Na$^+$,K$^+$-ATPase Phosphorylation in the Choroid Plexus: Synergistic Regulation by Serotonin/Protein Kinase C and Isoproterenol/cAMP-PK/PP-1 Pathways

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Abstract

Background: The ion pump Na$^+$,K$^+$-ATPase is responsible for the secretion of cerebrospinal fluid from the choroid plexus. In this tissue, the activity of Na$^+$,K$^+$-ATPase is inhibited by serotonin via stimulation of protein kinase C–catalyzed phosphorylation. The choroid plexus is highly enriched in two phosphoproteins which act as regulators of protein phosphatase-1 activity, DARPP-32 and inhibitor-1. Phosphorylation catalyzed by cAMP-dependent protein kinase on a single threonyl residue converts DARPP-32 and inhibitor-1 into potent inhibitors of protein phosphatase-1. Previous work has shown that in the choroid plexus, phosphorylation of DARPP-32 and I-1 is enhanced by isoproterenol and other agents that activate cAMP-PK. We have now examined the possible involvement of the cAMP-PK/protein phosphatase-1 pathway in the regulation of Na$^+$,K$^+$-ATPase.

Materials and Methods: The state of phosphorylation of Na$^+$,K$^+$-ATPase was measured by determining the amount of radioactivity incorporated into the ion pump following immunoprecipitation from ^{32}P-prelabeled choroid plexuses incubated with various drugs (see below). Two-dimensional phosphopeptide mapping was employed to identify the protein kinase involved in the phosphorylation of Na$^+$,K$^+$-ATPase.

Results: The serotonin-mediated increase in Na$^+$,K$^+$-ATPase phosphorylation is potentiated by okadaic acid, an inhibitor of protein phosphatases-1 and -2A, as well as by forskolin or the β-adrenergic agonist, isoproterenol, activators of cAMP-dependent protein kinase. Two-dimensional phosphopeptide maps suggest that this potentiating action occurs at the level of a protein kinase C phosphorylation site. Forskolin and isoproterenol also stimulate the phosphorylation of DARPP-32 and protein phosphatase inhibitor-1, which in their phosphorylated form are potent inhibitors of protein phosphatase-1.

Conclusions: The results presented here support a model in which okadaic acid, forskolin, and isoproterenol achieve their synergistic effects with serotonin through phosphorylation of DARPP-32 and inhibitor-1, inhibition of protein phosphatase-1, and a reduction of dephosphorylation of Na$^+$,K$^+$-ATPase at a protein kinase C phosphorylation site.

Introduction

The cerebrospinal fluid (CSF) provides the brain with a chemically stable environment necessary

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for normal neuronal function. Secretion of CSF occurs primarily at the level of the choroid plexus and is dependent on the activity of the ion pump Na$^+$,K$^+$-ATPase. Na$^+$,K$^+$-ATPase catalyzes the transport of Na$^+$ across the choroidal cell with a consequent movement of chloride and bicarbonate ions into the ventricle. This in

turn results in the creation of an osmotic gradient responsible for the diffusion of water into the ventricular cavity and the production of CSF (1,2). Although several hormones and neurotransmitters have been implicated in the regulation of the volume and composition of CSF (3–5), little is known about the mechanisms by which such regulation occurs.

It has been shown that stoichiometric phosphorylation of Na$^+$,K$^+$-ATPase by protein kinase C results in a 50% inhibition of activity (6). More recently, it was shown that in the choroid plexus, phorbol 12,13-dibutyrate inhibited the activity of Na$^+$,K$^+$-ATPase by about 30% and serotonin inhibited the activity by about 15%. It was calculated that these inhibitions were associated with a stoichiometry of phosphorylation of 0.6 and 0.3, respectively (7). These effects appeared to be mediated via activation of protein kinase C (7) and could be responsible for the decrease in CSF secretion observed after systemic administration of serotonin (5) or of its precursor, 5-hydroxytryptophan (8).

