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Protein Kinase Activation as an Early Event in the Trans-Synaptic Induction of Tyrosine 3-Monooxygenase in Adrenal Medulla

(cyclic AMP/cyclic GMP/catecholamine)

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ABSTRACT **An increase of cAMP/cGMP concentration ratio is the earliest stimulus-coupled biochemical change that has been measured in the adrenal medulla during the trans-synaptic induction of tyrosine 3-monooxygenase [EC 1.14.16.2; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating)]. In adrenal medulla of rats receiving reserpine alone (16 μmol/kg intraperitoneally) or reserpine and propranolol (40 μmol/kg intraperitoneally 30 min before reserpine), or exposed to 4° for 4 hr, the extent and duration of the increase of the cAMP/cGMP concentration ratio exceeds the critical value that is required to activate the protein kinases (EC 2.7.1.37; ATP:protein phosphotransferase). Gel filtration experiments indicate that during this activation, the catalytic subunit of the protein kinase (low-molecular-weight enzyme) is released from the holoenzyme. The activation of protein kinase lasts longer than the increase in the cAMP/cGMP concentration ratio and appears to be an obligatory early event that mediates the increase of tyrosine monooxygenase synthesis. The trans-synaptic induction of the monooxygenase in adrenal medulla appears to be due to an increased synthesis of the enzyme; the rate for monooxygenase degradation is proportional to the number of enzyme molecules that are present at various stages of the induction process.**

Appropriate environmental stimuli or administration of drugs that directly or indirectly activate the cholinergic nicotinic receptors in rat adrenal medulla cause a delayed long-lasting induction of tyrosine 3-monooxygenase [EC 1.14.16.2; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating)] (1–5). This induction of the rate-limiting enzyme for catecholamine synthesis (6) is mediated trans-synaptically (7).

We have reported several lines of evidence to support the hypothesis that in adrenal medulla the trans-synaptic induction of the monooxygenase is triggered by an early increase of the concentration ratio of 3′:5′-adenosine monophosphate (cAMP) and 3′:5′-guanosine monophosphate (cGMP), lasting 1 hr or longer (5, 8–12). Our findings have been confirmed (13, 14), but modifications of our procedure have led to experimental situations where the trans-synaptic induction of medullary tyrosine monooxygenase can occur in the absence of a stimulus-coupled increase of the cAMP content (13, 14). When the inducing stimulus elicits hypothermia, the lack of correlation between cAMP increase and monooxygenase induction is only apparent because the increase of cAMP is delayed (9, 11, 12, 15).

These discrepancies have suggested that a crucial role for cAMP and/or cGMP in the trans-synaptic induction of tyrosine monooxygenase cannot be definitely proved by merely confirming or denying that the medullary content of these nucleotides changes in coincidence with a stimulus that induces the enzyme. Since the receptor that mediates the action of cyclic nucleotides in eukaryotic cells is the regulatory subunit of protein kinase (EC 2.7.1.37; ATP:protein phosphotransferase) (16, 17), we have investigated the number of unsaturated cAMP binding sites and the susceptibility of the protein kinase to cAMP activation at various times after the inducing stimulus.

METHODS

Male Sprague–Dawley rats (Zivic Miller, Allison Park, Pa.) of about 100 g were used; their adrenals were either intact or monolaterally (left side) denervated 5–7 days before the experiment.

cAMP and cGMP Assay. The adrenal medulla was dissected from cortex (18), and the perchloric acid (0.4 M) homogenate was prepared (5). The medullary content of cAMP and cGMP was measured after the two nucleotides were purified and separated by column chromatography (19).

