ARPP-21, a Cyclic AMP-Regulated Phosphoprotein Enriched in Dopamine-Innervated Brain Regions. I. Purification and Characterization of the Protein from Bovine Caudate Nucleus

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ARPP-21 (cAMP-regulated phosphoprotein, $M_r = 21,000$ as determined by SDS/PAGE) is a major cytosolic substrate for cAMP-stimulated protein phosphorylation in dopamine-innervated regions of rat CNS (Walaas et al., 1983c). This acidic phosphoprotein has now been identified in bovine caudate nucleus cytosol and purified to homogeneity from this source. The purification procedure involved diethylaminoethyl-cellulose chromatography, ammonium sulfate fractionation, phenyl-Sepharose CL-4B chromatography, and fast protein liquid chromatography using Mono Q anion-exchange resin. Two isoforms of ARPP-21 (ARPP-21A and ARPP-21B) were obtained, which were present in approximately equal amounts in the starting material. ARPP-21A was purified 2610-fold with a final yield of 20% and ARPP-21B was purified 2940-fold with a final yield of 21%. The purified preparations of both isoforms were judged to be homogenous by SDS/PAGE. ARPP-21A and ARPP-21B yielded identical 2-dimensional thin-layer tryptic phosphopeptide maps, identical amino acid compositions and closely related, but distinct, reverse-phase high-pressure liquid chromatograms of tryptic digests. The amino acid composition of ARPP-21 showed a high content of glutamic acid/glutamine, and no methionine, tryptophan, tyrosine, phenylalanine, or histidine. ARPP-21 was stable to heat denaturation and to 50% (vol/vol) ethanol treatment and was partially soluble at pH 2. The M_r determined for ARPP-21 by SDS/PAGE was 21,000. The Stokes radius of ARPP-21 was 26.3 Å, and the sedimentation coefficient of ARPP-21 was 1.3 S; these values yield a calculated molecular mass of 13,700 Da and a frictional ratio of 1.7, indicative of an elongated tertiary structure. ARPP-21 was an excellent substrate for cAMP-dependent protein kinase and was either not phosphorylated or only poorly phosphorylated by cGMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase I, calcium/calmodulin-dependent protein kinase II, casein kinase II, or protein kinase C. The purified catalytic subunit of cAMP-

dependent protein kinase catalyzed the incorporation of 1.2 mol phosphate/mol purified ARPP-21. Phosphorylation occurred exclusively on seryl residues. Phospho-ARPP-21 was dephosphorylated effectively by protein phosphatase-1 or -2A, but not by protein phosphatase-2B or -2C. Rabbit polyclonal and mouse monoclonal antibodies were prepared to purified ARPP-21. These antibodies specifically immunoprecipitated ARPP-21, which was found to be highly enriched in the caudate nucleus and putamen of monkey brain. In an accompanying paper, we report the use of antibodies to ARPP-21 for the immunocytochemical localization of ARPP-21 in rat brain (Ouimet et al., 1989). The available evidence suggests that ARPP-21 is a cAMP-regulated phosphoprotein (ARPP) highly enriched in dopamine-innervated brain regions and may therefore play a role as an intracellular messenger in mediating some of the effects of dopamine and/or other neurotransmitters acting via cAMP in the basal ganglia.

Protein phosphorylation is a molecular mechanism by which many extracellular signals, including various neurotransmitters, appear to produce specific physiological effects in the nervous system (for reviews, see Greengard, 1978; Nestler and Greengard, 1984; Nairn et al., 1985; Hemmings et al., 1986a, 1987a). Certain of the physiological effects of dopamine appear to be mediated by the second-messenger cAMP (Kebabian et al., 1972; Kebabian and Calne, 1979; Stoof and Kebabian, 1984). The stimulation of adenylate cyclase by dopamine acting at D-1 dopamine receptors results in the activation of cAMP-dependent protein kinase and the resultant phosphorylation of specific substrate proteins (for review, see Hemmings et al., 1987b). The identification, purification, and characterization of these substrate proteins provides an approach to learning more about the biochemical mechanisms involved in the responses of cells that possess D-1 dopamine receptors to stimulation by dopamine.

A distinct subset of substrates for cAMP-dependent protein kinase, with highly restricted regional distributions similar to the distribution of axon terminals of the mesencephalic dopaminergic neurons, has been identified in rat CNS (Walaas et al., 1983b, c). At least 7 of these substrates (soluble phosphoproteins of $M_r = 98,000$; 96,000; 81,000; 40,000; 32,000; and 21,000; and a particulate phosphoprotein of $M_r = 39,000$) were found to be highly enriched in the forebrain basal ganglia, brain regions that are heavily innervated by dopaminergic neurons (for reviews, see Lindvall and Björklund, 1978; Moore and Bloom, 1978; Björklund and Lindvall, 1984) and that contain relatively high amounts of dopamine-sensitive adenylate cyclase activity (for review, see Miller and McDermid, 1979) and of D-1 do-

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pamine receptors (Boyson et al., 1986; Dawson et al., 1986; Dubois et al., 1986; Savasta et al., 1986). Furthermore, the state of phosphorylation of the soluble phosphoprotein of $M_r = 32,000$ (known as DARPP-32¹) has been found to be regulated by dopamine and by the cAMP analog 8-bromo-cAMP in intact cells of the rat caudatoputamen (Walaas et al., 1983a; Walaas and Greengard, 1984). It appears likely that DARPP-32 and other substrates for cAMP-dependent protein kinase that are concentrated within cells that possess D-1 dopamine receptors are involved in mediating certain of the physiological effects observed following stimulation by dopamine.

The purification and biochemical characterization of DARPP-32 from bovine caudate nucleus has been reported (Hemmings et al., 1984a-d; Hemmings and Greengard, 1986; Williams et al., 1986). DARPP-32 is a potent inhibitor, in its phosphorylated form, of the broad-specificity enzyme protein phosphatase-1. This effect may be an important component of the biochemical mechanisms involved in mediating some of the effects of dopamine acting on dopaminergic cells (Hemmings et al., 1984a, 1987c). ARPP-21, like DARPP-32, was found by Walaas et al. (1983c) to exhibit a highly restricted regional distribution in rat CNS corresponding to the distributions of dopamine-containing axon terminals, dopamine-sensitive adenylate cyclase activity and D-1 dopamine receptors. Recently, the state of phosphorylation of ARPP-21, like that of DARPP-32, has been found to be regulated by 8-bromo-cAMP in intact neurons (Girault et al., 1987, 1988; Shalaby et al., 1987). We now report the purification of ARPP-21 (cAMP-regulated phosphoprotein, $M_r = 21,000$). The purified preparation of bovine ARPP-21 has been biochemically characterized and used to prepare both rabbit antiserum and mouse monoclonal antibodies. These antibodies have been employed to determine the regional and cellular distribution of ARPP-21 in rat CNS by immunocytochemical methods, as described in the accompanying paper (Ouimet et al., 1989).

Materials and Methods

Materials. Materials were obtained from the following sources: Tris, glycine, ATP, BSA (fraction V powder), methylene bisacrylamide, 2-mercaptoethanol, Coomassie brilliant blue R250, 8-bromo-cAMP, and EGTA from Sigma Chemical Co.; deoxycholic acid (sodium salt), HEPES, and PMSF from Calbiochem-Behring; DTT from Boehringer Mannheim Biochemicals; leupeptin and pepstatin A from Chemicon (Los Angeles); ammonium sulfate from Bethesda Research Laboratories; triethanolamine, ammonium bicarbonate, trichloroacetic acid, and ethylene glycol from Fisher Scientific Co.; acrylamide and thin-layer cellulose sheets (type 13254) from Eastman Co.; DEAE-cellulose from Whatman, Ltd.; phenyl-Sepharose CL-4B, Mono-Q anion-exchange FPLC resin, Sephadex G-25 (fine) and DE Sephacel from Pharmacia; Liscint scintillation fluid from National Diagnostics; Bio-Rad Protein Assay Dye Reagent from Bio-Rad Laboratories; ampholytes from LKB; and γ -³²P-ATP, iodo-¹⁴C-acetic acid, and ¹²⁵I-Bolton Hunter reagent from New England Nuclear. All other chemicals used were of reagent grade.

