Phosphorylation of DARPP-32 and Protein Phosphatase Inhibitor-1 in Rat Choroid Plexus: Regulation by Factors Other Than Dopamine

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The molecular mechanisms underlying regulation of fluid production by secretory epithelia such as the choroid plexus are poorly understood. Two cAMP-regulated inhibitors of protein phosphatase-1, inhibitor-1 (I₁) and a dopamine- and cAMP-regulated phosphoprotein, $M_r = 32,000$ (DARPP-32), are enriched in the choroid plexus. We show here that these two phosphoproteins are colocalized in choroid plexus epithelial cells. We have developed a novel method for studying the phosphorylation state of DARPP-32 and I₁ in intact cells, using a phosphorylation state-specific monoclonal antibody. Several drugs and hormones that are known to alter fluid secretion and that increase cAMP levels (forskolin, isoproterenol, vasoactive intestinal peptide) or cGMP levels (atrial natriuretic peptide) or that may use additional second messenger pathways (5-HT), increase the phosphorylation of I₁ and DARPP-32 in rat choroid plexus. In contrast, dopamine does not alter cAMP and cGMP levels, or I₁ and DARPP-32 phosphorylation. Our results indicate that DARPP-32, known to be regulated by dopamine in a number of tissues, can be phosphorylated in response to non-dopaminergic factors, including hormones acting through non-cAMP-dependent pathways. Our results also raise the possibility that inhibition of phosphatase-1, as a result of I₁ and DARPP-32 phosphorylation, might be part of a final common pathway in the action of several factors that are known or thought to alter cerebrospinal fluid production.

Cerebrospinal fluid (CSF) is formed in and secreted from epithelial cells of choroid plexus (for review, see Cserr, 1971). Although the physiological events underlying CSF production are beginning to be understood (see Johanson, 1989, for recent review), the mechanisms that regulate this essential process re-

main unclear. There is some evidence that CSF production is under neural and hormonal control and that some of the neurotransmitters and hormones involved act through the regulation of cAMP. For example, drugs that directly increase cAMP levels by activating adenylyl cyclase (i.e., cholera toxin or forskolin) have been reported to alter the rate of CSF production in choroid plexus (Epstein et al., 1977; Feldman et al., 1979) and the rate of aqueous humor production from the physiologically analogous ciliary process (Gregory et al., 1981; Caprioli et al., 1984). Several neurotransmitters and hormones, including norepinephrine and vasoactive intestinal peptide (VIP), also stimulate cAMP formation in choroid plexus and have been hypothesized to influence CSF production by this mechanism (Nathanson, 1979, 1980; Crook et al., 1984; Lindvall et al., 1985; Crook and Prusiner, 1986). However, at present, the mechanisms by which cAMP might alter CSF formation are unknown.

Neurotransmitters and hormones acting through cAMP are known to modulate cellular processes by the reversible phosphorylation of proteins (Nestler and Greengard, 1984). One class of regulated phosphoproteins that has been postulated to play a role in the mediation of cAMP-associated hormone action is the protein phosphatase inhibitors, DARPP-32 (i.e., dopamine- and cAMP-regulated phosphoprotein, $M_r = 32,000$) (Walaas et al., 1983a,b; Hemmings et al., 1984b; Walaas and Greengard, 1984) and inhibitor-1 (I₁) (Ingebritsen and Cohen, 1983; Ingebritsen et al., 1983). Both proteins are phosphorylated by cAMP-dependent protein kinase on a single threonyl residue and, in phosphorylated form, are potent inhibitors of protein phosphatase-1 *in vitro* (Hemmings et al., 1984a). DARPP-32 is abundant in brain neurons possessing the dopamine D₁-receptor (e.g., medium-sized striatonigral neurons) (Ouimet et al., 1984; Hemmings and Greengard, 1986) and is phosphorylated in these cells in response to dopamine acting through cAMP-dependent protein kinase (Walaas et al., 1983a,b). DARPP-32 is also found in high concentration in the choroid plexus (Hemmings and Greengard, 1986; Steardo and Nathanson, 1987). However, prior reports suggest that dopamine is a poor activator of adenylyl cyclase in this tissue and there is no evidence for a distinct dopamine-sensitive adenylyl cyclase (Nathanson, 1980). Because DARPP-32 phosphorylation has previously been shown to be regulated only by dopamine, this finding raises the question of whether DARPP-32 is uniquely a dopamine-regulated protein and whether DARPP-32 and homologous phosphoproteins might be involved in the action of other hormones and neurotransmitters.

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Figure 1. Amino acid sequences surrounding the cAMP-dependent phosphorylation sites of bovine DARPP-32 (*upper sequence*) and rabbit or rat I₁ (*lower sequence*). CAMP-dependent protein kinase phosphorylates a threonyl residue in a homologous region of both proteins, Thr₃₄ in DARPP-32 (Hemmings et al., 1984c) and Thr₃₅ in I₁ (Cohen et al., 1977). The sequence of the synthetic peptide that was phosphorylated and used for immunization is indicated by a *box*. One letter amino acid codes are used: *A*, alanine; *D*, aspartate; *E*, glutamate; *F*, phenylalanine; *I*, isoleucine; *L*, leucine; *M*, methionine; *P*, proline; *Q*, glutamine; *R*, arginine; *T*, threonine; *V*, valine. The sequences are from bovine caudate DARPP-32 (Williams et al., 1986) and rabbit or rat skeletal muscle I₁ (Aitken et al., 1982; Elbrecht et al., 1990).

