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Regulation of transsynaptically elicited increase of 3′:5′-cyclic AMP by endogenous phosphodiesterase activator

(cyclic nucleotide phosphodiesterase/phosphorylation/protein kinase)

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ABSTRACT Experiments with cold exposure confirmed previous studies indicating that the endogenous protein activator of phosphodiesterase (PDEA) isolated by Cheung participates in the *in vivo* regulation of 3′:5′-cyclic adenosine monophosphate (cAMP) in adrenal medulla. This activator of cAMP phosphodiesterase (PDE) (3′:5′-cyclic-AMP 5′-nucleotidohydrolase, EC 3.1.4.17) is present in the particulate as well as the soluble fractions of rat brain. It was found that a purified cAMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37), in the presence of ATP and cAMP, stimulates 3-fold the release of PDEA from the particulate fraction of rat brain and adrenal medulla. The substrate for this phosphorylation could be either a membrane protein that binds PDEA or PDEA itself. *In vivo* evidence, however, obtained by injecting rats intraventricularly with [γ-^{32}P]ATP, indicates that the PDEA does not contain radioactive phosphate in its structure. Also, PDEA could not be phosphorylated by protein kinase *in vitro*.

The following mechanism is postulated: when the intracellular content of cAMP increases it activates a protein kinase which phosphorylates a PDEA-binding membrane protein and releases PDEA. In turn this binds to activator-deficient high-K_m PDE and decreases its K_m to facilitate the hydrolysis of the increased concentration of cAMP.

When the postsynaptic nicotinic receptors of rat adrenal medulla are activated transsynaptically the 3′:5′-cyclic adenosine monophosphate (cAMP) content of chromaffin cells increases (1–4). The onset of the response is coupled to the stimulus but its duration is not (5). We have reported that when the nicotinic receptors of adrenal medulla are stimulated by carbamylcholine the endogenous protein activator of cyclic 3′:5′-nucleotide phosphodiesterase (PDE) (3′:5′-cyclic-AMP 5′-nucleotidohydrolase, EC 3.1.4.17) increases in the soluble supernatant fraction (6). The present report shows that the increase of cAMP elicited in adrenal medulla by cold exposure is also associated with an increase of the endogenous PDE activator (PDEA) in the cytosol.

PDE, the only enzyme which hydrolyzes cAMP and 3′:5′-guanosine monophosphate (cGMP), exists in several molecular forms (7–10). The activity of a purified PDE that has only one K_m (350 μM) for cAMP and hydrolyzes cGMP (K_m – 5–9 μM) (11, 12) can be regulated by PDEA. The activator lowers the K_m of this PDE for cAMP and increases its V_{max} for cGMP (12, 13). The increase in cytosol PDEA activity which follows an increase in the cAMP content may be interpreted to indicate that cAMP triggers a compensatory mechanism that facilitates its own hydrolysis. To study the molecular nature of this regulation we have used as a model a membrane preparation from rat adrenal medulla or brain which contains bound PDEA.

Abbreviations: cAMP, cyclic 3′:5′-adenosine monophosphate; cGMP, cyclic 3′:5′-guanosine monophosphate; PDE, cyclic 3′:5′-nucleotide phosphodiesterase; PDEA, phosphodiesterase activator.

The present report shows that a phosphorylation by a cAMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) can regulate the release of PDEA from rat brain and adrenal medulla membranes.

METHODS

[^{3}H]cAMP (50 Ci/mmol) and [γ-^{32}P]ATP (22 mCi/mol) were purchased from New England Nuclear (Boston, Mass.). Unlabeled cAMP, ATP, and *Ophiophagus hannah* snake venom were purchased from Sigma Chemical Co. (St. Louis, Mo.). Histones (calf thymus) B grade were purchased from Calbiochem (La Jolla, Calif.).

Preparation of Enzymes. Activator-deficient high-K_m PDE was purified from rat brain by preparative polyacrylamide gel electrophoresis according to the method of Uzunov and Weiss (10). This enzyme, when rechromatographed on analytical gel electrophoresis, yielded a single protein band. The endogenous protein activator of PDE was purified to homogeneity from bovine brain as described by Lin *et al.* (13). This preparation showed only one protein band on analytical gel electrophoresis. Cyclic AMP-dependent protein kinase was prepared from bovine heart by the method of Kuo and Greengard (14).