Protein Kinase Activity Ratio. To measure the protein kinase activity, the adrenal medulla was homogenized (1/15 w/v) in 10 mM potassium phosphate buffer (pH 6.5) containing 10 mM EDTA and 5 mM aminophylline. The protein kinase activity of the clear supernatant (20,000 × *g*, 20 min at 4°) was assayed in the presence and in the absence of cAMP (0.7 μM) by adding 10 μl of the supernatant to an incubation mixture containing in a total volume of 140 μl: 10 μl of 0.1 M Mg acetate buffer (pH 6.0), 40 μl of 0.5 M Na acetate buffer (pH 6.0), 10 μl of 0.3 M NaF, 10 μl of 65 mM aminophylline, 20 μl of calf histone mixture (2 mg/ml), 30 μl of H_2O, and 10 μl of cAMP or H_2O. The reaction was initiated by adding 10 μl of 0.5 mM [^{32}P]ATP (specific activity, 100 μCi/μmol). After 5 min of incubation at 30°, the reaction was terminated by pipetting 50 μl of the reaction mixture onto filter paper discs previously soaked in saturated EDTA solution (20). The assay was run in duplicate, with boiled tissue as a blank. To evaluate the extent of interferences caused by ^{32}P incorporation into polynucleotides, we omitted the histones from the above described mixture for the protein kinase assay. After incubation, the polynucleotides were removed according to Kabat (21). The values obtained in this assay were comparable to those obtained with boiled tissue blank in the presence or in the absence of histones.

The susceptibility of the protein kinase of adrenal medulla to activation by cAMP was expressed by the ratio between the ^{32}P incorporated in the histones in the absence of cAMP (−cAMP) and in the presence of cAMP (+cAMP).

In some experiments the same measurements were performed in adrenal medulla homogenates prepared in potas-

sium phosphate, EDTA, aminophylline buffer (pH 6.5) with the addition of 0.5 M NaCl to prevent the possible reaggregation of the protein kinase subunits during homogenization (22).

In order to separate the protein kinase subunits, the supernatant fraction derived from adrenal medulla homogenates was chromatographed on a Sephadex G-200 column (0.34 × 11 cm). The column was equilibrated with 10 mM potassium phosphate buffer (pH 6.5) containing 10 mM EDTA and 0.5 M NaCl (22). Fifty microliters of the supernatant fraction obtained by homogenizing 40 adrenal medullae (about 40 mg) in 120 μl of 10 mM potassium phosphate buffer (pH 6.5) containing 10 mM EDTA, 5 mM aminophylline, and 0.5 M NaCl were added to the column. In 20 min, 15 fractions (50 μl each) were eluted with 2 ml of 10 mM potassium phosphate buffer (pH 6.5) containing 10 mM EDTA and 0.5 M NaCl. The enzyme activity (+ or −cAMP) was determined in 15 μl of each fraction. The total time interval between the homogenization and the termination of the assay was about 1 hr.

Tyrosine Monooxygenase Synthesis and Degradation. The rate of monooxygenase synthesis was measured by the immunoprecipitation technique 90 min after pulse labeling of the tissue amino-acid pools (23). The rate of monooxygenase degradation was determined by measuring the decay of radioactivity incorporated in the enzyme occurring between 9 and 100 hr after an intraperitoneal injection of [^{3}H]leucine (700 μCi per rat; specific activity 50 Ci/mmol). The ^{3}H incorporated into the monooxygenase was estimated by immunoprecipitation technique with a specific antibody to the enzyme (22). The first-order rate constant (k_d) for the degradation of the monooxygenase was calculated from the decay of the radioactivity incorporated into the enzyme. The rate constant for the enzyme synthesis (K_s) was calculated from the equation $K_s = k_d[E]$, where [E] is the specific activity of the enzyme. The antibody against the monooxygenase of rat tissues was prepared as described (23).

Tyrosine monooxygenase activity: Immediately after removal, the adrenal glands were homogenized in 500 μl of 50 mM Tris-acetate (pH 6) containing 0.2% Triton X100. The homogenate was dialyzed against the same buffer for 4 hr, centrifuged at 1.1 × 10^4 × g for 10 min at 4°, and 25 or 50 μl of the supernatant fraction were used for determination of enzyme activity by the method of Waymire *et al.* (24) as modified by Zivkovic *et al.* (25).

ATP measurement: ATP was assayed in the supernatant of the perchloric acid tissue extract or in the reaction mixture used to determine the protein kinases after purification on an alumina column (18) by the luciferin–luciferase method (26).

