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Induction of Tyrosine 3-Monooxygenase Elicited by Carbamylcholine in Intact and Denervated Adrenal Medulla: Role of Protein Kinase Activation and Translocation

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SUMMARY

KUROSAWA, A., GUIDOTTI, A. & COSTA, E. (1976) Induction of tyrosine 3-monooxygenase elicited by carbamylcholine in intact and denervated adrenal medulla: role of protein kinase activation and translocation. *Mol. Pharmacol.*, 12, 420-432.

In adrenal medullae of rats treated with carbamylcholine (9.2 μmoles/kg intraperitoneally) the adenosine cyclic 3',5'-monophosphate (cAMP) content increased promptly by about 10-fold. The increase lasted for about 1 hr and elicited a sequence of molecular events including the activation of cAMP-dependent protein kinase in cytosol, an increase in the protein kinase activity extracted from the particulate fraction, which is due to the transfer of catalytic subunits from cytosol (translocation), and the delayed induction of tyrosine 3-monooxygenase (tyrosine hydroxylase). The translocation of catalytic subunits of kinase always preceded the delayed induction of tyrosine hydroxylase. In rats with a unilaterally denervated adrenal, the injection of aminophylline (200 μmoles/kg) increased the cAMP content in both intact and denervated adrenals. In the cytosol of both adrenal medullae the cAMP-dependent protein kinase was activated, but the translocation of protein kinase and the delayed induction of tyrosine hydroxylase occurred only on the intact side. Gel filtration and experiments with purified regulatory subunits proved that the increase in cAMP dissociates the catalytic subunit from the holoenzyme of the cytosol. The administration of hexamethonium (45 μmoles/kg intraperitoneally) prior to carbamylcholine (9.2 μmoles/kg intraperitoneally) abated the increase in cAMP content, blocked the activation of the cytosol kinase, and prevented tyrosine hydroxylase induction, whereas the administration of atropine (4 μmoles/kg intraperitoneally) failed to block any of these responses elicited by carbamylcholine. These results strongly suggest that activation of the cAMP-dependent protein kinase and its translocation from cytosol to subcellular structures are obligatory intermediate processes triggered by the early increase in cAMP; the kinase translocation mediates the delayed induction of tyrosine hydroxylase.

INTRODUCTION

In rat adrenal medulla a delayed transsynaptic induction of tyrosine-3-monooxygenase (tyrosine hydroxylase) is elicited by injections of carbamylcholine or reserpine or by cold exposure (1-3). A stimulus-coupled increase in adenosine cyclic 3',5'-monophosphate content of the medulla (3-5), lasting 1-2 hr, precedes by several hours the increased synthesis of tyrosine hydroxylase and its induction (6, 7). In adrenal medulla cytosol of rats exposed to 4° or injected with reserpine, the activity of cAMP[1]-dependent protein kinase is de-

[1] The abbreviation used is: cAMP, adenosine cyclic 3',5'-monophosphate.

creased but that of cAMP-independent protein kinase is increased (7). This increase lasts about 4 hr and, in the case of reserpine, is dose-related (7). The threshold dose of reserpine for early activation of the protein kinase is identical with that for the delayed induction of tyrosine hydroxylase (7). When the adrenal medullae have been denervated 5 days before, cold exposure fails to cause the delayed induction of tyrosine hydroxylase and the early activation of cytosol protein kinase (7).

The present report gives further evidence that activation of protein kinase participates in the induction of medullary tyrosine hydroxylase elicited by carbamylcholine. Adrenal medulla denervation fails to inhibit the protein kinase activation and tyrosine hydroxylase induction elicited by this drug. The data presented suggest that the protein kinase activation and the successive transfer of catalytic subunits of the activated enzyme to subcellular structures are an obligatory step in eliciting the delayed induction of tyrosine hydroxylase.

MATERIALS AND METHODS

Animals

Normal fed male Sprague-Dawley rats weighing about 100 g (Zivic Miller, Allison Park, Pa.) were used. In some experiments these rats were splanchnicotomized unilaterally or bilaterally 5–7 days before the experiment.

Assay of Protein Kinase Activity

Adrenal medullae were stereomicroscopically dissected from the cortex (8). Four medullae were homogenized at 4° for 30 sec at 3000 rpm in a glass homogenizer with a Teflon pestle in 75 μl of 10 mM potassium phosphate buffer (pH 6.5) containing 10 mM EDTA and 5 mM aminophylline (standard buffer). After centrifugation at 20,000 × g for 20 min, the supernatant fraction was assayed for protein kinase activity within 30 min.

To measure the protein kinase activity in the 20,000 × g precipitate, the pellet was rehomogenized in 75 μl of 10 mM potassium phosphate buffer (pH 6.5) containing 0.5 M NaCl, 10 mM EDTA, 5 mM aminophylline, and 0.2% Triton X-100. The homogenate was centrifuge again for 20 min at 20,000 × g. The activity measured in the resultant supernatant is referred as the pellet extract protein kinase activity. The protein kinase assay was carried out as previously described by measuring the amount of [^{32}P]phosphate incorporated into calf thymus histone (7).

In gel filtration experiments, 40 adrenal medullae were homogenized at 0–4° with 120 μl of 10 mM potassium phosphate buffer (pH 6.5) containing 10 mM EDTA, 5 mM aminophylline, and 0.5 M NaCl. After centrifugation for 20 min at 20,000 × g at 0–4°, 50 μl of the supernatant fraction (900 μg of protein) were applied to a Sephadex G-200 column (0.2 × 50 cm) equilibrated with the same buffer. The column was eluted with 2 ml of this buffer. Twenty fractions of 80 μl were collected, and the protein kinase activity in each fraction was immediately measured.