Materials and Methods

Monoclonal antibodies were prepared against the phosphorylated peptide using the method of Kohler and Milstein (1975). The phosphopeptide conjugated to *Limulus* hemocyanin was used to immunize Balb/c mice. Spleen cells from mice whose serum was reactive with phospho-DARPP-32 were fused with SP 2/0 mouse myeloma cells. Screening of mouse sera and fusion supernatants was first carried out using a modified ELISA. Nunc II plates (96 well, Nunc, Sweden) were coated overnight with aliquots (1 μ g/well) of the 10 amino acid phosphorylated peptide (conjugated to BSA) that had been used for immunization of mice. After blocking with 1% BSA in PBS, supernatants from fusion hybrids (100 μ l) were added and incubated for 30 min at 37°C. The plates were washed three times with PBS containing 1% BSA and 0.1% Tween 80. Goat

anti-mouse IgG conjugated to horseradish peroxidase (100 μ l of a 1:100 dilution; Kirkegaard and Perry, Gaithersburg, MD) was added to each well, and incubation was carried out for an additional 30 min at 37°C, after which the plates were washed three times with 1% BSA and 0.1% Tween 80. Fifty microliters of a substrate solution containing a 1:1 mixture of hydrogen peroxide and ABTS (2,2-amino-di[3-ethyl-benzthiazoline sulfonate]; Kirkegaard and Perry, Gaithersburg, MD) were added to each well, and the resulting color reaction was quantified after 30 min using an SLT ER400 EIA reader (SLT Labinstruments, Austria). From approximately 2000 clones initially screened using this procedure, about 500 showed positive color reactions with the phosphorylated DARPP-32 peptide and were further tested with regard to their specificity for the phospho- versus dephospho-form of the native protein. The protocol for these experiments was essentially the same as that used for the primary screening except that plates were coated with aliquots of dephosphorylated or phosphorylated DARPP-32 purified and prepared as described above. Nearly 50% of these clones reacted preferentially with the phosphorylated DARPP-32, and 27 of these were selected for further use. The antibody produced by one of these clones (mAb 23) was shown to react strongly with the phospho-form but not the dephospho-form of DARPP-32 and was further assessed by immunoprecipitation of γ^{32} P-phospho-DARPP-32, as described previously (Hemmings and Greengard, 1986), and by dot-immunobinding assay. These cells were injected (intraperitoneally) into Balb/c mice primed with pristane (0.5 ml/mouse). Ascites was collected from these mice for use in the present experiments and stored at -70°C .

Immunoprecipitation of phospho-DARPP-32

Microcentrifuge tubes containing ^{125}I -labeled phospho-DARPP-32 (10,000 cpm/tube), 1:10 dilution of ascites, and various concentrations of an unlabeled competing antigen (dephospho-DARPP-32, phospho-DARPP-32, dephospho- I_1 , phospho- I_1) were incubated for 2 h at 4°C in 0.1 ml of NETF buffer, of the following composition 100 mM NaCl, 5 mM EDTA, 50 mM Tris HCl, pH 7.5, 50 mM NaF, 1% NP-40, 100 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mg/ml BSA. A 25 μ l aliquot of rabbit anti-mouse IgG was added to each tube, and these samples were incubated for an additional hour at 4°C . A solution of *Staphylococcus aureus* cells (SACs) bearing protein A (3.1 mg protein/ml) that had been washed and suspended in NETF buffer containing 25 mg/ml BSA was then added (50 μ l/tube), and the tubes were incubated for a 30 min period at 4°C . The tubes were centrifuged in a Beckman J-6M centrifuge at $1000 \times g$ for 15 min. The resulting SAC pellet was washed two times with 0.1 ml of NETF, and its radioactivity was measured using a Beckman 5500 gamma counter.

In vitro phosphorylation of proteins in acid extracts

Male Sprague-Dawley rats weighing 250–300 gm were killed by decapitation. The brains were placed on an ice-cold glass plate, and either corpus striatum or choroid plexus from lateral ventricles was dissected. Tissue was homogenized in 10 vol of cold 5 mM zinc acetate and centrifuged at $3000 \times g$ for 10 min, and the pellet was resuspended in 10 vol of 10 mM citric acid containing 0.1% Nonidet P-40. This acid extract was neutralized to pH 7.0 by addition of 0.1 vol of 0.5 M Na_2HPO_4 and boiled at 95°C for 1 min. This neutralized extract was centrifuged at $10,000 \times g$ for 10 min, and the supernatant was used for *in vitro* phosphorylation. Phosphorylation was carried out in a buffer containing 50 mM HEPES, pH 7.4, 10 mM magnesium acetate, 1 mM EGTA, 20 μM ATP, and 1 $\mu\text{g}/\text{ml}$ catalytic subunit of cyclic AMP-dependent protein kinase. The tubes were incubated in a shaking water bath at 30°C for 30 min. The incubation was terminated by the addition of an equal volume of "sample buffer" containing 50 mM Tris HCl, pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, and 0.01% bromophenol blue. Samples were heated at 95°C for 3 min and subjected to electrophoresis on 12% acrylamide gels (Laemmli, 1970).

In vitro phosphorylation of purified DARPP-32 and inhibitor-1

The time course of phosphorylation of DARPP-32 and I_1 by cAMP-dependent protein kinase as measured by immunoblotting with mAb 23 was compared with the time course of phosphate incorporation from γ^{32} P-ATP. Preparations of DARPP-32 and I_1 (10–20 μM) were phosphorylated *in vitro* as described above. In addition, the concentration

of catalytic subunit used was decreased by a factor of 1000 in order to reduce the rate of protein phosphorylation and facilitate measurement of time-dependent increases in phosphate incorporation. Parallel phosphorylation reactions were carried out in two tubes, one that received 1 mM unlabeled ATP and another that received 1 mM γ^{32} P-ATP (2 mCi/ml). Both tubes were incubated for 60 min. At 2.5, 5, 7.5, 10, 15, 30, 45, and 60 min, a 10 μ l aliquot of the phosphorylation mix was removed from each tube, added to a 50 μ l aliquot of sample buffer, and boiled for 3 min at 95°C . Proteins were separated by SDS-PAGE on a 12% acrylamide gel.

Immunoblotting

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose membranes was carried out according to Towbin et al. (1979). Dot blots were prepared by spotting 1 μ l of phospho- or dephospho-DARPP-32 at concentrations ranging from 1 to 100 $\mu\text{g}/\text{ml}$ on nitrocellulose strips. Proteins were fixed by a 10 min incubation in 25% isopropanol and 10% glacial acetic acid. Immunolabeling was performed as described for DARPP-32 and I_1 (Girault et al., 1989). Labeling of the antibodies was achieved by incubating the membranes sequentially in affinity-purified rabbit anti-mouse IgG (1:500, Cappel, when a mouse primary antibody was used) and ^{125}I -iodo-protein A as described previously (Girault et al., 1989).