Previous studies performed using renal preparations indicate that protein phosphatase-1 (PP-1) is also involved in the regulation of Na$^+$,K$^+$-ATPase activity (9). The choroid plexus is highly enriched in two phosphoproteins which act as regulators of PP-1 activity, a dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) and protein phosphatase inhibitor-1 (I-1) (10). Phosphorylation catalyzed by cAMP-dependent protein kinase (cAMP-PK) on a single threonyl residue converts DARPP-32 and I-1 into potent inhibitors of PP-1 (11). Previous work has shown that in the choroid plexus, phosphorylation of DARPP-32 and I-1 is enhanced by treatments that activate cAMP-PK (12). In the present study, we have examined the possible involvement of the cAMP-PK/PP-1 cascade in the control of the state of phosphorylation of Na$^+$,K$^+$-ATPase in the rat choroid plexus.

Materials and Methods

^{32}P-Labeling and Pharmacological Treatment of Choroid Plexus

Male Sprague-Dawley rats (200–250 g) were killed by decapitation and the choroid plexus was dissected from the lateral cerebral ventricles. Choroid plexus from one rat per treatment condition was incubated at 30°C in 2 ml of Krebs bicarbonate buffer (124 mM NaCl, 4 mM KCl, 26 mM NaHCO$_3$, 1.5 mM CaCl$_2$, 1.5 mM MgSO$_4$,

0.25 mM KH$_2$PO$_4$, 10 mM glucose) equilibrated with 95% O$_2$/5% CO$_2$ (v/v). After 15 min, medium was replaced with the same amount of fresh buffer containing 2.5 mCi of ^{32}P-orthophosphoric acid (DuPont NEN; specific activity 8500–9120 Ci/mmol) and the tissue was incubated for 60 min. The radioactive buffer was then removed, and ^{32}P-labeled choroid plexuses were washed twice with 2 ml of fresh buffer and incubated for an additional 10 min in the presence or absence of drugs, as described. After drug treatment, the buffer was removed and the tissue was rapidly frozen in liquid nitrogen and stored at −70°C until assayed.

Assay of Na$^+$,K$^+$-ATPase Phosphorylation in Choroid Plexus

^{32}P-labeled choroid plexuses were sonicated in 1 ml of lysis buffer [20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% bovine serum albumin (BSA); pH 8.0] containing 50 mM NaF to block phosphatase activity and the following protease inhibitors: 1 mM EGTA, 25 mM benzamidine, 100 μM phenylmethylsulfoxide, 20 μg/ml chymostatin, 20 μg/ml pepstatin A, 5 μg/ml leupeptin, and 5 μg/ml antipain (Peptide International). Aliquots of the homogenate (20 μl) were used for determination of total ^{32}P-incorporation into trichloroacetic acid–precipitated proteins. Ten milligrams of preswollen Protein A Sepharose CL-4B (Pharmacia Biotech) was added to each tube and the samples mixed for 30 min at 4°C. The Sepharose beads and associated, nonspecifically adsorbed proteins were removed by centrifugation for 10 sec at 15,000 rpm in a tabletop microcentrifuge. The supernatants were mixed for 2 hr at 4°C with 15 μl of mouse ascites fluid containing a monoclonal antibody specific for the α1 isoform of Na$^+$,K$^+$-ATPase (13) [the only isoform expressed in the choroid epithelium (14)], followed by a 1-hr incubation with 15 μl of affinity-purified rabbit anti-mouse antibody (Cappel, 1 mg/ml). The samples were then transferred to Eppendorf tubes containing 10 mg of preswollen Protein A Sepharose beads and incubated for 1 hr at 4°C. The beads were collected by centrifugation and washed once with 1 ml of lysis buffer, three times with 1 ml of a buffer containing 20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), and 0.2% BSA (pH 8.0), three times with 1 ml of a buffer containing 20 mM Tris-HCl, 500 mM NaCl, 0.5% Triton X-100, and 0.2% BSA (pH