Preparation of the Membrane Fraction. The particulate fraction used for measuring bound PDEA was prepared by homogenizing the rat tissue in 4 volumes of 0.32 M sucrose containing either 25 mM or 150 mM KCl. The homogenate was centrifuged for 1 hr at 105,000 × *g* in a Beckman ultracentrifuge. The 105,000 × *g* pellet was washed several times with 0.32 M sucrose and rehomogenized in 5 ml of buffer containing 32 mM Tris·HCl, pH 7.5, 1 mM $MgSO_4$, 20 μM $CaCl_2$, and 0.6 mM dithiothreitol. The homogenate was then dialyzed against 200 volumes of the above buffer for 3 hr with two changes. For measurement of total bound PDEA, the pellet was rehomogenized in 2 ml of 1% Triton X-100.

Assay of PDE Activity. PDE activity was assayed by the isotopic method of Filburn and Karn (15). The standard incubation mixture, in a final volume of 100 μl, contained 20 μM cAMP (150,000–180,000 cpm of ^{3}H), 32 mM Tris·HCl buffer, pH 8.0, 1 mM $MgSO_4$, 20 μM $CaCl_2$, 0.6 mM dithiothreitol, PDE (0.23 μg/μl), and PDEA. The incubation was carried out for 5 min at 37°.

Assay of PDEA Activity. PDEA was assayed by adding various tissue extracts, which were heated for 3 min at 95°, to an activator-deficient PDE and measuring the degree of enzyme activation. One unit of activity is defined as that amount of PDEA that will increase the activity of 23 μg of highly purified activator-deficient PDE by 50% using 20 μM cAMP under the standard conditions described for the PDE assay. PDEA was isolated from rat adrenal medulla accord-

ing to the procedure of Uzunov *et al.* (6).

Assay of Protein Kinase Activity. Protein kinase activity was measured in an assay containing 0.05 M sodium acetate buffer, pH 6.0, 0.05 M MgOAc, 0.7 μM cAMP, 25 μM [γ-^{32}P]ATP (1×10^6 cpm), protein kinase (0.58 μg/μl), and protein substrate in a final volume of 200 μl. The reaction was carried out for 5 min at 30° and was stopped by applying a 50 μl aliquot to a filter paper circle (Whatman 3MM), which was washed three times for 20 min each in the 5% trichloroacetic acid mixture used by Kuo and Greengard (14). The radioactivity was measured by counting the disk in 5 ml of Aquasol in a Beckman (LS-250) scintillation spectrometer.

Protein was measured by the method of Lowry *et al.* (16) using bovine serum albumin as a standard.

RESULTS

Increase of cAMP content and PDEA activity in adrenal medulla of rats exposed to 4°

The relationship between the concentrations of cAMP and the activity of PDEA in the soluble supernatant of adrenal medullae of rats killed at various times after exposure to 4° is presented in Fig. 1. The content of cAMP in adrenal medulla promptly increased upon exposure of the rats to the cold, reaching concentrations about 10-fold greater than normal. The concentration of cAMP began to decrease when PDEA activity in the cytosol increased significantly. The peak value of PDEA activity coincided with a sharp decline in cAMP concentration.

Distribution of PDEA in the particulate and soluble fractions of the cell

When adrenal medullae are stimulated transsynaptically the PDEA activity in cytosol rises promptly, suggesting that this increase in PDEA activity may not require protein synthesis (12). Furthermore, cycloheximide (215 μmol/kg) injected intravenously 3 hr before cold exposure failed to prevent the transsynaptically induced rise in soluble PDEA activity. It was, therefore, assumed that the PDEA might be released from storage sites by the increase in cAMP content. In order to look at this possibility we studied the distribution of PDEA between the particulate fraction and cytosol. We found that a large amount of PDEA is present in the particulate fraction of both adrenal medulla and brain homogenates (Table 1). In these studies the particulate fractions were solubilized by Triton X-100 and heated at 95° for 3 min [the activator is relatively heat stable (17)]; aliquots of these extracts were added to activator-deficient PDE to assay for PDEA (Table 1). The finding that a large amount of PDEA binds to the particulate fraction even after the homogenization with 0.15 M KCl, which minimizes nonspecific PDEA binding to membrane fractions (18), suggests that the PDEA is truly present in the membranes.

