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Cholinergic Regulation of Protein III Phosphorylation in Bovine Adrenal Chromaffin Cells

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Protein IIIa ($M_r \approx 74{,}000$) and protein IIIb ($M_r \approx 55{,}000$), referred to collectively as protein III, are synaptic vesicle-associated phosphoproteins found in all regions of the rat nervous system and in the rat adrenal medulla. In the present study, the presence of protein III and the regulation of its phosphorylation were examined in chromaffin cells isolated from bovine adrenal medullae.

Protein III was present in chromaffin cells isolated and purified from bovine adrenal medullae. The levels of protein III were moderately enriched in purified chromaffin cells compared with whole adrenal medullae. Preincubation of chromaffin cells with $^{32}PO_4$ led to the endogenous phosphorylation of protein III, and phosphopeptide maps of chromaffin cell protein III were similar to those of protein III from bovine cerebral cortex.

Treatment of the chromaffin cells with ACh produced calcium-dependent increases in both the phosphorylation of protein III and the release of ^{3}H-norepinephrine. These effects of ACh were mimicked by nicotine but not by muscarine. Other secretagogues (elevated K^+, veratridine, Ba^{2+}) also increased both the phosphorylation of protein III and the release of ^{3}H-norepinephrine. However, detailed characterization of the secretagogue-induced increases in protein III phosphorylation and ^{3}H-norepinephrine secretion suggested that protein III phosphorylation was more directly associated with an increase in intracellular calcium than with secretion per se.

Extensive evidence for the involvement of protein phosphorylation in neuronal function has accumulated in recent years. In addition to modulation of ion channels and neurotransmitter synthesis (for reviews, see Nestler and Greengard, 1984; Browning et al., 1985; Levitan, 1985), protein phosphorylation has recently been implicated in the control of neurotransmitter release at the squid giant synapse (Llinás et al., 1985). Protein phosphorylation has also been implicated in the release of catecholamines from adrenal medullary chromaffin cells (cf. Burgoyne, 1984).

Activation of the splanchnic nerve, which innervates the adrenal medulla, liberates ACh, which, in turn, stimulates the exocytotic secretion of catecholamines from chromaffin cells (cf. Douglas, 1968). Associated with this stimulation is the phosphorylation of $M_r \approx 100{,}000$ and $M_r \approx 60{,}000$ proteins that can be observed in extracts of total cellular protein after SDS-PAGE (Amy and Kirshner, 1981). The $M_r \approx 100{,}000$ protein was shown (Haycock et al., 1986) to be "100-kDa," a $M_r \approx 100{,}000$ phosphoprotein that is a specific substrate for calcium/calmodulin-dependent protein kinase III (Palfrey, 1983; Nairn et al., 1985). The $M_r \approx 60{,}000$ protein was shown (Haycock et al., 1982a, b) to be tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis (Nagatsu et al., 1964).

Activation of rat nervous tissue increases the phosphorylation of protein IIIa ($M_r \approx 74{,}000$) and protein IIIb ($M_r \approx 55{,}000$) (Forn and Greengard, 1978; Tsou and Greengard, 1982). Both protein IIIa and protein IIIb are present in the rat adrenal medulla (Browning et al., 1987), but the phosphorylation of these proteins was not detected in bovine adrenal medullary chromaffin cells (Amy and Kirshner, 1981). Using immunoprecipitation to isolate protein III from extracts of total chromaffin cell proteins, we have now demonstrated the presence of proteins IIIa and IIIb in chromaffin cells isolated from bovine adrenal medullae and characterized the regulation of their phosphorylation in intact cells by secretagogues. A preliminary account of these data has appeared previously (Haycock et al., 1984).

Materials and Methods

Materials. Fresh bovine adrenal glands were transported on ice from a local slaughterhouse to the laboratory, and chromaffin cells were prepared as previously described through either the initial cell fraction stage or the purified chromaffin cell fraction stage (Waymire et al., 1983). Chromaffin cells constituted ~60% (initial cell fraction) to ~95% (purified chromaffin cell fraction) of the cells as judged by neutral red staining. The cells were maintained either in suspension culture or as monolayers (on collagen-coated dishes) and used within 2 weeks of culture.

^{125}I-protein A, $^{32}PO_4$, [γ-^{32}P]-ATP, and L-[7-^{3}H(N)]-norepinephrine (^{3}H-NE) were purchased from New England Nuclear. ACh, nicotine, muscarine, and veratridine were purchased from Sigma. Pansorbin was purchased from Calbiochem-Behring. *S. aureus* V8 protease was from Miles. Trypsin and chymotrypsin were from Cooper. All other chemicals were reagent grade or better.