8.0), and one time with 1 ml of a buffer containing 50 mM Tris-HCl (pH 8.0). After the final wash, the beads were resuspended in 50 μl of SDS-PAGE sample buffer (50 mM Tris-HCl, 10% glycerol, 2% SDS, 10% 2-mercaptoethanol, 0.01% bromophenol blue, pH 6.8), vortexed, and centrifuged. The recovered proteins were separated by SDS-PAGE on 7.5% acrylamide gels (15). Gels were dried and ^{32}P-incorporation into Na$^+$,K$^+$-ATPase was quantified using a PhosphorImager 400B and ImageQuant™ software from Molecular Dynamics. Individual values of ^{32}P-incorporation into Na$^+$,K$^+$-ATPase were corrected for total ^{32}P-incorporation measured for each sample.

Determination of Phosphorylation of DARPP-32 and I-1

The state of phosphorylation of DARPP-32 and that of I-1 were determined as previously described, using an antibody able to recognize phospho[Thr[34]]-DARPP-32 and phospho-[Thr[35]]-I-1 (12).

Phosphorylation of Purified Na$^+$,K$^+$-ATPase

Phosphorylation of purified rat kidney Na$^+$,K$^+$-ATPase (16) by protein kinase C was carried out for 30 min at 22°C in a reaction volume of 100 μl containing 50 mM Hepes, pH 7.5, 10 mM MgCl$_2$, 1.2 mM CaCl$_2$, and ~2 μg of Na$^+$,K$^+$-ATPase. The final concentration of protein kinase C in the reaction mixture was 100 μg/ml. Reactions were initiated by the addition of 100 μM [γ-^{32}P]ATP (6 × 10^{5} cpm/nmol) and terminated after 30 min by the addition of SDS-PAGE sample buffer. Phosphorylation of Na$^+$,K$^+$-ATPase by cAMP-PK was performed as previously described (17). Samples were subjected to SDS-PAGE (15).

Two-Dimensional Phosphopeptide Mapping

Pieces containing ^{32}P-labeled Na$^+$,K$^+$-ATPase α1 subunit, either purified from rat renal cortex or immunoprecipitated from choroid plexus, were excised from dried gels, washed with two changes of 10% acetic acid/30% methanol, and three changes of 50% methanol, and lyophilized. One milliliter of 50 mM NH$_4$HCO$_3$, pH 8.0, containing trypsin (Calbiochem, 100 μg/ml), was added to the dried gel pieces, and the mixture was incubated at 37°C for 20 hr. The supernatants were removed and the gel pieces were washed with an additional 0.5 ml of 50 mM

NH$_4$HCO$_3$. The pooled supernatants were lyophilized. Dried samples were resuspended in electrophoresis buffer (10% acetic acid, 1% pyridine, pH 3.5) and spotted 10 cm from the right and 4 cm from the bottom on thin-layer cellulose sheets (20 × 20 cm, Eastman Kodak). Phosphopeptides were separated by electrophoresis at 400 V for 60 min in the first dimension (left, positive), followed by chromatography in the second dimension in a buffer containing pyridine:1-butanol:water:acetic acid (15:10:12:3, v/v). Dried sheets were subjected to autoradiography.

Results

In preliminary experiments, ^{32}P-prelabeled choroid plexuses were incubated for 10 min at 30°C in the presence of a range of serotonin concentrations between 100 nM and 1 mM. Serotonin increased Na$^+$,K$^+$-ATPase phosphorylation by 5-fold at a concentration of 100 nM. A maximal effect (1328 ± 397% of control) was observed at a concentration of 10 μM (data not shown).