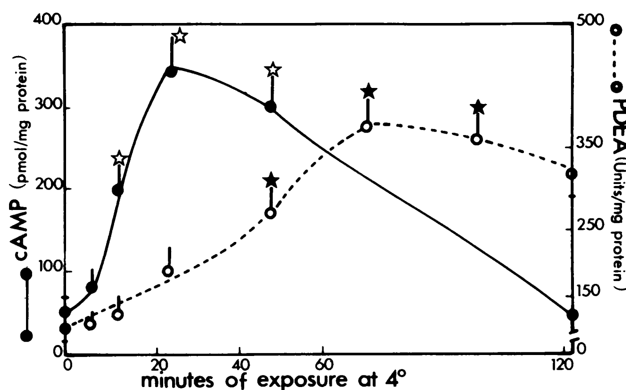

FIG. 1. Changes in cAMP concentration and PDEA activity in adrenal medullae of rats exposed to 4°. Male Sprague-Dawley rats (150–180 g) were kept at 4° in individual cages for different times. The adrenal glands were removed after decapitation and the medulla was separated from the cortex according to Guidotti and Costa (2). PDEA was isolated from the homogenate using the procedure of Uzunov *et al.* (6). PDEA activity in the cytosol was measured as described in *Methods*. The concentration of cAMP in the cytosol was measured by the method of Guidotti *et al.* (5). Each point represents the mean value of six experiments. Vertical bars indicate standard errors.

☆, ★: $P < 0.01$ that concentrations are the same as in control animals kept at 18°.

Release of PDEA from the membrane fraction

Since protein kinase is the only known mammalian receptor for cAMP, we studied the PDEA release from an enriched membrane fraction of brain homogenate in the presence of a purified cAMP-dependent protein kinase. We found that the protein kinase added to a membrane preparation in the presence of ATP and cAMP stimulates the release of PDEA from the membranes. The particulate fraction, homogenized in Tris·HCl buffer, pH 7.5, was incubated for 15 min alone or in the presence of ATP, protein kinase, cAMP, or the combination of these three. The mixture was centrifuged at

Table 1. Distribution of PDEA between particulate and soluble fractions of rat brain and adrenal medulla

Tissue	Cell fraction	KCl mM	PDEA units/ mg of protein	Total mg of protein	Total PDEA, units $\times 10^{-3}$	% of total PDEA
Brain	Particulate	25	4.3 ± 0.6	70	304	51
		150	3.7 ± 0.7	72	270	48
	Soluble	25	7.5 ± 1.1	39	292	49
		150	7.0 ± 0.9	40	285	52
Adrenal medulla	Particulate	150	0.74 ± 0.09	18	13.3	58
	Soluble	150	2.6 ± 0.3	3.7	9.6	42

Distribution of PDEA in particulate and soluble fractions of rat brain and adrenal medulla. A rat brain (1.2 g) was homogenized in 0.32 M sucrose containing either 25 mM or 150 mM KCl and spun as described in *Methods*. The pellet was rinsed with 0.32 M sucrose and rehomogenized in 2 ml of 1% Triton X-100. Aliquots containing a wide range of protein concentrations from both the homogenized pellet and the soluble supernatant fraction were exposed to 95° for 3 min and centrifuged and PDEA activity in the supernatants was determined by the degree of activation of activator-deficient PDE as described in *Methods*. Adrenal medulla of rat was homogenized in 0.32 M sucrose containing 150 mM KCl and treated as described for rat brain. The PDEA activity in the soluble supernatant and Triton-solubilized particulate fraction was determined as described in *Methods*. The figures for PDEA activity represent a mean value of six experiments (± SEM).

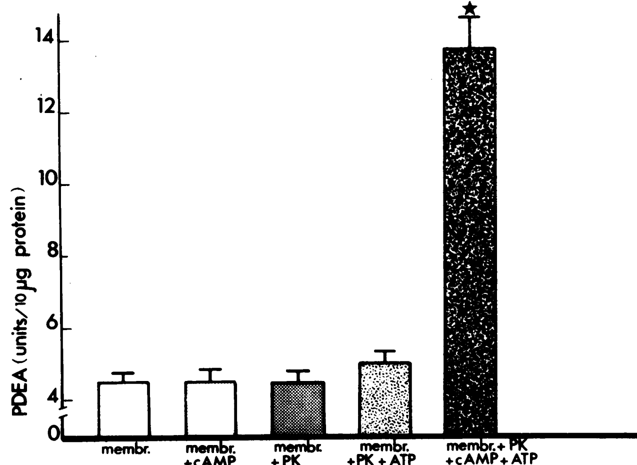

FIG. 2. Effect of phosphorylation on PDEA release. The particulate fraction was prepared as described in *Methods*. An aliquot of this fraction (40 μg) was incubated for 15 min at 30° alone or with combinations of 1 μM cAMP, protein kinase (PK) (0.58 μg/μl), and 25 μM ATP in a medium containing 0.05 M sodium acetate buffer, pH 6.0, and 0.05 M MgOAc in a total volume of 200 μl. The mixture was centrifuged to remove the membranes and the supernatant was boiled at 95° for 3 min. A 20 μl aliquot of the supernatant was used for PDEA determinations. Each column represents the mean value of eight experiments (± SEM).
★: $P < 0.01$ that value is the same as for membranes alone.