Fresh calf brains were obtained from a local slaughterhouse and either transported to the laboratory on ice or dissected and transported to the laboratory in liquid nitrogen. The caudate nuclei were stored frozen at -70°C for up to 1 year before use. Male Sprague-Dawley rats (150–200 gm body weight) were obtained from Charles River Laboratories. Frozen brain tissue from *Macaca mulatta* was a gift of Dr. Tamas Bartfai.

Enzymes. The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart as described previously (Kaczmarek et al., 1980). cGMP-dependent protein kinase was purified from bovine lung as described previously (Walter et al., 1980). Calcium/calmodulin-dependent protein kinase I was purified from bovine brain (Nairn and Greengard, 1987) and was a gift of A. C. Nairn of The Rockefeller University. Calcium/calmodulin-dependent protein kinase II was purified from rat brain (McGuinness et al., 1985) and was a gift of Y. Lai of The Rockefeller University. Protein kinase C was purified from bovine brain (Albert et al., 1984) and was a gift of K. A. Albert of The Rockefeller University. Casein kinase II was purified from rabbit reticulocytes (Hathaway and Traugh, 1983) and was a gift of J. Traugh of the University of California at Riverside. TPCK-trypsin was obtained from Cooper Biomedical (Malvern, PA).

Buffers. All solutions were made with distilled water which was further purified using a Barnstead Nanopure filtration system. Buffer pH values refer to measurements made at 4°C. Buffer A consisted of 10 mM potassium phosphate (pH 7.5), 1 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 0.1% (vol/vol) isopropanol (necessary as a solvent for PMSF), 5 μ g/ml pepstatin A, and 10 μ g/ml leupeptin. Buffer B consisted of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 0.1% (vol/vol) isopropanol, and 10 μ g/ml leupeptin. Buffer C consisted of buffer B containing 45% saturated (at 0°C) ammonium sulfate (0.258 gm/ml). Buffer D consisted of buffer B containing 50% (vol/vol) ethylene glycol. Buffer E consisted of 20 mM triethanolamine-HCl (pH 7.5) and 1 mM DTT.

SDS/PAGE. SDS/PAGE was carried out using slab gels prepared from 11.5% (wt/vol) acrylamide and 0.31% (wt/vol) methylene bisacrylamide in the presence of 2-mercaptoethanol by the method of Laemmli (1970). Gels were stained with 0.1% (wt/vol) Coomassie brilliant blue R250 in 50% (vol/vol) methanol/10% (vol/vol) acetic acid and destained in 25% (vol/vol) methanol/10% (vol/vol) acetic acid. Molecular-weight standards, gel drying, and autoradiography were performed as described previously (Hemmings et al., 1984b).

Two-dimensional PAGE with isoelectric focusing (IEF) in the first dimension and SDS/PAGE in the second dimension was performed as described by O'Farrell (1977) with the following modifications. Samples were phosphorylated as described below, and the reactions were terminated by the addition of trichloroacetic acid to a final concentration of 10% (wt/vol). The precipitated proteins were recovered by centrifugation at $12,400 \times g$ for 10 min in a Beckman Microfuge 12. The precipitate was washed 3 times with acetone at -20°C, dissolved in lysis buffer, and subjected to IEF in tube gels. The ampholytes used were 150 μ l of pH 3–10 and 100 μ l of pH 5–7. Following IEF, the tube gels were applied to slab gels (11.5% acrylamide) and separated in a second dimension as described above.

Endogenous phosphorylation of crude cytosolic extracts. Fresh tissue samples were homogenized in 10 vol (ml/gm) of buffer at 4°C with a Teflon-glass homogenizer using 20 up-and-down strokes at 2100 rpm. The homogenates were centrifuged in a Beckman L3-50 ultracentrifuge using an SW 50.1 rotor at $100,000 \times g$ for 1 hr at 4°C. The supernatants were dialyzed against buffer A for 2 hr at 4°C. Aliquots were phosphorylated by endogenous protein kinase in a reaction mixture (final volume, 0.1 ml) containing 50 mM sodium HEPES (pH 7.4), 1 mM EGTA, 10 mM magnesium acetate, and 2 μ M γ -³²P-ATP, in the absence or presence of 10 μ M 8-bromo-cAMP. The phosphorylation reactions were carried out at 30°C for 1 min and terminated by the addition of "SDS-stop solution" to yield the following final concentrations: 50 mM Tris-HCl (pH 7.4), 10% (vol/vol) glycerol, 2% (vol/vol) 2-mercaptoethanol, 1% (wt/vol) SDS, and 0.04 mg/ml pyronin Y. The phosphorylated samples were then heated to 95°C for 2 min and subjected to SDS/PAGE, followed by autoradiography of the dried gels.

Phosphorylation assay for ARPP-21. ARPP-21 was assayed by its ability to accept the γ -phosphoryl group from γ -³²P-ATP in a reaction catalyzed by the catalytic subunit of cAMP-dependent protein kinase. Samples for the assay were dialyzed against buffer A for 2 hr prior to phosphorylation. The reaction mixture (final volume, 0.1 ml) contained 50 mM sodium HEPES (pH 7.4), 1 mM EGTA, 10 mM magnesium acetate, 2–4 μ g/ml of the catalytic subunit of cAMP-dependent protein kinase, and 50 μ M γ -³²P-ATP (200–1000 cpm/pmol). The reaction mixtures, containing all components except the catalytic subunit of cAMP-dependent protein kinase and γ -³²P-ATP, were heated at 95°C for 2 min prior to phosphorylation to eliminate endogenous protein kinase and phosphoprotein phosphatase activities. The phosphorylation reactions were carried out for 60 min (unless indicated otherwise) at 30°C and

¹ Abbreviations used: ARPP-21, cAMP-regulated phosphoprotein, $M_r = 21,000$ as determined by SDS/PAGE; DARPP-32, dopamine- and cAMP-regulated phosphoprotein, $M_r = 32,000$ as determined by SDS/PAGE; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; IEF, isoelectric focusing; PMSF, phenylmethylsulfonyl fluoride.

were terminated by the addition of "SDS-stop solution." The phosphorylated samples were then heated at 95°C for 2 min and subjected to SDS/PAGE, followed by autoradiography of the dried gels. Phosphorylated bands corresponding to the $M_r = 21,000$ phosphoprotein were cut out of the gel and the amount of ^{32}P -radioactivity incorporated was determined by liquid scintillation spectrometry in 3 ml of Liquescent. The "substrate activity" of ARPP-21 is defined as the picomoles of ^{32}P -phosphate incorporated into the $M_r = 21,000$ band using this assay.

Two-dimensional thin-layer phosphopeptide mapping. Gel pieces containing phosphorylated ARPP-21 were subjected to proteolysis within the gel by a method described previously (Hemmings et al., 1984c) except that the protease used for digestion was 75 $\mu\text{g}/\text{ml}$ TPCK-trypsin. Electrophoresis and chromatography of the tryptic digests were performed as described previously (Hemmings et al., 1984c).

Analytical HPLC peptide mapping. ARPP-21A and ARPP-21B were reduced, S- ^{14}C -carboxymethylated and digested with TPCK-trypsin by a method described previously (Williams et al., 1986). The resulting tryptic peptides were analyzed by reverse-phase HPLC as described (Williams et al., 1986), except that a Vydak C-4 column was used. Peptides were eluted with linear gradients containing increasing concentrations of solvent B [0.05% (vol/vol) trifluoroacetic acid and 80% (vol/vol) acetonitrile] into solvent A [0.05% (vol/vol) trifluoroacetic acid] as follows: 0–90 min (0–37.5% B), 90–135 min (37.5–75% B).

