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Nuclear Translocation of Cyclic AMP-Dependent Protein Kinase Subunits During the Transsynaptic Activation of Gene Expression in Rat Adrenal Medulla

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SUMMARY

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In rat adrenal medulla gene expression can be activated by transsynaptic stimulation of nicotinic receptors via an increase of the adenosine 3′,5′ monophosphate content (cyclic AMP). This increase activates the cyclic AMP-dependent ATP: protein phosphotransferase (protein kinase) of adrenal medulla. Type I and II cyclic AMP-dependent protein kinases are present in the adrenal medulla cytosol; both enzymes were activated and dissociated into regulatory and catalytic subunits following the increase of medullary cyclic AMP content elicited transsynaptically. However only the cytosol content of Type I cyclic AMP-dependent protein kinase but not that of Type II enzymes decreases for several hours following persistent activation of nicotinic receptors. This decrease was associated with the appearance of catalytic subunits of cyclic AMP-dependent protein kinase in nuclei. The phosphorylation of endogenous nuclear protein was increased after the redistribution of the catalytic subunits of cyclic AMP-dependent protein kinase elicited by the persistent stimulation of nicotinic receptors. A similar increase of nuclear protein phosphorylation was elicited also "*in vitro*" by incubating nuclei of adrenal medulla with cyclic AMP and cyclic AMP-dependent Type I protein kinase; in contrast, the incubation of these nuclei with Type II protein kinase failed to increase the phosphorylation of nuclear proteins. For comparison, the fate of catalytic subunits of cyclic AMP-dependent protein kinase of pineal gland cytosol was studied following a sustained transsynaptic activation of protein kinase. Only Type II cyclic AMP-dependent protein kinase is present in pineal cytosol. This enzyme was activated and dissociated following the increase of cyclic AMP content elicited by isoproterenol, but its catalytic subunits failed to translocate into the pineal cell nuclei suggesting that translocation into nuclei is not a generalized property of cyclic AMP-dependent protein kinase.

INTRODUCTION

Several lines of evidence have suggested that the persistent release of acetylcholine from axons innervating adrenal medulla activates nicotinic and muscarinic receptors located on the plasma membrane of the chromaffin cells (1); only the stimulation of nicotinic receptors appears to control gene expression coding for tyrosine-3-monooxygenase (2). In rat adrenal medulla the activation of nicotinic receptors is associated

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with an elevation of 3',5'-adenosine monophosphate (cyclic AMP) (3) content, which is followed by the activation of cytosol cyclic AMP-dependent ATP:protein phosphotransferase (protein kinase) (4) and by the increase of nuclear protein kinase activity (5). The increase in nuclear protein kinase activity precedes the increase of mRNA (6) and tyrosine-3-monooxygenase synthesis and activity (7, 8). All the events are regulated transsynaptically (9, 10). In *in vitro* experiments using nuclei of cow adrenal medulla and purified cyclic AMP-dependent protein kinase, we have shown that the increase in nuclear protein kinase activity triggers phosphorylation of specific proteins (11). This phosphorylation is related to an increase of RNA transcription (12). Based on this evidence we have hypothesized that in rat adrenal medulla cyclic AMP could control the nuclear functions necessary to express an increase in tyrosine-3-monooxygenase (5, 6) by activating a cyclic AMP-dependent phosphorylation mechanism (4, 10).

A variety of molecular forms of protein kinase which phosphorylate histone and nonhistone proteins have been isolated from nuclei (11, 13–16). Since most of these nuclear protein kinases, including that of rat adrenal medulla cells, are cyclic AMP-independent (for reference see 13–16 and Table 1 of this paper), it has been particularly difficult to reconcile how an increase of intracellular cyclic AMP content could regulate nuclear protein phosphorylation. In several model systems (5, 14, 17–20) a persistent increase of cyclic AMP content not only dissociates the catalytic subunits of cytoplasmic cyclic AMP-dependent protein kinase but also increases the nuclear protein kinase activity. It has been proposed that this increase of nuclear protein kinase activity occurs because catalytic subunits of cytoplasmic cyclic AMP-dependent protein kinase can translocate from one to another cellular compartment (5, 9–11, 16, 17).

The present studies were undertaken to ascertain the time sequence of events leading to nuclear translocation of catalytic subunits of cyclic AMP-dependent protein kinase during the transsynaptic induction of tyrosine-3-monooxygenase in rat adrenal chromaffin cells. We measured the concentration of cyclic AMP and the activity of cytosol and nuclear protein kinase in adrenal medulla of rats exposed to cold stress or receiving reserpine. We used reserpine and cold exposure because these two treatments produce a persistent transsynaptic stimulation of nicotinic receptors (3, 4, 10). The results obtained following this transsynaptic activation of nicotinic receptors of adrenal gland were compared with those obtained following prolonged stimulation of β-adrenergic receptors in the pineal gland.

EXPERIMENTAL PROCEDURES

Methods

1. *Isolation of adrenal medulla and pineal gland.* Male Sprague Dawley rats (125–150 g) were used. The adrenal medullae and pineal gland were removed within 15 sec after decapitation. The adrenal medulla was dissected from the adrenal cortex at 0° (3). To determine the cyclic AMP content of the pineal gland the animals were sacrificed by microwave radiation (21).

2. *Preparation of cytosol and nuclear fractions.* Unless otherwise indicated, 100 rat adrenal medullae (approximately 100 mg tissue) or 50–60 pineal glands (approximately 50 mg tissue) were homogenized in 1 ml of chilled (4°) 0.32 M sucrose, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.4. The homogenate was centrifuged at 1,000 × *g* for 10 min. The supernatant was centrifuged for 60 min at 108,000 × *g* and the resultant supernatant was termed cytosol fraction.

The nuclear fraction was purified from the 1,000 × *g* pellet according to the method of Yasmineh and Yunis (22). This purified preparation was resuspended in 1 ml of 0.32 M sucrose, 10 mM potassium phosphate buffer (pH 6.5) containing 5 mM $MgCl_2$ and 0.2 mM $CaCl_2$. After centrifugation (5,000 × *g* for 10 min) the nuclear pellet was resuspended in the same buffer and used in various experimental procedures. The purity of the nuclei was determined biochemically by measuring DNA (23), RNA (23), protein (24) monoamine-oxidase activity (25), 5'-nucleotidase activity (26). The

monoamine-oxidase and the 5'-nucleotidase activity per unit of DNA was less than 0.1% of the value measured in the total homogenate.

The protein to DNA ratio of purified nuclei was 2.2 to 2.6; the recovery of DNA was 60–65%, that of RNA was approximately 2% and the RNA/DNA ratio was 0.20. Treatment of the nuclei with 0.1 or 0.2% Triton X-100 failed to change the DNA, RNA or protein content.

Within the time limits of our experiments, neither reserpine injection nor exposure of the rats to 4° for 2 hr changed the DNA, RNA or protein content of the medullary homogenates; moreover, the protein/DNA ratio or the DNA recovery in the nuclear fraction remained unchanged.