Preparation and treatment of striatal reaggregate cultures

Striatal reaggregate cultures were prepared from embryonic day 14 CD-1 mouse embryos as described previously (Hemmendinger et al., 1981) with a few modifications (Girault et al., 1988). Three-week-old reaggregate cultures were incubated for 1 hr in modified Krebs' buffer and further incubated in the presence or absence of forskolin as described previously (Girault et al., 1988). Incubations were stopped by aspiration of the buffer and immersing the tubes containing the aggregates in liquid nitrogen. Cells were stored at -70°C until analysis. Reaggregates were homogenized by sonication in 1% boiling SDS and placed for an additional 10 min in a protein boiling water bath. Equal amounts of protein (150–350 μg) in sample buffer were subjected to SDS-PAGE and immunoblotting as described above.

Pharmacological treatment of rat choroid plexus

Rats were decapitated, and choroid plexus was dissected from the lateral cerebral ventricles. Choroid plexus from five rats per treatment condition were incubated in 25 ml Ehrlenmeyer flasks containing 20 ml of oxygenated RPMI 1640 medium in a shaking water bath (30°C). After a 45 min preincubation period, medium was replaced with fresh normal RPMI 1640 or medium containing drug, and the tissue was incubated for an additional 5–45 min. After drug treatment, choroid plexuses were rapidly transferred to glass tubes using a Pasteur pipette and homogenized in 5 vol of cold 5 mM zinc acetate. The tissue was acid extracted and proteins separated by SDS-PAGE as described above (*In vitro* phosphorylation of proteins in acid extracts). In addition, a 0.5 ml aliquot of the incubation medium and a 0.1 ml aliquot of the zinc acetate homogenate were retained from control and drug-treated tissue, acidified with an equal volume of 0.2 N HCl, and used for cyclic AMP determinations using the method of Brooker et al. (1976).

Immunohistochemical identification and localization of DARPP-32, I_1 , and Na,K-ATPase in rat choroid plexus

Intact tissue. Intact lateral and fourth ventricular choroid plexuses from 8-week-old male Sprague-Dawley rats were dissected in Dulbecco's modified Eagle's medium (DMEM) and meticulously cleaned of any adhering meninges or brain under a high-power dissecting scope. Samples of lateral and fourth ventricular choroid were cut into 2–4 mm pieces, fixed for 1 hr in 1.5% or 2% paraformaldehyde, washed twice for 5 min in phosphate-buffered normal saline (PBS), and then stored at 0°C . Prior to immunostaining, the pieces of choroid were transferred to albumin-coated slides and air dried.

Epithelial and vascular cell fractions. Choroid epithelium was isolated and separated from choroid stroma as described previously (Nathanson, 1980; Nathanson and Chun, 1989). Briefly, pooled choroid plexuses (above) were further washed (3 $\times$ 10 min) in 10 ml of DMEM by gently tumbling (90 rpm) in a Rotovapor apparatus (in a 100 ml spherical flask), which allowed suspended cells to be separated and removed from intact tissue. Subsequently, tissue was tumbled in calcium-free artificial

Figure 2. mAb 23 detected increasing quantities of phospho-DARPP-32 but not dephospho-DARPP-32 in dot blot analysis. Various amounts of phosphorylated (solid symbols) or dephosphorylated (open symbols) purified DARPP-32 were spotted on nitrocellulose strips and detected by incubation with various dilutions of mAb 23 (triangles, 1:50,000; circles, 1:5000). Antibody binding was determined by sequential incubation with rabbit anti-mouse IgG (1:500 dilution) and 125 I-iodo-protein A (1:1000 dilution) and was quantitated by gamma radiation counting. Each point represents the mean of triplicate determinations.

CSF (Nathanson, 1980) containing 0.05% trypsin (Sigma) and 15 μ M EDTA. Every 15 min for 4 hr, choroid plexuses were gently triturated and allowed to settle, and supernatant fractions containing suspended cells were removed from intact tissue; in this manner, a large peak of highly enriched epithelial cells was obtained (usually fractions 5–10), prior to the onset of dissociation of the choroid plexus stroma (usually fractions 11–15). Cells from pooled fractions 5–10 were filtered through 150 μ m Nitex mesh to remove large cell aggregates, centrifuged and washed at low speed with PBS, and then fixed in 1.5% or 2% paraformaldehyde for 1 hr. Following two washes with PBS, approximately $1-5 \times 10^4$ cells were air dried on albumin-coated slides. The remaining, largely deepithelialized and fragmented choroid plexus present in the original dissociating flask were washed with PBS and fixed as above, and then aliquots were air dried on albumin-coated slides.

For pharmacological studies of cyclic nucleotide accumulation in isolated choroid epithelium, cells, isolated as above, were preincubated for 60 min at 34°C in DMEM containing 40 mM HEPES under 95% O_2 , 5% CO_2 . Aliquots of 1×10^5 cells were then transferred to tubes containing 0.2 ml of artificial CSF (Nathanson, 1980) supplemented with 0.1% bovine serum albumin and 2.5 mM ascorbic acid. Either dopamine (100 μ M) or ANF (1 μ M) was added, and cells were incubated for 3 min, following which an equal volume of 150 mM sodium acetate was added and the tubes heated to 90°C for 3 min. Tubes were then centrifuged, and cAMP and cGMP levels were determined in the supernatant by radioimmunoassay (Du Pont).

Immunostaining. Air-dried slides of whole choroid or choroid cell fractions were washed three times with PBS and once (for 10 min) with 4% goat serum, and incubated overnight at 4°C with rabbit polyclonal antibody (K2, 1:150) to the α -form of Na,K-ATPase, with mouse monoclonal antibody (1:300) to DARPP-32, or with rabbit polyclonal anti-serum (G 187, 1:50–1:150) to I_1 diluted in 0.1% Triton X-100 and 4% goat serum in PBS. Following five washes with PBS, slides were incubated for 1 hr at room temperature with the appropriate fluorescein- or rhodamine-labeled goat secondary antibody [$F(ab')_2$ fragment] (Cappel Labs, 1:100 dilution), washed five times with PBS, mounted in 67% glycerol containing 0.67% propyl gallate, and viewed by epifluorescence with an inverted microscope.