Figure 1 shows the effect of okadaic acid, an inhibitor of PP-1 and protein phosphatase-2A, used alone or in combination with serotonin, on the state of phosphorylation of Na$^+$,K$^+$-ATPase. Incubation of ^{32}P-prelabeled choroid plexus in the presence of 10 μM serotonin increased the amount of phosphorylated Na$^+$,K$^+$-ATPase by about 10-fold, compared with control, unstimulated tissue. In the presence of okadaic acid alone (1 μM, a concentration able to block both PP-1 and protein phosphatase-2A), a 4- to 5-fold increase in the level of phosphorylated Na$^+$,K$^+$-ATPase was observed. Moreover, incubation in the presence of okadaic acid plus 10 μM serotonin caused a synergistic increase in Na$^+$,K$^+$-ATPase phosphorylation.

The effect of forskolin, an activator of the adenylyl cyclase/cAMP-PK pathway, used alone or in combination with serotonin, on the state of phosphorylation of Na$^+$,K$^+$-ATPase is shown in Figure 2. Forskolin caused a slight increase in Na$^+$,K$^+$-ATPase phosphorylation and was able to potentiate by about 2.5-fold the stimulation exerted by serotonin. Forskolin and serotonin were also compared for their ability to affect the state of phosphorylation of DARPP-32 and I-1. In contrast to forskolin, which stimulated by several-fold the phosphorylation of DARPP-32 and I-1, serotonin, either alone or in combination with forskolin, did not produce any effect (Fig. 2C).
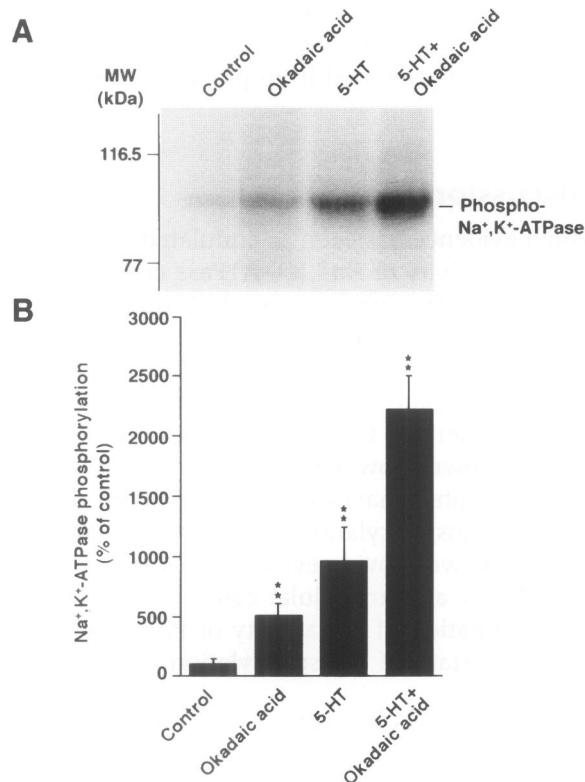
Fig. 1. Effect of okadaic acid and serotonin on the state of phosphorylation of Na$^+$,K$^+$-ATPase in choroid plexus. Phosphorylation of Na$^+$,K$^+$-ATPase was measured as incorporation of radioactivity into the protein after stimulation of ^{32}P-labeled choroid plexuses for 10 min at 30°C in the presence or absence of 1 μM okadaic acid and 10 μM serotonin (5-HT). (A) Autoradiogram obtained after immunoprecipitation of Na$^+$,K$^+$-ATPase and separation by SDS-PAGE, showing the incorporation of ^{32}P into Na$^+$,K$^+$-ATPase in a typical experiment. (B) Quantitative analysis of Na$^+$,K$^+$-ATPase phosphorylation determined in three experiments (data represent means ± S.E.M.); **$p < 0.01$ vs. control, Student's t test.

