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Regulation by cAMP and vasoactive intestinal peptide of phosphorylation of specific proteins in striatal cells in culture

(cAMP-dependent protein kinase/reaggregate cultures/forskolin/basal ganglia/caudate-putamen)

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ABSTRACT **We have studied three low molecular weight phosphoproteins, ARPP-16, ARPP-19, and ARPP-21 (cAMP-regulated phosphoproteins of M_r 16,000, 19,000, and 21,000, respectively) in reaggregate cultures from various regions of fetal mouse brain. ARPP-16 and ARPP-21 were detected only in striatal and cortical cultures. In contrast, ARPP-19, which is structurally related to ARPP-16, was also present in reaggregate cultures prepared from thalamus and ventral and dorsal mesencephalon, as well as in monolayer cultures of astroglial cells. In striatal aggregates cultured over a 3-week period, the relative levels of ARPP-16, ARPP-21, and synapsin I/protein IIIa (synaptic vesicle-associated phosphoproteins closely related to each other and treated as a single entity in the present study) increased with time, whereas the level of ARPP-19 decreased. Incubation of striatal aggregates with 8-Br-cAMP, forskolin, or vasoactive intestinal peptide increased the phosphorylation of all these proteins. We conclude that the state of phosphorylation of two proteins enriched in specific neurons (ARPP-16 and ARPP-21) and two more widely distributed proteins (ARPP-19 and synapsin I/protein IIIa) is regulated by cAMP and vasoactive intestinal peptide in striatal cells in culture. These phosphoproteins may therefore play a role in mediating some of the actions of vasoactive intestinal peptide in the caudate-putamen.**

Many of the actions of neurotransmitters are thought to be due to alterations in the state of phosphorylation of intracellular proteins (1). Among the substrates for cAMP-dependent protein kinase that have been characterized in rat brain, some have a restricted regional distribution within the central nervous system. In particular, several are enriched in the caudate-putamen and the substantia nigra (2–4). Two such low molecular weight proteins have recently been purified and antibodies directed against them have been obtained (5–7). The function of these proteins is not yet known and they are referred to as ARPP-16 and ARPP-21 (for cAMP-regulated phosphoproteins of M_r 16,000 and 21,000, respectively) (8). Another phosphoprotein, ARPP-19, has also been purified and shown to be highly homologous to ARPP-16 and to crossreact fully with antibodies raised against ARPP-16 (7). In fact, analysis of protein and cDNA sequences of these two proteins has revealed that ARPP-19 is identical to ARPP-16 except that it has an additional stretch of 16 amino acids on the N-terminal side (9). In contrast to the specific distribution of ARPP-16, which appears to be restricted to striatal and cortical neurons, ARPP-19 is found in all brain regions and in peripheral organs of the adult rat (8).

In the present study, using striatal cells in culture, we have investigated the regulation of the phosphorylation state of ARPP-16, ARPP-19, and ARPP-21 by cAMP and by vasoactive intestinal peptide (VIP), a highly potent activator of adenylate cyclase in such cells (10). For comparison, we have studied in the same experiments the regulation of two well-characterized synaptic vesicle-associated phosphoproteins, synapsin I and protein IIIa (see ref. 11 for review).

MATERIALS AND METHODS

Materials. Pregnant CD 1 mice were purchased from Charles River Breeding Laboratories. Chemicals and reagents were obtained from the indicated sources: minimal Eagle's culture medium, penicillin/streptomycin, trypsin, and heat-inactivated fetal bovine and horse sera (GIBCO); DNase (Cooper Biomedical, Malvern, PA); VIP (rat sequence) (Peninsula Laboratories, San Carlos, CA); 8-Br-cAMP (Sigma); Pansorbin (formaldehyde-treated cells of *Staphylococcus aureus*), forskolin, and phenylmethylsulfonyl fluoride (Calbiochem); leupeptin and pepstatin A (Chemicon, Los Angeles); aprotinin (Trasylol) (Mobay Chemical, New York); [γ-^{32}P]ATP (New England Nuclear); Nonidet P-40 (Particle Data, Elmhurst, IL); acrylamide (Serva, Heidelberg); protein dye reagent (Bio-Rad). Catalytic subunit of cAMP-dependent protein kinase, purified as described (12), was a gift of Angus Nairn and Atsuko Horiuchi (our laboratory). Anti-ARPP-16 rabbit antiserum (G 153), raised against purified bovine ARPP-16 (7), was a gift of Atsuko Horiuchi. Anti-ARPP-21 rabbit antiserum (G 154) was raised against bovine ARPP-21 purified as described (5).