Dephosphorylation of phosphorylated ARPP-21 by various purified protein phosphatases. Purified protein phosphatase-1, -2A, -2B, and -2C and phosphorylated phosphatase kinase were prepared, and dephosphorylation assays performed as described by Hemmings et al. (1984a). Purified ARPP-21 (A and B) was phosphorylated to a stoichiometry of 0.8–1.0 mol ^{32}P /mol protein and purified from unreacted $\gamma\text{-}^{32}\text{P}$ -ATP by dialysis and ion-exchange chromatography using DE-Sephacel.

Radioiodination of ARPP-21. Purified ARPP-21 (5 μg) was radioiodinated by the method of Bolton and Hunter (1973) using 2 mCi of diiodinated ^{125}I -Bolton-Hunter reagent. Iodination by the chloramine T method was ineffective due to the absence of tyrosine residues in ARPP-21 (see below). ^{125}I -labeled ARPP-21 was separated from the reaction mixture by gel filtration through Sephadex G-25 (fine) as described (Hemmings and Greengard, 1986) and stored at -70°C in small aliquots. This procedure resulted in a preparation of ^{125}I -labeled ARPP-21 in which 85–100% of the total ^{125}I radioactivity was contained in intact ARPP-21 as determined by SDS/PAGE.

Antibody detection assay. Rabbit and mouse sera, hybridoma culture supernatants and ascites fluid were analyzed for anti-ARPP-21 antibodies by their ability to precipitate ^{125}I -labeled ARPP-21 using fixed Protein A-bearing *Staphylococcus aureus* cells (Pansorbin, Calbiochem-Behring) as an immunoadsorbent as described previously for DARPP-32 (Hemmings and Greengard, 1986), with the following modification: rabbit anti-mouse antiserum (Cappel Laboratories) was added to the immunoprecipitation assay as a second antibody at a final dilution of 1:500.

Preparation of rabbit anti-ARPP-21 antibodies. Antiserum to ARPP-21 was raised in 3 female New Zealand white rabbits (1.5–2 kg) as described previously for DARPP-32 (Hemmings and Greengard, 1986), except that 100 μg per rabbit of purified ARPP-21 (A and B) emulsified in Freund's complete adjuvant was injected on day 0, and 50 μg per rabbit of purified ARPP-21 in Freund's incomplete adjuvant was injected on days 14 and 28. The rabbits were bled on day 38, and the antiserum was collected and stored at -70°C in small aliquots.

Preparation of mouse anti-ARPP-21 monoclonal antibodies. Monoclonal antibodies were produced in BALB/c mice essentially by the method of Köhler and Milstein (1975) as described previously for DARPP-32 (Hemmings and Greengard, 1986). This procedure yielded 10 stable anti-ARPP-21 antibody-producing hybrid cell lines, from 2 separate cell fusions, designated 21-1A, 21-3D, 21-4B, 21-6A, 21-7A, 21-7G, 21-7N, 21-14.1, 21-14.3, 21-14.5. Ascites was induced in pristane-primed BALB/c mice using each cell line, and the γ -globulin fractions were obtained by precipitation of the ascitic fluid with 45% saturated ammonium sulfate at 4°C . The precipitated proteins were dialyzed into PBS and stored at 70°C in small aliquots.

Miscellaneous methods. Determination of Stokes radius by gel filtration and of sedimentation coefficient by linear sucrose density gradient ultracentrifugation (Hemmings et al., 1984b), identification of phosphorylated amino acids (Hemmings et al., 1984c), amino acid analysis (Hemmings et al., 1984d), and protein determinations using a modification (Peterson, 1977) of the method of Lowry et al. (1951) were carried

Figure 1. 8-bromo-cAMP-stimulated endogenous phosphorylation of cytosolic extracts prepared from bovine caudate nucleus or cerebral cortex analyzed by SDS/PAGE. Samples containing 40 μg protein, determined by the method of Bradford (1976), were subjected to phosphorylation for 60 sec in the absence or presence of 10 μM 8-bromo-cAMP, as indicated, and analyzed by SDS/PAGE (11.5% acrylamide). An autoradiogram of the dried gel is shown. Two prominent low- M_r phosphoproteins, which are more concentrated in caudate nucleus than in cerebral cortex, are labeled (DARPP-32 and ARPP-21).

out as described previously. Protein determinations using the Coomassie blue-dye-binding assay were performed as described by Bradford (1976) using the Bio-Rad Protein Assay Dye Reagent. Initial rates of phosphorylation of ARPP-21 by various purified preparations of protein kinases were determined by a method described previously (Hemmings et al., 1984c) using modifications in the reaction mixtures necessary for maximal activation of each protein kinase as described in the references cited above for each protein kinase. Subcellular fractionation of bovine caudate nucleus homogenates by differential centrifugation was carried out by a modification of the method of Whittaker and Barker (1972) as described by Walaas and Greengard (1984).

Results

ARPP-21 in bovine brain

Identification of ARPP-21 in bovine caudate nucleus. When crude cytosolic fractions prepared from bovine caudate nucleus homogenates were subjected to endogenous phosphorylation stimulated by 8-bromo-cAMP, a prominent phosphoprotein with an apparent $M_r = 21,000$ was observed (Fig. 1). This phosphoprotein was named ARPP-21, for cAMP-regulated phosphoprotein of $M_r = 21,000$ as determined by SDS/PAGE. ARPP-21 was present at a much higher concentration in caudate nucleus than in cerebral cortex as determined by the endogenous phosphorylation assay (Fig. 1). Analysis of the subcellular localization of ARPP-21 in bovine caudate nucleus by differential

Figure 2. Endogenous phosphorylation of cytosolic extracts prepared from bovine caudate nucleus or hippocampus analyzed by 2-dimensional PAGE. Samples containing 100 μ g protein, determined by the method of Bradford (1976), were subjected to phosphorylation for 60 sec in the presence of 10 μ M 8-bromo-cAMP. The samples were analyzed by IEF in the horizontal dimension followed by SDS/PAGE (11.5% acrylamide) in the vertical dimension. Autoradiograms of the dried gels are shown. Two prominent acidic phosphoproteins are more highly concentrated in caudate nucleus than in hippocampus, with apparent M_r values of 32,000 (corresponding to DARPP-32 and indicated by the arrow pointing right) and 21,000 (corresponding to ARPP-21 and indicated by the arrow pointing left).

centrifugation revealed that the $M_r = 21,000$ phosphoprotein was highly enriched in the soluble fraction (data not shown).

The existence of a soluble phosphoprotein with a $M_r = 21,000$ that is highly concentrated in the caudatoputamen of rat CNS was reported previously (Walaas et al., 1983c). The distribution of this phosphoprotein in rat brain is similar to that of DARPP-32 (Hemmings et al., 1984b), a dopamine- and cAMP-regulated phosphoprotein with an apparent $M_r = 32,000$, which is also evident in Figure 1. Two other low molecular weight phosphoproteins of $M_r = 19,000$ and $M_r = 16,000$ are also seen to be enriched in the caudate nucleus (Fig. 1, Girault et al., 1987).

Analysis of ARPP-21 by 2-dimensional PAGE. Further analysis by 2-dimensional PAGE of ARPP-21 phosphorylated in bovine brain cytosolic fractions by the addition of 8-bromo-cAMP is shown in Figure 2. Two prominent 8-bromo-cAMP-stimulated phosphoproteins, which appeared to be highly concentrated in caudate nucleus, correspond to ARPP-21 and DARPP-32. Both phosphoproteins were present at much lower concentrations in the hippocampus (Fig. 2B). The isoelectric point of phosphorylated ARPP-21 determined using this technique was 4.5 and that of phosphorylated DARPP-32 was 4.6 (Hemmings et al., 1984b).