Figure 3. Immunoprecipitation of 125 I-labeled phospho-DARPP-32 by mAb 23 was competitively inhibited by the phospho- but not the dephospho-form of DARPP-32 or I_1 . 125 I-iodo-phospho-DARPP-32 was incubated with mAb 23 (1:10 dilution) in the absence or presence of the phospho- (solid symbols) or dephospho- (open symbols) forms of either DARPP-32 (circles) or I_1 (triangles). Bound antigen was precipitated by sequential incubation with rabbit anti-mouse IgG and SACs bearing protein A. The amount of labeled antigen precipitated by antibody under each condition was quantitated by counting radioactivity of the SAC pellet. Under these conditions, approximately 60% of the labeled antigen was precipitated in the absence of competing antigens. All data are expressed as a mean percentage of this value $\pm$ SEM of three determinations; when not apparent on the figure, SEM values were smaller than the size of the symbol).

Miscellaneous methods. Autoradiography was performed using Kodak XAR-5 film at room temperature or at -70°C with Du Pont Quanta II intensifying screens. Amounts of 125 I-iodo-protein A bound to the antigen on nitrocellulose filters, or of $\gamma^{32}\text{P}$ -phosphate incorporated into proteins, were determined by excising the radioactive bands using autoradiograms as guides. 125 I-iodine was then measured in a gamma counter, and $\gamma^{32}\text{P}$ -phosphate was quantified by scintillation spectrometry or by determination of Cerenkov radiation. Data were analyzed by ANOVA (for post hoc comparisons), with significance defined as $p < 0.05$. Protein concentrations were measured by the bicinchoninic acid method (Smith et al., 1985) or by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Results

In vitro characterization of a monoclonal antibody specific for DARPP-32 phosphorylated by cAMP-dependent protein kinase. In dot-immunobinding assays, mAb 23 reacted with phospho-DARPP-32, but not dephospho-DARPP-32 (Fig. 2). The ability of this antibody to distinguish between phospho- and dephospho- forms of DARPP-32 was demonstrated further by competitive immunoprecipitation experiments in which precipitation of holo- 125 I-iodo-phospho-DARPP-32 was inhibited effectively by unlabeled phosphorylated DARPP-32, but not dephosphorylated DARPP-32 (Fig. 3). Because of the similar primary sequences surrounding the cAMP-dependent phosphorylation sites of DARPP-32 and I_1 , the precipitation of radiolabeled substrate also was inhibited competitively by phosphorylated I_1 in a concentration-dependent manner (Fig. 3).

Figure 4. Time course of DARPP-32 and I_1 phosphorylation measured by $\gamma^{32}\text{P}$ -incorporation (*A*) or immunoblotting with mAb 23 (*B*). Purified preparations of DARPP-32 (open symbols) or I_1 (solid symbols) were phosphorylated *in vitro* in the presence of catalytic subunit of cAMP-dependent protein kinase and either $\gamma^{32}\text{P}$ -ATP (*A*) or cold ATP (*B*). Aliquots of these reaction mixtures were withdrawn at various times and subjected to SDS-PAGE. Gels containing samples phosphorylated with radiolabeled phosphate were directly autoradiographed. Proteins phosphorylated in parallel experiments using cold ATP were transferred to nitrocellulose membranes and immunoblotted using mAb 23, rabbit anti-mouse IgG (1:500 dilution), and ^{125}I -iodo-protein A (1:1000 dilution). Radioactivity was counted in excised segments of gel ($\gamma^{32}\text{P}$ -phosphate) or nitrocellulose membranes (^{125}I -iodine) in order to quantitate phosphate content of proteins at each time point.

Dephospho- I_1 , like dephospho-DARPP-32, had no significant effect on the immunoprecipitation of ^{125}I -iodo-phospho-DARPP-32 by mAb 23 (Fig. 3).

The correlation between DARPP-32 phosphorylation and the detection of the protein by mAb 23 was examined by following the time course of DARPP-32 phosphorylation in the presence of cAMP-dependent protein kinase (Fig. 4). The $\gamma^{32}\text{P}$ -phosphate content of DARPP-32 increased rapidly within the initial 15 min of exposure to the kinase, as did the immunoreactivity of the protein toward mAb 23. Under the same conditions, I_1 was phosphorylated at a slower rate than DARPP-32 as indicated both by phosphate content and immunoreactivity of the protein.

Further characterization of the phospho-DARPP-32 antibody using striatal extracts and striatal reaggregate cultures. The usefulness of mAb 23 for detection of phosphorylated DARPP-32 in brain and peripheral tissues first was assessed using striatum, a brain region in which DARPP-32 is highly enriched (Hemmings and Greengard, 1986). An immunoreactive band with an

Figure 5. DARPP-32 and phospho-DARPP-32 were detected in phosphorylated acid extracts of rat striatum and in striatal reaggregate cultures treated with forskolin. Acid extracts of rat striatum were phosphorylated by incubation with the catalytic subunit of cAMP-dependent protein kinase for 30 min (lanes 2 and 4), or were incubated for 30 min without addition of the protein kinase (lanes 1 and 3), a treatment that promotes dephosphorylation of DARPP-32. Proteins were separated by SDS-PAGE (70 μg protein/lane), transferred to nitrocellulose, and immunoblotted as described in the Figure 4 caption using as the primary antibody either a mixture of the DARPP-32 monoclonal antibody C24-6A and -5A (mAb 5,6; 1:1000 dilution; lanes 1 and 2) or (mAb 23; 1:100 dilution; lanes 3 and 4). Striatal cultures were prepared from embryonic mouse striatum as described in Materials and Methods. Three week aggregate cultures were incubated for 6 min in the absence (lane 5) or presence (lane 6) of forskolin (50 μM) in physiological buffer at 37°C. Incubations were stopped by freezing in liquid nitrogen. Cells were homogenized by sonication in 1% (w/v) hot SDS containing 5 mM zinc acetate and maintained for 5 min in a boiling water bath. Equal amounts of protein (270 μg) were loaded on SDS-polyacrylamide gels, separated by electrophoresis, transferred to nitrocellulose and immunoblotted with mAb 23 as described above. Antibodies were detected by ^{125}I -iodoprotein A overlay and autoradiography. In addition to phospho-DARPP-32 (arrow), the mAb 23 antibodies also reacted with unidentified bands of higher molecular weight that were apparent only in the SDS homogenates and not acid extracts phosphorylated *in vitro* with catalytic subunit of the cAMP-dependent protein kinase. The immunoreactivity of these bands with mAb 23 was not altered by forskolin.

apparent M_r of $\sim 34,000$ was revealed by this antibody in acid extracts of adult rat striatum that had been phosphorylated *in vitro* with catalytic subunit of cAMP-dependent protein kinase (Fig. 5, lane 4), but not in extracts incubated in the absence of the kinase (Fig. 5, lane 3). This protein band was identified as DARPP-32 using a mixture of C24-6A and -5A monoclonal antibodies (mAb 5,6, Fig. 5, lanes 1, 2).