Preparation of Cell Cultures. Corpus striatum and, in some experiments, cerebral cortex, thalamus, and ventral and dorsal mesencephalon were dissected from 14-day mouse embryos, and rotation-mediated reaggregate cultures were prepared as described by Hemmendinger *et al.* (13). Monolayer astroglial cultures were prepared from cerebral cortex of 4-day-old mice according to Hertz *et al.* (14). These latter cultures appeared to consist mainly of astrocytes and to be devoid of identifiable neurons based on light microscopy and immunoblots for glial fibrillary acidic protein, synapsin I, and synaptophysin (data not shown).

Pharmacological Treatments. Experiments were performed with aggregates that had been maintained in culture for 3 weeks. One day after a change of medium, aggregates from several flasks were pooled, washed three times in buffer (124 mM NaCl/5 mM KCl/25.9 mM $NaHCO_3$/1.4 mM $MgSO_4$/1.2 mM KH_2PO_4/1.5 mM $CaCl_2$/10 mM D-glucose/25 mM Hepes, pH 7.4), aliquoted into separate flasks, and preincubated for 1 hr in the rotary incubator shaker. Drug or vehicle was then added to the flasks [stock solutions of forskolin were prepared at 10 mM in 95% (vol/vol) ethanol and diluted as required in water; stock solutions of VIP were prepared at 0.1 mM in water containing 0.1 mg of bovine serum albumin per ml]. After a 10-min incubation, aggregates were rapidly transferred to ice-cold 15-ml conical polypropylene tubes. After removal of buffer, aggregates were frozen by dipping

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Abbreviation: VIP, vasoactive intestinal peptide.

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the tubes into liquid nitrogen and stored at −70°C until biochemical analysis. In time-course experiments, the aggregates were transferred directly from Erlenmeyer flasks to polypropylene tubes containing the drug or the vehicle in 2 ml of buffer. Incubation was extended at 37°C with frequent resuspension of the aggregates and stopped by removal of the buffer and freezing.

Measurement of the State of Phosphorylation of the Proteins. The state of phosphorylation of the proteins of interest was estimated by "back-phosphorylation" of acid-extracted samples as described for synapsin I (15), with some modifications. All experiments were carried out at 0–4°C. In pharmacological experiments, frozen aggregates were homogenized by sonication with a Kontes cell disrupter in 1.5 ml of ice-cold 5-mM zinc acetate. In experiments in which the total amount (phospho- plus dephospho-) of phosphoprotein was determined (Figs. 1 and 2), homogenization was carried out in 200 μl of buffer [10 mM Tris Cl, pH 7.4/2 mM EDTA/1 mM EGTA/1 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride/leupeptin (10 μg/ml)/aprotinin (5000 units/ml)/pepstatin A (4 μg/ml)] to allow dephosphorylation of substrates. Zinc acetate was then added to achieve a final concentration of 5 mM in a volume of 1.5 ml. The homogenates were centrifuged at 3000 × *g* for 15 min. The pellets were resuspended in 200 μl of 200 mM citric acid containing leupeptin (10 μg/ml), pepstatin A (4 μg/ml), aprotinin (5000 units/ml), and phenylmethylsulfonyl fluoride (1 mM) and were centrifuged at 12,000 × *g* for 15 min. Protein concentrations were measured in the supernatants by the method of Bradford (16) with bovine serum albumin as standard. Equal amounts of protein (50–150 μg) were transferred to polypropylene microcentrifuge tubes, volumes were equalized by adding citric acid with protease inhibitors, and the pH was brought to 7.0 with 1:10 (vol/vol) 0.5 M Na_2HPO_4. The acid extracts were heated at 95°C for 30 sec and clarified by centrifugation at 12,000 × *g* for 10 min. The supernatants were used for phosphorylation reactions. This brief heating, found to decrease the variability and the background during *in vitro* phosphorylation, resulted in virtually no loss of ARPP-16, ARPP-19, and ARPP-21 and in only limited loss of synapsin I/protein III that did not preclude its quantification (data not shown).