Preparation of Regulatory and Catalytic Subunits

Bovine brain protein kinase was purified with 70% ammonium sulfate precipitation and DE-52 column chromatography (9). In order to dissociate the regulatory and catalytic subunits, the protein kinase was incubated for 5 min at 30° in the presence of 5 μM cAMP. The dissociated regulatory and catalytic subunits were separated by Sephadex G-200 column chromatography. cAMP binding activity was measured by the procedure of Gilman (10).

cAMP Determination

cAMP in tissues was purified through alumina and Dowex column chromatography (8) and then assayed with a cAMP-dependent protein kinase by measuring the incorporation of [^{32}P]phosphate into histone according to Kuo and Greengard (11).

Tyrosine Hydroxylase Activity

Each adrenal gland was homogenized with 500 μl of 50 mM Tris-acetate buffer (pH 6.0) containing 0.2% Triton X-100. The homogenate was dialyzed against the same buffer for 4 hr and centrifuged at 11,000 × g for 10 min at 4°. Then 50 μl of

the supernatant were assayed for tyrosine hydroxylase activity according to a modification (12) of the method described by Waymire *et al.* (13).

Assay of ATP

The adenosine 5'-triphosphate content in the reaction mixture for the protein kinase assay was measured by the luciferin-luciferase method (14) following alumina column purification (8).

Dephosphorylation of Phosphoprotein

This activity was estimated by measuring the degradation of ^{32}P-containing histone in the mixture used to determine protein kinase activity. The histone containing ^{32}P, used as substrate, was prepared according to Maeno and Greengard (15).

Hydrolysis of cAMP

The cAMP hydrolysis occurring in the mixture for protein assay was estimated by measuring the decrease in [^{3}H]cAMP (4000 cpm/150 pmole) (7).

In all experiments the protein content in the sample was determined according to Lowry *et al.* (16), using bovine serum albumin as a standard.

Reagents

Sephadex G-200 was purchased from Pharmacia. ATP and cAMP were products of Sigma Chemical Company. Calf thymus histone (B grade) was purchased from Calbiochem. [γ-^{32}P]ATP and [^{3}H]cAMP were obtained from New England Nuclear Corporation. All other reagents were analytical grade and were used without further purification.

RESULTS

Increase in cAMP Content and Activation of Soluble Protein Kinase Elicited by Carbamylcholine

cAMP content. Carbamylcholine (9.2 mmoles/kg) injected intraperitoneally caused a rapid increase (about 10-fold) in the cAMP content of adrenal medulla (Fig. 1). This increase proceeded at an initial rate of about 20 pmoles/mg of protein per minute and reached a peak value at 24 min (Fig. 1). The cAMP content began to decline after 30 min but was still significantly elevated 60 min after the carbamylcholine injection (Fig. 1).

Properties of protein kinase in 20,000 × g supernatant of crude homogenate. In adrenal medullae of rats injected with carbamylcholine, the increase in cAMP content was followed by activation of the soluble protein kinase (Fig. 1). Since this activation is expressed by the ratio between the protein kinase activity detected in the absence and presence of cAMP (17), the maximal possible activation coincides with a value of 1. In the 20,000 × g supernatant of adrenal medullae of rats injected with carbamylcholine, the maximal activation was observed 30 min after injection, while a lower degree of protein kinase activation persisted for about 4 hr (Fig. 1). To obtain an appropriate control for events occurring during the homogenization, we measured the activation index of soluble protein kinase in the supernatant (20,000 × g) of medullae of rats injected with carbamylcholine (3 μmoles/kg intraperitoneally). As shown in Fig. 5 below, this dose of carbamylcholine increased the cAMP content by 12-fold. The protein kinase activation index (−cAMP/+cAMP) was 0.22 ± 0.05 (N = 4) and 0.20 ± 0.02 (N = 3) in carbamylcholine- and saline-treated rats, respectively. To establish whether sponta-

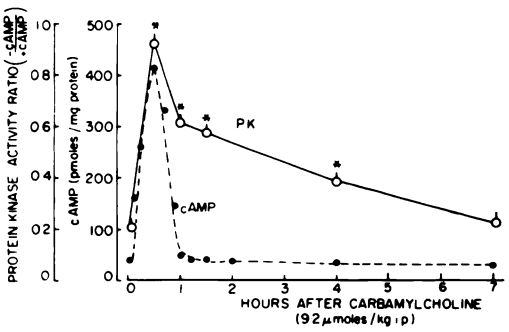

FIG. 1. *cAMP content and protein kinase activity (PK) ratio in adrenal medullae of rats injected with 9.2 μmoles/kg of carbamylcholine*

The protein kinase activity ratio was determined in the 20,000 × g supernatant fraction. Each value is the mean of five experiments. Vertical brackets represent the standard error of the mean.

* $p < 0.05$ compared with controls.

neous reassociation of the protein kinase subunits could change the ratio which we have taken as an index of activation, we determined whether the activity in the presence and absence of cAMP (0.7 μM) changed when the 20,000 × g supernatant of adrenal medulla was allowed to stand for various times at 0°.