Analysis of ARPP-21 by 2-dimensional thin-layer phosphopeptide mapping. The 8-bromo-cAMP-regulated $M_r = 21,000$ phosphoprotein (ARPP-21) present in endogenously phosphorylated bovine caudate nucleus cytosol (Fig. 1) was compared with the $M_r = 21,000$ phosphoprotein previously observed in rat caudatoputamen cytosol (Walaas and Greengard, 1984) by 2-dimensional thin-layer phosphopeptide mapping of tryptic digests. Both phosphoproteins yielded identical peptide maps consisting of a single, highly basic and hydrophilic phospho-

peptide (Fig. 3A), indicating that these 2 phosphoproteins represent the homologous forms of rat and bovine ARPP-21. Based upon these experiments, which demonstrated the homology of rat and bovine ARPP-21, bovine caudate nucleus cytosol was chosen as the starting material for the large-scale purification of ARPP-21.

Purification of bovine ARPP-21

All purification steps were performed at 0–4°C unless indicated otherwise. Centrifugations were carried out using Sorvall RC 2-B centrifuges.

Step 1: homogenization and centrifugation. Frozen bovine caudate nuclei (500 gm) at -70°C were equilibrated to -20°C and thawed in 1 liter of buffer A at room temperature. An additional liter of buffer A at 4°C was added, and the tissue was homogenized twice for 1 min using a Brinkman Polytron homogenizer at setting 5. The homogenate was centrifuged at $1500 \times g$ for 20 min in a GS-3 rotor. The supernatant was decanted through glass wool. The pellet was rehomogenized in 1 liter of buffer A at 4°C for 1 min and centrifuged at $1500 \times g$ for 20 min in a GS-3 rotor. The second supernatant was decanted through glass wool and combined with the first supernatant. The combined supernatants were then centrifuged at $27,000 \times g$ for 4 hr in a GSA rotor. The supernatant was used for the purification of ARPP-21 (below) and other soluble striatal phosphoproteins (A. Horiuchi, A. C. Nairn, H. C. Hemmings, Jr. and P. Greengard, unpublished observations).

Step 2: DEAE-cellulose chromatography. The clear, light red supernatant was adjusted to pH 7.5 with 1 N KOH and diluted to 2.5 liters with buffer A (final conductivity, about 240 μ S). The supernatant was then loaded at a flow rate of 4 ml/min

Figure 3. Two-dimensional thin-layer tryptic phosphopeptide mapping of phosphorylated ARPP-21. Phosphorylated proteins were digested using TPCK-trypsin as described in Materials and Methods. The tryptic digests were applied to thin-layer cellulose chromatography plates (origin at lower right, *o*) and separated in 2 dimensions, first by electrophoresis in the horizontal dimension (cathode, *left*; anode, *right*), followed by ascending chromatography in the vertical dimension. *A*, Comparison of the phosphopeptide maps obtained from ARPP-21 phosphorylated in crude cytosolic extracts of rat or bovine caudate nucleus by the addition of 8-bromo-cAMP. *B*, Comparison of the phosphopeptide maps obtained from the 2 peaks of ARPP-21 (ARPP-21A and ARPP-21B) observed upon chromatography of bovine caudate nucleus cytosolic extracts on DEAE-cellulose. All 4 phosphopeptide maps appear virtually identical.

onto a column (5 × 50 cm) of DEAE-cellulose (DE-52) previously equilibrated with buffer B. After washing the column with 1.5 liters of buffer B, the bound proteins were eluted at a flow rate of 4 ml/min with a 4 liter gradient of 0–0.5 M NaCl made up in buffer B. Two peaks of ARPP-21, which were present in approximately equal amounts, were detected and were pooled separately, as indicated in Figure 4. The first peak eluted at a conductivity of about 630 μ S and the second peak eluted at a conductivity of about 790 μ S. Comparison of the $M_r = 21,000$ phosphoproteins contained within each pool by 2-dimensional thin-layer phosphopeptide mapping of tryptic digests is shown in Figure 3*B*. The 2 phosphoproteins obtained from the DEAE-cellulose column yielded identical phosphopeptide maps; these

maps were also identical to those observed for ARPP-21 present in crude cytosolic extracts of rat or bovine caudate nucleus subjected to 8-bromo-cAMP-stimulated protein phosphorylation (Fig. 3*A*). This finding suggested that the 2 peaks of ARPP-21 observed represent closely related isoforms of a single phosphoprotein, which have been designated ARPP-21A and ARPP-21B. DARPP-32, a dopamine- and cAMP-regulated phosphoprotein with a distribution in brain similar to that of ARPP-21 (Hemmings et al., 1984b), eluted much later than ARPP-21 at a conductivity of about 1.25 mS (Fig. 4).

Step 3: ammonium sulfate fractionation. In preliminary experiments, ARPP-21 was found to be soluble in solutions containing up to 45% saturated ammonium sulfate (data not shown).

Figure 4. Chromatography of caudate nucleus cytosol on DEAE-cellulose (DE-52) at pH 8.0 (Step 2). The flow rate was 4 ml/min, and 15.0 ml fractions were collected. Aliquots of each fraction were assayed for ARPP-21 by the phosphorylation assay described in Materials and Methods. The elution positions of ARPP-21A, ARPP-21B, and DARPP-32 are indicated by arrows. The fractions of ARPP-21A and ARPP-21B pooled for further purification are indicated by the horizontal bars.

The 2 pooled fractions of ARPP-21A and ARPP-21B obtained by chromatography on DEAE-cellulose were fractionated with ammonium sulfate by the slow addition of solid ammonium sulfate with stirring to a saturation (at 0°C) of 45% (0.258 gm/ml). The solutions were stirred for 1 hr, and the precipitated proteins were removed by centrifugation at $27,000 \times g$ for 20 min using a GSA rotor.

Step 4: phenyl-sepharose CL-4B chromatography. The ammonium sulfate soluble proteins obtained from the 2 DEAE-cellulose column pools (Step 3) were each loaded at a flow rate of 1.5 ml/min directly onto separate columns (2.5×10 cm) of phenyl-Sepharose CL-4B previously equilibrated with buffer C. After washing each of the columns with 100 ml of buffer C, the bound proteins were eluted at a flow rate of 1.5 ml/min with 500 ml gradients from buffer C to buffer D (Fig. 5). Aliquots of each fraction were dialyzed against buffer A to remove ammonium sulfate and ethylene glycol prior to assay. The absorbance at 280 nm of the column fractions was not monitored due to interference from these solutes. Single peaks exhibiting slight tailing were obtained for both ARPP-21A and ARPP-21B; the tailing was more pronounced if ethylene glycol was not used in the gradients. Both ARPP-21A and ARPP-21B eluted at a conductivity of about 6.0 mS. The peak fractions of ARPP-21A and ARPP-21B were each pooled and dialyzed overnight against 3 changes of 4 liters of 10 mM NH_4HCO_3 . The dialyzed proteins were lyophilized and then solubilized in buffer E.