Phosphorylation reactions were carried out for 45 min at 30°C in a final volume of 200 μl containing 120 μl of acid extract, 50 mM Hepes (pH 7.4), 10 mM magnesium acetate, 1 mM EGTA, 5 μM [γ-^{32}P]ATP (specific activity, 10 Ci/mmol; 1 Ci = 37 GBq), and 0.1–0.2 μg of catalytic subunit of cAMP-dependent protein kinase. Reactions were stopped by the addition of 50 μl of 750 mM NaCl/75 mM EDTA/250 mM Tris Cl, pH 7.4/250 mM NaF/0.02% NaN_3 and 30 sec of heating at 95°C. Two hundred microliters of the phosphorylation mixture was used for immunoprecipitation, as described for DARPP-32 (17), using either 5 μl of anti-ARPP-16 rabbit antiserum, to precipitate ARPP-16 and ARPP-19, or 20 μl of anti-ARPP-21 rabbit antiserum, or a mixture of the two. This procedure of immunoprecipitation was quantitative for ARPP-16, ARPP-19, and ARPP-21 and the results were virtually identical whether the two sera were used separately or together (data not shown). The remainder of the phosphorylated extracts and the immunoprecipitates were subjected to polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ (18) (concentration of polyacrylamide in the gel was 13%, wt/vol) and analyzed by autoradiography.

The amount of ^{32}P incorporated into the proteins of interest was measured as radioactivity in the corresponding dried gel pieces by liquid scintillation spectrometry. With this method it was not possible to reliably separate the two forms of synapsin I (Ia and Ib) from each other or from protein IIIa, another protein enriched in nerve terminals and probably functionally related to synapsin I (11, 19). Therefore the quantification of synapsin I/protein IIIa reported below represents the sum of synapsin I and protein IIIa. The amounts of ^{32}P incorporated into the proteins studied in the drug-treated samples were expressed as percentages of the radioactivity incorporated in control samples. Statistical analysis was done either with the two-tailed Student's *t* test for comparisons of two means or with one-way analysis of variance followed by the two-tailed Dunnett's test for multiple comparisons (20).

RESULTS AND DISCUSSION

ARPP-16 and ARPP-21 were found in cortical and striatal cultures but not in cultures from other regions of the mouse brain (Fig. 1), indicating that the specific regional distribution of these proteins, observed in the adult rat (8), is already determined in 14-day-old embryos and is maintained during culture. ARPP-16 and ARPP-21 were not detected in cultures from ventral mesencephalon, which contains the embryonic substantia nigra (13), in agreement with the fact that, in the adult rat, the high levels of these proteins found in the substantia nigra are actually contained in the terminals of