Measurement of endogenous protein III levels by immunolabeling. Chromaffin cell and bovine brain tissue samples were solubilized in 1% SDS and subjected to SDS-PAGE in 7.5% gels according to Laemmli (1970). The samples were electrophoretically transferred to nitrocellulose sheets (BA 83, 0.2 μm; Schleicher and Schuell), which were then incubated sequentially with rabbit antibody to purified bovine cerebral cortex protein III (Huang et al., 1982; Browning et al., 1987) and ^{125}I-protein A (Burnette, 1981). Immunoreactive bands were localized by autoradiography and ^{125}I was quantitated after excision of the bands, in a gamma counter (Micromedic Systems, model 2/200). (Within the protein ranges analyzed, ^{125}I-protein A binding was linear with the amount of protein loaded. In addition, phosphorylation of protein III did not

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influence the immunolabeling). After calculation of cpm/mg protein for chromaffin cell samples and for bovine cerebral cortex standards run on the same gel, the chromaffin cell cpm/mg protein values were converted into protein III levels on the basis of previously established values (Huang et al., 1982; Browning et al., 1987) for bovine cerebral cortex (protein IIIa: 9 pmol/mg protein; protein IIIb: 19 pmol/mg protein).

Protein phosphorylation in intact chromaffin cells. Monolayers of chromaffin cells (10^6 cells/60-mm-diameter dish) were rinsed with HEPES-buffered saline (HBS, pH 7.4: 150 mM NaCl, 10 mM HEPES, 5.5 mM D-glucose, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$) and preincubated at 37°C in fresh HBS (2 ml) containing $^{32}PO_4$ (1 mCi, carrier free) for 60 min. [In one set of experiments, chromaffin cells were preincubated in 2 ml of a mixture of F-12/fetal calf serum (2 vol) and HBS (1 vol) containing $^{32}PO_4$ (1 mCi) for 24 hr.] The dishes were brought to room temperature and 0.5 ml of HBS containing the test substance was added. After incubation with the test substances, the incubation medium was aspirated and 0.5 ml of 1% (wt/vol) SDS was added. The samples were collected, after scraping the dishes, and heated for 5 min in a boiling water bath.

An aliquot (50 μl) from each sample was taken for analysis by SDS-PAGE of protein phosphorylation of total cellular proteins. Protein III in the remainder of the sample was immunoprecipitated from a final volume of 0.9 ml containing 0.5% SDS, 2.5% (wt/vol) Nonidet P40, 150 mM NaCl, 35 mM NaF, 10 mM HEPES, 2 mM EDTA, 2 mM EGTA (final pH, 7.4). For immunoprecipitation, the samples were precleared with Pansorbin, incubated with rabbit antibody to protein III (Browning et al., 1987), and then incubated with fresh Pansorbin. After centrifugation, the pellets were resuspended by sonication in HBS containing 0.5% (wt/vol) Nonidet P40. After 3 cycles of centrifugation and resuspension, the samples were then repelleted and sonicated in SDS-PAGE sample buffer (2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, in 62.5 mM Tris/HCl, pH 6.8). The efficacy of immunoprecipitation was ~100% as judged by immunolabeling.

The samples were subjected to SDS-PAGE and autoradiography. ^{32}P incorporation was quantitated either by AMBIS beta scanning (Advanced Microbiology Systems, Inc.), for analysis of total chromaffin cell protein, or by liquid scintillation counting after excision of the bands localized by autoradiography, for analysis of immunoprecipitates. Basal ^{32}P incorporation into proteins IIIa and IIIb increased in an approximately linear fashion for at least 2 hr under these conditions.

Back-phosphorylation of chromaffin cell extracts. Chromaffin cells were incubated (without added $^{32}PO_4$) and treated as described above, up through the aspiration of the incubation medium. The cells were then collected, after scraping, in ice-cold 5 mM zinc acetate (2 × 0.5 ml). After a brief (3 sec) sonication, the samples were centrifuged at 12,000 × *g* for 12 min, and each pellet was resuspended by sonication in 100 μl of 10 mM citric acid (pH 3.0) that contained 0.1% (wt/vol) Nonidet P40, 10 μg/ml pepstatin and 10 μg/ml phenylmethylsulfonyl fluoride. Particulate matter was removed by centrifugation, and the supernatant was collected and neutralized by the addition of 10 μl of 0.5 M $NaPO_4$ (pH 7.0). Particulate material was removed by centrifugation, and the supernatant (referred to as the "acid extract") was used in the back-phosphorylation assay of protein III. Immunolabeling revealed that >90% of both proteins IIIa and IIIb was recovered from the chromaffin cells in the acid extraction procedure. The phosphorylation state of protein III was assayed by determining the amount of radioactive phosphate incorporated into protein III in the acid extract in the presence of [γ-^{32}P]-ATP and the catalytic subunit of cAMP-dependent protein kinase. The assay mixture (final volume, 100 μl) contained 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, and 0.3 μg of the catalytic subunit of cAMP-dependent protein kinase (provided by Dr. A. Nairn). The phosphorylation reaction was initiated by the addition of ~1 nmol of [γ-^{32}P]-ATP (~3 × 10^7 dpm) in a volume of 10μl. After 60 min at 37°C, the reaction was terminated by the addition of 10 μl of 10% SDS. Protein III was then immunoprecipitated as described above. (Under the assay conditions used, phosphorylation of proteins IIIa and IIIb was proportional to the amount of acid extract added to the assay.) As described previously (Forn and Greengard, 1978; Tsou and Greengard, 1982; cf. Nestler and Greengard, 1984), this back-phosphorylation assay accurately reflects the phosphorylation state of proteins IIIa and IIIb.