Phosphoprotein phosphatase activity: The phosphoprotein phosphatase (EC 3.1.3.16) activity was measured by the decay of ^{32}P incorporated in the histones (27). [^{32}P]Histones were prepared by incubating for 10 min at 30° a partially purified beef heart protein kinase (28), [^{32}P]ATP, and calf histone mixture (Calbiochem, Calif.). After addition of cold ATP and EDTA (27), the adrenal medulla 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°.

cAMP hydrolysis: The rate of cAMP hydrolysis in the reaction mixture used to determine the protein kinase activity was measured by adding [^{3}H]cAMP (4000 cpm/0.2 pmol). After 5 min of incubation, the cAMP of the reaction mixture was purified (19) and the radioactivity was measured.

Protein was determined by the method of Lowry *et al.* (29).

TABLE 1. *Protein kinase activity of intact and denervated adrenal medulla of rats exposed to 4° for 1.5 hr*

Addition of cAMP (0.7 μM)	^{32}P Incorporated in histone mixture (pmol × 10^{-2}/μg of protein per min)			
	Intact		Denervated	
	23°	4°	23°	4°
−cAMP	2.6 ± 0.35	4.2 ± 0.48*	1.9 ± 0.25	2.3 ± 0.32
+cAMP	13 ± 2.8	7.6 ± 0.90*	11 ± 1.8	10 ± 0.91
−cAMP/ +cAMP	0.20	0.56	0.17	0.22

Each value is the mean ±SE of five to eight experiments. The conditions of the assay are described in *Methods*. The left adrenal was denervated 5 days before the experiment; the right intact adrenal was used as a control. The protein kinase activity was measured in 2 × 10^4 × g supernatant of homogenate of four pooled medullae prepared as described in *Methods*.

* $P < 0.02$ when compared with intact adrenals obtained from rats kept at 23°.

RESULTS

Activation of Adrenal Medulla Protein Kinases by Cold Exposure. In adrenal medulla of rats exposed to 4° the cAMP content was increased for about 90 min (see Fig. 3); in contrast, the protein kinase activity measured in the absence of cAMP was increased for several hours (Table 1, Fig. 3). Denervation inhibits the increase of cAMP content elicited by cold (8) and the protein kinase activation (Table 1). In the supernatant of adrenal medulla homogenates from rats exposed to 4°, the ratio of the ^{32}P incorporated into histone with or without the addition of cAMP was higher than that in medulla from rats kept at 23° (Table 1). When the adrenal had been denervated 5 days before the experiment, this ratio failed to change during exposure to 4° (Table 1).

The data of Table 1 show that in conditions of maximal stimulation (addition of 0.7 μM cAMP) the total protein kinase activity of adrenal medulla from rats exposed to cold was about 30% lower than that of rats kept at 23°. Since the phosphodiesterase activity was almost completely inhibited by aminophylline, it is unlikely that a change in the cAMP metabolism was operative. The ATP concentration and the histone dephosphorylation were similar in the intact and denervated adrenal medulla from rats kept at 4° or at 23°. Moreover, the apparent K_m of the protein kinase for cAMP was the same (10^{-7} M) in adrenal medulla from rats kept at 23° or at 4°.

The exposure to 4° increased the protein kinase activity measured in the absence of cAMP also when the homogenates were prepared in the presence of 0.5 M NaCl. Moreover, in intact medulla from rats exposed to cold, the total enzyme activity measured in the presence of 0.7 μM cAMP and 0.5 M NaCl was reduced by about 30% when it was compared to either denervated medulla of rats exposed to cold or to intact medulla of rats kept at 23°.