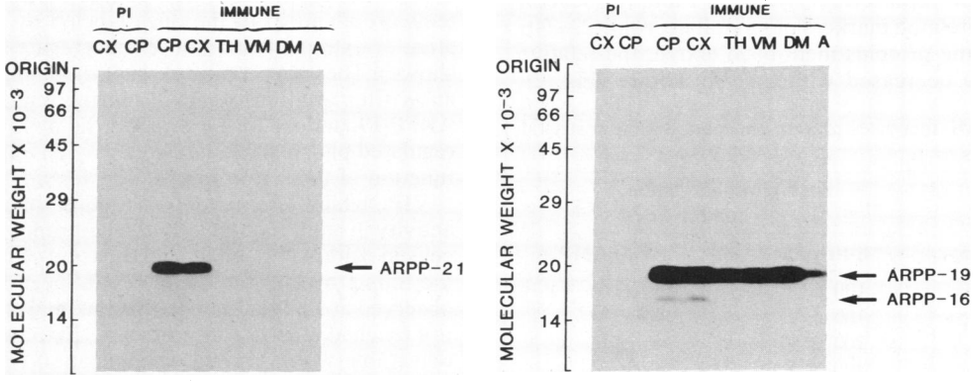

FIG. 1. Autoradiograms showing the presence of ARPP-21, ARPP-19, and ARPP-16 in cultures from various regions of embryonic mouse brain. Reaggregate cultures from caudate-putamen (CP), cerebral cortex (CX), thalamus (TH), ventral mesencephalon (VM), and dorsal mesencephalon (DM) and monolayer astroglial cultures (A) were prepared. After 3 weeks (aggregates) or 12 days (monolayers) in culture, equal amounts of protein from each type of culture were phosphorylated *in vitro* with cAMP-dependent protein kinase and [γ-^{32}P]ATP, immunoprecipitated with rabbit antiserum against ARPP-21 (*Left*) or rabbit antiserum against ARPP-16/ARPP-19 (*Right*), and analyzed by polyacrylamide gel electrophoresis. In the two left lanes of each autoradiogram, preimmune serum (PI) was used instead of immune serum. ARPP-16 and ARPP-21 were detected only in cortical and striatal cultures, whereas ARPP-19 was found in cultures from all brain regions studied and, at a lower level, in astroglial cultures.

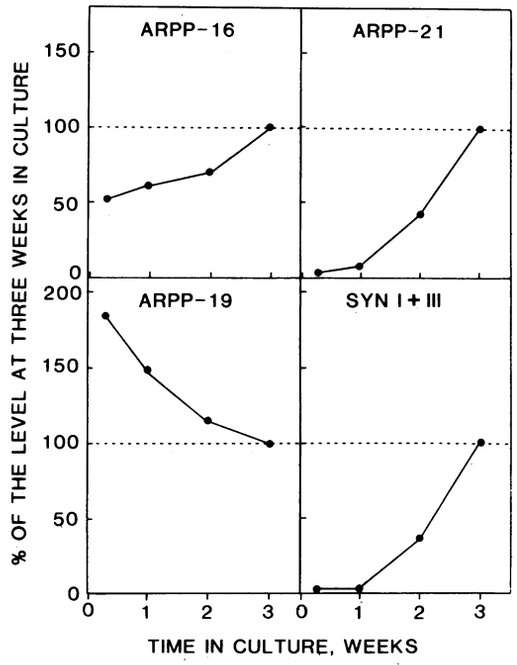

FIG. 2. Changes in the relative levels of ARPP-16, ARPP-19, ARPP-21, and synapsin I/protein III with time in culture. Reaggregate striatal cultures were frozen after various periods of time in culture. The levels of ARPP-16, ARPP-19, and ARPP-21 per mg of total protein were measured by back-phosphorylation. The levels of synapsin I/protein III were estimated by immunoblotting with ^{125}I-labeled protein A and measuring the bound radioactivity by γ-ray scintillation counting as described (21). Data are expressed as percentage of the levels at 3 weeks in culture and represent the means of two experiments.

striatonigral neurons (6, 8). In contrast, ARPP-19 was present in cultures prepared from ventral and dorsal mesencephalon, thalamus, cerebral cortex, and caudate-putamen and, albeit at a lower concentration, in astroglial cultures (Fig. 1). The relative levels of ARPP-16, ARPP-21, and synapsin I/protein IIIa increased with time in culture (Fig. 2), probably as a consequence of neuronal differentiation, although the amounts of ARPP-16 always remained very low as compared to adult tissue [in which the concentrations of ARPP-16 and ARPP-19 are comparable (8)] and could not be reliably determined in every experiment. A similar increase with time in culture has been reported for synapsin I and for several neurotransmitter-related markers of striatal neurons (22–24). In contrast with the proteins mentioned above, the relative level of ARPP-19 decreased with time in culture (Fig. 2),