It should be noted that cAMP-dependent protein kinase and calcium/calmodulin-dependent protein kinase I both phosphorylate protein III at the same site (A. Nairn, M. Browning, and P. Greengard, unpublished observations). Thus, the effects of ACh *in situ*, as measured in the back-phosphorylation assay, could reflect an action by either protein kinase.

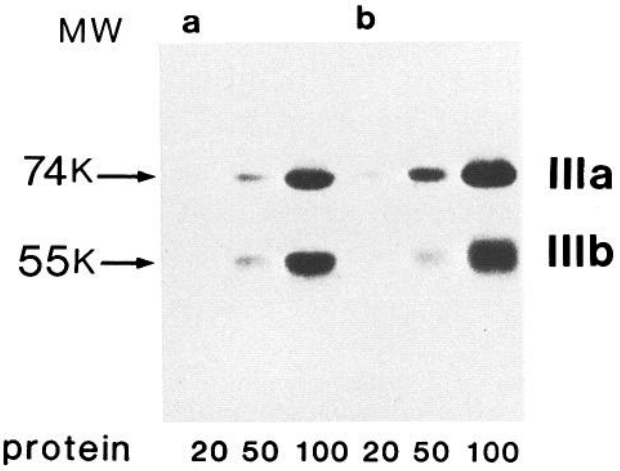

Figure 1. Immunolabeling of protein III in whole adrenal medulla and isolated chromaffin cells. Samples of bovine adrenal medulla and purified chromaffin cells were subjected to SDS-PAGE and then electrophoretically transferred to a nitrocellulose sheet. The sheet was incubated with anti-protein III antibody and ^{125}I-protein A, and the resultant autoradiogram is shown. *a,* Whole adrenal medulla; *b,* purified chromaffin cells; *74K* and *55K,* the location of cerebral cortex protein IIIa and protein IIIb, respectively.

Phosphopeptide and phosphoamino acid analysis. Some of the excised ^{32}P-protein III bands were subjected to 1-dimensional phosphopeptide analysis after partial digestion with *S. aureus* V8 protease (Cleveland et al., 1977) or to 2-dimensional phosphopeptide analysis after limit digestion with trypsin/chymotrypsin (Elder et al., 1977). Purified bovine cerebral cortex proteins IIIa and IIIb (Browning et al., 1987), phosphorylated *in vitro* as described above and separated on SDS-PAGE, served as standards for comparison. Some of the tryptic/chymotryptic digests were also subjected to phosphoamino acid analysis after hydrolysis in 6 *N* HCl for 1 hr at 110°C (Bylund and Huang, 1976; Haycock et al., 1985).

Release of ^3H-catecholamine from chromaffin cells. Monolayers of chromaffin cells were rinsed and preincubated, as described above for $^{32}PO_4$, with ^3H-NE (2 μCi/2 ml HBS; ~50 nM). The samples were brought to room temperature and washed with 5 ml of HBS 3 times (10 min each). The cells were given successive 2 ml washes at 2 min intervals, and aliquots were taken for liquid scintillation counting in Tritosol (Fricke, 1975). Basal efflux of ^3H-NE approached a constant level after 4–5 of the 2 ml washes. Test substances were included in the 2 ml washes starting at the 9th wash. (In one set of experiments, 30 sec collection periods were used after the addition of the test substances.) After the last addition (and removal) of test substance, 2 ml of 0.1 *N* HCl was added to each dish (to extract the tissue radioactivity), and an aliquot was taken for counting. Similar procedures have been used by several other laboratories, and the release of ^3H-NE has been shown to reflect the release of endogenous catecholamines (e.g., Livett et al., 1979; Kilpatrick et al., 1980; Trifaro and Lee, 1980). Efflux was calculated as the fractional rate constant (per collection period) and expressed as a percentage.

Data analysis. For most of the data presented, triplicate determinations were made for each data point, and each experiment was performed with at least 3 different cell preparations.