In another group of experiments the protein kinase activity

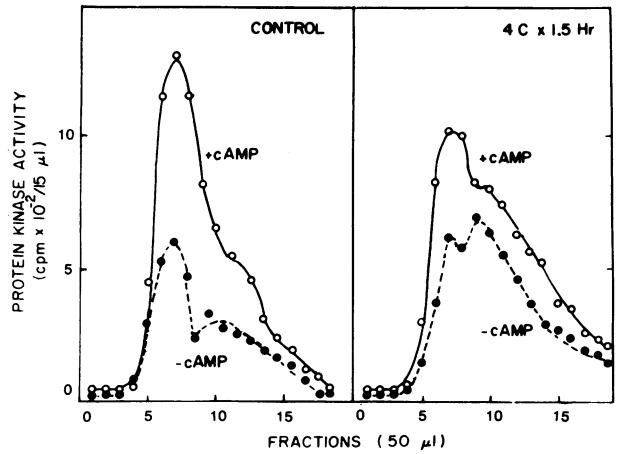

FIG. 1. Sephadex G-200 (0.3 × 11 cm) elution profile of the protein kinase activity from adrenal medulla of rats kept at 23° (*left*) or from adrenal medulla of rats exposed to 4° for 1.5 hr (*right*). Fifty microliters of $2 \times 10^4 \times g$ clear supernatant containing 450 µg of protein (see *Methods* for details) were applied to each column. Fractions (50 µl) were collected, and the protein kinase activity was determined in each fraction in the absence (●) and in the presence (○) of 0.7 µM cAMP.

of the supernatant of adrenal medulla homogenates was measured after gel filtration on a Sephadex G-200 column. The elution profiles of Fig. 1 show that the homogenates of intact medulla from rats kept at 23° tested without cAMP contained two major peaks. The second peak contained the enzyme activity associated with low-molecular-weight proteins. In the elution profile of adrenal medulla homogenates from rats exposed to 4°, the second peak was broader and taller than that of homogenates from rats kept at 23°. In both homogenates the addition of 0.7 µM cAMP increased the protein kinase activity of the first peak by an extent greater than that observed in the second peak. Moreover, the increase of the protein kinase activity elicited by cAMP in the first peak was greater in adrenal medulla of rats kept at 23° than in that of rats kept at 4° for 1.5 hr. Fig. 2 shows that the elution profile of the protein kinase in medulla homogenates from rats exposed to cold (panel C) is similar to that of homogenates from normal rats preincubated with 50 µM cAMP (panel B).

Biochemical Events Preceding the Trans-Synaptic Induction of Tyrosine Monooxygenase in Adrenal Medulla. Some biochemical changes occurring in adrenal medulla of rats exposed to 4° for 4 hr and then returned to 23° were studied at various times after the beginning of cold exposure (Fig. 3). The body temperature of the rats remained within normal range throughout the experiment. The cAMP content increased after a few minutes of exposure to cold (panel A); this increase lasted about 90 min and was associated with a decrease of cGMP content (panel B). The ratio of the protein kinase activity measured with and without the addition of cAMP

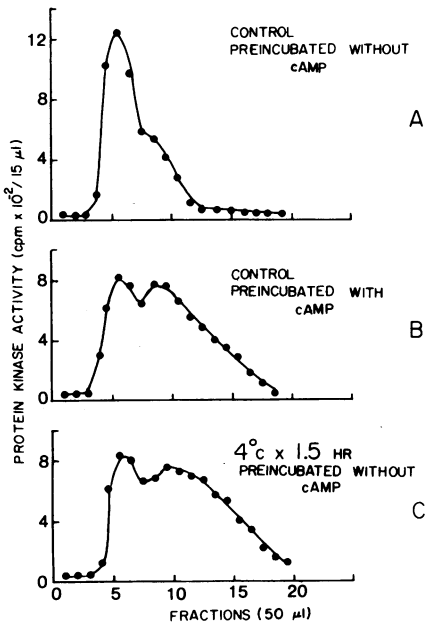

FIG. 2. Effect of exogenous cAMP (50 µM) and cold exposure on the Sephadex G-200 elution profile of the protein kinase from rat adrenal medulla. Adrenal medullae (about 50 mg) were homogenized in 0.5 M NaCl potassium phosphate EDTA aminophylline buffer (pH 6.5) as described in *Methods*. The homogenate was then incubated for 5 min at 30° in the presence or in the absence of cAMP (50 µM). After centrifugation at $2 \times 10^4 \times g$, 50 µl of the supernatant containing 260 µg of protein were applied to a Sephadex G-200 column (0.3 × 11 cm). Successive 50-µl fractions were collected, and the protein kinase activity was assayed in the presence of 0.7 µM cAMP. Panels A and B, homogenates of adrenal medullae obtained from rats kept at 23°. Panel C, homogenate of adrenal medullae obtained from rats exposed to 4° for 1.5 hr.