Table 1. 8-Br-cAMP increases protein phosphorylation in striatal reaggregate cultures

Phosphoprotein	Back-phosphorylation, % control	
	− 8-Br-cAMP	+ 8-Br-cAMP
ARPP-16	100 ± 7	56 ± 6*
ARPP-19	100 ± 9	48 ± 3*
ARPP-21	100 ± 9	45 ± 6*
SYN I/IIIa	100 ± 5	42 ± 6*

Three-week-old striatal reaggregate cultures were incubated in the presence of vehicle or 4 mM 8-Br-cAMP for 10 min, and the phosphorylation state of the proteins was estimated by back-phosphorylation. The amount of ^{32}P incorporated into the proteins in treated samples (+ 8-Br-cAMP, $n = 9$) is expressed as percentage of the amount incorporated in control samples (− 8-Br-cAMP, $n = 8$). Data represent means ± SEM.
*$P < 0.001$ (two-tailed Student's t test).

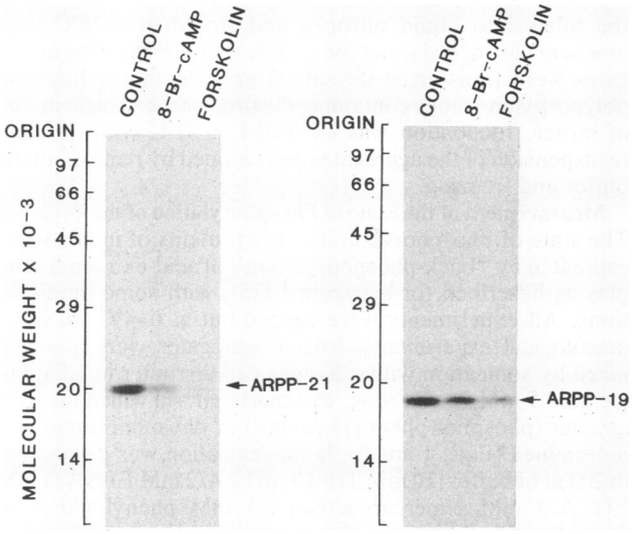

FIG. 3. Autoradiograms showing the effects of 8-Br-cAMP and forskolin on the phosphorylation of ARPP-21 and ARPP-19. Three-week-old striatal reaggregate cultures were incubated for 10 min in the presence of vehicle (control), 4 mM 8-Br-cAMP, or 10 μM forskolin. Extracts were back-phosphorylated and ARPP-21 and ARPP-19 were immunoprecipitated as described in the text. By this method, increases in the levels of phosphorylation of the proteins in intact cells are manifested as decreases in the amount of ^{32}P incorporated *in vitro*. ARPP-16 was immunoprecipitated together with ARPP-19 (see Fig. 1) but was not visible on this autoradiogram, which was exposed for only 4 hr.

suggesting that ARPP-19 is more abundant in nondifferentiated than in differentiated nervous tissue.

8-Br-cAMP increased the level of phosphorylation of ARPP-16, ARPP-19, ARPP-21, and synapsin I/protein IIIa (Table 1 and Fig. 3). Forskolin, a naturally occurring diterpene that stimulates adenylate cyclase directly (see ref. 25 for review), also enhanced the phosphorylation of all four proteins (Figs. 3 and 4). These results are in agreement with data obtained in adult rat striatal slices for synapsin I (26) and demonstrate that the three low molecular weight proteins, which were identified and purified on the basis of their phosphorylation by cAMP-dependent protein kinase *in vitro*, are also substrates for this kinase in intact cells. In addition, phospho amino acid analyses and two-dimensional tryptic phosphopeptide maps indicate that the sites phosphorylated by cAMP-dependent protein kinase on purified bovine proteins are identical to the ones labeled by back-phosphorylation of extracts from mouse reaggregate cultures (data not shown). These observations, suggesting that the phosphorylation sites are conserved in murine and bovine ARPP-16, ARPP-19, and ARPP-21, support the hypothesis that cAMP-regulated phosphorylation sites play an important role in the function of these proteins.