The β-adrenergic agonist isoproterenol (10 μM) also activates the adenylyl cyclase/cAMP-PK pathway in rat choroid plexus (18,19), thereby increasing the levels of phosphorylated DARPP-32 and I-1 (12). We therefore tested this substance for its ability to modulate the state of phosphorylation of Na$^+$,K$^+$-ATPase. Isoproterenol significantly potentiated the serotonin-induced increase in Na$^+$,K$^+$-ATPase phosphorylation, without affecting the basal phosphorylation of Na$^+$,K$^+$-ATPase (Fig. 3).

Both protein kinase C and cAMP-PK phosphorylate Na$^+$,K$^+$-ATPase in vitro, thereby inhibiting the activity of the ion pump (6). Phosphorylation by these two protein kinases is
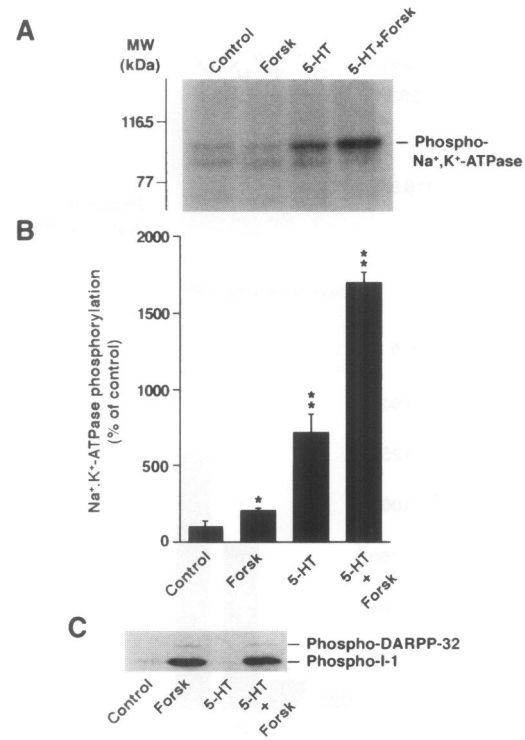

Fig. 2. Effect of forskolin and serotonin on the state of phosphorylation of Na$^+$,K$^+$-ATPase, DARPP-32, and I-1 in choroid plexus. ^{32}P-labeled choroid plexuses were incubated for 10 min at 30°C in the presence or absence of 10 μM forskolin (Forsk) and 10 μM serotonin (5-HT). Phosphorylation of Na$^+$,K$^+$-ATPase was measured as incorporation of radioactivity into the protein. Phosphorylation of DARPP-32 and I-1 was determined in the same samples using a phosphorylation state–specific antibody (see Materials and Methods). (A) Autoradiogram obtained after immunoprecipitation of Na$^+$,K$^+$-ATPase and separation by SDS-PAGE, showing the incorporation of ^{32}P into Na$^+$,K$^+$-ATPase in a typical experiment. (B) Quantitative analysis of Na$^+$,K$^+$-ATPase phosphorylation determined in three experiments (data represent means ± S.E.M.); *$p < 0.05$ and **$p < 0.01$ vs. control, Student's t test. (C) Western blot showing phospho-DARPP-32 and phospho-I-1.