Results

Presence of protein III in chromaffin cells

As previously described for rat adrenal medulla (Browning et al., 1987), protein III was also detected in extracts of whole bovine adrenal medulla (Fig. 1*a*). Isolation and purification of chromaffin cells from whole adrenal medulla moderately increased (20–40%) the specific activity of protein III (Fig. 1*b*, Table 1). The specific activity of protein III in bovine adrenal medullae was much smaller than that in brain (Table 1). In

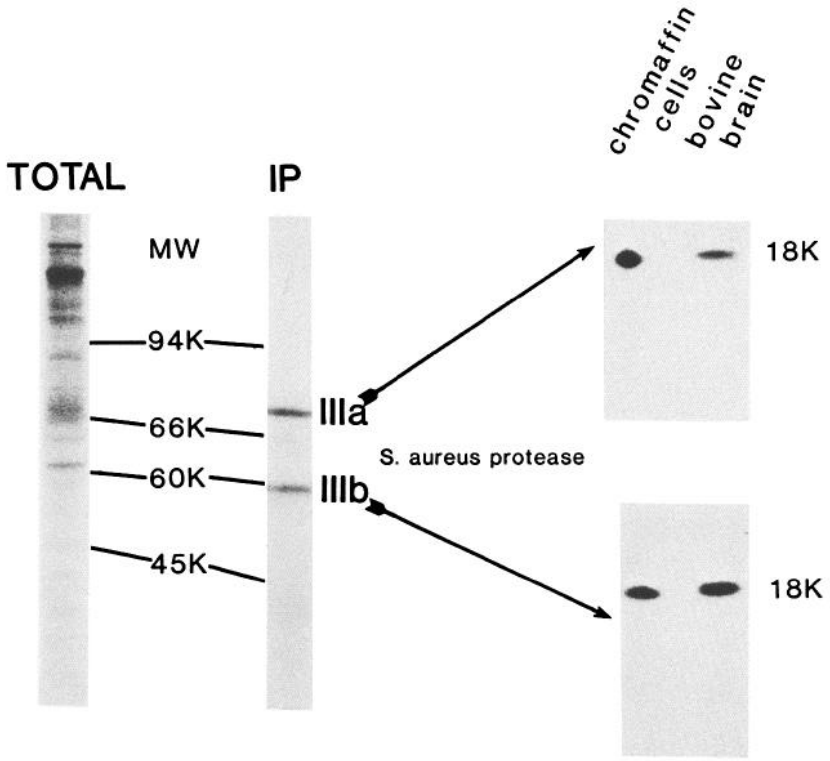

Figure 2. Phosphorylation of protein III in intact chromaffin cells. Isolated chromaffin cells were incubated with $^{32}PO_4$ and solubilized as described in Materials and Methods. A 50 μl aliquot was taken for analysis of total chromaffin cell protein phosphorylation (*TOTAL*) and the remainder (450 μl) of the sample was immunoprecipitated with antibodies to protein III (*IP*). *Left,* Autoradiograms showing 1-dimensional SDS-PAGE. The sample labeled TOTAL was exposed to X-ray film for 20 min at room temperature. The sample labeled IP was exposed to X-ray film for 4 hr at −70°C with a Trimax 12 intensifying screen. *Right,* Autoradiograms showing 1-dimensional phosphopeptide analysis in 15% SDS-PAGE gels, after partial proteolysis with *S. aureus* V8 protease, of the immunoprecipitated protein IIIa and protein IIIb bands. For comparison, bovine brain protein III standards, also subjected to partial proteolysis with 1 μg *S. aureus* V8 protease, are shown.