VIP, which is a potent stimulator of adenylate cyclase activity in adult rodent striatal membranes (27) and in striatal cells in culture (10), enhanced the level of phosphorylation of the four proteins studied. A pronounced effect of VIP was observed at 1 μM, although the maximal effect was probably not reached at that concentration (Fig. 5*A*). This effect was detectable within 45 sec of application (Fig. 5*B*). Although VIP is thought to act on target cells, in brain as well as in the periphery, mainly by activating adenylate cyclase, there is also evidence that VIP may increase the breakdown of phosphatidylinositol in some tissues (28, 29). Nevertheless, it seems likely that the effects of VIP observed in the present study were related to the enhancement of cAMP levels, since the technique of back-phosphorylation specifically measures the degree of phosphorylation of the cAMP-dependent phos-

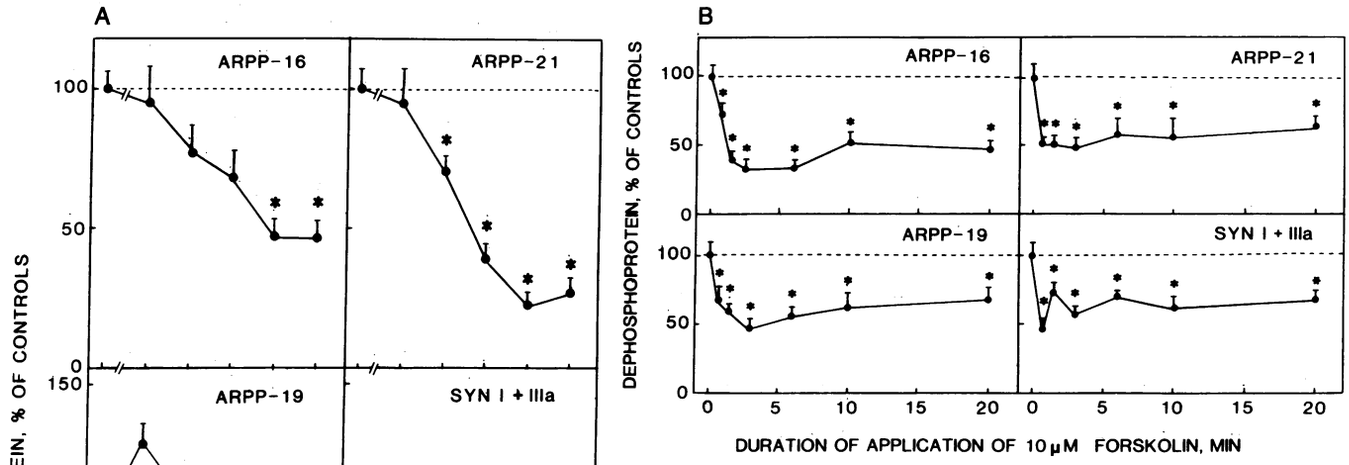

FIG. 4. Forskolin increases protein phosphorylation in striatal reaggregate cultures. Three-week-old striatal reaggregate cultures were incubated for 10 min in the presence of the indicated concentration of forskolin or vehicle (*A*) or for various periods of time in the presence of 10 μM forskolin (*B*). The phosphorylation state was estimated by back-phosphorylation. Data are expressed as percentage of control and represent means ± SEM (n = 5–8). Statistical analyses were done by one-way analysis of variance. (*A*) ARPP-16, $F = 4.64$, $P = 0.004$; ARPP-19, $F = 9.49$, $P < 0.001$; ARPP-21, $F = 22.34$, $P < 0.001$; synapsin I/protein IIIa, $F = 4.97$, $P = 0.003$. (*B*) ARPP-16, $F = 12.43$, $P < 0.001$; ARPP-19, $F = 4.86$, $P = 0.001$; ARPP-21, $F = 3.86$, $P = 0.004$; synapsin I/protein IIIa, $F = 5.10$, $P = 0.001$. Comparisons between each point and controls were done by the two-tailed Dunnett's test (*, $P < 0.05$).