3. *Characterization of cytosol cyclic AMP-dependent protein kinase by Whatman DE-52 column chromatography.* A 200 μl aliquot of adrenal medulla or pineal cytosol (approximately 3 mg of protein) was dialyzed 12 hr at 4° against 1 liter of 5 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The dialyzed sample was loaded on a Whatman DE-52 column (0.3 × 3 cm) equilibrated with 5 mM Tris-HCl (pH 7.5), 1 mM EDTA. The flow rate of this column was adjusted to approximately 3 ml per hr. The column was eluted with a linear gradient of NaCl generated by a reservoir containing 4 ml of 0.5 M NaCl in the equilibration buffer and a mixing chamber containing 4 ml of the equilibration buffer alone. Fractions of 300 μl eluate were collected. The protein kinase activity was determined in the crude cytosol fraction or in the eluate of the Whatman DE-52 column with a modification of a previously described procedure (4). In brief, an incubation volume of 140 μl contained: enzyme 10–50 μl; Mg acetate buffer (pH 6.0), 1 μmole; Na acetate buffer (pH 6.0), 5 μmole; NaF, 1.4 μmole; aminophylline, 0.65 μmole; calf histone mixture, 40 μg and cyclic AMP 100 pmole (when indicated). The reaction was initiated by adding 15 nmole of [γ-^{32}P]ATP (S.A. 100 μCi/μmole). After 5 minutes of incubation at 30° the reaction was terminated by pipetting 50 μl of the reaction mixture onto filter paper disc previously soaked in 10% trichloroacetic acid and 100 mM EDTA solution. The filter paper disc was then extensively washed with 5% trichloroacetic acid solution containing 1.2% NaOH, 0.25% Na tungstate, 0.2% H_2SO_4 (27). The assay was run in triplicate. Boiled or EDTA (100 mM) treated samples were used as a blank. The recovery of the protein kinase activity from homogenates of adrenal medulla or pineal after DE-52 column was consistently better than 110%. The high recovery can be explained by the removal of some inhibitory material during the column purification. However, when 150 units of partially purified Type I or Type II cyclic AMP-dependent protein kinases were added to the column the recovery was consistently better than 80%.

One unit of protein kinase activity is that amount of enzyme that in 1 min at 30° catalyzes the transfer of 1 pmole of PO_4^{-3} from [γ-^{32}P]-ATP to calf histone mixture or to another endogenous protein functioning as a PO_4^{-3} acceptor.

4. *Measurement of protein kinase activity in nuclear extracts.* Purified nuclear fraction from 100 adrenal medullae (see paragraph 3) was suspended in 500 μl of 0.5 M NaCl, 10 mM potassium phosphate buffer (pH 6.5), 1 mM EDTA, 5 mM aminophylline, 0.2% Triton X-100. After sonication (4 burst of 15 sec each), the sample was centrifuged for 20 min at 20,000 × g and the supernatant "nuclear extract" was dialyzed for 12 hr against 10 mM potassium phosphate buffer (pH 6.5), 1 mM EDTA, 1 mM 2-mercaptoethanol. About 90% of the nuclear protein kinase activity was solubilized by this treatment. The protein kinase reaction was carried out at 30° × 2 min and was terminated using the same conditions described for cytosol protein kinase. To remove ^{32}P bound to nonprotein material, the filter paper was washed with chloroform and hot (90°) 5% trichloroacetic acid (28). The phosphorylation reaction was linear up to 10 min and proportional to the concentration of nuclear extract.

5. *Measurement of endogenous phosphorylation in intact nuclei of adrenal medulla.* The intact nuclei (300 to 500 μg/protein) were suspended in 270 μl of 0.32 M sucrose, 10 mM potassium phosphate buffer (pH 6.5), 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 30 mM NaF. The mixture was incubated for 1 minute at 30° and then mixed

with 30 μl of 500 μM [γ-^{32}P]ATP (S.A. 100 μCi/μmole).

At different time intervals (0.5, 1, 2, 5 min), 50 μl aliquot of the mixture was placed on filter paper discs and the samples were then washed as described in paragraph 4 of this section. Boiled samples, 5% trichloroacetic acid-treated samples and/or 100 mM EDTA-treated samples were used as blank. The presence of 30 mM NaF in the assay medium favored a linear rate of phosphorylation. In some experiments, aliquots of the nuclear fraction obtained from normal or reserpine-treated rats were washed before the assay with 0.2% Triton. This treatment did not change significantly the extent of nuclear phosphorylation. The protein kinase activity was calculated from the initial linear rate of the protein phosphorylation which usually extend for a 2 min period.

6. *Partial purification of regulatory subunits of cyclic AMP-dependent protein kinase.* Regulatory subunits of cyclic AMP-dependent protein kinase were obtained from rat adrenal glands. The soluble cyclic AMP-dependent protein kinase obtained from this tissue was first purified with 70% ammonium sulfate precipitation and Whatman DE-52 column chromatography (27). The crude protein kinase preparation (about 200 units) was then incubated for 5 min at 30° in the presence of 5 μM cyclic AMP, 0.5 M NaCl, 10 mM potassium phosphate buffer (pH 6.5), and 1 mM EDTA. The dissociated regulatory subunits of cyclic AMP-dependent protein kinase were separated from the catalytic subunits by Sephadex G-200 chromatography (0.2 × 50 cm; equilibrated with 10 mM potassium phosphate buffer (pH 6.5), 0.5 M NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol and 5 μM cyclic AMP). The fractions (80 μl) eluted from the Sephadex column were extensively dialyzed against 10 mM potassium buffer (pH 6.5), 1 mM EDTA, and 1 mM 2-mercaptoethanol. The protein kinase activity of each fraction was measured in the presence and in the absence of cyclic AMP. The [^{3}H]cyclic AMP binding activity was determined according to Gilman's method (29) except that 0.05 M potassium phosphate buffer, pH 7, was substituted for 0.05 M sodium acetate buffer, pH 4. A typical regulatory subunit preparation from Sephadex G-200 was eluted between fractions 5 and 10 and could bind a significant amount of cyclic AMP but it was virtually devoid of protein kinase activity. The potency of the regulatory subunit to inhibit the activity of catalytic subunits of cyclic AMP-dependent protein kinase was tested in enzyme recombination experiments: different aliquots of regulatory subunit preparation were mixed with a constant amount (10 units) of purified catalytic subunits of cyclic AMP-dependent protein kinase (10). The mixtures were incubated for 60 min at 4°. Aliquots of the mixtures were then assayed for cyclic AMP-dependent protein kinase activity by the method described in paragraph 3 of this section. In these conditions 2.5 μg of the regulatory protein completely blocked the catalytic activity of 5 units of protein kinase.