A comparable protein band was revealed in SDS homogenates prepared from 3-week-old striatal aggregate cultures that had been incubated for 6 min in the presence of forskolin (50 μM), a drug known to stimulate cAMP formation (see Seamon and Daly, 1986) and thereby activate endogenous cAMP-dependent protein kinase. Forskolin treatment increased phospho-DARPP-32 immunoreactivity in striatal cells (Fig. 5, lane 6), whereas no detectable immunoreactivity was present in homogenates from untreated striatal cultures (Fig. 5, lane 5). Additional immunoreactive bands with apparent M_r $\sim 75,000$ and $\sim 100,000$ were observed. These immunoreactive bands were also found

Figure 6. DARPP-32 immunostaining in whole-mount of rat choroid plexus. Whole-mount of tissue slice of rat choroid plexus from lateral ventricle shown by phase contrast (*A*); by fluorescein optics, immunostained for DARPP-32 (*B*); and by rhodamine optics, immunostained for Na,K-ATPase (*C*). Note presence and localization of DARPP-32 immunoreactivity in choroid plexus villae, which are covered with CSF-

in tissues from adult rat (data not shown). However, these protein bands were not affected by forskolin treatment, and were not recognized by other antibodies specific for DARPP-32 or I_1 (Fig. 5, and data not shown; see also below).

Immunohistochemistry of DARPP-32 and I_1 in rat choroid plexus. The increased formation of cAMP observed in choroid plexus in response to pharmacological treatment of this tissue occurs primarily in the epithelial cells (Nathanson, 1980). In the present studies, we examined whether DARPP-32 and I_1 were localized to this cell type. Tissue slices of rat choroid plexus from both lateral and fourth ventricle showed bright immunoreactive staining for DARPP-32. Figure 6 shows an example of lateral ventricular choroid that consists of numerous epithelial-covered capillary loops (villae) arising from a veil-like ependymal membrane that floats within the ventricle. These can be visualized by phase contrast in Figure 6*A*, but are much more easily seen in Figure 6*B*, which shows that the bright immunoreactive staining for DARPP-32 is localized primarily to the thicker epithelial-covered loops. Other loops, below the plane of focus, appear fainter. Staining was brightest in epithelial cells, the nuclei of which were unstained (Fig. 6*B*). The pattern of staining for the $\alpha 1$ (kidney) form of Na,K-ATPase, an enzyme known to be present in very high concentration in choroid plexus and other transporting epithelium (Milhorat, 1976; Sweadner and Gilkeson, 1985; Nathanson and Chun, 1989), was nearly identical to that seen for DARPP-32 (Fig. 6*C*).

Choroid plexus from the lateral and fourth ventricles was subjected to cell separation to produce two fractions: (1) a purified preparation of epithelial cells (Fig. 7*C–F*), and (2) a largely deepithelialized vascular fraction consisting of collapsed capillaries, arterioles, and stroma (Fig. 7*A,B*). The absence of staining of the latter fraction demonstrates the selective localization of DARPP-32 to choroidal epithelium. As shown in Figure 7*A* (phase), a few typical large, round epithelial cells remained attached to the vascular choroidal surface following cell isolation. In Figure 7*B*, these, but not the remainder of the choroid, stain brightly for DARPP-32. Cells from the epithelial cell fraction (Fig. 7*C,D*) demonstrate bright immunoreactive staining both for Na,K-ATPase (Fig. 7*C*) and DARPP-32 (Fig. 7*D*). Figure 7*E* (phase) shows another group of isolated epithelial cells, used as a staining control, in which nonimmune mouse serum was substituted for the mouse monoclonal DARPP-32 antibodies. No secondary antibody immunofluorescence was observed (Fig. 7*F*).

The immunohistochemical localization of I_1 is shown in Figure 8. Figure 8*A* shows by phase contrast a fragment of fourth ventricle choroid plexus, which, in one area (arrows), has been partially deepithelialized by treatment with trypsin, exposing capillaries underneath. Antiserum to I_1 results in bright staining of the epithelium (Fig. 8*B*) but not the deepithelialized area. Figure 8*C* is a control section of isolated epithelial cells, in which nonimmune rabbit serum was substituted for the rabbit antiserum against I_1 . No immunofluorescence is observed. Figure 8*D* shows, by phase contrast, another piece of choroid plexus from the lateral ventricle that has also been deepithelialized in some areas (thin arrows). In most other areas, the epithelium remains intact and some detached epithelial cells (thick arrows)

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secreting epithelial cells known to contain high concentrations of Na,K-ATPase. See Results for details. Scale bar, 30 μ m.

Figure 7. Isolated cell and tissue preparations from choroid plexus showing localization of immunostaining for DARPP-32 in secretory epithelial cells. *A*, As viewed by phase contrast, a piece of choroid plexus vascular stroma is almost entirely denuded of epithelium except for a few epithelial cells (barely visible) above and slightly to left of center. *B*, The same tissue, seen by fluorescein optics, shows bright immunostaining of DARPP-32 in the adhering epithelial cells. The vascular and connective tissue portion of choroid shows little staining. Area of comet-shaped staining is due to autofluorescence. *C* and *D*, A group of isolated choroid epithelial cells immunostained for Na,K-ATPase (*C*) and the same group of cells double-labeled for DARPP-32 (*D*), showing that all of these cells colabel for the phosphoprotein. *E* and *F*, A control group of isolated epithelial cells (phase; *E*), showing a lack of immunostaining when nonimmune serum is substituted for specific antibody (*F*). Scale bar, 20 μ m.