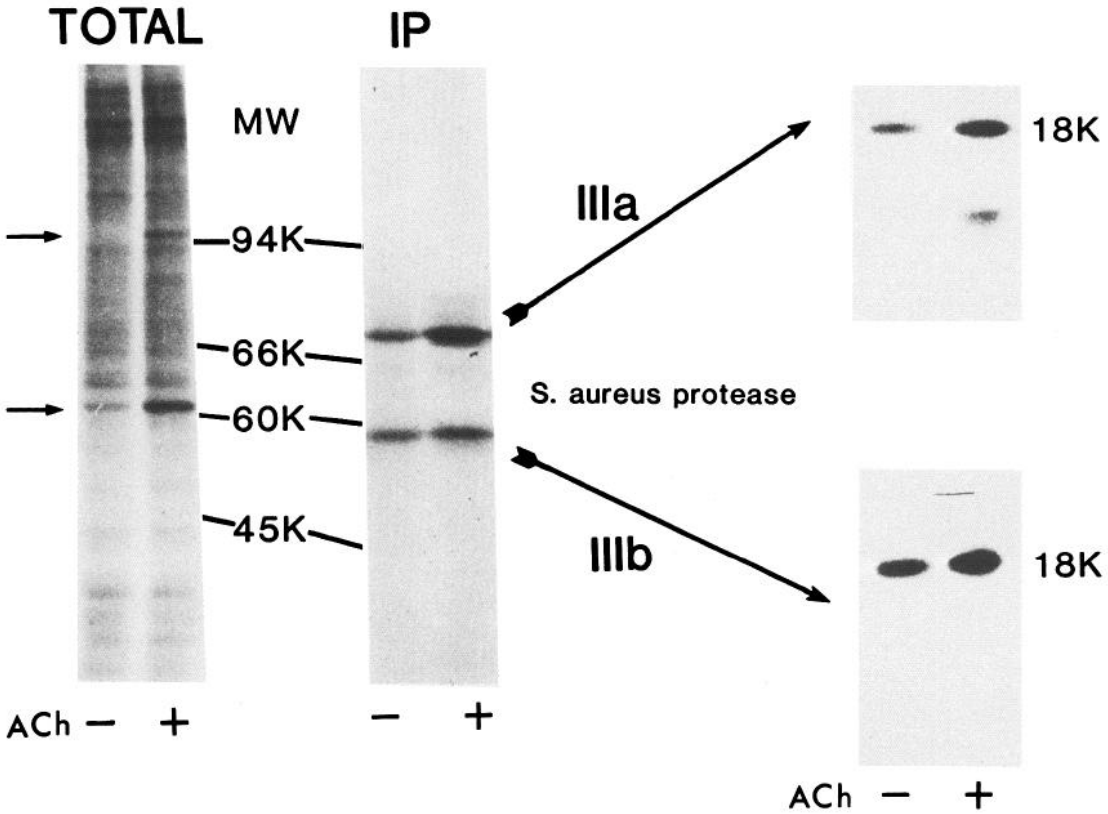

Figure 3. Cholinergic regulation of protein III phosphorylation in intact chromaffin cells. Experimental details were as described in the legend to Figure 2, with the exception that some of the cells were treated with 100 μM ACh for 2 min prior to being solubilized. *Arrows* (left) point to the $M_r \approx 100{,}000$ and $M_r \approx 60{,}000$ phosphoproteins, the phosphorylation of which is increased by ACh. At *right* are autoradiograms showing 1-dimensional phosphopeptide analysis in 15% SDS-PAGE gels, after partial proteolysis with *S. aureus* V8 protease, of the immunoprecipitated protein IIIa and protein IIIb bands.

Table 1. Comparison of protein III levels in bovine cerebral cortex, adrenal medulla, and chromaffin cell fractions

	Protein III levels (pmol/mg protein)			
			Chromaffin cell fraction	
Substrate	Cerebral cortex	Adrenal medulla	Initial	Purified
Protein IIIa	9[a] (100)	1.0 (11)	1.2 (13)	1.3 (14)
Protein IIIb	19[a] (100)	0.8 (4.1)	1.0 (5.2)	1.1 (6.0)

Values were determined as described in Materials and Methods from immunoblots as shown in Figure 1. Values in parentheses indicate the percentage relative to bovine cerebral cortex.

[a] Values taken from Browning et al. (1987).

addition, the ratio of protein IIIa to protein IIIb was at least 2-fold larger than in brain. The reason for the higher ratio of protein IIIa to protein IIIb in bovine adrenal medulla and chromaffin cells than in bovine brain is not presently understood, but this higher ratio has also been observed in rat adrenal medulla, olfactory bulb, and posterior pituitary (Browning et al., 1987).

In situ *phosphorylation of protein III in chromaffin cells*

Incubation of chromaffin cells in $^{32}PO_4$ resulted in the labeling of numerous cellular proteins in extracts of total chromaffin cell protein (Fig. 2, TOTAL). Although none of the major bands exhibited an M_r appropriate for either proteins IIIa or IIIb, antibodies to bovine brain protein III did immunoprecipitate 2 ^{32}P-labeled proteins (Fig. 2, IP) with mobilities in SDS-PAGE corresponding to those observed with immunolabeling. The ^{32}P incorporation into these bands was minor relative to the incorporation into the prominent phosphoproteins evident in extracts of total chromaffin cell protein; however, this is to be expected for proteins, such as protein III, which constitute only 0.01–0.02% of total protein.

Partial proteolysis of the protein III bands with *S. aureus* V8 protease produced an 18,000 M_r phosphopeptide characteristic of purified bovine brain protein III phosphorylated *in vitro* with [γ-^{32}P]-ATP and the catalytic subunit of cAMP-dependent protein kinase (Fig. 2, right). In addition, 2-dimensional phosphopeptide maps after tryptic/chymotryptic digestion of the bands produced a pattern similar to that of the purified bovine brain protein III (data not shown; see Browning et al., 1987).

Table 2. Comparison of the effects of cholinergic agonists on protein III phosphorylation and ^{3}H-NE secretion

	^{32}P incorporation (% control)		^{3}H-NE secretion (% efflux)
Test substance	IIIa	IIIb	
Control	100	100	1.2
ACh (100 μM)	238	175	7.6
Nicotine (50 μM)	222	172	7.9
Muscarine (200 μM)	105	108	1.4[a]

Chromaffin cells were preincubated for 60 min with either $^{32}PO_4$ or ^{3}H-NE, processed as described in Materials and Methods, and incubated with the indicated test substances for 2 min.

[a] Additional 2 min treatments did not increase percentage efflux.