Step 5: Mono Q FPLC. ARPP-21A and ARPP-21B were further purified by anion-exchange FPLC using a Mono Q HR 16/10 column previously equilibrated with buffer E (Fig. 6). ARPP-21 was eluted at a flow rate of 3 ml/min with increasing gradients of NaCl in buffer E; elution was monitored continuously at 280 nm. Neither ARPP-21A nor ARPP-21B exhibited any absorbance at 280 nm, which is consistent with their amino acid compositions (see below). ARPP-21A and ARPP-21B were clearly resolved by FPLC using the Mono Q anion-exchange resin. ARPP-21A eluted at a concentration of NaCl of about 0.15 M, while ARPP-21B eluted at a concentration of NaCl of

Figure 5. Chromatography of 45% saturated ammonium sulfate-soluble proteins on phenyl-Sepharose CL-4B at pH 8.0 (Step 4). The flow rate was 1.5 ml/min, and 5.0 ml fractions were collected. Aliquots of each fraction were dialyzed and assayed for ARPP-21 by the phosphorylation assay described in Materials and Methods. The chromatograms obtained for ARPP-21A (above) and ARPP-21B (below) are shown.

about 0.17 M. The ARPP-21A pool from the DEAE-cellulose column was contaminated with a small amount of ARPP-21B, while the ARPP-21B pool from the DEAE-cellulose column was contaminated with a small amount of ARPP-21A. The peak fractions of ARPP-21A or ARPP-21B from each run were combined and dialyzed against 2 changes of 4 liters of 10 mM NH_4HCO_3 . The dialyzed proteins were lyophilized and solubilized in 20 mM sodium HEPES (pH 7.4) containing 1 mM DTT and were stored at -70°C . Repeated freezing and thawing of ARPP-21 over a 6 month period resulted in the formation of higher-molecular-weight aggregates detected by SDS/PAGE that could not be reduced by high concentrations of 2-mercaptoethanol or DTT.

A summary of the purification procedure developed for ARPP-21 is presented in Table 1. ARPP-21 was assayed using a phosphorylation assay employing $\gamma\text{-}^{32}\text{P}\text{-ATP}$ and the catalytic subunit of cAMP-dependent protein kinase. Interfering endogenous protein kinase and phosphoprotein phosphatase activity was eliminated by heating the samples prior to assay since control experiments revealed that ARPP-21 is stable to heat treatment (data not shown). This assay appeared to be unreliable in assaying the 45% saturated ammonium sulfate supernatants, possibly because of the presence of an endogenous heat-stable pro-

Figure 6. FPLC of Step 4 pools on a Mono Q 16/10 column at pH 7.5 (Step 5). The flow rate was 3 ml/min, and 1 min fractions were collected. Aliquots of each fraction were assayed for ARPP-21 by the phosphorylation assay described in Materials and Methods. The elution positions of ARPP-21A and ARPP-21B are indicated by arrows. The chromatograms obtained for ARPP-21A (left) and ARPP-21B (right) are shown.

tein kinase inhibitor (see, for example, Walsh et al., 1971). About 0.7 mg each of ARPP-21A and ARPP-21B were obtained with a final yield of about 20%. ARPP-21 was purified 2610-fold and ARPP-21B was purified 2940-fold. This purification procedure has been carried out a total of 6 times, with no significant variation in the results.

Figure 7A shows the protein staining pattern and Figure 7B shows an autoradiogram of phosphorylated aliquots from each step of the purification procedure of ARPP-21A and ARPP-21B analyzed by SDS/PAGE. Protein staining using either Coomassie brilliant blue R250 (Fig. 7A) or a silver staining technique (data not shown) of the purified preparations of both ARPP-21A and ARPP-21B (lane 4) yielded a single band of apparent $M_r = 21,000$, suggesting homogeneity of the 2 preparations. Similarly, autoradiography indicated that all of the ^{32}P -radioactivity comigrated with the protein staining bands. Purified ARPP-21A and ARPP-21B comigrated with the ARPP-21 detected by 8-bromo-cAMP-stimulated protein phosphorylation in crude rat or bovine caudate nucleus cytosolic extracts. The electrophoretic migration of ARPP-21A and ARPP-21B upon SDS/PAGE were not detectably different. Two-dimensional thin-layer phosphopeptide maps of purified ARPP-21A and ARPP-21B (data not shown) were identical to those of the ARPP-21A or ARPP-21B present in the DEAE-cellulose column fractions and to endogenously phosphorylated ARPP-21 (Fig. 3).

Characterization of purified bovine ARPP-21

Comparison of ARPP-21A and ARPP-21B by HPLC peptide mapping. The purified preparations of ARPP-21A and ARPP-21B were compared using analytical HPLC peptide mapping of tryptic digests (Fig. 8) by the methods described by Hemmings et al. (1984d) and Williams et al. (1986). Although the overall elution profiles of the tryptic peptides derived from ARPP-21A and ARPP-21B were very similar, there were several clear differences in the peptides eluting later in the chromatograms (after 70 min). These differences did not appear to be due to variation in the enzymatic digests since they were reproducible. There were no significant differences between the HPLC tryptic peptide

maps of phosphorylated and dephosphorylated ARPP-21B (data not shown).

Amino acid analyses. The amino acid compositions of purified ARPP-21A and ARPP-21B are shown in Table 2. The compositions of the 2 forms of ARPP-21 are not significantly different given the errors of about 10% in these determinations. The compositions are remarkable for the absence of methionine, tyrosine, phenylalanine, histidine, and tryptophan. The absence of any aromatic amino acids accounts for the very low absorbance of ARPP-21 at 280 nm. There is a very high content of glutamic acid/glutamine, which accounts for 25 mol % of the amino acids in ARPP-21, which is consistent with the acidic isoelectric point of ARPP-21 (Fig. 2). The partial specific volume of ARPP-21 calculated from its amino acid composition using values from Cohn and Edsall (1943) is 0.718 ml/gm.

Stability to denaturation. ARPP-21 is heat stable. Heating of crude or purified ARPP-21 to 95°C for 5 min did not reduce the ability of ARPP-21 to be phosphorylated by exogenous cAMP-dependent protein kinase at the same site, nor was the purified protein precipitated. In addition, ARPP-21 in crude cytosol was soluble in solutions containing up to at least 50% (vol/vol) ethanol. However, ARPP-21 was only partially soluble at pH 2 (data not shown).

Hydrodynamic measurements. The Stokes radius of purified ARPP-21 was determined by gel filtration on Ultrogel AcA 44 (LKB) by the method of Laurent and Killander (1964) as described previously (Hemmings et al., 1984b). The Stokes radius of both ARPP-21A and ARPP-21B was $26.3 \pm 1.1 \text{ \AA}$. For a typical globular protein, this Stokes radius corresponds to a molecular mass of 30,500 Da.

The sedimentation coefficient of purified ARPP-21 was determined by velocity sedimentation using linear sucrose density gradients by the method of Martin and Ames (1961) as described previously (Hemmings et al., 1984b). The sedimentation coefficient of both ARPP-21A and ARPP-21B was $1.3 \pm 0.2 \text{ S}$.

The molecular mass of ARPP-21 was calculated as described by Siegel and Monty (1966) using values of $26.3 \text{ \AA}$ for the Stokes radius, 1.3 S for the sedimentation coefficient, and 0.718 ml/gm (calculated from the amino acid composition) for the partial

A. Protein Stain

B. Autoradiogram

Figure 7. Purification of ARPP-21 monitored by SDS/PAGE. Coomassie brilliant blue R250 protein staining pattern (A) and autoradiogram (B) of the dried gel are shown. Samples were heated in a boiling water bath for 2 min to inactivate endogenous protein kinases and phosphoprotein phosphatases and were then assayed with exogenous catalytic subunit of cAMP-dependent protein kinase as described in Materials and Methods. The lane containing the starting material (Step 1) is indicated by S and contained 50 μ g of protein. Aliquots from the various stages in the purification of ARPP-21A and ARPP-21B are 1, 40 μ g of DEAE-cellulose column pool (Step 2); 2, 35 μ g of ammonium sulfate-soluble fraction (Step 3); 3, 4 μ g of phenyl-Sepharose CL-4B column pool (Step 4); and 4, 3 μ g of Mono Q HR 16/10 column pool (Step 5). ARPP-21 is indicated by the double-headed arrow. Protein values were determined by the method of Peterson (1977).

specific volume. A value of $13,700 \pm 1000$ Da was obtained, which is 65% of the apparent M_r determined by SDS/PAGE (see above). The frictional ratio calculated from the hydrodynamic properties of ARPP-21 according to equation 2 of Siegel and Monty (1966) was 1.7, indicative of an elongated tertiary structure.

