many of the actions of cholinergic agents are mediated by nitric oxide (NO), which plays important roles in fluid homeostasis. The inhibition of Na⁺-K⁺-ATPase was blocked by the NO synthase inhibitor [N⁵-nitro-L-arginine methyl ester] and was quantitatively mimicked by the NO agonists sodium nitroprusside (SNP) and diethylenetriamine NO. Inhibition by SNP correlated with an increase in tissue cGMP and was abolished by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, an inhibitor of soluble guanylate cyclase. Inhibition was mimicked by the kinase G activator 8-bromo-cGMP and by okadaic acid, an inhibitor of protein phosphatases 1 and 2A. cGMP-dependent protein kinase inhibitors Rp-8-pCPT-cGMP (0.5–5 μM) and KT-5823 (2.0 μM) did not block the effects of SNP, but higher concentrations of the more selective inhibitor (Rp-8-pCPT-cGMP) had a pharmacological inhibitory effect on Na⁺-K⁺-ATPase. The data suggest that cholinergic regulation of the Na⁺-K⁺-ATPase is mediated by NO and involves activation of guanylate cyclase and elevation of cGMP.

Nitric oxide; guanosine 3',5'-cyclic monophosphate

The choroid plexuses are highly vascularized tissues located in the large cavities of the brain: the lateral, third, and fourth ventricles. They are the major producers of cerebrospinal fluid (CSF). The choroid plexus epithelial cells are highly enriched in Na⁺-K⁺-ATPase, located at the cell’s apical surface. It has been demonstrated that the α₁-, β₁-, β₂-, and β₃-Na⁺-K⁺-ATPase isoforms are expressed in rat choroid plexus epithelium (5, 19, 29, 36, 42, 46, 47). The Na⁺-K⁺-ATPase catalyzes the transfer of 2 K⁺ from the extracellular space into the cell and the extrusion of 3 Na⁺ while hydrolyzing ATP to ADP and Pᵢ. In fact, choroid plexus Na⁺-K⁺-ATPase catalyzes (in concert with ion channels and carriers) the bulk secretion of Na⁺ followed by Cl⁻ and water (1, 4, 24). The Na⁺-K⁺-ATPase also plays a major role in controlling the composition of CSF, particularly the concentration of K⁺, which is lower in CSF than in plasma (2, 34).

Just as renal Na⁺-K⁺-ATPase is regulated by complex hormonal mechanisms to maintain plasma electrolytes during dietary and pathological fluctuations of electrolytes and pH, the choroid plexus Na⁺-K⁺-ATPase is also subject to several known forms of hormonal regulation. Functionally important sympathetic innervation of choroid vascular bed and epithelium is known (21), and inhibition based on the activation of protein kinase C (15) and protein kinase A (14) has been documented. However, little attention has been paid to the cholinergic innervation of the choroid plexus (10). It has been reported that cholinergic stimulation is associated with increases in the levels of the second messenger cGMP (39). Other physiological studies have demonstrated that intraventricular injection of cholinomimetic agents, such as carbachol and acetylcholine, inhibits CSF secretion (27).

The actions of acetylcholine in relaxing blood vessels are mediated by the free radical nitric oxide (NO) (17). NO is a key paracrine and autocrine regulator in a number of organs and tissues, including blood vessels, immune cells, smooth muscle, nervous system, and some endothelium. Many of the physiological actions of NO are mediated by activation of soluble guanylate cyclase and elevation of cGMP. There is growing evidence that the Na⁺-K⁺-ATPase is regulated by NO (for review, see Ref. 11) and other cGMP-generating agents such as carbon monoxide (32) and atrial natriuretic peptide (38). This regulation may be inhibition or stimulation of the Na⁺-K⁺-ATPase, depending on the tissue.