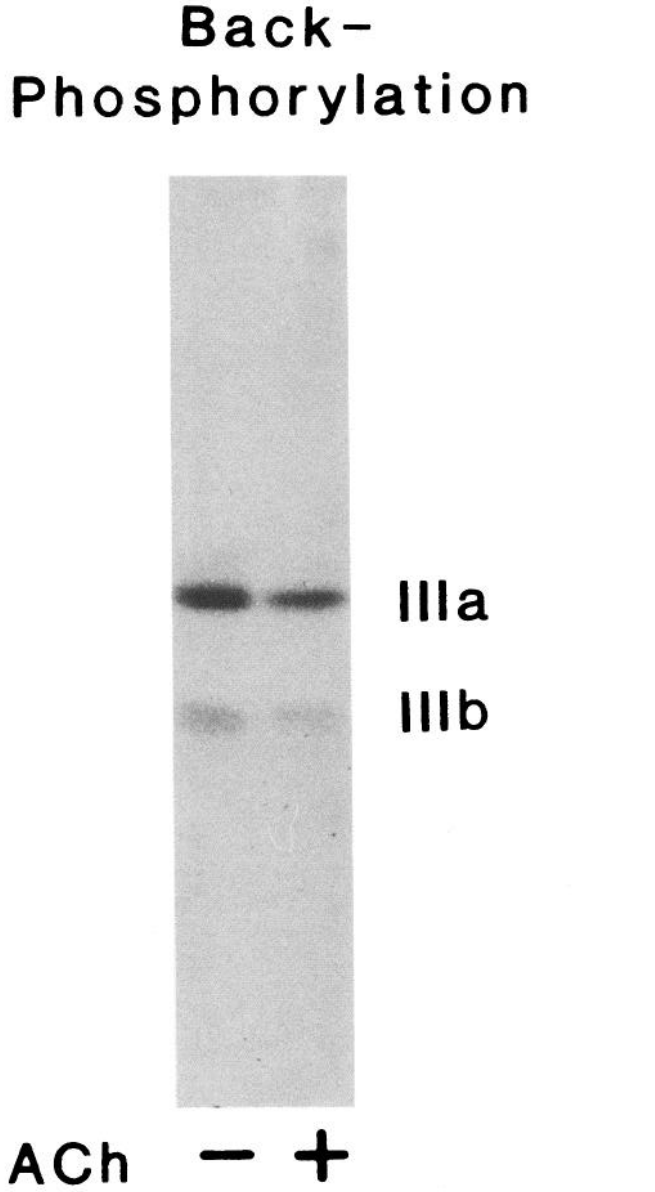

Figure 4. **Back-phosphorylation of protein III. Chromaffin cells were incubated for 60 min in HBS without $^{32}PO_4$ and then treated with or without 100 μM ACh for 2 min prior to precipitation with 5 mM zinc acetate. Protein III was extracted, phosphorylated with [γ-$^{32}PO_4$]-ATP and the catalytic subunit of cAMP-dependent protein kinase, immunoprecipitated, and subjected to SDS-PAGE as described in Materials and Methods. A decrease in phosphorylation *in vitro* indicates that the treatment increased the amount of phospho-protein III *in situ*.**

Cholinergic regulation of protein III phosphorylation and catecholamine secretion.

ACh (100 μM), when added to chromaffin cells previously incubated with $^{32}PO_4$, produced a rapid and selective increase in the ^{32}P content of the $M_r \approx 100{,}000$ and $M_r \approx 60{,}000$ proteins seen in extracts of total chromaffin cell protein (Fig. 3, TOTAL). Immunoprecipitation with antibodies to protein III also revealed an ACh-induced increase in ^{32}P incorporation into proteins IIIa and IIIb (Fig. 3, IP). Comparable effects were also observed either in suspension cultures treated as in Figure 3 or in monolayer cultures that had been preincubated with $^{32}PO_4$ in culture medium for 24 hr (data not shown). The latter finding,

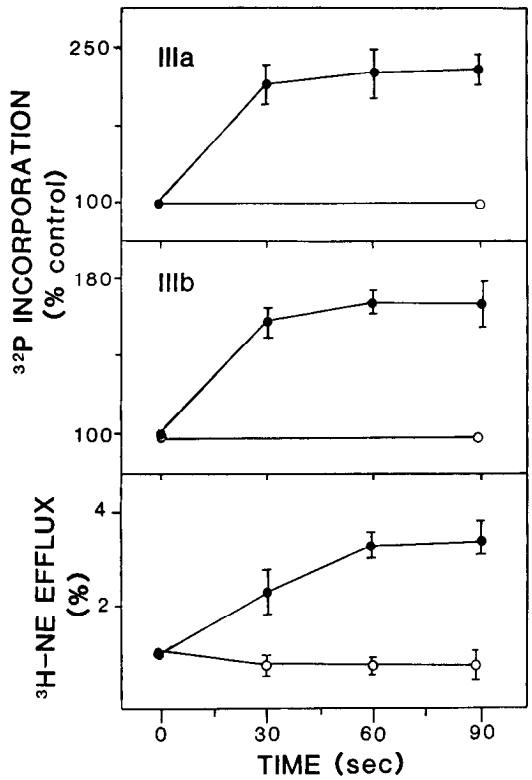

Figure 5. Effects of ACh on protein III phosphorylation and ^{3}H-NE secretion at short incubation times. Chromaffin cells were preincubated as described in the legend to Table 2 and were untreated (*open circles*) or treated with (*closed circles*) 50 μM ACh.