7. *Preparation of the heat-stable inhibitor of cyclic AMP-dependent protein kinase.* The inhibitor was isolated from rabbit skeletal muscle by the method of Walsh *et al.* (30). After trichloroacetic precipitation step, the dialyzed sample was loaded onto a Sephadex G-100 column (1 × 70 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7, containing 100 mM NaCl (31). This column separates the heat-stable inhibitor of cyclic AMP-dependent protein kinase from a heat-stable inhibitor of other protein kinases (31). The purified inhibitor of cyclic AMP-dependent protein kinase did not accept $^{32}PO_4^{-3}$ from [γ-^{32}P]ATP when tested as a substrate in the protein kinase assay. In a typical preparation, 3 μg of this protein completely blocked 10 units of cyclic AMP-dependent protein kinase.

8. *Studies of cyclic AMP-dependent protein kinase activity: Use of regulatory subunits, heat-stable inhibitor of cyclic AMP-dependent protein kinase and ω-aminohexyl agarose.* To differentiate the activity of cyclic AMP-dependent protein kinases (holoenzyme and catalytic subunits) from the activity of the kinases which are not regulated by cyclic AMP, we have used three different methods.

a) Experiments with regulatory subunits: the protein kinase of cytosol or nuclear extracts was first dialyzed at 0° × 12 hr against 10 mM potassium phosphate buffer pH 6.5, 1 mM EDTA, 1 mM 2-mercaptoeth-

anol. An aliquot (20 μl) of the cytosol fraction (2–3 units of protein kinase in 10–20 μg of proteins) or an aliquot (20 μl) of the nuclear extract (2–3 units of protein kinase in 15–30 μg of protein) was incubated at 0° for 1 hr in the presence of 10 μg of partially purified regulatory subunit of cyclic AMP-dependent protein kinase (see paragraph 6 of this section). The protein kinase activity of this mixture was determined in the presence or in the absence of cyclic AMP using the same reaction conditions described in paragraph 3 of this section. The amount of cyclic AMP-dependent protein kinase (holoenzyme and/or C) was calculated by substracting from the total activity measured in the presence and in the absence of 0.7 μM cyclic AMP, the activity which remained after the addition of regulatory subunits of cyclic AMP-dependent kinase.

b) Experiments using heat-stable inhibitor of cyclic AMP-dependent protein kinase: aliquot of cytosol suspension (usually containing 2–3 units of protein kinase activity) were assayed for protein kinase activity in the presence or in the absence of 3 μg of heat-stable inhibitor prepared from rabbit hind leg muscle as described in paragraph 7 of this section.

Since this heat-stable inhibitor selectively inhibits the catalytic activity of cyclic AMP-dependent protein kinase, the activity due to catalytic subunits or to cyclic AMP-dependent holenzyme was determined by measuring the disappearance of protein kinase activity in the presence or in the absence of cyclic AMP (0.7 μM) when the inhibitor was present.

c) Experiments using ω-aminohexyl-agarose: in a series of preliminary experiments, using partially purified cyclic AMP-dependent (27) and independent protein kinase (10) from rat adrenal medulla or pineal gland, we have established that ω-aminohexyl-agarose resin selectively retains the molecular forms of protein kinase that are cyclic nucleotide-independent and thereby provides a rapid and efficient method to isolate the cyclic AMP-dependent protein kinase present in a given sample.

In these experiments, except when otherwise indicated, the samples were homogenized in 25 mM potassium phosphate buffer (pH 7), 1 mM EDTA and 150 mM NaCl. Aliquots of the 100,000 × *g* supernatant (usually 250 μl, 25–50 units of protein kinase, 300–500 μg of protein) were applied directly to 100 μl of settled ω-aminohexyl agarose equilibrated with the same buffer. After gentle mixing for 15 min at 4° the resin was separated by centrifugation (500 × *g* for 5 min). The supernatant fluid was assayed for protein kinase activity in the presence and in the absence of cyclic AMP with and without the addition of the heat-stable inhibitor of cyclic AMP-dependent protein kinase or purified regulatory subunits of cyclic AMP-dependent protein kinase. Within the range of the concentrations of tissue extract used the recovery of cyclic AMP-dependent protein kinase (holoenzyme or C) in the supernatant was about 70–80%. In contrast, only 10–20% of the cyclic nucleotide-independent protein kinase activity was recovered in the eluate.

Using these three different procedures, we have established that in medullary cytosol the cyclic AMP-dependent protein kinase activity (holoenzyme + C) is approximately 90–95% of the total protein phosphorylating activity and that in pineal cytosol only 55 to 60% of the total protein kinase activity is cyclic AMP-dependent. In contrast, a cyclic AMP-dependent protein kinase activity is apparently absent in the nuclear fractions of either tissue.

9. *Determination of the cyclic AMP content.* The tissue was homogenized in 0.4 M perchloric acid. The content of cyclic AMP was determined by the activation of a specific cyclic AMP-dependent protein kinase after purification on alumina and Dowex column (3, 21).

10. *Measurement of phosphoprotein phosphatase activity.* The phosphoprotein phosphatase (EC 3.1.3.16) activity was measured by the release of ^{32}P from phosphorylated histones (32). ^{32}P histones were prepared by incubating for 10 min at 30° a partially purified beef heart protein kinase (27) with [γ-^{32}P]ATP and calf histone mixture. After the addition of non-radioactive ATP and EDTA (32), the tissue supernatant, prepared as described for protein kinase assay, was added and 50 μl aliquots of the reaction were applied to filter paper discs after various times of incubation at 30°.

11. *Hydrolysis of cyclic AMP.* The rate of cyclic AMP hydrolysis in the reaction mixture used to determine the protein kinase activity was measured by adding cyclic [^{3}H]AMP (4000 cpm/0.2 pmol). After 5 min of incubation, the cyclic AMP of the reaction mixture was purified (21) and the radioactivity was measured. Protein was determined by the method of Lowry *et al.* (24).

Materials

Sephadex G-200 or G-100 was obtained from Pharmacia Fine Chemicals. DEAE-cellulose (DE-52) was purchased from Whatman. Histone H2B and H1, l-isoproterenol-HCl, reserpine and aminophylline were obtained from Sigma Chemical Co. Reserpine was dissolved in saline containing 0.1% acidic acid; the pH of the solution was adjusted to approximately 6 with NaOH. Control rats received a saline solution containing 0.1% neutralized acidic acid. This solution was defined "solvent" [γ-^{32}P]ATP (40 Ci/mmol) and cyclic (8-^{3}HO-adenosine 3',5'-monophosphate [cyclic AMP]) (23–27 Ci/mmol) was obtained from New England Nuclear. Cyclic-AMP was purified by the method of Guidotti *et al.* (21). ω-Aminohexyl agarose was purchased from Miles-Yeda Laboratories. Calf histone mixture was purchased from Calbiochem.