Stoichiometry of phosphorylation. The stoichiometry of phosphorylation of purified ARPP-21A and ARPP-21B catalyzed by cAMP-dependent protein kinase was measured using a reaction mixture containing 2 μ M ARPP-21A or ARPP-21B (as determined by amino acid analysis), 8 μ g/ml of the catalytic subunit of cAMP-dependent protein kinase, and 200 μ M γ - 32 P-ATP. A stoichiometry of 1.2 ± 0.1 mol phosphate/mol phosphoprotein was determined for both ARPP-21A and ARPP-21B after a 60 min incubation (data not shown), based on a molecular mass of 13,700 Da. Longer incubation times did not result in any significant increase in the amount of phosphate incorporated.

Phosphoamino acid analysis. In order to determine the amino acid(s) in ARPP-21 phosphorylated by cAMP-dependent protein kinase, 32 P-phospho-ARPP-21A and 32 P-phospho-ARPP-21B were proteolyzed with trypsin in gel pieces as described above, and the digests were subjected to partial acid hydrolysis (Bylund and Huang, 1976). The hydrolysates were analyzed for 32 P-phosphoamino acids by thin-layer electrophoresis. As shown

in Figure 9, phosphorylation of both ARPP-21A and ARPP-21B occurred exclusively on seryl residues. Likewise, phosphorylation of ARPP-21 by 8-bromo-cAMP-stimulated endogenous protein kinase in crude extracts occurred only on seryl residues.

Protein kinase specificity of ARPP-21. A purified preparation of ARPP-21, containing both ARPP-21A and ARPP-21B, was tested as a substrate for a variety of purified protein kinase preparations. ARPP-21 was not phosphorylated by calcium/calmodulin-dependent protein kinase I, protein kinase C, or casein kinase II. ARPP-21 was phosphorylated poorly by calcium/calmodulin-dependent protein kinase II, at a rate <5% of that observed for synapsin I (Ueda and Greengard, 1977), an excellent substrate for this enzyme (Nairn and Greengard, 1987). Similarly, ARPP-21 was phosphorylated poorly by cGMP-dependent protein kinase at a rate <10% of that observed for DARPP-32, a good substrate for this enzyme (Hemmings et al., 1984c). However, ARPP-21 was an excellent substrate for the catalytic subunit of cAMP-dependent protein kinase compared with DARPP-32 and synapsin I, 2 well-characterized neuronal substrates for cAMP-dependent protein kinase. The rates of phosphorylation observed at a substrate concentration of 1.5 μ M for each protein were 1.6 μ mol/min/mg for ARPP-21, 1.0 μ mol/min/mg for DARPP-32, and 2.6 μ mol/min/mg for synapsin I.

Protein phosphatase specificity of ARPP-21. The dephos-

Figure 8. Analytical HPLC peptide mapping of tryptic peptides from 760 pmol of reduced and S-¹⁴C-carboxymethylated dephospho-ARPP-21A (above) or dephospho-ARPP-21B (below). A Vydak C-4 column was eluted at a flow rate of 0.7 ml/min as described in Materials and Methods, and the absorbance of the effluent was monitored at 210 nm.

phorylation of phospho-ARPP-21 by various purified protein phosphatase preparations was compared with the dephosphorylation of the standard phosphoprotein substrate phosphorylase kinase as described previously (Hemmings et al., 1984a). Protein phosphatase-1 and -2A dephosphorylated phospho-ARPP-21 at 12 and 22%, respectively, of the rate at which they dephosphorylated phosphorylase kinase, while protein phosphatase-2B and -2C each dephosphorylated phospho-ARPP-21 at <2% of the rate at which they dephosphorylated phosphorylase kinase.

A summary of the physical and chemical properties of ARPP-21 is presented in Table 3. Since the only detectable differences between ARPP-21A and ARPP-21B were their elution positions from DEAE-cellulose and Mono Q and their HPLC tryptic peptide maps, the values listed in Table 3 for ARPP-21 apply to both ARPP-21A and ARPP-21B.

Characterization of anti-ARPP-21 antibodies. The specificity

Table 2. Amino acid compositions of ARPP-21A and ARPP-21B

Amino acid	ARPP-21A		ARPP-21B	
	Mol %	Residues/mol ^a	Mol %	Residues/mol ^a
Cys ^b	0.78	1.0	0.62	0.8
Asx	6.3	8.1	5.9	7.6
Thr ^c	5.5	7.0	5.4	7.0
Ser ^c	9.8	12.5	10.0	12.9
Glx	25.0	32.0	25.7	33.3
Pro	5.1	6.5	4.9	6.3
Gly	9.8	12.5	10.4	13.5
Ala	6.7	8.6	6.4	8.3
Val	4.4	5.6	4.2	5.4
Met	0	0	0	0
Ile	1.9	2.4	1.8	2.3
Leu	9.9	12.7	10.3	13.3
Tyr	0	0	0	0
Phe	0	0	0	0
His	0	0	0	0
Lys	7.1	9.1	6.9	8.9
Arg	7.8	10.0	7.6	9.9
Trp ^d	0	0	0	0
Total		128.0		129.5

Hydrolyses were carried out for 24, 48, and 72 hr at 115°C in 6 N HCl containing 0.02% (wt/vol) phenol.

^a The number of amino acid residues was calculated assuming a molecular weight of 13,700 calculated from hydrodynamic measurements (see Results).

^b Determined as cysteic acid after performic acid oxidation (Moore, 1963).

^c Obtained by extrapolation to zero time.

^d Determined spectrophotometrically (Goodwin and Morton, 1946). Since the absorbance of ARPP-21 (0.8 mg/ml) at 280 nm was <0.05 AU, it is concluded that the protein is devoid of tryptophan.

of antibodies to ARPP-21 could not be adequately analyzed by conventional methods of immunolabeling electrophoretic transfers of SDS-polyacrylamide gels to nitrocellulose membranes, since ARPP-21 failed to bind efficiently to nitrocellulose (of 0.1,

Table 1. Purification of ARPP-21 from 500 gm of bovine caudate nucleus

Step	Volume (ml)	Protein ^a (mg)	ARPP-21 ^b (nmol)	Specific activity (nmol/mg)	Purification ^c (fold)	Yield ^c (%)
1. 27,000 × g supernatant ARPP-21A	2500	9625	346	0.036	1	100
2A. DEAE-cellulose chromatography	142	397	87	0.22	12.2	50
3A. Ammonium sulfate fractionation	155	108	(13) ^d	(0.12)	(6.6)	(7.5)
4A. Phenyl-Sepharose 4B chromatography	80	5.8	40	6.9	383	23
5A. Mono Q HR 16/10 FPLC ARPP-21B	15	0.74	35	47	2610	20
2B. DEAE-cellulose chromatography	172	413	95	0.23	12.8	55
3B. Ammonium sulfate fractionation	190	122	(45) ^d	(0.37)	(20)	(26)
4B. Phenyl-Sepharose 4B chromatography	98	6.8	58	8.5	472	34
5B. Mono Q HR 16/10 FPLC	9	0.67	36	53	2940	21

^a Determined by the method of Lowry et al. as modified by Peterson (1977). Protein values for purified ARPP-21A or ARPP-21B determined by Bradford's method (1976) were 75–80% lower when BSA was used as a standard.

^b Determined by a phosphorylation assay using γ -³²P-ATP and the catalytic subunit of cAMP-dependent protein kinase as described in Materials and Methods. A phosphorylation stoichiometry of 1.0 mol phosphate/mol ARPP-21 is assumed.

^c Calculated assuming that the amount of ARPP-21 measured in the supernatant consisted of 50% ARPP-21A and 50% ARPP-21B.