The addition of 0.5 M NaCl virtually prevented any reassociation of the protein kinase subunits (Fig. 2); however, in the absence of NaCl the activity ratio declined with time. The slope of the decline in the absence of NaCl was accelerated during drug-induced activation of the protein kinase (compare data for carbamylcholine and controls of Fig. 2). When the 20,000 × g supernatant of adrenal medullae obtained from saline- or carbamylcholine-treated rats was challenged with various concentrations of regulatory subunits, purified from bovine brain, the protein kinase activity measured in the presence of cAMP (0.7 μM) did not change but the activity measured in the absence of cAMP was greatly reduced (Table 1). The addition of regulatory subunits reduced the protein kinase activity measured in the absence of cAMP from 21% of control activity to 9.3%, and from 80% of the total activity to 10%, in saline- and carbamylcholine-treated rats, respectively (Table 1).

A physical separation of the cAMP-dependent, high molecular weight holoenzyme from the cAMP-independent, low molecular weight catalytic subunit of protein kinase can be achieved by Sephadex column chromatography (17). When 50-μl aliquots of the 20,000 × g supernatant of adrenal medulla from NaCl-treated rats were applied to a Sephadex G-200 column, the elution profile of protein kinase included two peaks of activity (Fig. 3). The activity of the first peak was related to a high molecular weight protein and was stimulated by the addition of cAMP (0.7 μM). The activity of the second peak was associated with low molecular weight protein and was not stimulated by cAMP

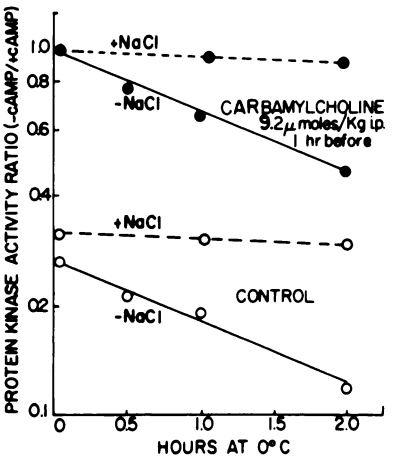

FIG. 2. *Reassociation of protein kinase subunits in 20,000 × g supernatant of adrenal medulla homogenate kept at 0°*

Twenty-four adrenal medullae were homogenized in 400 μl of potassium phosphate-EDTA-aminophylline buffer with or without 0.5 M NaCl. Aliquots (10 μl) of the supernatant fraction were assayed for protein kinase activity at different time intervals in the presence and absence of 0.7 μM cAMP. Each point is the mean value of three determinations. Total protein kinase activity without incubation was 14.9 pmoles of ^{32}P incorporated per 0.1 mg of protein per minute in control rats and 9.7 in rats treated intraperitoneally with 9.2 μmoles/kg of carbamylcholine 1 hr before. After 2 hr at 0° the activity was 15.7 in controls and 10.8 in carbamylcholine-treated rats.

TABLE 1

Protein kinase activity in 20,000 × g supernatant from adrenal medullae of saline- and carbamylcholine-treated rats: effects of the addition of regulatory subunits purified from bovine brain

The regulatory subunits, prepared from purified bovine brain protein kinase as described in MATERIALS AND METHODS, were incubated at 0° for the times indicated with adrenal medulla supernatant (20,000 × g for 20 min at 4°) in the presence and absence of cAMP (0.7 μM). Medullae were dissected 90 min after the intraperitoneal injection of saline (2.5 ml/kg) or carbamylcholine (9.2 μmoles/kg) and homogenized in 15 volumes of the standard buffer mixture without NaCl. Each value is the mean of triplicate determinations.

Regulatory subunit	Incubation	Protein kinase activity			
		NaCl		Carbamylcholine	
		+cAMP	-cAMP	+cAMP	-cAMP
μg	*min*	*pmoles/mg protein/min*		*pmoles/mg protein/min*	
0	0	150	32	100	80
0	60	152	25	101	63
1.5	60	155	19	102	20
3.0	60	148	14	99	10

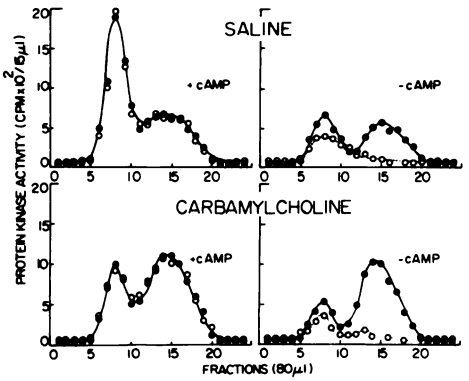

FIG. 3. *Sephadex G-200 elution profile of protein kinase in 20,000 × g supernatant from adrenal medulla homogenates of rats injected with saline (upper) or with carbamylcholine (9.2 μmoles/kg intraperitoneally) 1.5 hr before (lower)*

Adrenal medullae (about 50 mg) were homogenized with 120 μl of NaCl-potassium phosphate-EDTA-aminophylline buffer (pH 6.5). After centrifugation for 20 min at 20,000 × *g*, 50 μl of the clear supernatant (900 μg of protein) were gel-filtered with the same buffer. The protein kinase activity in each fraction of the eluate after dialysis for 3 hr against potassium phosphate buffer and 1 mM 2-mercaptoethanol was determined in the absence (○· · · ·○) and presence (●——●) of 3 μg of bovine brain regulatory subunit (see MATERIALS AND METHODS). The left panels depict the elution profiles of the activity measured in the presence of 0.7 μM cAMP; the right panels depict the profiles in the absence of cAMP.