RESULTS

A. *Studies with Adrenal Medulla*

1. *Effect of trans-synaptic stimuli on the protein kinase activity of cytosol and nuclei of rat adrenal medulla.* The characteristics of the protein kinase activity present in cytosol and in nuclear extracts from rat adrenal medulla are shown in Table 1. The phosphorylating activity of nuclear extracts measured without cyclic AMP addition was about twice that of cytosol. However, the protein kinase activity of the nuclear fraction was approximately one-third of that measured in the cytosol when assayed in the presence of 0.7 μM cyclic AMP. When the protein kinase activity of cytosol preparations or nuclear extracts was assayed in the presence of an excess of either regulatory subunits or of heat-stable inhibitors of cyclic AMP-dependent protein kinase or after purification with ω-aminohexyl agarose, 80 to 90% of the cytosol enzyme was

TABLE 1

Characterization of the protein kinase activity in cytosol and nuclear extract of rat adrenal medulla

Cytosol or nuclear extract, obtained as described in the paragraph 3 and 4 of the EXPERIMENTAL PROCEDURES were dialyzed at 0° for 12 hr against 10 mM potassium phosphate buffer pH 6.5, 1 mM EDTA, 1 mM 2-mercaptoethanol. Aliquots of the dialyzed cytosol fraction (20 μl, 2.0 units of protein kinase activity, 15 μg of protein) or aliquots of the dialyzed nuclear extract (20 μl, 10 units of protein kinase, 20 μg protein) were assayed for protein kinase activity in presence or absence of 0.7 μM cyclic AMP. For details on the experiments with regulatory subunits, inhibitory protein (heat-stable inhibitor of cyclic AMP-dependent protein kinase) see paragraph 8 of the EXPERIMENTAL PROCEDURES. For the experiment with ω-aminohexyl agarose resin the cytosol fraction was prepared in 25 mM potassium phosphate pH 7 and 150 mM NaCl. The values given in the table were corrected for the recovery (70% in this experiment). The activity recovered from the ω-amino hexyl agarose was proven to be cyclic AMP-dependent with the use of heat-stable inhibitor of cyclic AMP-dependent protein kinase or with the use of regulatory subunits. Each value is the mean of triplicate determinations.

Fraction	Protein kinase activity (pmole/mg protein/min)							
	Total activity		Activity in presence of:				Activity after ω-aminohexyl agarose	
	−cyclic AMP	+Cyclic AMP[a]	Regulatory subunits		Inhibitor of cAMP-DPK[b]		−Cyclic AMP	+Cyclic AMP
			−Cyclic AMP	+Cyclic AMP	−Cyclic AMP	+Cyclic AMP		
Cytosol	32	145	15	152	12	20	20	118
Nuclei	53	48	52	54	50	49	Undetectable	Undetectable

[a] Cyclic AMP indicates that the assay was carried out in the presence of 0.7 μM of cyclic AMP.

[b] cAMP-DPK = cyclic AMP-dependent protein kinase.

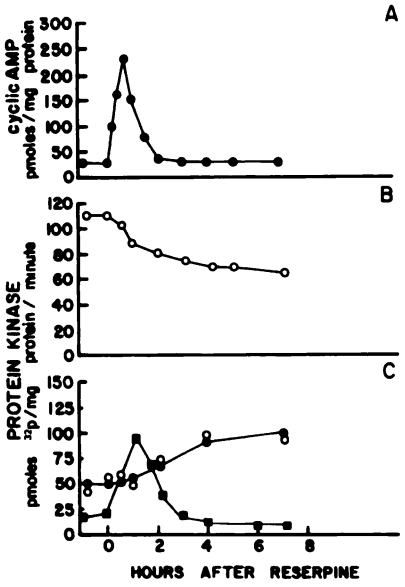

FIG. 1. *Change in the cyclic AMP content (panel A), cytosol cyclic AMP-dependent protein kinase activity (panel B), cytosol catalytic subunits of cyclic AMP-dependent protein kinase and nuclear cyclic AMP-independent protein kinase activity (panel C) in adrenal medulla of rats receiving reserpine (16 μmole/kg, i.p.)*

At the times indicated on the abscissa, adrenal medullae were dissected and cytosol and nuclear extracts were assayed for cyclic AMP and protein kinase activity as described in the EXPERIMENTAL PROCEDURES. Panel A refers to changes of cyclic AMP content. Panel B depicts the changes of the total (measured in presence of 0.7 μM cyclic AMP) cytosol cyclic AMP-dependent protein kinase activity. Panel C indicates the changes in the activity of catalytic subunits of cyclic AMP-dependent protein kinase in the cytosol (■——■) and the concomitant changes of nuclear protein kinase activity measured in presence (●——●) or absence (○——○) of 0.7 μM cyclic AMP. The assay of cytosol and nuclear protein kinase activity was run in samples obtained from the same animal. The cytosol cyclic AMP-dependent protein kinase activity was calculated by subtracting from the activity measured in presence or absence of cyclic AMP (0.7 μM) the activity which remains after addition of the heat-stable inhibitor of cyclic AMP-dependent protein kinase or by subtracting from the activity measured in the absence of cyclic AMP the activity remaining after addition of regulatory subunits (see METHODS section for details). The last measurement was performed in samples dialyzed against 10 mM potassium phosphate, pH 6.5, 1 mM EDTA, 1 mM 2-mercaptoethanol. The protein kinase in the nuclear extract was not stimulated by addition of cyclic AMP to the incubation media. Cyclic AMP failed to stimulate the nuclear cyclic AMP-dependent whereas virtually all of the protein kinase activity of nuclear extracts appeared to be cyclic AMP-independent (see Table 1).

Figure 1 shows the time course of the change in the cyclic AMP content and in the phosphorylating activity of cytosol and nuclear extracts of adrenal medulla from rats killed at various times after reserpine (16 μmole or 10 mg/kg, i.p.). During the first hour, the cyclic AMP content and the activity of free catalytic subunits of cyclic AMP-dependent protein kinase in cytosol was increased by several fold (Figs. 1A and C). In contrast, the nuclear protein kinase activity failed to change at this time (Fig. 1C). However, two hours after the reserpine injection, the cyclic AMP-dependent protein kinase activity of cytosol was decreased (Fig. 1B) and the phosphorylating activity of nuclear extracts was increased (Fig. 1C). At four and seven hours following reserpine injection, the total cytosol protein kinase activity was decreased by 40–50% (see Fig. 1B) and the activity of free catalytic subunits of cyclic AMP-dependent protein kinase was also below normal levels (Fig. 1C). The difference in five separate experiments was approximately 40% and was statistically significant ($p < 0.05$). At this time the ratio between holenzyme and free catalytic subunits of cyclic AMP-dependent protein kinase was similar to that of control adrenal medulla. In contrast the protein kinase activity of nuclear extracts was increased by 80–90% (Fig. 1C). If the data at seven hours after reserpine were expressed in units of protein kinase activity per adrenal gland, the cytosol protein kinase declined from 8–10 units to 5–6 units while the nuclear protein kinase increased from 0.5–0.6 to 1–1.2 units. Under the conditions used for the determination of protein kinase activity (large dilution, aminophylline and NaF), the marked decrease in the activity of cytosol cyclic AMP-dependent protein kinase and the increase in nuclear kinase activity could not be accounted for by changes in either phosphoprotein phosphatase or phosphodiesterase activities (see also 4, 10).

extracts also after extensive dialysis of the extracts against 10 mM potassium phosphate pH 6.5, 1 mM EDTA, 1 mM 2-mercaptoethanol.