known to occur at distinct sites on Na$^+$,K$^+$-ATPase (17,20,21). Tryptic cleavage of purified Na$^+$,K$^+$-ATPase (16) phosphorylated in vitro using protein kinase C or cAMP-PK generates distinct phosphopeptide maps (Fig. 4A and B). Therefore, we have used two-dimensional phosphopeptide mapping to identify the site(s) of phosphorylation affected by serotonin, serotonin plus okadaic acid, or serotonin plus forskolin, in intact cells. Tryptic cleavage of ^{32}P-labeled Na$^+$,K$^+$-ATPase immunoprecipitated from cho-
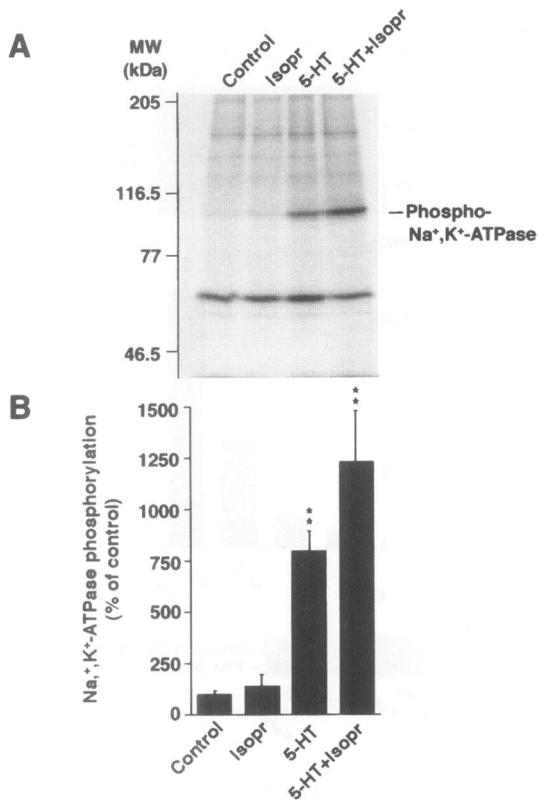
Fig. 3.   Effect of isoproterenol and serotonin on the state of phosphorylation of Na^+,K^+-ATPase in choroid plexus. Phosphorylation of Na^+,K^+-ATPase was measured as incorporation of radioactivity into the protein after stimulation of ^{32}P-labeled choroid plexuses for 10 min at 30°C in the absence or presence of 10 μM isoproterenol (Isopr) and 10 μM serotonin (5-HT). (A) Autoradiogram obtained after immunoprecipitation of Na^+,K^+-ATPase and separation by SDS-PAGE, showing the incorporation of ^{32}P into Na^+,K^+-ATPase in a typical experiment. (B) Quantitative analysis of Na^+,K^+-ATPase phosphorylation determined in three experiments (data represent means $\pm$ S.E.M.); $**p < 0.01$ vs. control, Student's t test.

roid plexuses incubated with serotonin alone (Fig. 4C), serotonin plus okadaic acid (Fig. 4D), or serotonin plus forskolin (Fig. 4E) generates radioactive peptides that are present in the tryptic map obtained from Na^+,K^+-ATPase phosphorylated in vitro using protein kinase C (Fig. 4A) but not cAMP-PK (Fig. 4B). It should be noted that some of the minor peptides present in the tryptic map generated from protein kinase C–phosphorylated purified Na^+,K^+-ATPase (Fig. 4A, arrows) are not always visible in the maps of immunoprecipitated ^{32}P-labeled Na^+,K^+-ATPase obtained from prelabeled tissue.

This is due to the lower level of ^{32}P-incorporation into Na^+,K^+-ATPase obtained when the protein is immunoprecipitated from prelabeled tissue.

Discussion

Much evidence has been accumulated indicating that the activity of Na^+,K^+-ATPase can be regulated by controlling its state of phosphorylation (6,7,17,22). Studies performed using intact cell systems have shown that protein kinase C is able to phosphorylate the catalytic α-subunit of this ATPase, thereby inhibiting its activity (7,22). Less is known, however, about the contribution of protein phosphatases to the regulation of the state of phosphorylation of Na^+,K^+-ATPase. In this study, we provide evidence that in the choroid plexus, an intracellular cascade that leads to the modulation of the activity of PP-1 may regulate the state of phosphorylation of Na^+,K^+-ATPase.

The involvement of PP-1 in the dephosphorylation of Na^+,K^+-ATPase is suggested by the effect of okadaic acid, which dramatically increases the serotonin-stimulated phosphorylation of the ion pump. These results, together with the previous finding of very high levels of the PP-1 inhibitors DARPP-32 and I-1 in the choroid plexus, indicate a possible role for these two proteins in the control of the state of phosphorylation of Na^+,K^+-ATPase. In agreement with previous studies (12), stimulation of cAMP-PK using either forskolin or the β-adrenergic agonist isoproterenol causes an increase in the state of phosphorylation of DARPP-32 and I-1. Such an increase seems likely to result in a significant inhibition of PP-1 activity and a consequent increase in the levels of phosphorylated Na^+,K^+-ATPase.