(compare right with left panels, solid circles, Fig. 3). The elution profile of the protein kinase activity in the medullary supernatant from rats injected with carbamylcholine differed from that of saline-treated rats. In the presence of cAMP the enzyme activity related to the high molecular weight protein was reduced in comparison with that of saline-treated rats; moreover, in both the presence and absence of cAMP, the low molecular weight fraction of medulla supernatant from rats injected with carbamylcholine contained a greater amount of protein kinase activity than the corresponding peak from saline-treated rats. In both the absence and presence of cAMP, the activity of the first peak showed insignificant changes when the various fractions were incubated in the presence of regulatory subunits (compare right and left panels, solid circles vs. open circles, Fig. 3). However, after the addition of the regulatory subunits the fractions of the second peak displayed no activity if cAMP was absent (compare solid and open circles, right panels, Fig. 3).

Time course of protein kinase activation in supernatant fraction. Since the presence of 0.5 M NaCl depressed the protein kinase activity of the supernatant and produced a slight dissociation of the catalytic and regulatory subunits (see ref. 17; confirmed in Fig. 2), the effect of carbamylcholine on the activity of supernatant protein kinase was determined in the absence of NaCl within 30 min after preparation of the supernatant. As shown in Fig. 1, carbamylcholine caused a rapid, long-lasting activation of the protein kinase in the medullary supernatant. This activation was almost maximal at 45 min, 60% of maximal at 1.5 hr, and 40% of maximal 4 hr after carbamylcholine. After 1.5 hr the tissue levels of cAMP in medullae of rats injected with carbamylcholine were identical with those measured in the medullae of saline-treated animals (Fig. 1). During the first 2 hr after carbamylcholine, the increase in the protein kinase activation index was due to an increase in the protein kinase activity measured in the absence of cAMP (Fig. 4) and to a decrease in the protein kinase activity measured in the presence of cAMP (0.7 μM) (Fig. 4, lower panel). The increase in the protein kinase activity measured in the absence of cAMP returned to normal values at 4 hr, while the activity measured in the presence of cAMP was still reduced 7 hr following carbamylcholine injection (Fig. 4, lower panel).

This marked decrease in the cAMP-dependent protein kinase activity could not be accounted for by changes in ATPase, phosphoprotein phosphatase, or phosphodiesterase activity in the 20,000 × *g* supernatant of adrenal medulla. The K_m of cAMP (0.1 μM) for protein kinase in the supernatant of adrenal medulla homogenates from carbamylcholine-treated rats was identical with that from normal rats.

When various dilutions of a fresh or a heat-inactivated (90°, 3 min) supernatant fraction of adrenal medulla homogenates

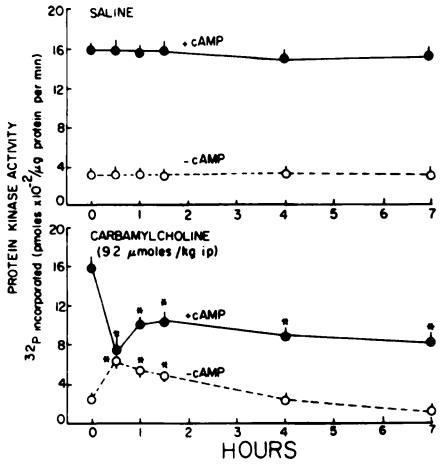

FIG. 4. *Protein kinase activity in 20,000 × g supernatant of rat adrenal medulla homogenates at different times after carbamylcholine (9.2 μmoles/kg) i.p.*

The protein kinase activity was measured in the supernatant fraction as described in MATERIALS AND METHODS. Each point indicates the mean value of six experiments. Vertical brackets represent the standard error of the mean.

* $p < 0.05$ compared with controls.

from carbamylcholine-treated rats were added to the supernatant of adrenal medullae from rats receiving NaCl, we failed to detect significant changes in an endogenous inhibitor of protein kinase, which could account for the protein kinase activation found in the supernatant of adrenal medullae from carbamylcholine-treated rats. Moreover, when the adrenal medulla homogenates from rats injected with carbamylcholine were prepared with 10 mM potassium phosphate buffer (pH 6.5) containing 10 mM EDTA, 150 mM NaCl, and 5 mM aminophylline, we still observed the long-lasting decrease in cAMP-dependent protein kinase activity.

Protein Kinase Translocation from Cytosol to Subcellular Fractions in Adrenal Medullae of Rats Receiving Carbamylcholine

The data presented in Table 2 show that the protein kinase activity in the 20,000 × *g* pellet extract of adrenal medulla homogenates prepared with 0.5 M NaCl–0.2% Triton X-100 buffer was higher than in those prepared with buffer containing 0.2% Triton X-100 or 0.5 M NaCl alone. Pellet extracts prepared with 0.5 M NaCl and 0.2% Triton X-100 in potassium phosphate buffer, pH 6.5 (see MATERIALS AND METHODS for details), from adrenal medullae of saline- and carbamylcholine-injected rats phosphorylated the endogenous protein at comparable rates in the absence and presence of exogenous cAMP (Table 3). However, the pellet extract prepared from adrenal medullae of rats receiving carbamylcholine 1.5 or 7 hr before (Tables 3 and 4) phosphorylated a mixture of calf thymus histones more rapidly than the corresponding extract from saline-treated rats. Carbamylcholine reduced the total histone kinase activity (difference between lines A and B of Table 3) in the supernatant by an extent comparable to the increase in histone kinase activity measured in the pellet extract. In agreement with previous re-

TABLE 2

Effects of NaCl and/or Triton X-100 on protein kinase activity in fractions of adrenal medulla homogenates

Twenty adrenal medullae from normal rats were homogenized in 350 μl of phosphate-EDTA-aminophylline buffer solution (see MATERIALS AND METHODS). After centrifugation at 20,000 × *g* for 20 min at 4°, the supernatant and pellet fractions (the pellets were resuspended in 350 μl of the same buffer and again centrifuged at 20,000 × *g* for 20 min) were divided into four aliquots and prepared for the enzymatic assay as indicated. Each value is the mean of triplicate assays with 10 μl of supernatant. The total activity in the homogenate prepared in the presence of 0.5 M NaCl and 0.2% Triton X-100 was 23.3 pmoles/min/10-μl sample, which corresponded to the summation of the activity measured in cytosol and pellet fractions.