The apparent *Km* of cytosol protein kinase for cyclic AMP (80 nM) was identical in the adrenal medulla of rats injected with solvent or reserpine. When 10 units of protein kinase obtained from cytosol or nuclear extracts of adrenal medulla of rats exposed to cold or receiving reserpine were mixed with an equal number of protein kinase units obtained from cytosol or nuclear extracts of solvent or saline-treated rats, the resulting mixtures exhibited protein kinase activities equivalent to the algebric sum of the activity present in each extract. These results exclude changes of endogenous protein kinase activators or inhibitors (30, 31).

As shown in Table 2 and Fig. 1C the enzyme activity of nuclear extracts from medullae of solvent-treated rats was unaffected by the addition of regulatory subunits. In contrast, the increase in the protein kinase activity of nuclear extracts of rats treated with reserpine (5, 10) or exposed to cold (4° for 2 hr) (see Table 2) was nullified by the addition of regulatory subunits of cyclic AMP-dependent protein kinase.

TABLE 2

Effect of addition of exogenous regulatory subunits of cyclic AMP-dependent protein kinase on the protein kinase activity of nuclear extract prepared from adrenal medulla of control and cold exposed rats

Nuclear extracts prepared as described in the EXPERIMENTAL PROCEDURES section were dialyzed for 18 hr against 10 mM potassium phosphate buffer, pH 6.5, 1 mM EDTA, 1 mM 2-mercaptoethanol. The dialyzed extract (2–3 units of protein kinase activity, approximately 20–30 μg protein) was then incubated at 0° for 1 hr in presence of 10 μg of partially purified regulatory subunits. The assay was performed in absence and presence of 0.7 μM cyclic AMP (see EXPERIMENTAL PROCEDURES). Rats (120 g body weight) were exposed at 4° for two hours, one per cage. After exposure to cold the animals were left at 22° and sacrificed five hours later. Values represent the average of triplicate assay.

Treatment	Regulatory subunit (μg/tube)	^{32}P Incorporated (pmole/mg protein/min)	
		+cAMP	−cAMP
Control	0	55	49
	10	53	54
4° for 2 hr	0	110	115
	10	100	50

The increase in nuclear protein kinase activity was not the result of cytoplasmic or other subcellular contamination during the homogenization procedure because the enzyme activity measured in nuclei washed once with 0.2% Triton X-100 was unchanged. To further examine the specificity of the increase of protein kinase activity in nuclei, adrenal medulla homogenates from rats killed 7 hr after solvent or reserpine were incubated for 5 min at 30° in the presence or in the absence of 0.7 μM cyclic AMP. In the supernatant of both homogenates incubated with cyclic AMP, the cyclic AMP-dependent protein kinase was completely dissociated into regulatory and catalytic subunits; however, in the nuclear pellet the specific activity of the cyclic AMP-independent protein kinase (42 ± 5 pmoles ^{32}P/mg protein/min) for solvent-treated rats and for reserpine-treated rats (89 ± 10 pmoles ^{32}P/mg/min; n = 3) was not significantly different from the specific activity present in the nuclear pellet of aliquots of the same homogenates incubated without the addition of exogenous cyclic AMP.

We also studied whether the increase in nuclear phosphorylating activity caused by reserpine or cold exposure could be expressed when the endogenous nuclear protein was the PO_4^{-3} acceptor. Table 3 shows that 30 minutes after reserpine injection

TABLE 3

Endogenous protein phosphorylation in intact nuclei prepared from adrenal medulla of rats killed at various times after reserpine (16 μmoles/kg i.p.)

Intact nuclei (approximately 400 μg of protein per sample) were prepared from 30 adrenal medullae and were incubated with 500 μM [γ-^{32}P]ATP. The activity was estimated measuring protein kinase activity at different time intervals (0–5 min) as described in EXPERIMENTAL PROCEDURES. Each value is the mean value of 2–3 experiments. Single values range between 8–12% of the mean. When cAMP was added the final concentration was 0.7 μM.

Hours after reserpine	Protein kinase activity (pmole/mg protein/min)	
	−cAMP	+cAMP
0	58	55
0.5	55	60
4	95	90
7	105	110

the protein kinase activity of intact nuclei prepared from adrenal medulla of reserpine or solvent-treated rats was identical. In contrast, from four to seven hours following reserpine injection we obtained an increase of about 2-fold in the nuclear protein phosphorylation. This increase paralleled the increase of nuclear protein kinase activity measured in nuclear extracts (see Fig. 1C).

2. *Fractionation of cytosol protein kinase by ion exchange chromatography.* As shown in Fig. 2, the protein kinase activity of adrenal medulla cytosol was resolved in two enzyme forms after chromatography on Whatman DE-52 (Fig. 2). The major protein kinase peak (referred according to the nomenclature proposed by Corbin *et al.* (33) as Type II protein kinase) was eluted between 0.15 and 0.25 M NaCl. The smaller protein kinase peak (referred to as Type I protein kinase) was eluted between 0.05 and 0.1 M NaCl. Both types of protein kinases were cyclic AMP-dependent. In five identical experiments by comparing the elution areas of the two peaks, it was observed that in the cytosol of control adrenal medulla the amount of Type II protein kinase was consistently twice that of Type I protein kinase (Fig. 2, top panel). In the cytosol of adrenal medulla prepared from reserpine-treated rats the elution profile of Type I and II protein kinase failed to change in the first hour (data not shown). However, seven hours after reserpine, the ratio (approximately 5) between the elution areas of Type I and II protein kinase activities was greater than in solvent-treated rats (approximately 2). This increase in the peak ratios was due to a decrease in the activity of Type I protein kinase while Type II protein kinase remained virtually unchanged (compare top versus bottom panel of Fig. 2). In addition to the two major cyclic AMP-dependent protein kinase peaks, another small peak of protein kinase activity appeared in the flow-through portion of the eluate (see fractions 1-5 of the eluate in Fig. 2). This peak was not stimulated by exogenous cyclic AMP and failed to change in the Whatman DE-52 chromatogram of cytosol prepared 60 min or 7 hr after reserpine treatment (see Fig. 2).

The decrease in the activity of Type I

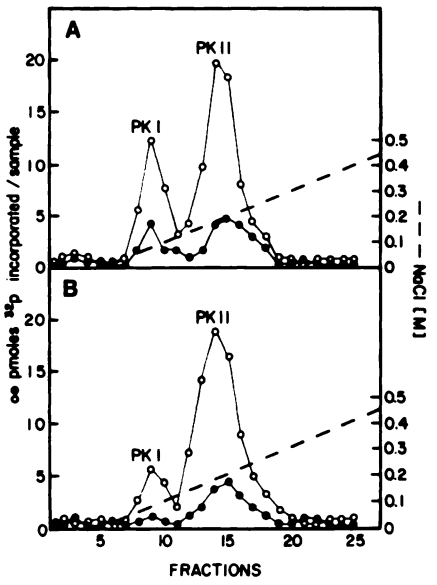

FIG. 2. *Whatman DE-52 column chromatography of adrenal medulla cytosol prepared from adrenal medulla of rats treated i.p. with solvent (A) (5 ml/kg) or (B) reserpine (16 μmole/kg) 7 hr before the experiment*

A 200 μl aliquot of cytosol fraction of rat adrenal medulla (450 units, 3 mg protein) prepared as described in the EXPERIMENTAL PROCEDURES was dialyzed for 12 hr with 5 μM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol. The dialyzed cytosol was then applied to the DE-52 Whatman column (0.3 × 3 cm) equilibrated with 5 mM tris-HCl (pH 7.5), 1 mM EDTA. The column was developed with a linear gradient from 0 to 0.5 M NaCl.