There is evidence that the choroid plexus contains a rich distribution of NO synthase (NOS) as demonstrated by NADPH-diaphorase histochemical localization (44) and immunoreactivity for the neuronal NOS isoform (26). Choroid plexus epithelium also contains cGMP, and its target, cGMP-dependent protein kinase (PKG) (7). Previous studies showed that intraventricular injection of atrial natriuretic peptide (known to activate the membrane guanylate cyclase-cGMP system) caused a significant decrease in CSF production that correlated with an increase in cGMP levels in...
choroid plexus (41). The former observations raise the possibility that cholinergic stimulation and subsequent formation of NO might be capable of altering membrane ion gradients through direct effects on choroid plexus epithelium. This study investigates the role of carbachol, NO, soluble guanylate cyclase, and cGMP in inhibiting the Na\(^+\)-K\(^+\)-ATPase activity in bovine choroid plexus. Bovine choroid plexus tissue slices were treated with carbachol and other agents, and Na\(^+\)-K\(^+\)-ATPase activity was measured by the hydrolysis of ATP after permeabilization of the slices.

**METHODS**

**Tissue preparation.** Bovine brain was obtained from an abattoir, and the choroid plexus was dissected from the fourth ventricle. Tissue slices (0.4 \(\times\) 0.4 \(\times\) 1 mm) were prepared on a Brinkmann chopper cooled to 4°C and suspended (25–30 mg/ml wet weight) in microdissection buffer containing (in mM) 137 NaCl, 5 KCl, 0.8 MgSO\(_4\), 0.25 CaCl\(_2\), 1.0 MgCl\(_2\), 10 HEPES, and 2 NaOH to adjust pH to 7.4 at 34°C.

Na\(^+\)-K\(^+\)-ATPase and cGMP measurements. Drugs used in these experiments were added to cultures that contained 1-ml aliquots of slice suspension. Tubes were incubated for 15 min at 34°C and then frozen at −80°C. In studies using inhibitors, the inhibitors were added 3 min before addition of the drug. Tubes were thawed and centrifuged (1,700 \(\times\) g) and supernatant (containing drug) was removed. The supernatant was heated for 5 min at 90°C, after which 75 mM sodium acetate was added and the sample was dried and stored for cGMP assay by RIA (Biomedical Technologies, Stoughton, MA).

Na\(^+\)-K\(^+\)-ATPase activity was determined using two methods to check the validity of both protocols. The first method involved assaying activity in suspended tissue slices and the colorimetric ATPase assay: ATP is hydrolyzed, and the released \(\text{P}_i\) is measured by forming a complex with molybdate. The pellet was then added to tubes that contained 1-ml aliquots of resuspension buffer containing (in mM) 85 NaCl, 20 KCl, 4 MgCl\(_2\), 0.2 EGTA, and 30 histidine adjusted to pH 7.2. Tubes were thawed on ice water. For further permeabilization of tissue slices, saponin (20 \(\mu\)g/ml) was added and slices were incubated for 10 min at 34°C. Aliquots of tissue slices (50–150 \(\mu\)l) were centrifuged (10,000 \(\times\) g) for 15 min, and the supernatant was removed. We resuspended the particulate fraction containing Na\(^+\)-K\(^+\)-ATPase activity by addition of 1 ml of resuspension buffer containing (in mM) 85 NaCl, 20 KCl, 4 MgCl\(_2\), 0.2 EGTA, and 30 histidine adjusted to pH 7.2, with or without 100 \(\mu\)M ouabain. There were no differences between ouabain-insensitive activity in samples treated with 100 \(\mu\)M to 3 mM ouabain. Because there was only a 5% reduction in the slope at 30 min compared with 20 min, Na\(^+\)-K\(^+\)-ATPase activity was measured at 30 min at 37°C. The reaction was terminated by addition of a quenching solution (0.6 ml) containing 1N H\(_2\)SO\(_4\) and 0.5% ammonium molybdate. Formation of phosphomolybdate complex was determined spectrophotometrically at 700 nm (12).