^d The values determined for the amount of ARPP-21A and ARPP-21B in the ammonium sulfate supernatants using the phosphorylation assay proved to be unreliable, possibly due to the presence of an endogenous protein kinase inhibitor (see text).

Figure 9. Identification of the phosphorylated amino acids in ^{32}P -phospho-ARPP-21 by thin-layer electrophoresis. Electrophoresis was carried out in the vertical dimension with the anode above and the cathode below. Lane 1, Autoradiogram of purified ARPP-21 phosphorylated by the addition of 8-bromo-cAMP to bovine caudate nucleus cytosol; lane 2, autoradiogram of purified ARPP-21A phosphorylated by the purified catalytic subunit of cAMP-dependent protein kinase; lane 3, autoradiogram of purified ARPP-21B phosphorylated by the purified catalytic subunit of cAMP-dependent protein kinase; and lane 4, ninhydrin stain of internal phosphoamino acid markers. P_i , inorganic ^{32}P -phosphate; (p)Ser, phosphoserine; (p)Thr, phosphothreonine.

Figure 10. Immunoprecipitation of ARPP-21 from phosphorylated extracts prepared from various regions of bovine and monkey brain. Crude cytosol was prepared from the brain regions indicated and phosphorylated using cAMP and $\gamma\text{-}^{32}\text{P}$ -ATP as described previously (Hemmings and Greengard, 1986). The phosphorylated proteins (150 μg) were immunoprecipitated with 1 μl each of monoclonal antibodies 21-3D and 21-6A. The immunoprecipitated proteins were then subjected to SDS/PAGE (11% acrylamide) and analyzed by autoradiography.

Table 3. Summary of physical and chemical properties of ARPP-21

Property	Method of determination	Value
Molecular weight (M_r)	SDS/PAGE	21,000
	Stokes radius and sedimentation coefficient ^a	13,700
Isoelectric point (pI) of phospho-form	Polyacrylamide gel IEF	4.6
Stokes radius (a)	Gel filtration	26.3 Å
Sedimentation coefficient ($s_{20,w}$)	Sucrose density gradient centrifugation	1.3 S
Frictional ratio (f/f_0) ^b	Stokes radius and sedimentation coefficient	1.7
Axial ratio ^c	Stokes radius and sedimentation coefficient	13.5
Amino acid composition	Amino acid analysis	High Glx; no Met, Tyr, Phe, His, Trp
Partial specific volume (v)	Amino acid analysis	0.718 ml/gm

^a The molecular weight was calculated using the Stokes radius determined by gel filtration, the sedimentation coefficient by sucrose density gradient centrifugation, and the partial specific volume by the amino acid composition according to the following equation (Siegel and Monty, 1966): $M = 6\pi\eta N a s / (1 - v\rho)$, where η = viscosity of water at 20°C, N = Avogadro's number, a = Stoke's radius, s = sedimentation coefficient, v = partial specific volume, and ρ = density of water at 20°C.

^b The frictional ratio was calculated according to the following equation (Siegel and Monty, 1966): $f/f_0 = \frac{a}{(3vM/4\pi N)^{1/3}}$.

^c Value for a prolate ellipsoid (Schachman, 1959).

0.2, or 0.4 μ m pore size). The antibodies were analyzed for specificity by an immunoprecipitation assay using fixed Protein A-bearing *Staphylococcus aureus* cells (Pansorbin, Calbiochem-Behring) as an immunoadsorbent as described previously for DARPP-32 (Hemmings and Greengard, 1986). Using cytosolic extracts of rat or bovine caudate nucleus phosphorylated by the addition of γ - 32 P-ATP and 8-bromo-cAMP (Hemmings and Greengard, 1986), the rabbit antisera and mouse monoclonal antibodies 21-1A, 21-4B, 21-7A, 21-7G, 21-7N, 21-14.1, 21-14.3, 21-14.5 were found to immunoprecipitate bovine ARPP-21 efficiently but not rat ARPP-21. Mouse monoclonal antibodies 21-3D and 21-6A were found to immunoprecipitate both rat and bovine ARPP-21, although rat ARPP-21 was immunoprecipitated less efficiently than bovine. No other immunoprecipitated proteins were detected in phosphorylated rat or bovine brain cytosol. All of the antibodies produced were able to react equally well with both purified isoforms of bovine ARPP-21 (A and B) (data not shown).

The use of monoclonal antibodies 21-3D and 21-6A to immunoprecipitate ARPP-21 from various regions of bovine and monkey brain is shown in Figure 10. ARPP-21 was highly enriched in the basal ganglia, including the caudate nucleus, putamen, and globus pallidus. The amount of ARPP-21 present in the cerebral cortex was about 15% of that present in the caudate nucleus, as determined by liquid scintillation counting of the immunoprecipitated phosphoproteins (data not shown). ARPP-21 was undetectable in the cerebellum and was present at a very low concentration in the hippocampus.

Discussion

The purification and characterization of neurotransmitter-regulated neuronal phosphoproteins and the elucidation of their physiological roles provides an approach to determining the molecular mechanisms by which neurons respond to extracellular signals. Recent work has resulted in the identification in rat brain of a subset of substrates for cAMP-dependent protein kinase that are enriched in the forebrain basal ganglia (for review, see Hemmings et al., 1987b), brain regions that are densely innervated by mesencephalic dopaminergic neurons. The association of these cAMP-regulated phosphoproteins with the basal ganglia suggests that they may be involved in mediating certain of the physiological effects of dopamine acting through the D-1 dopamine receptor, a receptor subtype coupled to the stimulation of adenylate cyclase (Kebabian and Calne, 1979; Stoof and Kebabian, 1984). The phosphorylation of one of these phosphoproteins (DARPP-32) has been shown previously to be regulated by dopamine or by 8-bromo-cAMP in intact neurons (Walaas et al., 1983a; Walaas and Greengard, 1984; for review, see Walaas et al., 1986). This paper describes the purification to homogeneity and biochemical characterization of ARPP-21, a phosphoprotein that, like DARPP-32, is highly enriched in the basal ganglia and is regulated by cAMP in intact neurons.

In preliminary experiments, the cAMP analog 8-bromo-cAMP was found to stimulate the phosphorylation of a phosphoprotein of $M_r = 21,000$ in crude cytosol prepared from bovine caudate nucleus; this phosphoprotein was found by 2-dimensional thin-layer phosphopeptide mapping studies to be identical to ARPP-21, a phosphoprotein of $M_r = 21,000$ that had been identified previously in rat caudatoputamen as being highly concentrated in dopamine-innervated brain regions (Walaas et al., 1983c) and that has recently been shown to be regulated by cAMP in intact

neurons (Girault et al., 1987, 1988; Shalaby et al., 1987). In the present study, ARPP-21 was found to be highly enriched in caudate nucleus cytosol compared with cytosol from several other brain regions in bovine and monkey brain, results similar to those obtained previously in the rat. ARPP-21 was purified to homogeneity from bovine caudate nucleus cytosol and biochemically characterized.

Fractionation of bovine caudate nucleus cytosol on DEAE-cellulose revealed the existence of 2 separate peaks of ARPP-21, termed ARPP-21A and ARPP-21B. These 2 peaks contained approximately equal amounts of ARPP-21, and were purified separately. The 2 peaks of ARPP-21 obtained by DEAE-cellulose chromatography can be combined and copurified through subsequent steps, and re-separated by FPLC using a Mono Q column as a final step (J.-A. Girault, H. C. Hemmings, Jr., and P. Greengard, unpublished observations), although this procedure results in a considerable decrease in the purity of ARPP-21B obtained.