Fractions of 300 μl were collected and 50 μl was used to assay protein kinase activity in the presence (○) or absence (●) of 0.7 μM cyclic AMP. Results similar to those reported in the figure were obtained in five separate experiments. PK I = Type I protein kinase; PK II = Type II protein kinase.

cyclic AMP-dependent protein kinase found in the cytosol of reserpine-treated rats could not be accounted for by changes in either phosphoprotein phosphatase or phosphodiesterase activity. The #5 to #10 eluates from DE-52 column loaded with cytosol preparations from adrenal medulla of solvent or reserpine-treated rats failed to hydrolyze cyclic AMP or dephosphorylate histones. In addition, when the elution fractions obtained from medulla of reserpine-treated rats were mixed 1:1 with the corresponding fractions obtained from control medulla, the protein kinase activities were

additive. Moreover, we also found that reserpine treatment failed to change the apparent affinity of Type I and II medullary protein kinase for cyclic AMP, ATP or histone. The cyclic AMP concentration necessary for half maximal activation of Types I and II protein kinase was approximately the same (50 nM). The apparent affinity constants for ATP (20 μM) and for H2b histone (150 μM) were also the same. The affinity of Type I protein kinase (150 μM) or Type II protein kinase (250 μM) for H_1 histone was also unaffected by reserpine or cold treatment. Both Types I and II protein kinase were inhibited to the same extent (approximately 95%) by the addition of heat-stable endogenous inhibitor of cyclic AMP-dependent protein kinase.

3. *Phosphorylation of nuclear proteins after incubation of nuclei with Type I and II protein kinase.* Figure 3 shows that when nuclei prepared from rat adrenal medulla were incubated with Type I cyclic AMP-dependent protein kinase and 0.7 μM cyclic AMP the incorporation of $^{32}PO_4^{-3}$ into nuclear material proceeded at an initial rate of about 150 pmole/mg protein/min. This rate was twice that measured in nuclei incubated without exogenous protein kinase or with exogenous Type II cyclic AMP-dependent protein kinase and 0.7 μM cyclic AMP. The protein kinase activity remaining in the supernatant of the pelleted nuclei was also measured using a calf histone mixture as PO_4^{-3} acceptor protein. Only 30% of the initial protein kinase activity was recovered from the supernantant of nuclei incubated with Type I cyclic AMP-dependent protein kinase and cyclic AMP (0.7 μM), whereas about 70% of the activity was recovered from the supernatant of nuclei incubated with Type II cyclic AMP-dependent protein kinase and 0.7 μM cyclic AMP.

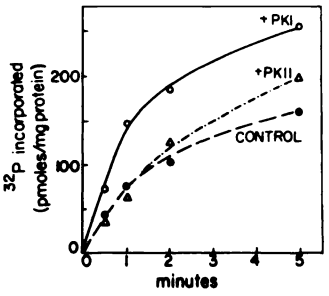

FIG. 3. *Protein phosphorylation in adrenal medulla nuclei incubated with protein kinase Type I or Type II and cyclic AMP*

One hundred microliter of nuclear suspension (0.32 M sucrose, 5 mM MgCl, 0.2 mM CaCl, 10 mM potassium phosphate, pH 6.5, approximately 300 μg of protein; for details see paragraph 3 of EXPERIMENTAL PROCEDURES) was mixed with 10 μl cyclic AMP (final concentration 5 μM) and 20 μl of partially purified cytosol Type I protein kinase (20 units/25 μg protein, ○) or Type II protein kinase (20 units/20 μg protein, △). After 60 min at 0°, the mixture was centrifuged at 5,000 × *g* for 10 min. The pellet nuclei were washed once with 100 μl of sucrose-buffer, then suspended in 270 μl of the same buffer containing 30 mM NaF and after addition of 30 μl of 500 μM [γ −^{32}P]ATP (S.A. 100 μCi/μmole) used to measure the incorporation of ^{32}P into nuclear protein at different time intervals. The supernatants obtained from the last two centrifugations were pooled and the protein kinase activity remaining was determined in order to monitor the recovery of protein kinase Type I and Type II added to the nuclear suspension. Control nuclei (●) (no Type I or Type II protein kinase added) were run through the different steps.

B. *Studies with pineal gland.*

Adrenal medulla and pineal gland have the following similarities with regard to their innervation and second messenger responses elicited by stimulation of postsynaptic receptors: 1) both tissues are innervated by only one type of afferent neurons; 2) in both tissues, the injection of postsynaptic receptor agonists causes a stimulus-coupled increase in the cyclic AMP content of postsynaptic cells (3, 34). However, these two tissues present important differences: the cytosol preparations of adrenal medulla contain Type I and Type II cyclic AMP-dependent protein kinase (Fig. 2); those of pineal contain mainly Type II cyclic AMP-dependent protein kinase (Fig. 4). In pineal extracts the major peak of Type II protein kinase was 9–10 times larger than that of Type I protein kinase peak eluted between 0.05 and 0.1 M NaCl. The properties cyclic AMP-dependent protein kinase activity present in pineal cytosol of rats treated 2 hr before with isoproterenol (1 or 4 injections) were practically identical to those of controls.

The data reported in Fig. 5 show that

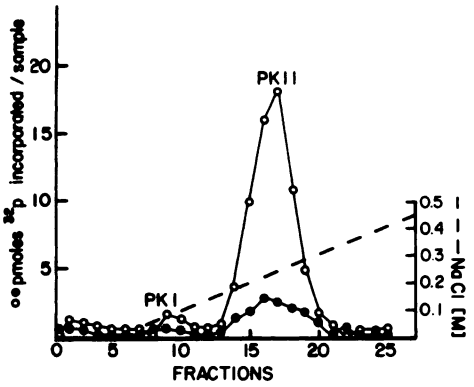

FIG. 4. *DE-52 Whatman column chromatography of cytosol obtained from pineal glands*

For details, see Fig. 2. Fractions of 300 μl are collected and assayed in presence (○) and absence (●) of 0.7 μM cyclic AMP. PK I = Type I protein kinase, PK II = Type II protein kinase.