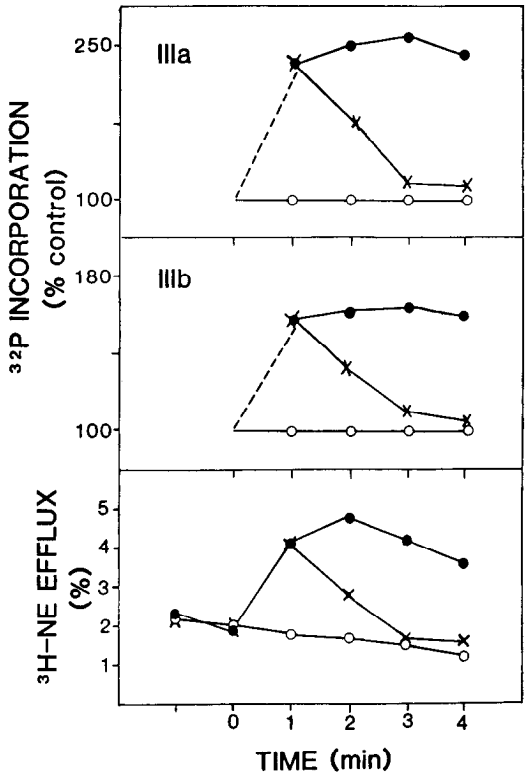

Figure 6. Temporal decay of protein III phosphorylation and ^{3}H-NE secretion after EGTA quench. Chromaffin cells were preincubated as in the legend to Table 2 and were untreated (*open circles*) or treated with (*closed circles*) 50 μM ACh. Half of the samples treated with ACh were also treated with 1.2 mM EGTA after 1 min of exposure to ACh (*crosses*).

using physiological phosphate concentrations and equilibrium isotopic labeling, reinforce the interpretation that the ^{32}P incorporation into protein III observed with the standard labeling conditions accurately reflects the phosphorylation state of protein III. For both protein IIIa and protein IIIb, 1-dimensional phosphopeptide maps after partial proteolysis with *S. aureus* V8 protease showed that the increased ^{32}P incorporation was present in the 18,000 M_r phosphopeptide (Fig. 3, right). Also, as previously shown for bovine brain protein III (Browning et al., 1987), phosphoamino acid analysis revealed that the ^{32}P incorporation into protein IIIa and protein IIIb was associated with phosphoserine (data not shown).

An ACh-dependent increase in the phosphorylation state of protein III was also detected by back-phosphorylation with [γ-^{32}P]-ATP and the catalytic subunit of cAMP-dependent protein kinase (Fig. 4). ACh produced an approximately 40% decrease in dephospho-protein IIIa and in dephospho-protein IIIb. These effects were observed whether cells were treated in HBS or in culture medium.

Nicotine (50 μM) and muscarine (200 μM) were compared to ACh (100 μM) for their effects on protein III phosphorylation and ^{3}H-NE release in prelabeled cells. As shown in Table 2, nicotine, but not muscarine, produced increases in protein III phosphorylation and ^{3}H-NE secretion that were comparable to those produced by ACh. Similar effects on the phosphorylation of the $M_r \approx 60{,}000$ protein (tyrosine hydroxylase) were also observed (data not shown). The effects of ACh (Table 3) and nicotine (data not shown) both required extracellular calcium.

The time course of the effects of ACh was also investigated. ACh (50 μM) produced a rapid increase in protein IIIa and protein IIIb phosphorylation (Fig. 5, top and middle). This effect was nearly maximal within 30 sec. In contrast, the rate of ACh-stimulated ^{3}H-NE secretion did not reach a maximum until 60 sec (Fig. 5, bottom). The ACh-induced increases in both protein III phosphorylation and ^{3}H-NE secretion did, however, decay with a similar time course when EGTA was added to chelate free calcium (Fig. 6). In both cases, reversal was complete within 2–3 min after addition of EGTA. The ACh-induced phosphorylation of tyrosine hydroxylase, although dependent upon extracellular calcium (Haycock et al., 1982a, b), showed little or no decrement after EGTA addition (data not shown).

Effects of other secretagogues on protein III phosphorylation and catecholamine secretion

Several noncholinergic depolarizing agents also promote the secretion of catecholamines from adrenal chromaffin cells. Veratridine, elevated K^+, and barium were evaluated for their effects, relative to ACh, on both protein III phosphorylation and ^{3}H-NE secretion (Table 4). Veratridine, elevated K^+, and barium produced increases in protein III phosphorylation that were comparable to those produced by ACh. However, each of 3 secretagogues varied in its ability to stimulate ^{3}H-NE secretion. The effects of ACh, veratridine, and elevated K^+ on both protein III phosphorylation and ^{3}H-NE secretion were calcium-dependent, whereas the effects of barium were only marginally so.