Substantially the same assay has been performed in the past but with \(^{32}\)P-labeled ATP instead of colorimetric measurement of ATP hydrolysis (30, 32, 38). Na\(^+\)-K\(^+\)-ATPase activity was measured as the difference between ouabain-treated and ouabain-untreated samples.

The second method involved the pyruvate kinase-lactate dehydrogenase assay that couples the generation of ADP and oxidation of NADH (33). For this, the tissue slice pellets were resuspended in resuspension buffer and homogenized in a glass/glass homogenizer. The homogenate was fractionated by centrifugation at 10,000 \(\times\) g for 15 min, and the supernatant was removed. We resuspended the particulate fraction of the homogenate in resuspension buffer. Na\(^+\)-K\(^+\)-ATPase activity assays were performed using both supernatant and particulate fractions; however, we observed ouabain-sensitive Na\(^+\)-K\(^+\)-ATPase activity only in the particulate fraction. The ATPase reaction medium contained (in mM) 3 ATP, 140 NaCl, 6.25 KCl, 3.6 MgCl\(_2\), 1.5 phosphoenolpyruvate, 0.5 NADH, 15 Tris-HCl (pH 7.2), and 40 \(\mu\)g/ml pyruvate kinase and 40 \(\mu\)g/ml lactate dehydrogenase. The reaction was initiated by addition of \(\sim\)10–15 \(\mu\)M of the enzyme particulate fraction to a cuvette thermostated at 37°C. Na\(^+\)-K\(^+\)-ATPase activity was calculated from the difference between the slopes in the time course of absorption change at 340 nm in the absence and in the presence of 100 \(\mu\)M ouabain.

Protein concentrations were determined by the Lowry method (28).

**Purification of Na\(^+\)-K\(^+\)-ATPase.** Bovine choroid plexus Na\(^+\)-K\(^+\)-ATPase was partially purified according to the modified method of Jørgensen (22). Tissue was homogenized in buffer containing (in mM) 30 histidine, 1 EDTA, and 250 sucrose (pH 7.2) (HES). Microsomal fractions were obtained, and tissue pellets were resuspended in equal volume of HES buffer and buffer containing (in mM) 60 histidine, 2 EDTA, and 6 ATP (pH 7.5), which stabilizes Na\(^+\)-K\(^+\)-ATPase activity for SDS extraction. Low concentrations of SDS were added, which allowed for the removal of contaminating proteins, followed by centrifugation on sucrose gradients to separate the membrane fraction containing Na\(^+\)-K\(^+\)-ATPase. The enzyme was stored in HES buffer. Protein concentration was determined (as indicated above) using bovine serum albumin as a standard. Na\(^+\)-K\(^+\)-ATPase α-bands were verified with polyclonal antibody ETTY, which recognizes all three Na\(^+\)-K\(^+\)-ATPase α-isoforms, while SpETb1 and SpETb2 polyclonal antibodies were used to verify β1- and β2-isoforms, respectively. The specific activity was 300 μmol P\(_i\)/h/mg protein.

**Phosphorylation.** Phosphorylation experiments to test the efficacy of PKG inhibitors were performed by a modification of the methods of Fotis et al. (16). Histone 1A (0.5 \(\mu\)g) was incubated for 15 min at room temperature in buffer containing (in mM) 30 Tris-HCl (pH 7.4), 5 MgCl\(_2\), 100 NaCl, 10 KCl, 1 EDTA, 1 dithiothreitol, and 1 phenylmethylsulfonyl fluoride. Protein phosphorylation was initiated at room temperature by the addition of PKG (200 U) and 25 μM [\(^{32}\)P]ATP (final activity 1,000 cpm/pmol) in the presence or absence of cGMP (50 μM) in a final volume of 25 μl. The reaction was terminated after 15 min by the addition of an equal volume of Laemmli sample buffer. The phosphorylation reaction mixture was analyzed on 10% gels by SDS-PAGE and transferred to nitrocellulose membrane electrophoretically. Phosphoproteins were visualized by autoradiography.