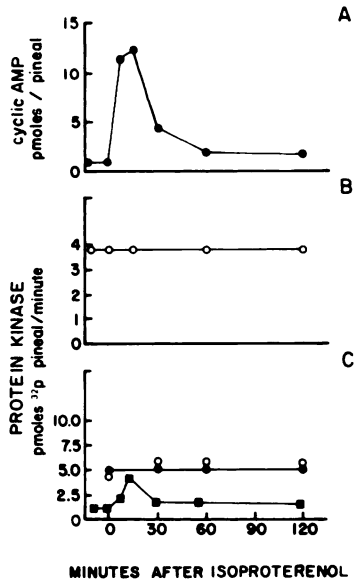

FIG. 5. *Changes of cyclic AMP concentration (panel A), cytosol protein kinase activity (panel B), cytosol catalytic subunits of cyclic AMP-dependent protein kinase and nuclear cyclic nucleotide independent protein kinase activity (panel C) in pineal gland of rats receiving isoproterenol (60 μmole, i.p.)*

At the times indicated on the abscissa, pineal glands were removed from the brain and assayed for cyclic AMP and protein kinase activity as described in the EXPERIMENTAL PROCEDURES. Panel A refers to changes in cyclic AMP content. Panel B depicts the changes of the total (measured in the presence of 0.7 μM cyclic AMP) cytosol cyclic AMP-dependent protein kinase activity. Panel C indicates the changes of catalytic subunits of cyclic AMP-dependent protein kinase in cytosol (■——■) and the changes of nuclear protein kinase activity measured in presence (●——●) or absence (○——○) of 0.7 μM cyclic AMP.

While the assay of cytosol and nuclear protein kinase was run in samples obtained from the same animals, the cyclic AMP determination was done in a separated group of rats sacrificed with microwave irradiation.

The cytosol cyclic AMP-dependent protein kinase activity was calculated by using three different criteria (see paragraph 8 of the METHODS section). The cytosol cyclic nucleotide independent activity (45%) (which failed to change after isoproterenol treatment) was subtracted from the total cytosol activity. Based on the same criteria, nuclear extracts did not contain appreciable amounts of cyclic AMP-dependent protein kinase. Addition of cyclic AMP (0.7 μM) to the nuclear extracts, also after extensive dialysis, failed to stimulate the protein kinase activity.

following the injection of a single dose of isoproterenol (60 μmole/kg, i.p.), the cyclic AMP content in the pineal gland was maximally elevated in 10-15 min and returned toward basal levels in about 30 min. The activity of free catalytic subunits of cyclic AMP-dependent protein kinase present in cytosol increased during the first 30 min following isoproterenol but returned to basal levels by 45 min and remained at control levels during the successive 90 min (see Fig. 5C). In contrast the cyclic AMP-dependent protein kinase (measured in the presence of 0.7 μM cyclic AMP) of cytosol (Fig. 5B) or the protein kinase activity of nuclear extracts (see Fig. 5, panel C) from pineal of rats treated with isoproterenol were equal to those of control rats.

To simulate in pineal the increase of cyclic AMP content elicited in medulla by postsynaptic receptor activation, it was necessary to give four successive injections of isoproterenol (60 μmole/kg i.p.) every 30 min (Fig. 6). The response of pineal adenylate cyclase after repeated isoproterenol injections showed rapid tolerance; nevertheless, the pineal concentration of the cyclic nucleotide after the dosage schedule mentioned above (see Fig. 6) was maintained elevated for about 2 hr. Although as in the adrenal medulla (see Fig. 1) the activity of catalytic subunits of cyclic AMP-

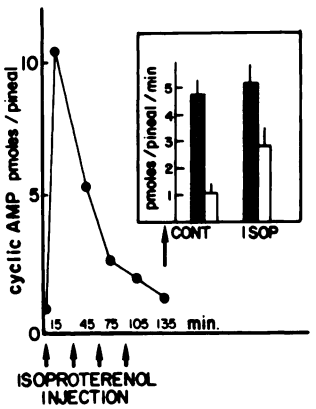

FIG. 6. *Cyclic AMP concentration and cytosol cyclic AMP-dependent protein kinase after repeated administration of isoproterenol*

Isoproterenol (60 μmole/kg, i.p.) was injected every 30 min for 4 times. For the cyclic AMP determination, rats were microwaved at 15 minutes after each isoproterenol injection. For the cyclic AMP-dependent protein kinase assay the animals were sacrificed 30 min after the last isoproterenol injection and the data are reported in the insert.

All the conditions were the same as those used in the experiment reported in Fig. 5. Hatched column: cytosol cyclic AMP-dependent protein kinase = assay in presence of 0.7 μM cyclic AMP; white column: cytosol cyclic AMP-dependent protein kinase = assay in absence of exogenous cyclic AMP. Each bar represents the mean of six determinations. Vertical line over the bars represents the standard error of the mean. CONT = rats receiving saline; ISOP = rats receiving isoproterenol.

dependent protein kinase present in the cytosol of pineal (see Fig. 6) remained elevated longer than 2 hr, unlike in the medulla the total cyclic AMP-dependent protein kinase activity measured in the cytosol failed to decrease (compare Fig. 1 and Fig. 6). Moreover, the protein kinase activity of nuclear extracts measured at 4 and 7 hr after isoproterenol in the presence or absence of 0.7 μM of cyclic AMP was 4.7 and 4.9 pmole $^{32}PO_4^{-3}$/pineal/min, respectively, and very similar to that of pineal nuclei of control rats (5.2 pmole of $^{32}PO_4^{-3}$/pineal/min).

DISCUSSION

In highly specialized mammalian cells the interaction of transmitters or hormones with their receptor sites may induce functional modifications of intracellular regulatory proteins through a cyclic AMP-mediated phosphorylation process (4, 13, 14, 17, 35).

From various studies on cyclic AMP system it emerges that particle-bound adenylate cyclase and protein kinase are part of an overall compartmentalized system (36). Thus the proposal of a cyclic AMP-mediated protein kinase translocation (5, 17, 37) acquires a particular physiological significance in order to explain the specificity of the changes in nuclear metabolism in response to hormones and neurotransmitters.