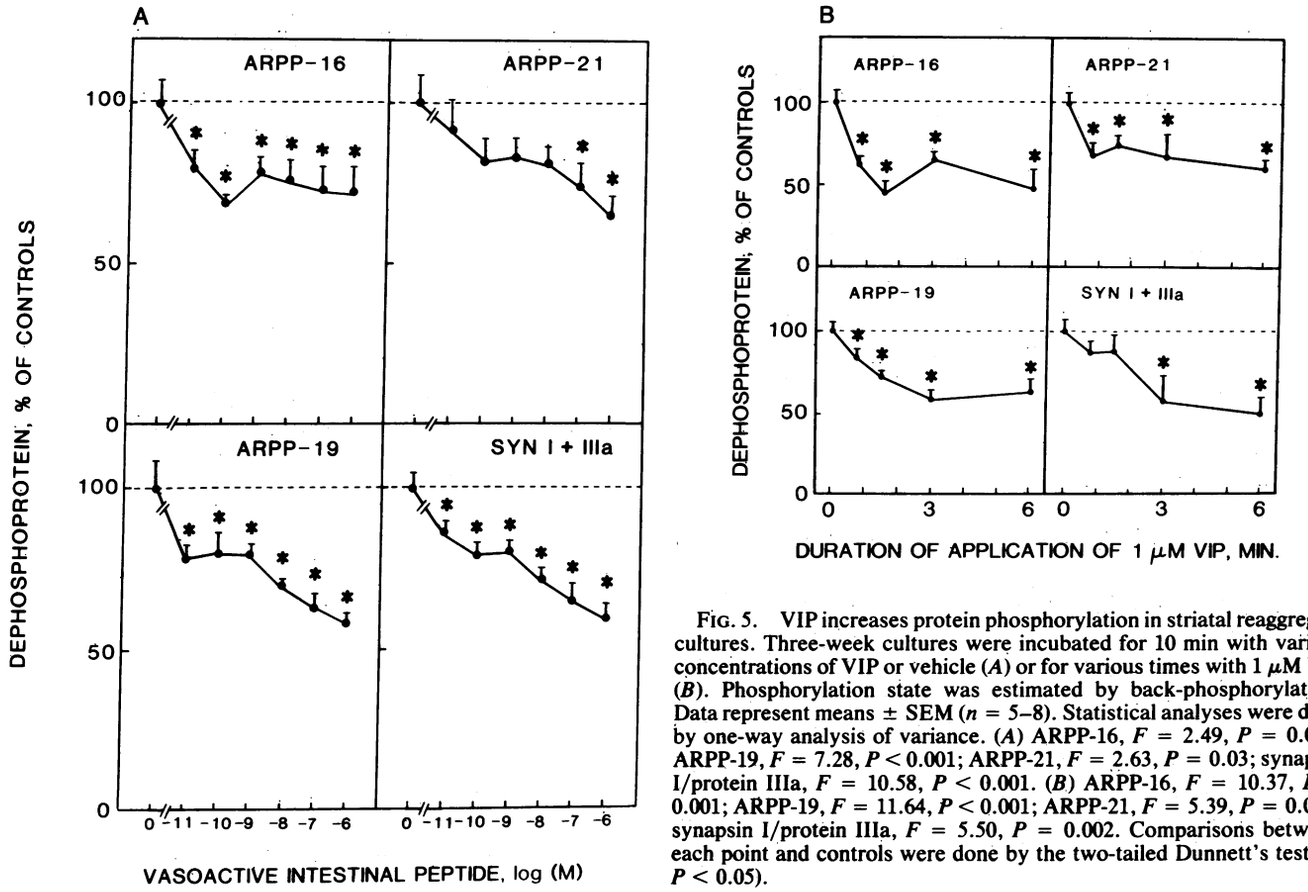

FIG. 5. VIP increases protein phosphorylation in striatal reaggregate cultures. Three-week cultures were incubated for 10 min with various concentrations of VIP or vehicle (*A*) or for various times with 1 μM VIP (*B*). Phosphorylation state was estimated by back-phosphorylation. Data represent means ± SEM (n = 5–8). Statistical analyses were done by one-way analysis of variance. (*A*) ARPP-16, $F = 2.49$, $P = 0.036$; ARPP-19, $F = 7.28$, $P < 0.001$; ARPP-21, $F = 2.63$, $P = 0.03$; synapsin I/protein IIIa, $F = 10.58$, $P < 0.001$. (*B*) ARPP-16, $F = 10.37$, $P < 0.001$; ARPP-19, $F = 11.64$, $P < 0.001$; ARPP-21, $F = 5.39$, $P = 0.001$; synapsin I/protein IIIa, $F = 5.50$, $P = 0.002$. Comparisons between each point and controls were done by the two-tailed Dunnett's test (*, $P < 0.05$).

phorylation sites. Further, the four proteins studied are poor substrates for protein kinase C *in vitro* (A. C. Nairn, H. C. Hemmings, A. Horiuchi, and P.G., unpublished observations). Finally, the relatively high concentrations of VIP necessary to achieve a maximal change in phosphorylation were similar to those (≥ 1 μM) necessary to achieve maximal stimulation of cAMP accumulation in brain tissues (10, 27, 30).

In rat caudate-putamen, neurons containing VIP-like immunoreactivity are sparse, medium-size, aspiny interneurons, often found in the vicinity of fiber bundles (31). VIP receptors, on the other hand, are present in relatively high levels in caudate-putamen as indicated by specific binding of ^{125}I-labeled VIP (32) and by VIP-induced accumulation of cAMP (27). Comparison of the amplitudes of the effects of forskolin and of VIP in the present experiments indicates that a large proportion of the cells containing