**Statistics.** Statistical comparisons were performed by ANOVA followed by Fisher’s protected least significant difference and Scheffe’s F-test for comparison of significant difference among different means.

**Chemicals.** Routine reagents, sodium nitroprusside (SNP), ouabain, saponin, N\(^\circ\)-nitro-l-arginine methyl ester (l-NAME), histone, and carbachol were purchased from Sigma (St. Louis, MO). Others were obtained as follows: [\(^{32}\)P]ATP tetra(triethylammonium) salt from DuPont-New England Nuclear (Boston, MA); 8-bromo-cGMP sodium salt, 1H-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one (ODQ), diethylenetriamine NO (DETA-NO) from Sigma-RBI (Natick, MA); 1- and 2-(p-chlorophenyl)pentane (PCP), Rp-8-pCPT-cGMP, and KT-5823 from Calbiochem (La Jolla, CA).
The presence of NOS and Na\(^{+}\)-K\(^{+}\)-ATPase in choroid plexus epithelium and the known effects of cGMP in this tissue suggest that the activation of the NOS system, with subsequent cGMP synthesis, might regulate the Na\(^{+}\)-K\(^{+}\)-ATPase. For these experiments, the coupled assay method was used (see METHODS for preparation of tissue). After a 15-min exposure of bovine tissue slices to the NO donors SNP (100 μM) (18) and DETA-NO (9), there was a marked reduction of Na\(^{+}\)-K\(^{+}\)-ATPase activity (Fig. 2). The effects of SNP and DETA-NO were specific to Na\(^{+}\)-K\(^{+}\)-ATPase, because no measurable changes were observed in the ouabain-insensitive (Mg-ATPase) activity. Compared with the activity in control choroid plexus, Na\(^{+}\)-K\(^{+}\)-ATPase activity in slices treated with either SNP (100 μM) or DETA-NO (100 μM) was inhibited 30–35%.

The same result was obtained whether Na\(^{+}\)-K\(^{+}\)-ATPase activity was measured in tissue slices or the particulate fraction of homogenate. Thus the stability of the inhibition to homogenization and fractionation indicates that it does not require the cell's integrity. The ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase activity was somewhat greater in the particulate fraction (7.4 ± 0.4, mean for 6 experiments) than in tissue slices (3.4 ± 0.14, mean for 6 experiments) (Fig. 3), consistent with loss of cytoplasmic protein during particulate fraction preparation.

Action of SNP on Na\(^{+}\)-K\(^{+}\)-ATPase involves soluble guanylate cyclase activation. Many of the actions of NO involve activation of the guanylate cyclase system. As such it was important to determine if the physiological action of SNP on Na\(^{+}\)-K\(^{+}\)-ATPase activity in choroid plexus was a result of the release of NO and activation

**Fig. 1.** Carbachol (Carb) inhibits ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase activity. Choroid plexus tissue slices were incubated without drugs or with N\(^{\circ}\)-nitro-L-arginine methyl ester (L-NAME; 300 μM) for 3 min at 34°C followed by incubation with carbachol (100 μM) for 15 min at 34°C. After removal of the drugs and permeabilization of tissue slices, ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase activity was determined by the colorimetric assay. Activity is expressed as μmol P\(_{i}\) h\(^{-1}\) mg protein\(^{-1}\). Values for ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase activity represent means ± SE for an average of 4 samples in each of 6 experiments. *Significantly different from the corresponding control at P < 0.05 [by ANOVA and Fisher’s protected least significant difference test (PLSD)]. **Significantly different from carbachol-treated samples at P < 0.05 (by ANOVA, Fisher PLSD, and Scheffe’s F-test).