25,000 × *g* for 25 min. The supernatant was treated at 95° for 3 min to destroy any remaining PDE or protein kinase activity. An aliquot (0.02–20 μl) from the supernatant was added to activator-deficient PDE using 20 μM cAMP as substrate. Fig. 2 shows that in 15 min, 4.5 units/10 μg of protein of PDEA are released from the membrane fraction upon incubation in the presence or in the absence of a purified protein kinase. Moreover, cAMP or ATP added in the absence of protein kinase failed to accelerate the release rate of PDEA from the membrane fraction. However, when ATP, protein kinase, and 1 μM cAMP were added the rate of release of PDEA increased from 4 to about 13 units/10 μg of protein. The optimal cAMP concentration to release PDEA from brain membranes was 1 μM. When the cAMP concentration was increased to 10 μM and 100 μM, the PDEA release from the membrane preparation was decreased (Fig. 3). In a preparation of adrenal medulla membranes a spontaneous release of 2.0 units/10 μg of protein of PDEA was

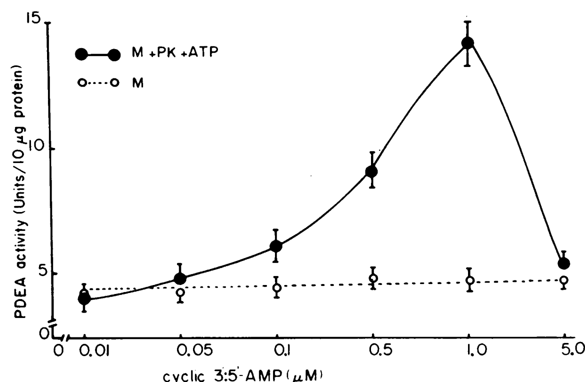

FIG. 3. Effect of cAMP on PDEA release from membranes (M) by protein kinase (PK). The conditions of the reaction were as described under Fig. 2. Each point represents the mean value of five experiments (± SEM).

increased to 4.2 units/10 μg of protein in the presence of protein kinase, ATP, and 1 μM cAMP.

The phosphate acceptor that triggers the PDEA release could be either the PDEA itself or a membrane protein that binds PDEA. Not only was PDEA not an *in vitro* substrate for the protein kinase, but PDEA was a weak inhibitor of the cAMP-dependent and -independent phosphorylation of histones by protein kinase.

In order to see whether PDEA can be phosphorylated *in vivo* rats were injected intraventricularly with [γ-^{32}P]ATP (12.5 μCi per rat) and decapitated 4 hr later. The brains were homogenized in 0.32 M sucrose and the PDEA fraction was separated on preparative polyacrylamide gel electrophoresis. This fraction was then concentrated and subjected to analytical disk gel electrophoresis. One set of gels was stained while a duplicate was sliced and the radioactivity was counted. The band corresponding to that of purified PDEA did not contain ^{32}P. This excludes the possibility that PDEA is a phosphate acceptor in the phosphorylation reaction that triggers its release from an enriched membrane preparation.

DISCUSSION

The transsynaptic activation of nicotinic receptors of adrenal medullae increases the cAMP content of chromaffin cells by about 10-fold (1, 2, 4, 6). When an exposure to 4° is the stimulus, this second messenger increase persists for only about 1 hr even though the rats are kept in the cold for a longer time interval (5). The present study has shown that in adrenal medulla the PDEA activity increases shortly after the cAMP has accumulated. Moreover, the decline of the cAMP content is accelerated when the PDEA activity has reached a peak value. The rise in cytosolic PDEA activity could not be prevented by cycloheximide; therefore, it is unlikely that new synthesis of PDEA causes the increases of PDEA activity.

To investigate the mechanism whereby the cytosol PDEA activity increases when the cAMP content is elevated we have used as a model enriched membrane preparations from brain and adrenal medullae of rats. The present report shows that a large amount of PDEA is present in the particulate fraction of brain homogenates prepared with 0.15 M KCl. Thus PDEA is actually bound to the membrane and is not held nonspecifically into the particulate fraction. The presence of PDEA in the brain membrane fraction suggested to us a model for studying the factors that could regulate the release of PDEA from the membrane. Because the activator-deficient PDE is a soluble enzyme, PDEA must be released from the membrane in order to interact with it. Since the increase of cAMP precedes the increase of PDEA activity in medulla and since protein kinase is the receptor for cAMP we tested whether a cAMP-dependent protein kinase could effect this release in brain and adrenal medulla.