ARPP-16, -19, and -21 are responsive to VIP. Given the fact that ARPP-21 (6) and ARPP-16 (E. Gustafson and P.G., unpublished observations) are contained mainly in medium-size spiny neurons, this observation is in agreement with the suggestion of synaptic contacts between VIP-containing terminals and medium-size spiny neurons (31). Further, the effect of VIP on the phosphorylation of synapsin I and protein IIIa suggests that VIP receptors are present on nerve terminals in striatal cultures. In support of this conclusion, in some experiments the phosphorylation of protein IIIb, another synaptic vesicle-associated phosphoprotein, was measured and found to give results similar to those obtained for synapsin I/protein IIIa (data not shown). It will be of interest to determine whether VIP also regulates the phosphorylation of ARPP-16, ARPP-19, ARPP-21, and the synaptic vesicle-associated phosphoproteins in the cerebral cortex, where all these proteins as well as VIP-immunoreactive terminals are present (see ref. 33 for a review of VIP in the cortex).

In conclusion, we have shown that the phosphorylation state of two proteins present in all brain regions, ARPP-19 and synapsin I/protein IIIa, and of two proteins specifically enriched in striatal and cortical neurons, ARPP-16 and ARPP-21, is regulated in intact striatal cells by cAMP and VIP. These proteins may therefore play a role in mediating some of the physiological effects of VIP in the caudate-putamen and, possibly, in other regions of the brain. Further work is required to determine whether the phosphorylation of ARPP-16, ARPP-19, and ARPP-21 is also regulated by other neurotransmitters known to enhance cAMP levels in the caudate-putamen, such as dopamine.

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