Preparation of tissue extract with	^{32}P incorporated into histone and endogenous proteins	
	Supernatant	Pellet
	pmoles/min/10 μl	
Standard buffer	15.2 ± 0.50	3.4 ± 0.10
+0.5 M NaCl	14.5 ± 0.45	2.4 ± 0.12[a]
+0.2% Triton X-100	14.0 ± 0.40	6.2 ± 0.22[a]
+0.5 M NaCl + 0.2% Triton X-100	14.1 ± 0.35	9.2 ± 0.27[a]

[a] $P < 0.05$ compared with results with standard buffer.

TABLE 3

Protein kinase activity in supernatant and pellet of adrenal medullae of rats treated with carbamylcholine

Four adrenal medullae were homogenized with 15 volumes of phosphate-EDTA-aminophylline buffer and centrifuged at 20,000 × *g* for 20 min. The supernatant and pellets (resuspended in the original volume of potassium phosphate buffer) were treated with NaCl (final concentration, 0.5 M) and Triton X-100 (final concentration, 0.2%) and centrifuged at 20,000 × *g* for 20 min. Each value is the mean ± standard error of 10 experiments.

Substrate	Carbamylcholine (9.2 μmoles/kg 1.5 hr before)				Saline (2.5 ml/kg)			
	Supernatant		Pellet		Supernatant		Pellet	
	+cAMP	−cAMP	+cAMP	−cAMP	+cAMP	−cAMP	+cAMP	−cAMP
	pmoles ^{32}P incorporated/0.1 mg protein/min				*pmoles ^{32}P incorporated/0.1 mg protein/min*			
A. Histone + endogenous substrates	11.6 ± 1.0[a]	4.9 ± 0.6[a]	15.0 ± 1.4[a]	11.8 ± 0.8[a]	17.4 ± 2.5	2.8 ± 0.6	11.3 ± 0.5	8.5 ± 0.9
B. Endogenous substrates	1.3 ± 0.3	1.1 ± 0.3	4.0 ± 0.5	3.5 ± 0.5	1.4 ± 0.3	1.3 ± 0.2	5.2 ± 0.5	4.3 ± 0.7
Histone kinase activity (A−B)	10.2 ± 1.0[a]	4.0 ± 0.7[a]	10.9 ± 1.0[a]	7.8 ± 0.6[a]	16.0 ± 2.3	1.5 ± 0.6	6.0 ± 0.8	5.0 ± 0.8

[a] $p < 0.05$ compared with the corresponding value for saline-treated rats.

TABLE 4

Protein kinase activity in pellet extract of adrenal medullae after addition of regulatory subunits purified from bovine brain

Rats were given intraperitoneal injections of 9.2 μmoles/kg of carbamylcholine or 2.5 ml/kg of NaCl. The pellet extracts of adrenal medulla homogenates were prepared 7 hr after the carbamylcholine injection as described in MATERIALS AND METHODS. The regulatory subunit (200 μl, 60 μg of protein) was mixed with an equal volume of pellet extract (500 μg of protein) and dialyzed for 4 hr at 4° against 1 liter of 10 mM potassium phosphate buffer (pH 6.5) containing 1 mM 2-mercaptoethanol; 20 μl of the dialysate were assayed for protein kinase activity by the standard method with or without calf thymus histone as substrate. Each value is the mean of triplicate determinations.

Substrate	Carbamylcholine				NaCl			
	−Regulatory subunit		+Regulatory subunit		−Regulatory subunit		+Regulatory subunit	
	+cAMP	−cAMP	+cAMP	−cAMP	+cAMP	−cAMP	+cAMP	−cAMP
	pmoles ^{32}P incorporated/0.1 mg protein/min				*pmoles ^{32}P incorporated/0.1 mg protein/min*			
A. Histone + endogenous substrates	13.3	11.9	12.4	6.2	9.2	8.1	9.2	5.8
B. Endogenous substrates	5.0	4.9	5.1	5.0	5.2	4.8	5.0	4.9
Histone kinase activity (A−B)	8.3	7.0	7.3	1.2	4.0	3.3	4.2	0.9

ports (18–20), the transfer of protein kinase from supernatant to pellet extracts was termed translocation. The quantitative relationships of this translocation were not changed when the protein kinase in the cytosol and pellet was estimated as a unit of activity per adrenal medulla. In the supernatant the activity was 7.8 ± 0.7 pmoles/medulla/min for saline-treated and 4.5 ± 0.3 pmoles/medulla/min for carbamylcholine-treated rats. In the pellet extract the activity was 3.2 ± 0.2 for saline-treated and 6.0 ± 0.5 pmoles/medulla/min for carbamylcholine-treated rats. Since histones have a low molecular weight, the endogenous histone present in the pellet extracts could be separated from the protein kinase by filtration through a Sephadex G-200 column. After this procedure the enzyme from adrenal medulla pellets of carbamylcholine-treated rats also phosphorylated histones more rapidly than medullary pellets from saline-treated rats. When an excess of purified regulatory subunits prepared from brain protein kinase was added to the pellet extract from saline- or carbamylcholine-treated rats, the protein kinase activity measured in the presence of endogenous substrate remained virtually unchanged (Table 4). In contrast, the activity resulting from the addition of the histone mixture was practically abolished in both saline- and carbamylcholine-treated rats (Table 4).