We showed that the PDEA release from the membrane fraction of a brain or adrenal medulla homogenate can be accelerated after incubation with cAMP, ATP, and a purified (14) cAMP-dependent protein kinase. The phosphate acceptor in the phosphorylation catalyzed by cAMP-dependent protein kinase could be PDEA itself or a PDEA-binding protein present in the particulate fraction. Johnson *et al.* (19) found that the synaptic membrane fractions of brain homogenates were good substrates for exogenous cAMP-dependent protein kinase. Ueda *et al.* (20) also found endogenous cAMP-dependent phosphorylation of two specific pro-

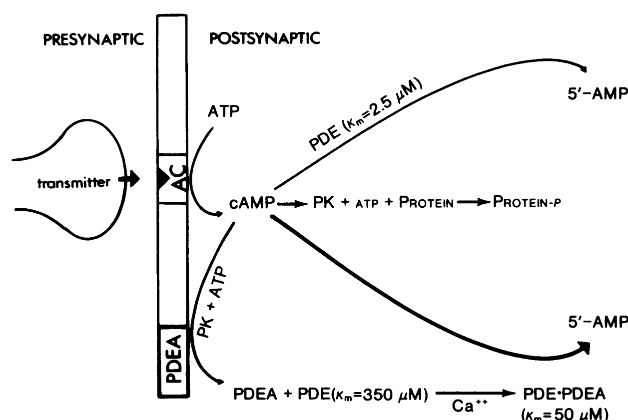

FIG. 4. The participation of PDEA in the regulation of the transsynaptically elicited increase in 3′:5′-cyclic AMP. AC = adenylyl cyclase, PK = protein kinase.

teins in synaptic membranes. It is possible that one of the membrane proteins that functions as phosphate acceptor could be a protein which binds PDEA. We propose that when this protein is phosphorylated, the PDEA is released into the soluble fraction. In preliminary experiments we have injected rats intraventricularly with [γ-^{32}P]ATP. The soluble PDEA of their brains did not incorporate ^{32}P, suggesting that the substrate for protein kinase is probably a membrane protein that binds PDEA.

In the presence of protein kinase a concentration of cAMP optimal to release PDEA from the particulate fraction is 1 μM. Very little release of PDEA was obtained with 0.1 μM cAMP. We found that the K_m for cAMP of the same protein kinase preparation was 10 nM using 40 μg of histones as substrate. There are several possible reasons for the higher cAMP concentration required to stimulate the release of PDEA from the particulate fraction with the protein kinase. The membranes may contain a cAMP-dependent protein phosphatase (21) which opposes the action of the cAMP-dependent protein kinase. A low K_m phosphodiesterase (K_m = 10 μM) is present in membrane; this enzyme could hydrolyze low concentrations of cAMP. Uedo *et al.* (20), working with brain membranes, found that 5 μM cAMP elicited a maximal stimulation of the phosphorylation of Protein I and Protein II in the synaptic membranes and that increasing the concentration of cAMP above this level caused a progressively lower stimulation of phosphorylation. Donnelly *et al.* (22) found that cAMP acts as a competitive inhibitor of ATP in protein phosphorylation. In the present report we also demonstrated that when the cAMP concentration is greater than 1 μM, the release of membrane bound PDEA by the protein kinase is reduced.

In conclusion, we have shown that PDEA is present in a particulate cell fraction of brain homogenates and its release into the soluble fraction is facilitated by a cAMP-dependent protein kinase. The results from this study allow us to postulate the following mechanism to explain the regulation of cAMP content in postsynaptic cells of adrenal medulla following transsynaptic stimulation of adenylyl cyclase (Fig. 4). This stimulus activates the membrane-bound adenylyl cyclase, and the intracellular content of cAMP is increased (Fig. 1). When the concentration of cAMP reaches a certain level, it activates a specific or specifically located protein kinase which phosphorylates a membrane protein that binds PDEA. This phosphorylation releases PDEA from the membrane fraction into the cytoplasm where the activator-sensitive PDE is located. In the presence of Ca^{++} (13, 23, 24) the PDEA binds to activator-deficient high-K_m PDE and decreases its K_m for cyclic AMP (12), thus facilitating the hydrolysis of cAMP when it accumulates. This scheme might become of value in explaining postsynaptic receptor super- and subsensitivity to transmitters. Moreover, it may offer a molecular basis to explain the mechanisms whereby drugs facilitate or inhibit transsynaptic activation of second messenger responses.

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