**RESULTS**

A cholinergic agonist, carbachol, inhibits ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase activity. Figure 1 shows that 15 min incubation of choroid plexus tissue slices with the acetylcholine analog carbachol (100 μM) (27) resulted in a significant inhibition of ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase activity compared with that of slices treated with vehicle. Ouabain-insensitive Mg-ATPase activity was not affected. Carbachol at 10 μM had the same effect as at 100 μM, indicating that the effect is saturated (data not shown). Because stimulation of the cholinergic system has been reported to result in an increase in cGMP (39) and many cholinergic effects are mediated by NO it was of interest to determine if the cholinergic and NO systems are linked in choroid plexus. Exposure of bovine choroid plexus tissue slices to L-NAME (300 μM), a specific inhibitor of NOS, in the presence of carbachol largely blocked the carbachol-induced decrease in ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase (Fig. 1). These data demonstrate that the action of carbachol is upstream from NO and predict that stimulation of the cholinergic system in choroid plexus results in the release of NO.

Cholinergic inhibition of ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase activity is mimicked by SNP and DETA-NO.

**Fig. 2.** Sodium nitroprusside (SNP) and diethylenetriamine nitric oxide (DETA-NO) mimicked the effects of carbachol on ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase activity in bovine choroid plexus. Tissue slices were incubated (15 min at 34°C) with SNP (100 μM) and DETA-NO (100 μM) or without drugs. The particulate fraction of the homogenate was assayed for Na\(^{+}\)-K\(^{+}\)-ATPase activity using the coupled assay method. SNP and DETA-NO inhibited ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase activity by 30–35% after removal from the incubation buffer. Data are expressed as μmol P\(_{i}\) h\(^{-1}\) mg protein\(^{-1}\). Values are means ± SE for 3 experiments. *Significantly different from the control group: P < 0.01 (by ANOVA and Fisher’s PLSD).
Exposure of bovine choroid plexus tissue slices to an inhibitor selective for soluble guanylate cyclase, ODQ (1 mM) (18), partially blocked the SNP-induced inhibition of the Na\(^{+}\)-K\(^{+}\)-ATPase (Fig. 4A). Although a number of physiological actions of NO are mediated by activation of soluble guanylate cyclase, recent evidence suggests that NO may affect Na\(^{+}\)-K\(^{+}\)-ATPase activity directly by modification of sulfhydryl groups on the Na\(^{+}\)-K\(^{+}\)-ATPase molecule (37). That this was not the case here is demonstrated in Fig. 4B. Incubation of SDS-extracted bovine choroid Na\(^{+}\)-K\(^{+}\)-ATPase with SNP (100 \(\mu\)M) or ODQ (1 \(\mu\)M) did not alter ouabain-sensitive Na\(^{+}\)-K\(^{+}\)-ATPase activity. This would suggest that modulation of the Na\(^{+}\)-K\(^{+}\)-ATPase by SNP is under the regulation of a second messenger system and that direct effects such as nitrosylation of sulfhydryl groups were not responsible for the observations.

Increases in cGMP are correlated with NO inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase. Because SNP inhibition of the Na\(^{+}\)-K\(^{+}\)-ATPase involved activation of soluble guanylate cyclase, it was of interest to determine if changes in Na\(^{+}\)-K\(^{+}\)-ATPase activity corresponded with alterations in cGMP levels. cGMP was measured in the supernatants of the same samples that were subsequently assayed for Na\(^{+}\)-K\(^{+}\)-ATPase. As shown in Fig. 5, SNP (100 \(\mu\)M) caused substantial increases in cGMP levels. ODQ, in addition to blocking the SNP-induced alteration in Na\(^{+}\)-K\(^{+}\)-ATPase, also caused a decrease in cGMP generation in response to the drug (Fig. 5). These results not only provide evidence for the possible involvement of cGMP in mediating the SNP-induced inhibition of the Na\(^{+}\)-K\(^{+}\)-ATPase activity, but also...
further support the involvement of soluble guanylate cyclase (vs. membrane bound guanylate cyclase) in mediating the SNP response in bovine choroid plexus.