During homogenization, free catalytic subunits might bind to acidic groups in the pellet. To assess the extent of such an artifact, we incubated the adrenal medulla homogenate for 5 min at 30° in the presence of 10 μM cAMP. After centrifugation the protein kinase activity in pellet extracts increased by only 10–13%. This increase could be prevented by the addition of 150 mM NaCl to the incubation medium.

Relationship between Increase in cAMP Content, Protein Kinase Activation and Translocation, and Tyrosine Hydroxylase Induction

To elucidate whether the histone kinase activation and the translocation are related to the delayed induction of tyrosine hydroxylase two groups of rats were injected intraperitoneally with different doses of carbamylcholine. The higher dose (9 μmoles/kg) increased the cAMP content in medulla for longer than 60 min and induced tyrosine hydroxylase 24 hr later

(Fig. 5). The smaller dose (3 μmoles/kg) increased the cAMP content of medulla by a similar extent, but this increase was shorter than 30 min and failed to induce tyrosine hydroxylase 24 hr later (Fig. 5). Only the higher dose of carbamylcholine translocated the protein kinase from the cytosol to the pellet (middle panel, Fig. 5). Although the maximal cAMP increase was the same after 3 or 9 μmoles/kg of carbamylcholine, the protein kinase was translocated to the pellet and the tyrosine hydroxylase was induced only after the higher dose (see Fig. 5).

An intraperitoneal injection of 45 μmoles/kg of hexamethonium (a nicotinic receptor antagonist) 15 min before carbamylcholine (9.2 μmoles/kg) curtailed the increment in medullary cAMP content and shortened its duration from 1 hr to less than 30 min (Fig. 6). Hexamethonium prevented the activation of cAMP-dependent protein kinase by carbamylcholine and the delayed induction of tyrosine hydroxylase. In an analogous experiment atropine (4 μmoles/kg intraperitoneally), a muscarinic receptor blocker, failed to prevent the increase in cAMP content, the activation of cAMP-independent protein kinase, and the induction of tyrosine hydroxylase elicited by carbamylcholine (Fig. 6).

In denervated adrenal medullae, carbamylcholine activated the cAMP-dependent protein kinase in the cytosol, translocated the catalytic subunits to the pellets, and caused the delayed induction of tyrosine hydroxylase.

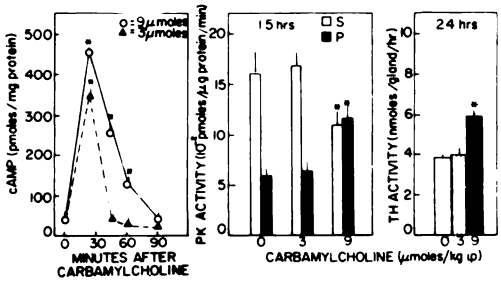

FIG. 5. *cAMP content and protein kinase (PK) activity in adrenal medullae of rats receiving different doses of carbamylcholine*

Protein kinase activity was determined in the supernatant (S) and pellet extract (P), obtained by centrifugation at 20,000 × *g* for 20 min, 1.5 hr after carbamylcholine injection, using 300 mg/ml of calf thymus histones and in the presence of 0.7 μM cAMP. Tyrosine hydroxylase (TH) was measured 24 hr after carbamylcholine injection. Each value is the mean of six experiments. Vertical brackets represent the standard error of the mean. cAMP content was measured at the intervals shown after the intraperitoneal administration of 9 (○——○) or 3 (▲- - -▲) μmoles/kg of carbamylcholine. Notice that in rats given 9 μmoles/kg of carbamylcholine the cAMP content of adrenal medullae increased for about 1 hr (left panel). The protein kinase activity in the supernatant was decreased and that in the pellet was increased (middle panel). Tyrosine hydroxylase was induced 24 hr later (right panel).

* $p < 0.05$ compared with corresponding controls.

Activation and Translocation of Protein Kinase by Aminophylline in Intact and Denervated Adrenal Medullae

Aminophylline (200 μmoles/kg intraperitoneally) increased the cAMP content of both intact and denervated adrenal medullae for longer than 1 hr (Table 5). The protein kinase activity of the supernatant, measured in the absence of cAMP, was increased in intact and denervated adrenals 2 hr after aminophylline (Table 5). The protein kinase activity in the pellet extract prepared from the adrenal medullae of a rat treated 7 hr before was increased on the intact but not the denervated side (Table 5). Other reports (4) have shown that aminophylline causes a delayed induction of tyrosine hydroxylase in intact but not in denervated adrenal medullae.