Figure 8. Choroid plexus preparations showing localization of I_1 immunoreactivity to secretory epithelial cells. *A* and *D*, Phase-contrast views of partially deepithelialized pieces of choroid plexus from fourth (*A*) and lateral (*D*) rat ventricle, showing areas of underlying capillaries (*thin arrows* in *A* and *D*) and free dissociated epithelial cells (*thick arrows* in *D*). *B* and *E*, The same pieces of tissue viewed by fluorescein optics show bright immunostaining for I_1 in the epithelium. Capillaries show little staining. *F*, Same tissue as in *E*, seen by rhodamine optics, immunostained for DARPP-32 showing similar distribution of DARPP-32 immunoreactivity. *C*, Seen by fluorescein optics, a control group of isolated epithelial cells shows a lack of immunostaining for I_1 when nonimmune serum was substituted for specific antibody. Scale bar, 80 μ m.

can be seen free above the piece. Figure 8*E* shows that antiserum to I_1 immunostains the intact epithelium and free epithelial cells, with little or no localization to blood vessels. In Figure 8*F*, the same piece of choroid has been colabeled for DARPP-32, showing a similar distribution of staining intensity. It is important

to note that the DARPP-32 antibodies used for immunohistochemistry showed little (1:3000) cross-reactivity with I_1 . Likewise, the G-187 antibody used for histochemistry of I_1 showed no cross-reactivity to DARPP-32 (see Fig. 9). In this slide, one of the capillaries shows some immunoreactivity to DARPP-32,

Figure 9. Forskolin stimulated an increase in phospho-DARPP-32 and phospho-I₁ in rat choroid plexus. Acid extracts of untreated (lane 1) or forskolin-treated (lane 2) choroid plexus were subjected to SDS-PAGE (100 μ g total protein/lane) and immunoblotting on nitrocellulose membrane with mAb 23 revealing protein bands of apparent M_r ~29,000 and ~34,000 only in forskolin-incubated tissue. The upper band was identified as DARPP-32 (lane 3) using the selective DARPP-32 antibody C24-4D7, which does not cross-react with I₁. The lower band was identified as I₁ (lane 4), using a selective rabbit antiserum, G-187. Note that mAb 23 detected an additional band (M_r ~70,000) that was unaltered by forskolin treatment, similar to a band detected by this antibody in SDS-homogenized striatal cell aggregates (see Fig. 5).

although in most instances (e.g., Fig. 7B) choroid vascular fractions did not stain with DARPP-32 immunoreactivity.

Phosphorylation of DARPP-32 and I₁ in rat choroid plexus. Two bands of M_r ~29,000 and ~34,000 were detected by mAb 23 in acid extracts from whole rat choroid plexus that had been incubated *in vitro* with forskolin (10 μ M) but not in extracts prepared from untreated choroid plexus (Fig. 9, lanes 1, 2). These two bands were identified as DARPP-32 (M_r ~34,000) and I₁ (M_r ~29,000) using either a monoclonal antibody (C24-4D7) that reacts specifically with DARPP-32 (Fig. 9, lane 3) or a rabbit antiserum (G-187) that detects specifically I₁ (Fig. 9, lane 4). Forskolin markedly stimulated cAMP accumulation in extirpated choroid plexus (Fig. 10). The forskolin-stimulated phosphorylation (Figs. 9, 10) appeared within 5 min but was undetectable 30–45 min after drug treatment (data not shown). A protein band of apparent M_r ~70,000 also was revealed by mAb 23 in acid extracts of choroid plexus (Fig. 9, lanes 1, 2). Like the cross-reactive band of comparable molecular weight found in striatum, this band was not apparently regulated by forskolin (Fig. 9, lanes 1, 2), nor was it detected by other DARPP-32- and I₁-selective antibodies (Fig. 9, lanes 3, 4, respectively).

Isoproterenol, a β -adrenergic agonist (10 μ M), and VIP (1 μ M) stimulated phosphorylation of I₁ and DARPP-32 (Fig. 10). These drugs also elevated cAMP levels in choroid plexus, an observation that is consistent with the previously reported effects of these treatments on cAMP accumulation in bovine, cat, and rabbit choroid plexus (see Nathanson, 1979, 1980; Crook et al., 1984; Crook and Prusiner, 1986). Propranolol (10 μ M), a β -adrenergic antagonist, blocked both the rise in cAMP levels and the increased phosphorylation of DARPP-32 and I₁ produced by isoproterenol. Dopamine (10 μ M) failed to elevate cAMP

Figure 10. Levels of phospho-forms of DARPP-32 (solid bars) and I₁ (hatched bars) and of cAMP (open bars) in tissue extracts prepared from rat choroid plexus incubated for 10 min in the absence (control) or presence of drugs. Control and drug-treated choroid plexus (lateral ventricle tissue from five rats/condition) were acid extracted and subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose and immunoblotted as described in Materials and Methods. Nitrocellulose pieces identified by autoradiography were excised and their radioactivity measured to provide a quantitative measure of antibody binding. These data are expressed as mean cpm $\pm$ SEM per band. Background levels averaged 99 $\pm$ 7 cpm per band. cAMP was measured in aliquots (0.1 ml) of choroid plexus homogenized in zinc acetate buffer, and results are expressed as picomoles of cAMP (mean $\pm$ SEM). Means correspond to the results of four to eight experiments. Means were different from antibody binding or cAMP levels in control extracts: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, ANOVA (post hoc comparisons). The cAMP increase following forskolin treatment was 262 ± 64 pmol. C, Control; FORS, 10 μ M forskolin; VIP, 1 μ M VIP; I, 10 μ M isoproterenol; P, 10 μ M propranolol; DA, 10 μ M dopamine; 5-HT, 10 μ M 5-HT; ANF, 1 μ M ANF.

levels significantly in choroid plexus and produced no detectable changes in DARPP-32 or I₁ phosphorylation in this tissue. Incubation of choroid plexus with 5-HT (10 μ M) increased the phosphorylation state of both DARPP-32 and I₁. 5-HT also produced a small, but statistically insignificant, rise in cAMP levels in choroid plexus (Fig. 10).

DARPP-32 and I₁ are phosphorylated *in vitro* by cGMP-dependent protein kinase on the same threonyl residue phosphorylated by cAMP-dependent protein kinase (Hemmings et al., 1984b). Choroid plexus epithelial cells express relatively high concentrations of cGMP and cGMP-dependent protein kinase (Cumming et al., 1981). Since ANF has been shown to stimulate cGMP formation in these cells (Steardo and Nathanson, 1987), we examined whether ANF treatment might also be involved in regulating protein phosphorylation in extirpated choroid plexus. ANF (1 μ M) increased the immunoreactivity of DARPP-32 and I₁ to mAb 23 (Figs. 10, 11), as did incubation of intact choroid plexus with the cGMP analog 8-bromo-cyclic GMP (4 mM) (Fig. 11). In whole choroid plexus, a very slight increase in cAMP level was also observed in the presence of ANF (Fig. 10). However, in experiments using isolated epithelial cells, ANF (1 μ M) caused no increase in cAMP content in contrast to a substantial increase in cGMP content (Fig. 12). Figure 12 also shows that dopamine (100 μ M) caused no increase in either cAMP or cGMP in isolated choroid epithelial cells.