Possible involvement of protein phosphorylation in inhibition of Na\(^{+}-K\(^{+}\)-ATPase activity. The nonhydrolyzable analog of cGMP and potent activator of PKG, 8-bromo-cGMP, mimicked the actions of SNP. Figure 6 shows that 8-bromo-cGMP inhibited ouabain-sensitive Na\(^{+}-K\(^{+}\)-ATPase activity in bovine choroid plexus slices. Other evidence of the involvement of protein phosphorylation in regulating the Na\(^{+}-K\(^{+}\)-ATPase activity was obtained from observations in which okadaic acid at 400 nM, a concentration known to inhibit both protein phosphatase type 1 and 2A, mimicked the actions of SNP in inhibiting the Na\(^{+}-K\(^{+}\)-ATPase activity in bovine choroid plexus (Fig. 6).

In many systems, elevated cGMP activates PKG, which then affects the regulation of enzymes either by directly phosphorylating them or by phosphorylating (and thereby activating) protein phosphatase inhibitors such as DARPP-32 (40, 45). Attempts to test for this pathway using pharmacological inhibitors of PKG proved to be problematic, however. KT-5823 (2.0 \(\mu\)M), Rp-8-pCPT-cGMP (5.0 \(\mu\)M), and KT 5720 (0.5 \(\mu\)M), a specific inhibitor of protein kinase A, did not block the effects of SNP (Fig. 7A). The efficacy of Rp-8-pCPT-cGMP and KT-5823 was tested in vitro on a known substrate of PKG, histone 1A. We were able to effectively inhibit PKG (200 U) phosphorylation of histone 1A (0.5 \(\mu\)g) with Rp-8-pCPT-cGMP (50 \(\mu\)M) and KT-5823 (2.0 \(\mu\)M) (data not shown). As with platelets (6), the possibility exists that there may be a high level of NO-pathway constituents in choroid plexus that may require higher concentrations of inhibitors. To test this, bovine choroid plexus tissue slices were exposed to 1 mM Rp-8-pCPT-cGMP alone or in the presence of SNP (100 \(\mu\)M). The higher concentration of Rp-8-pCPT-cGMP paradoxically inhibited Na\(^{+}-K\(^{+}\)-ATPase activity by itself, however, and treatment at the same time with SNP did not cause any further change in Na\(^{+}-K\(^{+}\)-ATPase activity (Fig. 7B). We were concerned that Rp-8-pCPT-cGMP, being a nucleotide analog, could conceivably inhibit the Na\(^{+}-K\(^{+}\)-ATPase directly by binding to its active site, and so we tested the effect of various concentrations of Rp-8-pCPT-cGMP (0.5 \(\mu\)M–1 mM) on Na\(^{+}-K\(^{+}\)-ATPase activity in the test tube. Results are shown in Fig. 8. High concentrations of these inhibitors have an inhibitory effect on the Na\(^{+}-K\(^{+}\)-ATPase activity. Figure 8 shows the drug concentration during preincubation. It was then diluted 60-fold into each ATPase assay mixture, which contained 3 mM ATP, to give final inhibitor concentrations of 8.33 nM to 16.67 \(\mu\)M during the assay.

DISCUSSION

Our studies in choroid plexus demonstrated that stimulation of the cholinergic system caused a significant decrease in ouabain-sensitive Na\(^{+}-K\(^{+}\)-ATPase activity. Furthermore, the NOS inhibitor L-NAME blocked the cholinergic agonist-induced inhibition of the Na\(^{+}-K\(^{+}\)-ATPase, suggesting that activation of the NOS system and subsequent formation of NO mediate
that NO release follows stimulation of cholinergic nerves in a number of tissues (13, 17). The NO agonists SNP and DETA-NO caused significant inhibition of the ouabain-sensitive Na⁺-K⁺-ATPase activity in choroid plexus. The ability of NO to cause alterations in ouabain-sensitive Na⁺-K⁺-ATPase activity is corroborated by previous reports demonstrating NO’s regulation of ouabain-sensitive Na⁺-K⁺-ATPase activity in other tissues, including stimulation in rat proximal trachea (8) and inhibition in rat kidney medulla (30).