The effect of veratridine on secretion develops more slowly than that of either ACh or elevated K^+ (e.g., Wada et al., 1985). Therefore, we also compared the time course of effects of ACh and veratridine on protein III phosphorylation and ^{3}H-NE secretion (Table 5). Both ACh (100 μM) and veratridine (150 μM)

Table 3. Calcium-dependence of the effects of ACh on protein III phosphorylation and ^{3}H-NE secretion

Test substances		^{32}P incorporation (% control)		^{3}H-NE secretion (% efflux)
ACh (μM)	$CaCl_2$ (mM)	IIIa	IIIb	
0	0	100	100	1.1
0	1.0	99	95	1.2
100	0	110	108	1.0
100	1.0	254	182	7.2

Chromaffin cells were preincubated as in Table 2 (but in calcium-deficient HBS) for 60 min with either $^{32}PO_4$ or ^{3}H-NE, processed as described in Materials and Methods, and incubated with the indicated test substances for 2 min.

produced a sustained increase in protein III phosphorylation. In contrast, ACh produced a transient increase in the rate of ^{3}H-NE secretion, while veratridine continued to increase the rate of ^{3}H-NE secretion throughout the incubation period.

Discussion

Conditions that promote stimulus–secretion coupling increase the phosphorylation of protein III in chromaffin cells. Three lines of evidence indicate that the ACh-stimulated increase in ^{32}P incorporation into protein IIIa and IIIb in chromaffin cells reflects a net increase in their phosphorylation states. First, ACh produced comparable percentage increases in protein III phosphorylation whether cells were preincubated for 1 or 24 hr. Second, as indicated by the selective increase in 100 kDa and tyrosine hydroxylase phosphorylation in extracts of total chromaffin cell proteins, ACh did not produce a nonspecific increase in the specific activity of [γ-^{32}P]-ATP. Third, ACh treatment decreased the back-phosphorylation of protein III.

In secretory cells such as neurons and chromaffin cells, one major effect of secretagogues is to stimulate calcium influx to trigger secretion. However, second messengers, such as calcium, are also expected to initiate a number of additional cellular responses, each of which with its own characteristic time course. Changes in protein III phosphorylation showed certain similarities to changes in NE secretion, but such an association does not necessarily imply that the phosphorylation of protein III is involved in stimulus-secretion coupling. For example, tyrosine hydroxylase is phosphorylated in response to secretagogue treatment, but this effect is not thought to be involved in the secretory response per se. Moreover, certain discrepancies were observed between the effects of secretagogues on protein III phosphorylation/dephosphorylation and their effects on catecholamine secretion. Thus, an obligatory relationship between protein III phosphorylation and secretion seems unlikely.

In summary, it would appear that protein III phosphorylation in chromaffin cells is triggered by the same stimulus that triggers secretion—an increase in intracellular calcium. The secretagogue-dependent phosphorylation of protein III (unlike that of tyrosine hydroxylase) appears to correlate well with secretagogue-dependent transients in intracellular calcium. Localization of protein III within the chromaffin cell, by subcellular fractionation and immunocytochemical methods, should help elucidate the functional significance of this phosphoprotein.

Table 4. Comparison of the effects of various secretagogues on protein III phosphorylation and ^{3}H-NE secretion

Test substances		^{32}P incorporation (% control)		^{3}H-NE secretion (% efflux)
Secretagogue	EGTA	IIIa	IIIb	
None	+	108	104	1.8
	−	100	100	1.7
ACh (100 μM)	+	98	104	1.8
	−	245	171	8.4
Veratridine (150 μM)	+	96	108	1.7
	−	224	168	3.9
KCl (40 mM)	+	108	105	1.9
	−	231	162	7.9
$BaCl_2$ (5 mM)	+	191	158	3.9
	−	215	156	5.5

Chromaffin cells were preincubated for 60 min with either $^{32}PO_4$ or ^{3}H-NE and processed as described in Materials and Methods. To half of the samples EGTA was then added to a final concentration of 1.2 mM. After 30 sec, the chromaffin cells were treated with the indicated secretagogues for 2 min.

Table 5. Time course of the effects of ACh and veratridine on protein III phosphorylation and ^{3}H-NE secretion

Test substances	Incubation time (min)	^{32}P incorporation (% control)		^{3}H-NE secretion (% efflux)
		IIIa	IIIb	
ACh (100 μM)	0	100	100	1.5
	2	242	172	8.3
	4	228	162	6.4
	6	238	167	4.3
Veratridine (150 μM)	0	102	98	1.7
	2	195	156	3.2
	4	220	168	4.8
	6	210	165	5.2

Chromaffin cells were preincubated for 60 min with either $^{32}PO_4$ or ^{3}H-NE and processed as described in Materials and Methods. Chromaffin cells were then incubated with ACh or veratridine for the indicated times. (In the case of ^{3}H-NE secretion, the values represent the percentage efflux during the 2 min period immediately preceding the indicated time.)

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