Discussion

The possibility of preparing phosphorylation state-selective antibodies had been demonstrated in the case of phospho-G-sub-

Figure 11. Effect of 8-bromo-cGMP (4 mM) and ANF (1 μ M) on the phosphorylation of DARPP-32 and I_1 . Results are shown for a representative experiment in which whole choroid plexus (tissue from three rats/treatment) was incubated for 5 min in the absence (CONTROL) or the presence of either 8-bromo-cGMP (4 mM) or ANF (1 μ M). Tissue was processed for SDS-PAGE (125 μ g protein/lane) and immunoblotted as described for Figure 5. Immunoblotting with mAb 23 shows increased amounts of phospho-DARPP-32 and phospho- I_1 in choroid plexus incubated either with 8-bromo-cGMP or with ANF.

strate (Nairn et al., 1982), a cGMP-regulated phosphoprotein that is enriched in cerebellar Purkinje cells (Detre et al., 1984). The present study demonstrates that this strategy is generally applicable and provides an excellent tool for studying the regulation of phosphorylation of specific proteins under physiological conditions. Phospho-specific antibodies have now been generated against the cAMP-dependent protein kinase phosphorylation site (site 1) and the Ca^{2+} /calmodulin-dependent protein kinase II sites (sites 2 and 3) in synapsin I, a nerve terminal-associated protein (Czernik et al., 1989, 1991). In the present study, the recognition of phospho-DARPP-32 and phospho- I_1 by the same antibody is not surprising due to the high degree of sequence homology existing between the cAMP-dependent phosphorylation sites of the two proteins (Hemmings et al., 1984c; Williams et al., 1986). mAb 23 cross-reacted with two unidentified protein bands of higher molecular weight present in brain and in choroid plexus in a manner that was independent of cAMP stimulation. These cross-reacting species did not appear to be otherwise related to DARPP-32 and/or I_1 since they were not detected using antibodies specific for these proteins. This cross-reactivity may preclude immunocytochemical applications of mAb 23. However, when combined with electrophoretic protein separation techniques, this antibody proved to be a valuable tool in studying the regulation of DARPP-32 and I_1 phosphorylation in choroid plexus.

Choroid plexus contains high concentrations of DARPP-32 (Hemmings and Greengard, 1986) and of I_1 (H. C. Hemmings, J. A. Girault, A. C. Nairn, G. Bertuzzi, and P. Greengard, unpublished observations). The present study demonstrates that DARPP-32 and I_1 are colocalized in epithelial cells, which also

Figure 12. Effect of ANF (1 μ M) and dopamine (100 μ M) on levels of cGMP and cAMP in isolated, purified choroid plexus epithelial cells incubated in artificial CSF with drug for 3 min. Results are expressed as a percentage of control cyclic nucleotide levels (9.5 ± 2.3 fmol/80,000 cells for cGMP and 87 ± 14 fmol/80,000 cells for cAMP). Values shown are the mean $\pm$ SD for duplicate tubes each assayed for cGMP and cAMP in triplicate. The results are similar to those seen in two other experiments.

contain high levels of Na,K-ATPase. Similarly, striatonigral neurons that contain high levels of DARPP-32 (Ouimet et al., 1984; Walaas et al., 1984; Hemmings and Greengard, 1986) also contain relatively high levels of I_1 (Nairn et al., 1988; Gustafson et al., 1991), while many other brain regions and tissues contain high levels of I_1 but no DARPP-32 (Gustafson et al., 1991; see also Huang and Glinsman, 1976). This suggests that, in addition to their common property of being cAMP-regulated inhibitors of phosphatase-1 (for a review, see Shenolikar and Nairn, 1991), DARPP-32 and I_1 may have distinct properties that would require their coexpression at high levels in specific cell types.

Isoproterenol and VIP increased levels of phospho-DARPP-32 and phospho- I_1 and also elevated cAMP levels in intact choroid plexus. Isoproterenol is known to increase cAMP formation by stimulating a β_2 -adrenergic-sensitive adenylyl cyclase of high specific activity present in the choroid plexus (Nathanson, 1979, 1980; see also Rudman, 1977). Consistent with the observed localization of DARPP-32 and inhibitor-1 to choroid epithelial cells (Figs. 6–8), β_2 -adrenergic-sensitive adenylyl cyclase also is highly enriched in the secretory epithelium. VIP also is known to increase cAMP levels in choroid plexus and in choroid plexus epithelial cells (Crook et al., 1984; Lindvall et al., 1985; Crook and Prusiner, 1986) as well as in the epithelium of the related ciliary process (Mittag et al., 1985). The above data indicate an association between hormonal activation of adenylyl cyclase in choroid plexus (particularly in choroid epithelium) and hormonal stimulation of phosphorylation of DARPP-32 and I_1 . This association between cAMP and phosphorylation state is further supported by the observed stimulation of phosphorylation by forskolin, a compound known to activate adenylyl cyclase directly in a variety of tissues (Seamon and Daly, 1986). Collectively, these observations suggest that several neurotransmitters and hormones that stimulate adenylyl cyclase can stimulate cAMP-dependent phosphorylation of DARPP-32.

Dopamine did not increase cAMP levels in choroid plexus and also failed to increase the phosphorylation of DARPP-32 and I_1 . These observations are consistent with the results of

several studies that have failed to demonstrate a dopamine-sensitive adenylate cyclase activity in choroid plexus (Rudman et al., 1977; Nathanson, 1979, 1980) and with several recent reports that failed to demonstrate specific binding to dopamine D_1 receptors in rat choroid plexus (Nicklaus et al., 1986; Yamamoto and Keabian, 1989). This finding is of particular significance since DARPP-32 is highly enriched in brain regions possessing high levels of D_1 receptors (Ouimet et al., 1984). The present study demonstrates the expression of DARPP-32 in cells lacking dopamine-sensitive adenylate cyclase. These results suggest that although DARPP-32 is abundant in dopaminergic cells, the expression of this phosphoprotein alone cannot be accepted as a reliable marker for dopamine receptors.