Nitrovasodilators, including SNP, can activate soluble guanylate cyclase in a number of tissues, presumably through release of NO. The ability of the specific soluble guanylate cyclase inhibitor ODQ to antagonize the actions of SNP on the Na⁺-K⁺-ATPase would suggest that a direct consequence of NOS stimulation is activation of soluble guanylate cyclase. The inhibition of Na⁺-K⁺-ATPase by SNP was consistently associated with an increase in cGMP, as determined from parallel measurements of cGMP and Na⁺-K⁺-ATPase activity in SNP-exposed choroid plexus, and this action was mimicked by 8-bromo-cGMP. Further evidence for the involvement of cGMP as a mediator in the action of SNP on Na⁺-K⁺-ATPase activity was obtained with agents capable of elevating choroid plexus cGMP levels through a SNP-independent pathway (Nathanson and Ellis, unpublished data). As with SNP, ouabain-sensitive Na⁺-K⁺-ATPase activity was inhibited by carbon monoxide and atrial natriuretic peptide, with concomitant increases in cGMP levels. These observations suggest that cGMP may play a wide role in regulating

the cholinergic response in choroid plexus. These results are consistent with earlier findings that demonstrated cholinergic involvement in regulating CSF secretion (27). Other independent studies have shown

Fig. 7. Effects of PKG inhibitors KT-5823 and Rp-8-pCPT-cGMP (Rp-8) on SNP-induced inhibition of ouabain-sensitive Na⁺-K⁺-ATPase activity in bovine choroid plexus. A: samples were incubated with KT-5823 (2.0 μM), Rp-8-pCPT-cGMP (5.0 μM), or KT-5720 (0.5 μM) (PKA inhibitor) for 5 min at 34°C and then with and without SNP (100 μM) for 15 min at 34°C. B: samples were incubated with Rp-8-pCPT-cGMP (1.0 mM) for 3 min at 34°C and then with and without SNP for 15 min at 34°C. Values for ouabain-sensitive Na⁺-K⁺-ATPase activity represent means ± SE for triplicate samples of 5 experiments. *Significantly different from control group: \( P \leq 0.005 \) (by ANOVA).

Fig. 8. Rp-8-pCPT-cGMP’s inhibition of the Na⁺-K⁺-ATPase is concentration dependent. Detergent-purified Na⁺-K⁺-ATPase from bovine choroid plexus (specific activity, 300 μmol Pi h⁻¹ mg protein⁻¹) was exposed to various concentrations of Rp-8-pCPT-cGMP (0.5 μM–1 mM) (34°C for 15 min). Na⁺-K⁺-ATPase activity was determined using the colorimetric method. Data is expressed as percent of control (the control group was incubated with vehicle). *Significantly different from control group: \( P \leq 0.05 \) (by ANOVA).
Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in many cell types. Our studies, however, do not preclude the involvement of other second messengers, such as protein kinase A or protein kinase C, in modulating Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in secretory epithelia.

Because 8-bromo-cGMP is known to be an activator of PKG, the observation that 8-bromo-cGMP mimics the actions of SNP would suggest that PKG might be involved in the inhibition of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase by NO. For technical reasons, we were unable to demonstrate in vivo regulation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase by PKG in choroid plexus tissue, however. Choroid plexus tissue slices were exposed to either KT-5823 (2.0 \mu M), a competitive inhibitor at the binding site for ATP (23), or Rp-8-pCPT-cGMP (5.0 \mu M), which binds to the cGMP binding site (6). Neither drug blocked SNP's effect on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity, although similar concentrations have been effective in protecting Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in other systems (30).