DISCUSSION

In adrenal medulla the delayed transsynaptic induction of tyrosine hydroxylase (1, 2) is preceded by an increase in cAMP content (3-5) which is coupled with the activation of cholinergic nicotinic receptors (8). If the extent of nicotinic receptor activation is estimated by the duration of the cAMP increase, the threshold signal for eliciting the delayed induction of tyrosine hydroxylase is an increase in cAMP content lasting about 1 hr (3 -5, 8). In the present experiments we have shown that the increase in cAMP content elicited by carbamylcholine activates cAMP-dependent protein kinase in cytosol and translo-

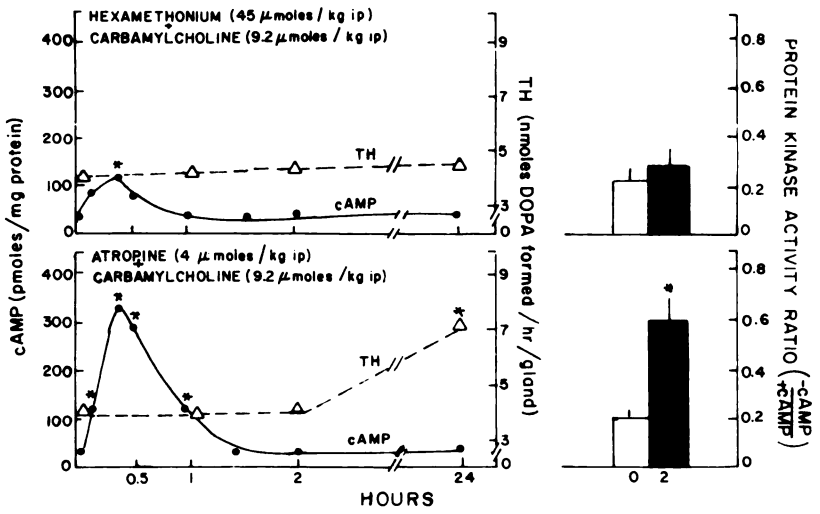

FIG. 6. *cAMP, tyrosine hydroxylase (TH) activity, and protein kinase activity ratio in adrenal medullae of rats receiving 9.2 μmoles/kg of carbamylcholine*

Upper: Rats were treated with hexamethonium 15 min before carbamylcholine. Lower: Rats were treated with atropine 15 min before carbamylcholine. Each value is the mean of five or six experiments. Vertical brackets represent the standard error of the mean. Hexamethonium and atropine failed to change the basal levels of cAMP, tryosine hydroxylase activity, or the protein kinase activity ratio.

* $p < 0.05$ compared with corresponding controls.

TABLE 5

cAMP content and protein kinase activity in intact and denervated adrenal medullae from aminophylline-treated rats

Adrenals were unilaterally denervated 5 days before the experiment, and rats were given 200 μmoles/kg of aminophylline intraperitoneally. The cAMP content of adrenal medullae and the protein kinase activities in the 20,000 × *g* (20 min at 4°) supernatant and pellet extract (prepared as described in Table 2) were measured at the times indicated. No cAMP was added. Each value represents the mean ± standard error of five experiments.

Splanchnic nerve	Aminophil-line	cAMP content at 1 hr	Protein kinase activity	
			Supernatant at 2 hr	Pellet extract at 7 hr
		pmoles/mg protein	*pmoles/0.1 mg protein/min*	
Intact	–	28 ± 2	1.6 ±0.1	6.2 ± 0.5
	+	350 ± 90[a]	3.6 ± 0.5[a]	13.4 ± 0.9[a]
Denervated	–	25 ± 2	1.8 ± 0.3	6.2 ± 0.6
	+	190 ± 12[a]	8.2 ± 0.9[a]	7.2 ± 0.6

[a] $p < 0.05$ compared with the control (no aminophylline).

cates the catalytic subunit from the cytosol to subcellular structures. The evidence presented indicates that the translocation is a necessary intermediary step in eliciting the trans-synaptic induction of tyrosine hydroxylase.

Prior treatment with hexamethonium blocks the delayed induction of medullary tyrosine hydroxylase, the early increase in cAMP content, and the activation of cAMP-dependent protein kinase elicited by carbamylcholine. In contrast, atropine, which blocks the carbamylcholine activation of muscarinic receptors, fails to block these biochemical responses. Denervation blocks the cAMP increase, the activation of cytosol protein kinase, and the tyrosine hydroxylase induction elicited by cold (7), but, as shown in the present report, denervation fails to block these three responses

elicited by carbamylcholine; this suggests that these responses are interrelated and are elicited by activation of nicotinic receptors.

In order to elucidate the nature of the protein kinase activation, regulatory subunits purified from bovine brain protein kinase were added to the supernatant of adrenal medulla homogenates (Table 4) and their gel filtrate (Fig. 3). The results of these experiments suggested that the increase in medullary cAMP content induced by carbamylcholine can activate cytosol protein kinase, thereby dissociating a low molecular weight catalytic subunit from the regulatory subunit of the cytosol holoenzyme. Based on this observation and in agreement with other reports (17), the activation state of the protein kinase in the tissue was measured as the ratio of protein kinase activity in the crude extract in the absence and presence of cAMP (0.7 μM). Carbamylcholine increased this ratio close to the maximal activation level (ratio = 1) from the normal state (ratio = 0.2). Thus, under basal conditions, only 20% of the protein kinase is dissociated but after carbamylcholine almost all the cytosol enzymes are dissociated. After carbamylcholine, the activation of cytosol protein kinase remains elevated for at least 4 hr, and therefore this activation outlasts by several hours the increase in cAMP concentration elicited by carbamylcholine.