The present study also provides evidence that cAMP-independent effector systems may regulate DARPP-32 and I_1 phosphorylation in choroid plexus. Thus, ANF caused a pronounced stimulation of the phosphorylation of DARPP-32 and I_1 . It also caused a very small elevation of cAMP content in the intact choroid, but no cAMP elevation in isolated epithelial cells, indicating that the slight increase occurred in some nonepithelial cell location. This latter observation is consistent with prior experiments with choroid plexus membrane preparations showing that ANF fails to activate adenylate cyclase (Steardo and Nathanson, 1987). On the other hand, ANF (but not dopamine) elevated choroid epithelial cell cGMP levels, and it has previously been demonstrated that choroid plexus membranes contain an ANF-activated guanylate cyclase of high specific activity enriched in the epithelium (Steardo and Nathanson, 1987). DARPP-32 and I_1 are phosphorylated *in vitro* by cAMP-dependent protein kinase and cGMP-dependent protein kinase on the same threonine residue (Hemmings et al., 1984d). Because cGMP-dependent protein kinase is known to be enriched in choroid epithelial cells (Cumming et al., 1981), our results strongly suggest that ANF increases DARPP-32 and I_1 phosphorylation in choroid plexus through elevation of cGMP levels and consequent activation of cGMP-dependent protein kinase. Consistent with this hypothesis was the observation, in the present experiments, that 8-bromo-cGMP, an analog of cGMP able to penetrate cells, enhanced DARPP-32 and I_1 phosphorylation.

In the present experiments, 5-HT also induced substantial phosphorylation of DARPP-32 and I_1 in choroid plexus, but only a small increase in cAMP (as reported previously; see Rudman et al., 1977; Crook et al., 1984). 5-HT receptors are present in the choroid plexus, and it has been shown that the 5-HT_{1c} receptor that is expressed in high density in epithelial cells (Pazos et al., 1985; Yagaloff and Hartig, 1985; Nicklaus et al., 1988) is linked to mechanisms stimulating phosphatidylinositol hydrolysis and not adenylate cyclase (Conn et al., 1986; for review, see Curzon and Kennett, 1990; but see Rudman et al., 1977; Crook et al., 1984; present results). In broken cell preparations, we have observed that 5-HT also causes a small stimulation ($V_{max} = 29\%$; $K_d = 0.1 \mu M$) of choroid plexus guanylate cyclase. Whether 5-HT affects DARPP-32 and I_1 phosphorylation as a consequence of guanylate cyclase activation or as a result of phosphatidylinositol hydrolysis will require further study.

The rate of CSF secretion from choroid plexus is decreased by intravenous injection of timolol and by intraventricular injection of 5-HT and norepinephrine, the latter effect being blocked by intraventricular propranolol (Epstein et al., 1977; Feldman et al., 1979; Lindvall et al., 1979; Vogh and Godman, 1982, 1984). These observations suggest that cAMP-dependent mechanisms may decrease CSF secretion from the choroid plexus

although intracerebroventricular injection of cholera toxin stimulated CSF production (see Epstein et al., 1977; for discussion, see Nathanson, 1982). Norepinephrine- and VIP-containing fibers terminate in proximity to the choroid secretory epithelium, and sympathetic stimulation or denervation alters the rate of CSF production (Lindvall et al., 1978; Lindvall and Owman, 1981). Likewise, in the ocular ciliary process, where DARPP-32 is also found (Stone et al., 1986), VIP, forskolin, cholera toxin, and β -adrenergic agonists all regulate cAMP levels and decrease aqueous humor secretion (Gregory et al., 1981; Nathanson, 1981; Caprioli et al., 1984; Mittag et al., 1985; Nilsson et al., 1986; Stone et al., 1986; reviewed in Nathanson, 1985).

CAMP-independent mechanisms also appear to be involved in regulating CSF production in the choroid plexus. For instance, ANF decreases CSF production in the brain as well as aqueous humor secretion in the eye (Mittag et al., 1987; Steardo and Nathanson, 1987; Nathanson, 1988; Korenfeld and Becker, 1989). Conversely, focal increases in intracranial pressure cause an increase in circulating ANF (which has potential access to choroid epithelium through fenestrated choroidal capillaries) (J. A. Nathanson and T. Miller, unpublished observations). The effects of ANF on fluid secretion are associated with the ability of this peptide to increase cellular levels of cGMP (Hamet et al., 1984; Waldman et al., 1984) and, in the eye, are mimicked by local application of direct activators of guanylate cyclase such as nitroglycerin (Nathanson, 1988).

It remains to be established what role the regulation of DARPP-32 and I_1 phosphorylation plays in the function of the choroid plexus. However, it is quite interesting that DARPP-32 has been localized to non-neural epithelial cells in other tissues that, like choroid plexus, are involved in fluid formation and transport, including those in the rabbit ciliary processes (Stone et al., 1986) and in the renal tubule cells of rat and monkey (Meister et al., 1989). It is intriguing that ANF-activated guanylate cyclase and ANF regulation of fluid secretion also occur in all these epithelia. It is striking that several pathways that decrease CSF secretion (e.g., epinephrine, ANF, 5-HT), but use different second messenger systems, all enhance DARPP-32 and I_1 phosphorylation. Since these two proteins, when phosphorylated, are potent inhibitors of protein phosphatase-1, our observations suggest that inhibition of phosphatase-1 may be part of a final common pathway involved in the control of fluid secretion. It is therefore of particular interest that a phosphopeptide derived from DARPP-32, which inhibits phosphatase-1, was able to mimic dopamine-induced inhibition of Na,K-ATPase in the renal tubules of the thick ascending limb of the loop of Henle (Aperia et al., 1991). It was also shown that cAMP-dependent protein kinase and protein kinase C each phosphorylate Na,K-ATPase, and that the phosphorylation is correlated with inhibition of enzyme activity (Bertorello et al., 1991). Since Na,K-ATPase, DARPP-32, and I_1 are all colocalized in choroid plexus epithelial cells, it is tempting to speculate that inhibition of Na,K-ATPase may be involved in the neural and hormonal inhibition of CSF production. Further studies of DARPP-32 and I_1 regulation in choroid plexus and in other secretory epithelia may provide important insights on the functions of these phosphoproteins.

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