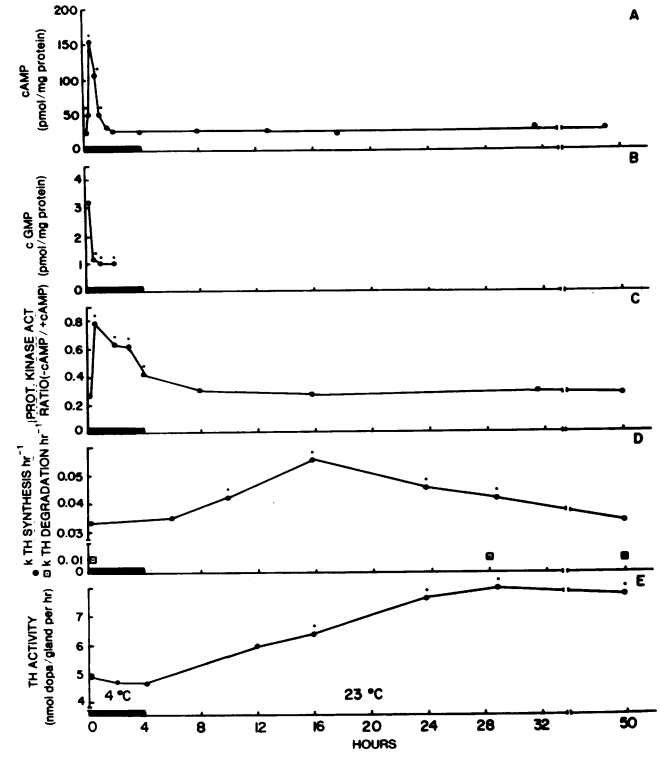

FIG. 3. Time course of biochemical changes preceding the delayed induction of tyrosine monooxygenase (TH) activity in adrenal medulla of rats exposed to 4°. (A) Changes of cAMP concentration; (B) changes of cGMP concentration; (C) protein kinase activity ratio; (D) fractional rate constant of monooxygenase synthesis and degradation; (E) monooxygenase activity. Each point represents the mean of at least five experiments. * $P < 0.05$ when compared with animals kept at 23°. ■, time at 4°C.