The data reported here suggest that the adaptive processes of rat adrenal medulla cells following stimulation of nicotinic receptors (2–8) are mediated by an increase of cyclic AMP and a cyclic AMP-mediated intracellular translocation of cyclic AMP-dependent protein kinase subunits. In rat adrenal medulla drug or cold exposure-induced stimulation of nicotinic receptors is consistently associated with a decrease of total cytosol protein kinase and an increase of particulate bound protein kinase activity (see Fig. 1 and 2–10). Assuming that the synthesis rate of protein kinase is constant, calculations show that during translocation, the enzyme that has disappeared from medullary cytosol can be recovered in other subcellular fractions (10, 38). More precisely the data presented here allow us to estimate that 4 to 7 hr after reserpine or cold exposure the increase in cyclic AMP-dependent protein kinase found in the nuclear fraction accounts for 20-30% of the protein kinase activity lost from the cytosol. This increase in the nuclear content of catalytic subunits of cyclic AMP-dependent protein kinase presumably reflects nuclear translocation of the catalytic subunits of cytosol cAMP-dependent protein kinase, not only because the time courses of the decrease and increase (10) are coincident and the increased enzyme activity detected in the nuclei transfers PO_4^{-3} from ATP to histone preferentially, but because the protein kinase that increases in nuclei after reserpine or cold exposure is selectively inhibited by the addition of regulatory subunits of cyclic AMP-dependent protein kinase (see Table 2). The occurrence of a

selective intracellular translocation of "mobile" catalytic subunits of cyclic AMP-dependent protein kinase of Type I (33) as shown in Fig. 2 and Table 2 was never reported before. However it is reminiscent of the translocation of protein kinase proposed to occur in uterus after beta-adrenergic stimulation (18) and in liver after glucagon treatment (17). In heart tissue, the physiological significance of such a translocation process has been challenged because the original experiments were performed using low ionic strength buffers, which would have allowed artificial binding of free catalytic subunits of cyclic AMP-dependent protein kinase during the homogenization procedure (39). More recently, however, operating with isotonic buffers, the possibility that the "mobile" catalytic subunit of cyclic AMP-dependent protein kinase can diffuse through the soluble portion of the cells and can catalyze the hormone direct control of compartmentalized protein phosphorylation has been demonstrated by Corbin *et al.* in heart (36) and by Spielvogel *et al.* in the ovary (14).

In our experiments the increase in the number of free catalytic subunits of cyclic AMP-dependent protein kinase in cytosol is terminated at 2 hr; thus, it is improbable (see Fig. 1C) that the increase in the catalytic subunits measured in nuclear extracts 5 hr later (Fig. 1C) is artifactual as suggested for experiments in which the changes of protein kinase activity occur simultaneously in the two compartments (39). In fact, the increase in nuclear phosphorylation due to accumulation of catalytic subunits of cyclic AMP-dependent enzyme was measured several hours after the activity of the free catalytic subunits of cyclic AMP-dependent protein kinase present in cytosol had returned to basal values (Fig. 1). Since the increase in phosphorylating activity persisted when the nuclei were treated with Triton X-100, a cytoplasmic contamination or changes in nuclear membrane permeability could be excluded (11). Moreover the present results (see Table 3) suggest that when the catalytic subunits of cyclic AMP-dependent protein kinase are translocated into the nucleus they can express their phosphorylating activity using endogenous nuclear proteins as PO_4^{-3} acceptors.

Since rat adrenal medulla are very small in size, the nature of the phosphorylated nuclear proteins was explored using nuclei isolated from cow adrenal medulla and enriched in cytosol cyclic AMP-dependent protein kinase (11). Among the endogenous nuclear proteins the best substrate for the cyclic AMP-dependent protein kinase taken up by the nuclei were the "non-histone proteins" tightly bound to chromatin (11).

A point which has not been considered in the results section but that requires attention is whether regulatory subunits are also translocated. Experiments are now in progress to elucidate this point. It is worth mentioning that the protein kinase activity extracted from nuclei of rat treated with reserpine is not influenced by cyclic AMP or by extensive dialysis in low salt buffer, a condition that notably facilitates reaggregation of catalytic and regulatory subunits (see Table 2). It could be argued that the procedure used to extract the nuclear protein kinase is not sufficient to remove regulatory subunits from chromatin. This is unlikely in light of the observation[2] that [^{3}H] cyclic AMP binding to cytosol or nuclei is the same in control and reserpine-treated rats. Based on these considerations we propose that regulatory subunits fail to follow the nuclear translocation of their catalytic subunits stechiometrically. This concept is in line with observations in ovary (14), heart (36) and bovine adrenal medulla (11).

Chromatography of rat adrenal medulla cytosol on Whatman DE-52 column yielded two major peaks of cyclic AMP-dependent protein kinase activity eluting at 0.1 and 0.2 M NaCl, respectively (Fig. 2). This elution position suggests that the cytosol of adrenal medulla contains Type I and Type II cyclic AMP-dependent protein kinases as recently defined by Corbin *et al.* (33).

Assuming that the rate of synthesis of Type I and II cyclic AMP-dependent protein kinase is not changed during translocation it can be inferred that in adrenal

[2] Unpublished results.

medulla only catalytic subunits of Type I protein kinase are translocated to the nucleus (see Fig. 2). In contrast, in the pineal gland, which contains mainly Type II cyclic AMP-dependent protein kinase, activation of the cytosol enzyme by beta adrenergic receptor stimulation fails to be associated with the translocation of catalytic subunits by cyclic AMP-dependent protein kinase from cytosol to the nuclei. Lack of nuclear protein kinase translocation in pineal after release of catalytic subunits to cyclic AMP has also been recently reported by Winters *et al.* (40).

To confirm the results of the *in vivo* studies, we have carried out experiments on the phosphorylation of nuclear protein following incubation of medullary nuclei with cyclic AMP and the two types of protein kinase present in medullary cytosol. The nuclei were washed after the incubation with Type I or II protein kinase and the phosphorylation of nuclear protein measured thereafter. These experiments (Fig. 3) have shown that the phosphorylation of nuclear protein increases after incubation with Type I but not with Type II protein kinase.

This finding raised the question of the specificity and functional significance of these multiple forms of protein kinase. In the ovary of the chinese hamster, during the cell cycle, Costa *et al.* (41) obtained evidence for cell cycle specific expression of Types I and II protein kinase. More recently, a differential role for Type I and II protein kinase in lymphocyte mitogenesis has been reported by Buys *et al.* (42). Using the cytosol and the particulate fraction of adrenal medulla or pineal gland, we have shown another condition that distinguishes Type I from Type II cyclic AMP-dependent protein kinase.

Since work from several laboratories has shown that various protein kinases contain identical catalytic subunits, but the properties of the regulatory subunits are different, it has been suggested that the specific properties of the various cyclic AMP-dependent protein kinases reside in differences in their regulatory subunits (43–46). Hence, it is tempting to speculate that the regulatory subunits will control the rate of nuclear uptake of the catalytic subunits by controlling their rate of reassociation, which is regulated by factors such as tissue content; compartmentalization of cyclic AMP and ATP (36, 44); binding of ATP to regulatory subunits of Type I protein kinase (47); autophosphorylation of Type II protein kinase (44); presence of endogenous inhibitory proteins (30). It is possible that in rat adrenal medulla *in vivo* these factors will influence differentially the reassociation rate of Type II and Type I cyclic AMP-dependent protein kinase. Alternatively, it may be possible that the difference in the surface charge of the regulatory subunits of Type I and II enzymes could allow for separate compartmentation of the enzymes. Finally, it may be speculated that nuclear membrane permeability changes as a result of the specific interaction with only one type of regulatory subunit, but not with the other two regulatory subunits.

Based on the present experiments we can propose that nuclear translocation and retention of catalytic subunits of cyclic AMP-dependent protein kinase is neuronally mediated and might regulate transcription of messenger RNA. However, the molecular mechanisms involved in the nuclear uptake of catalytic subunits are not yet understood in their specific regulatory features.

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