To further test this hypothesis we analyzed the effect of stimulation of cAMP-PK on Na^+,K^+-ATPase phosphorylation. cAMP-PK has been shown to directly phosphorylate and inhibit the activity of Na^+,K^+-ATPase in vitro (6). Our data, however, indicate that incubation of choroid plexus with forskolin or isoproterenol causes only a modest increase in Na^+,K^+-ATPase phosphorylation. Even this small increase cannot be ascribed to phosphorylation of Na^+,K^+-ATPase catalyzed by cAMP-PK, as two-dimensional phosphopeptide mapping experiments indicate that forskolin-induced activation of cAMP-PK affects a protein kinase C phosphorylation site (data not shown). In spite of the modest effect on
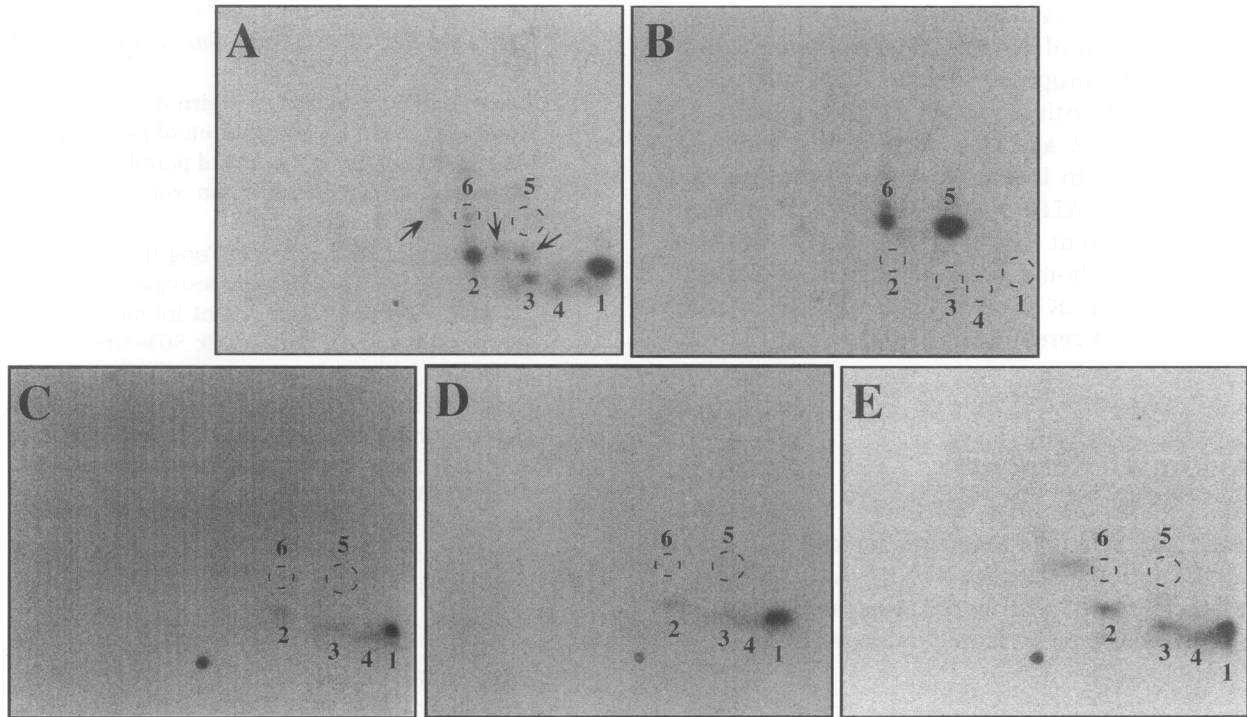
Fig. 4. Autoradiograms of two-dimensional phosphopeptide maps of Na$^+$,K$^+$-ATPase α-subunit after phosphorylation in vitro or in intact cells. (A) Purified Na$^+$,K$^+$-ATPase phosphorylated with protein kinase C in the presence of 100 μM [γ-^{32}P]ATP. (B) Purified Na$^+$,K$^+$-ATPase phosphorylated with cAMP-PK in the presence of 100 μM [γ-^{32}P]ATP. (C) Na$^+$,K$^+$-ATPase immunoprecipitated from ^{32}P-prelabeled choroid plexus incubated with serotonin. (D) Na$^+$,K$^+$-ATPase immunoprecipitated from ^{32}P-prelabeled choroid plexus incubated with serotonin plus okadaic acid. (E) Na$^+$,K$^+$-ATPase immunoprecipitated from ^{32}P-prelabeled choroid plexus incubated with serotonin plus forskolin. The more prominent phosphopeptides generated by tryptic digestion of Na$^+$,K$^+$-ATPase phosphorylated with protein kinase C and cAMP-PK are designated (1–4) and (5–6), respectively. Where absent, their position are indicated by dashed circles.