To exclude the possibility that the activation of protein kinase might occur during tissue homogenization, we measured the activation index of cytosol protein kinase after 3 μmoles of carbamylcholine. Although this dose increases the cAMP content by an extent comparable to that elicited by 9.2 μmoles/kg, the increase lasts for only 30 min (Fig. 5). The smaller dose of carbamylcholine fails to activate cytosol protein kinase; therefore this enzyme is not translocated and tyrosine hydroxylase is not induced (Fig. 5). Perhaps the signal for activation of protein kinase involves both the extent and duration of the increase in medullary cAMP. To establish whether the dissociation of the catalytic subunit, and therefore activation of the protein kinase system, depends on changes in protein binding during homogenization, we studied the reassociation of protein kinase subunits in homogenates of adrenal medulla from saline- and carbamylcholine-injected rats after incubation for various times at 0°. We found that 0.5 M NaCl slows the changes in subunit reassociation *in vitro*. In both the presence and absence of high salt concentration, dissociation of the catalytic subunits of protein kinase from medullae of carbamylcholine-treated rats was higher than that of saline-treated rats. Moreover, the data presented in Fig. 2 and Table 1 indicate that at the dilution used (15-fold), between 30 and 60 min after preparation of the extract, very little dissociation or reassociation of the protein kinase subunits occurred *in vitro* as a consequence of the homogenization procedure. The data reported in Fig. 3 and Tables 1 and 4 show that the activation of cytosol protein kinase elicited by carbamylcholine was due to dissociation of catalytic subunits; however, Fig. 4 shows that although the increase in catalytic subunits in the cytosol returned to normal in 4 hr, total kinase activity remained low for several hours thereafter.

Recently cytoplasm protein kinase has been shown to migrate and bind to subcellular organelles, including nuclei (18–21). Neither the biological significance nor the precise mechanism of this migration, which has been termed translocation, is presently understood. Tables 3 and 4 show that after carbamylcholine (9.2 μmoles/kg) the protein kinase activity lost from the cytosol can be recovered from the particulate fraction in both intact and denervated adrenal medullae. When purified regulatory subunits are added to the pellet extract, the amount of protein kinase activity that can be observed in the absence of cAMP is greatly reduced (Table 4). The decrease in ^{32}P incorporation observed when regulatory subunits were added to pellet extracts of saline-treated rats was 2.4 pmoles/0.1 mg of protein per minute; the corresponding decrease in pellets prepared 7 hr after carbamylcholine was 5.8 pmoles/0.1 mg of protein per minute (Table 4). These data show that after carbamylcholine the number of catalytic sub-

units in the pellet extract is twice that present in the extract from saline-treated rats. We suggest that carbamylcholine translocates catalytic subunits of protein kinase from the cytosol to the particulate fraction of the chromaffin cells.

Recently Keely *et al.* (22) concluded that in heart preparations the binding of protein kinase to particulate fractions does not always indicate a process of physiological significance. According to these authors, when the cAMP content of a given tissue increases and the ionic strength of the medium is low, the catalytic subunits of protein kinase that are dissociated from the holoenzyme readily bind to subcellular particles or other proteins. Since this binding can be prevented by increasing the ionic strength of the medium (22), the process cannot be termed translocation, and has no physiological role. However, we have found that after carbamylcholine, the cAMP-dependent histone kinase activity in the cytosol of medullae is decreased even when the tissue is homogenized with a high ionic strength medium (Fig. 2). Moreover, in the cytosol of medullae from carbamylcholine-treated rats, the decrease in kinase activity measured in the presence of cAMP lasts longer than the increase in kinase activity measured in the absence of cAMP (Fig. 4). The increase in the number of catalytic subunits of pellet extracts persists for several hours after the number of catalytic subunits in the cytosol has returned to normal. This lack of time relationship argues against the possibility that the catalytic subunits of protein kinase released by the increase in endogenous cAMP bind to subcellular particles during homogenization of the tissue. Hence we believe that carbamylcholine, in doses that induce tyrosine hydroxylase, translocates catalytic subunits in the particulate fraction of the medulla.

The relevance of protein kinase translocation to the mediation of tyrosine hydroxylase induction is supported by experiments with denervated adrenal medulla. Carbamylcholine and aminophylline increase the cAMP content, activate and translocate the protein kinase of cytosol, and cause a delayed induction of tyrosine hydroxylase in intact adrenal medullae by different mechanisms. Carbamylcholine causes this sequence of events by activating nicotinic postsynaptic receptors, and aminophylline, by inhibiting phosphodiesterase. In denervated medulla, provided that the denervation has been performed 5 days before the experiment (5), carbamylcholine responses are similar to those of intact medulla. In contrast, aminophylline increases the cAMP content and activates cytosol protein kinase in intact and denervated medullae but fails to translocate the catalytic subunits (see Table 5) and to induce tyrosine hydroxylase in denervated medulla. These data indicate that the translocation mediates tyrosine hydroxylase induction, but they also reveal that the activation of nicotinic postsynaptic receptors plays a permissive role in the translocation. Furthermore, a role for kinase translocation in tyrosine hydroxylase induction is supported by studies with carbamylcholine showing a dose-response relationship between extent and duration of cAMP increase, protein kinase translocation, and tyrosine hydroxylase induction (Fig. 5).

Although in Table 3 and 4 we have termed histone kinase the protein kinase that is translocated into the pellet, we do not intend to imply that histones are the natural substrates for this enzyme. However, the selectivity of the translocated enzyme for histone as phosphate acceptor suggests that there is some degree of selectivity in the process of kinase translocation; by a mechanism yet unknown, some kinases may be translocated preferentially. These considerations indicate that the increase in cAMP content elicited trans-synaptically may regulate expression of the nuclear metabolic code via phosphorylation of nuclear proteins. Perhaps phosphorylation of a given nuclear protein accounts for the increase in DNA transcription elicited trans-synaptically.

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