The purification procedures described for ARPP-21A and ARPP-21B did not involve any potentially denaturing techniques and could be carried out in 6 d. The use of FPLC proved to be a particularly useful method for achieving the final purification of ARPP-21A and ARPP-21B. Using the techniques described above, ARPP-21A has been purified 2610-fold to apparent homogeneity with a 20% recovery, while ARPP-21B has been purified 2940-fold to apparent homogeneity with a 21% recovery. Based on these yields, ARPP-21A and ARPP-21B comprise roughly 0.038% and 0.034%, respectively, of the total cytosolic protein present in a bovine caudate nucleus extract. ARPP-21 (ARPP-21A and ARPP-21B) is about 3-fold less abundant than DARPP-32 in bovine caudate nucleus cytosol (Hemmings et al., 1984b). More accurate quantitation of ARPP-21 in crude tissue fractions must await the development of methods more sensitive than the phosphorylation assay, such as radioimmunoassay, a technique that has been used successfully to quantitate DARPP-32 in various tissues (Hemmings and Greengard, 1986).

The 2 peaks of ARPP-21 (A and B) detected initially by chromatography on DEAE-cellulose or Mono Q were found to be identical by 2-dimensional thin-layer phosphopeptide mapping. This finding indicated that whatever feature is responsible for the chromatographic differences between ARPP-21A and ARPP-21B is not evident in the electrophoretic mobilities of their tryptic phosphorylation site peptides, and therefore most probably exists in another region of the molecule. The biochemical relationship between ARPP-21A and ARPP-21B was further analyzed by HPLC peptide mapping of tryptic digests of the purified preparations of ARPP-21A and ARPP-21B. A comparison of their tryptic HPLC maps indicated a marked degree of similarity between these 2 proteins; however, several specific differences were observed in the tryptic peptides eluting later in the chromatograms. These results are consistent with minor structural differences between ARPP-21A and ARPP-21B, possibly due to posttranslational modification or to genetic polymorphism and the presence of 2 different allelic forms of ARPP-21. We conclude that ARPP-21 consists of 2 closely related isoforms (ARPP-21A and ARPP-21B), with identical M_r values, hydrodynamic properties, isoelectric points, amino acid compositions, phosphorylated amino acid residues, and immunologic reactivity with polyclonal and monoclonal antibodies but with different chromatographic mobilities on DEAE-cellulose or Mono Q and slightly different HPLC tryptic peptide maps.

The amino acid sequences of ARPP-21A and ARPP-21B are probably very similar, and we are currently addressing this question by determining these sequences.

ARPP-21 is a highly acidic phosphoprotein. The amino acid composition of ARPP-21 is notable for the high content of glutamic acid/glutamine (25 mol %) and for the absence of any tyrosine, phenylalanine, tryptophan, histidine, or methionine. cAMP-dependent protein kinase catalyzes the phosphorylation of ARPP-21 to a stoichiometry of approximately 1 mol phosphate/mol ARPP-21. The solubility and ability of ARPP-21 to be phosphorylated were stable to heat or ethanol treatment and partially stable to acid treatment, suggesting that native ARPP-21 possesses little ordered tertiary structure. This possibility is further supported by studies of the hydrodynamic properties of ARPP-21. ARPP-21 exhibited a high frictional ratio, which is consistent with an elongated, asymmetric tertiary structure. Furthermore, the molecular weight of ARPP-21 calculated from the hydrodynamic measurements was considerably less than the relative molecular weight determined by SDS-PAGE. Anomalous migration in SDS-polyacrylamide gels has been described previously for proteins with relatively decreased binding of SDS and extended conformations (Tung and Knight, 1972), conditions that appear to hold for ARPP-21, a highly acidic protein with a very asymmetric tertiary structure.

The stability of ARPP-21 to denaturation and its hydrodynamic properties are reminiscent of several other acidic phosphoproteins that appear to function as intracellular regulatory proteins. Thus, DARPP-32 (Hemmings et al., 1984b), skeletal muscle protein phosphatase inhibitor-1 (Nimmo and Cohen, 1978), adipose tissue protein phosphatase inhibitor-1 (Nemoff et al., 1983), protein phosphatase inhibitor-2 (Huang and Glinsmann, 1976a), and G-substrate (Aswad and Greengard, 1981) are each stable to heat and acid treatments, and possess asymmetric tertiary structures. DARPP-32 (Hemmings et al., 1984a), protein phosphatase inhibitor-1, protein phosphatase inhibitor-2 (Huang and Glinsmann, 1976b), and G-substrate (A. C. Nairn, H. C. Hemmings, Jr., and P. Greengard, unpublished observations) are each capable of inhibiting specific protein phosphatases. However, neither phospho- nor dephospho-ARPP-21 was capable of inhibiting either protein phosphatase-1, -2A, -2B, or -2C (H. C. Hemmings, Jr., P. Cohen, and P. Greengard, unpublished observations). The acidic isoelectric point and high glutamic acid/glutamine residue content suggest that ARPP-21 may function as a Ca^{2+} -binding protein, since clusters of acidic amino acid residues are characteristic of many such proteins (Klee and Vanaman, 1982). Preliminary experiments have failed to reveal any Ca^{2+} -binding activity in ARPP-21 (unpublished observations). Determination of the complete amino acid sequence of ARPP-21 may provide important clues as to its biochemical functions, such as identifying a putative Ca^{2+} -binding site or additional phosphorylation sites.

ARPP-21 appears to be a specific substrate for cAMP-dependent protein kinase, and phospho-ARPP-21 a substrate for protein phosphatase-1 and -2A. None of the other purified protein kinase preparations tested displayed significant activity toward ARPP-21. The observation that phospho-ARPP-21 is a substrate for protein phosphatase-1 suggests that the state of phosphorylation of ARPP-21 may be regulated indirectly by DARPP-32 via its inhibition of protein phosphatase-1 (Hemmings et al., 1984a; see also Hemmings et al., 1987c) in medium-sized spiny neurons, which contain both ARPP-21 and DARPP-32 (see

below). The efficiency with which ARPP-21 is phosphorylated *in vitro* by cAMP-dependent protein kinase is comparable to that observed for DARPP-32 or synapsin I, 2 neuronal phosphoproteins that have previously been shown to be regulated in intact cells by dopamine acting through cAMP (Nestler and Greengard, 1980; Tsou and Greengard, 1982; Walaas et al., 1983a; Walaas and Greengard, 1984).

The regional distribution of ARPP-21, as determined by a phosphorylation assay (Walaas et al., 1983c) and by phosphorylation and immunoprecipitation (this study), has been shown to be quite similar to that of DARPP-32 in its restriction primarily to dopamine-innervated brain regions. Furthermore, immunocytochemical studies employing specific antibodies to ARPP-21 indicate that ARPP-21 is contained within many of the same cell types as DARPP-32, including the medium-sized spiny neurons of the caudatoputamen (Ouimet et al., 1983, 1984, 1989). The phosphorylation of DARPP-32 has previously been found to be regulated by dopamine and cAMP in slices prepared from rat caudatoputamen (Walaas et al., 1983a; Walaas and Greengard, 1984), while the phosphorylation of ARPP-21 has been found to be regulated by cAMP in slices and cultured cells prepared from rodent caudatoputamen (Girault et al., 1987, 1988; Shalaby et al., 1987). Taken together, these findings suggest that ARPP-21, which appears to be present in many of the same cells as DARPP-32, may also be regulated by dopamine acting through cAMP in these cells. Experiments to demonstrate the regulation of the state of phosphorylation of ARPP-21 in intact cells by dopamine or other neurotransmitters acting through cAMP are in progress. If the regulation of ARPP-21 by dopamine and/or another neurotransmitter can be demonstrated experimentally, ARPP-21 will join DARPP-32 as a prime candidate for an intracellular messenger involved in mediating some of the physiological effects of neurotransmitters acting through cAMP. Interestingly, the phosphorylation of ARPP-21 has recently been shown to be regulated by vasoactive intestinal peptide in cultured mouse striatal cells (Shalaby et al., 1987; Girault et al., 1988).

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