basal phosphorylation, forskolin and isoproterenol strongly potentiate the increase in Na$^+$,K$^+$-ATPase phosphorylation induced by serotonin.

These results support a model (Fig. 5) in which stimulation of cAMP-PK affects the state of phosphorylation of Na$^+$,K$^+$-ATPase via inhibition of dephosphorylation of the protein kinase C phosphorylation site. According to this model, in choroid epithelial cells serotonin and noradrenaline regulate the state of phosphorylation of Na$^+$,K$^+$-ATPase through complementary mechanisms: on the one hand, activation of 5-HT$_{2C}$ serotonin receptors stimulates the protein kinase C–catalyzed phosphorylation of Na$^+$,K$^+$-ATPase (7); on the other hand, activation of β-adrenergic receptors stimulates cAMP-PK which phosphorylates DARPP-32 and I-1, thereby inhibiting the activity of PP-1 and reducing the dephosphorylation of the ion pump.
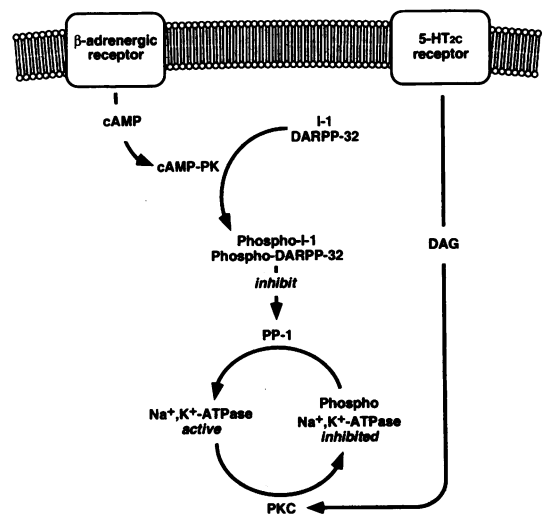
Previous studies have shown that serotonin

Fig. 5. Model to account for the synergistic effects of serotonin and a β-adrenergic agonist on the state of phosphorylation of Na$^+$,K$^+$-ATPase. For details see Discussion. DAG, diacylglycerol; PKC, protein kinase C.

(5,8,23) and noradrenaline (24,25) each reduce the production of cerebrospinal fluid. According to the model proposed (Fig. 5), these effects are the result of distinct actions on the activities of protein kinase C and PP-1, respectively, resulting in both cases in increased levels of phosphorylated Na^+,K^+-ATPase. One prediction stemming from the present model is that the two neurotransmitters should have synergistic inhibitory effects on Na^+,K^+-ATPase activity and on the production of cerebrospinal fluid.

Acknowledgments

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