The problem may be one of using a tissue with a high level of NO-pathway constituents. For example, platelets have an unusually high concentration of PKG, therefore 1 mM Rp-8-pCPT-cGMP was required to inhibit it (6). Consequently, concentrations of the inhibitors that work in renal or other tissue preparations may be insufficient for choroid plexus. We did not attempt to use a higher concentration of KT-5823 because of its cross-reactivity with protein kinase A. A higher concentration of Rp-8-pCPT-cGMP (1 mM) inhibited Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in tissue slices by itself, and treatment at the same time with SNP did not cause any further change in activity. Curiously, the same phenomenon was reported by de Oliviera Elías et al. (8), who used only 2.0 \mu M KT-5823 in studies of the stimulation of rat tracheal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. Because these inhibitors have an inhibitory effect on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity by an unknown mechanism, they are not suitable reagents for assessing the participation of PKG in the NO pathway. Direct inhibition of purified Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in the test tube was seen after preincubation in Rp-8-pCPT-cGMP (50 \mu M), and this may have contributed to the inhibition seen in slices. Such cross-reactivity is not implausible for two proteins with nucleotide binding sites. It is also possible that the cGMP pathway has effects through two different, interacting mechanisms that we do not understand at present.

Other evidence for the role of protein phosphorylation in regulating Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in choroid plexus was obtained from studies using the types 1 and 2A protein phosphatase inhibitor okadaic acid. The action of SNP and 8-bromo-cGMP in inhibiting ouabain-sensitive Na\textsuperscript{+}-K\textsuperscript{+}-ATPase was mimicked by okadaic acid. Other studies have demonstrated that okadaic acid mimicked the action of SNP, atrial natriuretic peptide, and 8-bromo-cGMP in renal medulla in inhibiting ouabain-sensitive Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, without affecting cGMP synthesis (Nathanson, unpublished observations). This interpretation is consistent with the presumed action of okadaic acid to bypass endogenous cGMP/PKG-dependent activation of the protein phosphatase inhibitors DARPP-32 and inhibitor 1 by inhibiting protein phosphatase directly (40, 45). Studies in the kidney have shown that DARPP-32 and inhibitor 1 are involved in the regulation of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (3). Alternatively, okadaic acid could be blocking a protein phosphatase 2A in choroid plexus. The fact that okadaic acid works also implies that there is a basal kinase activity in the absence of added agonists in our experimental protocols.

It has been known for years that the luminal localization of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase in choroid plexus epithelium plays a pivotal role in CSF production. The physiological implications for the role of the cholinergic-NOS system in choroid plexus are intriguing. Because cGMP-generating compounds and acetylcholine have the ability to decrease CSF production, this suggests that stimulation of cholinergic neurons and subsequent release of NO may decrease CSF production in part via inhibition of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity.

NO's inhibition of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in bovine choroid plexus epithelium may be more complex due to the multiple Na\textsuperscript{+}-K\textsuperscript{+}-ATPase isoforms that exist in this species (Ellis and Sweadner, unpublished observations). It should be noted that the directionality of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase modulation by carbobach, SNP, and other cGMP-generating agents may not be the same for all isoforms of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, which are differentially distributed among various tissues and species (43). For example, it has been observed that in rat kidney, which also contains the \(\alpha_1\)-isoform of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, SNP and cGMP cause inhibition of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (30). Studies using mouse brain vessel endothelial cells, which express Na\textsuperscript{+}-K\textsuperscript{+}-ATPase \(\alpha_1\)-, \(\alpha_2\)-, and \(\alpha_3\)-isoforms, have demonstrated that the cGMP-induced inhibition of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase is due mainly to the inhibition of the \(\alpha_1\)-isoform (35). Other studies in rat nervous system, which also expresses Na\textsuperscript{+}-K\textsuperscript{+}-ATPase \(\alpha_1\)-, \(\alpha_2\)-, and \(\alpha_3\)-isoforms, show that cGMP-generating agents caused a marked stimulation of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (20, 32). The present studies also do not rule out parallel regulation of Na\textsuperscript{+} transport through other mechanisms such as bumetanide-sensitive K\textsuperscript{+} cotransport (25) or Na\textsuperscript{+}-H\textsuperscript{+} exchanger (31), which by regulating Na\textsuperscript{+} entry to the cell may alter Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in intact tissue.

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