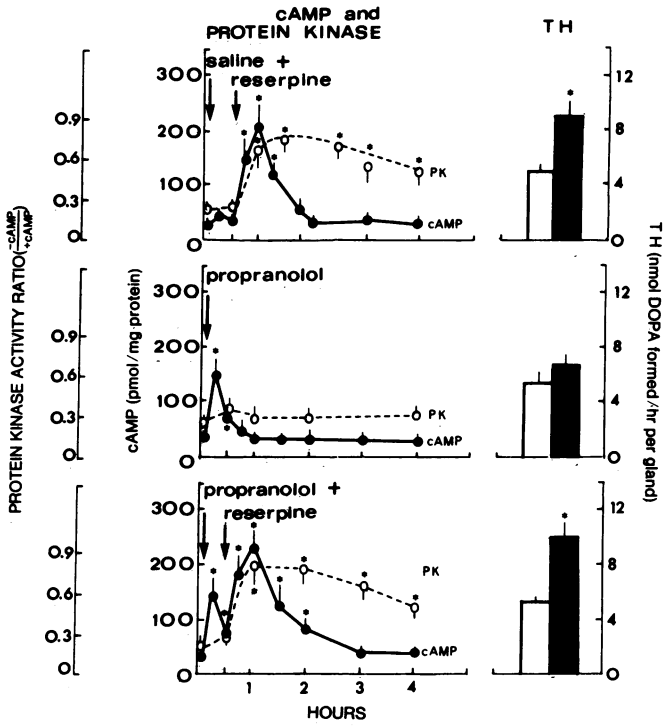

FIG. 4. cAMP concentration and protein kinase and tyrosine monooxygenase (TH) activity in adrenal medullae of rat after injection of reserpine, propranolol, and propranolol + reserpine. Reserpine (16 μmol/kg intraperitoneally) was injected 30 min after saline or propranolol (40 μmol/kg intraperitoneally). cAMP concentration and protein kinase (PK) activity in the absence or in the presence of 0.7 μM cAMP were measured in the first 4 hr after reserpine administration. Monooxygenase was measured 48 hr after reserpine or propranolol injection. $*P < 0.05$ when compared with control animals. Monooxygenase: (□) control, (■) treated. Each value is the mean ±SE of at least 10 determinations.

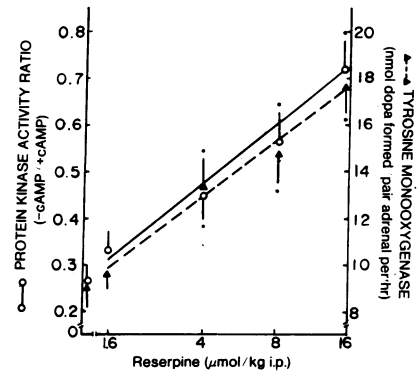

FIG. 5. Activation of protein kinase and induction of tyrosine monooxygenase in adrenal medulla of rat receiving various doses of reserpine. Reserpine was administered intraperitoneally; protein kinase activity ratio and monooxygenase were measured 1.5 and 48 hr after reserpine administration, respectively. Each point is the mean ±SE of eight protein kinase assays and five monooxygenase assays. Details of the calculation of +cAMP/−cAMP activity ratio and of assay procedures are given in *Methods*. Vertical bars represent standard error. $*P < 0.05$ when compared with solvent-treated rats. The slope of the line describing the dose-effect relationship for the change of kinetic state of protein kinase and for monooxygenase induction were statistically calculated (covariance analysis test), plotting the logarithm of the dose against the effect. Regression analysis shows that the two slopes are equal.

(0.7 μM) was increased, and this increase lasted longer than the changes of medullary cAMP content (panel C). The zero-order rate constant for monooxygenase synthesis was increased 10 hr after the beginning of cold exposure. It continued to increase for the successive 20 hr, and it returned to normal value at about 40 hr (panel D). Panel D also shows that the first-order rate constant for the degradation of the monooxygenase was unchanged at various times after exposure to 4°. The monooxygenase activity, which in this case measures the number of enzyme molecules, was increased at 16 hr after cold exposure (panel E) and reached a new steady state, which was maintained for at least 48 hr. The data of Fig. 3 also show that the monooxygenase activity began to increase after the K_s for synthesis of the enzyme was increased.

cAMP Content and Protein Kinase and Tyrosine Monooxygenase Activities in Adrenal Medulla of Rats Injected with Reserpine and Propranolol. Reserpine causes an immediate increase of the cAMP/cGMP concentration ratio (11, 12) and a delayed induction of monooxygenase activity in adrenal medulla (3). It was reported by Otten *et al.* (13) that when reserpine (16 μmol/kg intraperitoneally) was given 30 min after an injection of propranolol (40 μmol/kg intraperitoneally), the medullary cAMP content failed to increase; however, the induction of adrenal tyrosine monooxygenase remained unimpaired (13). We have repeated the experiment described by Otten *et al.* (13), but we could not confirm that propranolol blocks the increase of cAMP content produced by reserpine (Fig. 4A and C). Indeed, propranolol alone produced a short-lasting increase of cAMP content in adrenal medulla (Fig. 4B). This transitory increase of cAMP content elicited by propranolol failed to change the kinetic state of protein kinase and the monooxygenase activity of adrenal medulla (Fig. 4B). In contrast, the increase of cAMP produced by reserpine (16 μmol/kg intraperitoneally) lasted for about 90 min and was followed by an increase of the protein kinase activity measured in the absence of cAMP. The ratio of the protein kinase activities measured in the presence and in the absence of cAMP (0.7 μM) was increased for longer than 4 hr (Fig. 4A). Similar responses were also observed when reserpine was given 30 min after propranolol (40 μmol/kg intraperitoneally) (Fig. 4C). The change of the kinetic state of protein kinase of adrenal medulla homogenates from rats receiving either reserpine alone or reserpine and propranolol resembled that described after cold exposure (Table 1, Fig. 3).

The early activation of the protein kinase and the delayed induction of medullary tyrosine monooxygenase was measured after various doses of reserpine. It was found that 4 μmol/kg intraperitoneally of reserpine were about a threshold dose to elicit the early activation of protein kinase and the delayed induction of monooxygenase (Fig. 5). Moreover, the slope of the regression line defining the activation of protein kinase and the increase of monooxygenase activity after different doses of reserpine was the same (Fig. 5).

DISCUSSION

In the supernatant of adrenal medulla homogenates the activity of the protein kinase measured in the absence of exogenous cAMP is increased during cold exposure or after reserpine injection. The early activation of the protein kinase

and the delayed induction of tyrosine monooxygenase elicited by reserpine appear to be dose related. The protein kinase activation is concomitant with an increase of the cAMP/cGMP concentration ratio lasting 1 hr or longer. The data of Figs. 3 and 4 show that in adrenal medulla the duration and extent of the cAMP increase must reach a critical value before it changes the kinetic state of the protein kinase. Since the increase of the protein kinase activity reported in this paper is not due to an artifact occurring during the homogenization (Figs. 3 and 4), we suggest that it reflects an activation of this enzyme *in vivo*. This activation is characterized by an increase of the protein kinase activity measured in the absence of exogenous cAMP and by a certain decrease of the enzyme's susceptibility to the stimulation by cAMP. Our results show that this resistance to cAMP stimulation cannot be ascribed to an increase of the cyclic nucleotide phosphodiesterase activity nor can it be explained by an artifact due to the activation of the enzymes that dephosphorylate histones or degrade ATP. Since the affinity of the protein kinase for cAMP is not changed, one might speculate that the decreased susceptibility to cAMP stimulation is due to a decrease in the number of enzyme molecules.

The data on Sephadex gel filtration presented in Figs. 1 and 2 indicate that in homogenates of intact adrenal medulla from rats kept at 23° the activation of protein kinase by exogenous cAMP is due to a stimulation of the activity of the high-molecular-weight enzymes. In contrast, the increase of protein kinase activity measured in the absence of cAMP in intact medulla of rats exposed to cold appears related to an increase in the activity of the low molecular weight enzymes. In agreement with similar observations in other tissues (22, 30), these results suggest that also in adrenal medulla the increase of cAMP content can produce a dissociation of the catalytic subunit from the less active molecule of the holoenzyme. The data of Table 1 show that this increase of the basal protein kinase activity, as well as the decrease of the protein kinase activity stimulated by cAMP, failed to occur in adrenals that were denervated 5 days before exposure to 4°. Thus, the mechanism of this activation appears to be trans-synaptic. The transitional activation of protein kinase appears to be related not only to the extent, but also to the duration of the increase in the cAMP (see Fig. 4). As suggested by early reports (11, 12), the medullary content of cAMP should remain elevated for longer than 1 hr and reach a peak value of about 10-fold the normal level (Figs. 3 and 4) in order to induce tyrosine monooxygenase. Both after reserpine injection (11) and after cold exposure (Fig. 3) the content of cGMP in medulla is decreased. It appears possible that this decrease of cGMP is operative in the sequence of events leading to induction of the monooxygenase, but the mechanism involved is not clarified by the present experiments.

The loss of about 30% of the total protein kinase activity in the $2 \times 10^4 \times g$ supernatant of adrenal medulla homogenates of rats exposed to 4° or receiving reserpine injections requires further consideration. One might surmise that the activated protein kinase has been translocated to other cell structures such as the nuclei. Although this hypothesis has been proposed to explain similar changes in protein kinase occurring in other tissues (31, 32), we have only preliminary data to suggest that such a hypothesis applies to our experiments with adrenal medulla.

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