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Activation and nuclear translocation of protein kinase during transsynaptic induction of tyrosine 3-monooxygenase

(3′:5′-cyclic AMP/adrenal medulla/reserpine)

E. COSTA, A. KUROSAWA, AND A. GUIDOTTI

Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeths Hospital, Washington, D.C. 20032

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ABSTRACT **The tyrosine-3-monooxygenase activity [L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2] of rat adrenal medulla is induced 20–24 hr after the injection of reserpine (16 μmol/kg intraperitoneally). This and other inducing stimuli increase the 3′:5′-cyclic AMP (cAMP) content in the medulla for longer than 60 min and activate the cAMP-dependent protein kinase (ATP:protein phosphotransferase; EC 2.7.1.37) for several hours. Corticotropin (ACTH), dopamine, and propranolol do not induce the monooxygenase, but elicit an increase in the cAMP content of the medulla which fails to activate protein kinase and lasts less than 1 hr. A high- and low-molecular-weight protein kinase are separated by gel filtration from the 20,000 × *g* pellet extract of adrenal medulla homogenate. The activity of the low-molecular-weight enzyme is expressed as its ability to phosphorylate histone. The protein kinase activity of the pellet is increased between 3 and 17 hr after reserpine injection. Our evidence indicates that this increase is due to a translocation from cytosol to subcellular structures of a kinase that utilizes lysine-rich histone as phosphate acceptor. The protein kinase activity that is extracted from a purified nuclear fraction prepared from the adrenal medulla of rats injected 7 hr previously with reserpine is greater than that extracted from medulla of saline-treated rats.**

In neural tissue, intracellular communication is mediated by hormones and neurotransmitters. The latter not only regulate the polarity of the neuronal membranes, but they also influence the expression of the metabolic code in postsynaptic cells (1). In the rat adrenal medulla, the biosynthesis of tyrosine-3-monooxygenase [EC 1.14.16.2; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating)] is regulated transsynaptically (2–4). In chromaffin cells that are presistently (1 hr or more) stimulated by the acetylcholine released by nerve impulses from presynaptic terminals, the monooxygenase is induced 18–24 hr later (2–7). We have adopted this transsynaptic induction of tyrosine monooxygenase as a model to study the molecular mechanisms whereby the release of acetylcholine regulates the expression of the metabolic code in postsynaptic cells. The first event in this induction is an increase in the 3′:5′-cyclic AMP (cAMP) content, presumably by the activation of adenylate cyclase (4, 7, 8). When the synthesis rate of cAMP is enhanced, the number of cytosol protein kinase (EC 2.7.1.37; ATP:protein phosphotransferase) molecules that are activated is also increased (3). In the present paper, we report that in the adrenal medulla the transsynaptic regulation of the expression of the metabolic code is critically dependent on the translocation from cytosol into the nucleus of a low-molecular-weight catalytic subunit of protein kinase. Moreover, we show that this translocation, which begins a few hours after the increase in the release of acetylcholine elicited by reserpine, persists for a critical time period of 10–14 hr. After translocation, mRNA synthesis (9) and tyrosine monooxygenase synthesis (3) are also increased.

Abbreviation: cAMP, 3′:5′-cyclic AMP.

MATERIALS AND METHODS

Animals. The adrenal medulla of Sprague-Dawley male rats (Zivic Miller Laboratories, Allison Park, Pa., weighing about 120 g) was dissected from the adrenal cortex at 0° as described (10). The rats were kept at 24° in circadian illumination (14 hr light, 10 hr dark), beginning at 6 a.m., for at least 6 days before the experiments.

Subcellular Location of Protein Kinase. To separate soluble from particulate bound protein kinase, the following procedure was used: four medullae were homogenized with 80 μl of 10 mM potassium phosphate buffer (pH 6.5) containing 0.15 M NaCl, 10 mM EDTA, 5 mM aminophylline. High salt concentration prevents binding of protein kinase to particulate matter. This homogenate was centrifuged for 20 min at 20,000 × *g*; the pellet was rehomogenized with 80 μl of 10 mM potassium phosphate buffer (pH 6.5) containing 0.5 M NaCl, 10 mM EDTA, 5 mM aminophylline, 0.2% Triton X-100 and centrifuged again for 20 min at 20,000 × *g*. This second supernate was termed "pellet extract" and was used to measure protein kinase activity. The nuclear fraction was prepared by homogenizing and centrifuging adrenal medullae as recommended by Yasmineh and Yunis (11). The nuclei were extracted with 0.5 M NaCl, 0.2% Triton X-100 to measure the protein kinase activity.

Gel Filtrations. The 20,000 × *g* supernate of the adrenal medulla homogenate and the corresponding pellet extract were applied to a Sephadex G-200 column (0.2 × 50 cm). The Sephadex was equilibrated with 0.5 M NaCl, 10 mM potassium phosphate buffer (pH 6.5), 10 mM EDTA. The protein was eluted with 80-μl fractions of the above mentioned buffer. The protein composition of the effluent from various Sephadex columns was normalized as suggested by Laurent and Killander (12).

Protein Kinase Assay. Protein kinase activity was determined by measuring the transfer of radioactive phosphate from ATP onto histone (3). The reaction was carried out for 5 min at 30° and terminated by pipetting a 50-μl aliquot onto a filter paper disc. After the filter papers were washed with hot trichloroacetic acid according to Wastila *et al.* (13), their radioactivity was determined. We have ascertained that our kinase measurement did not include acid-labile phosphate incorporation by hydrolyzing the reaction mixture for 10 min at 60° in 0.1 M HCl and then washing the samples in cold trichloroacetic acid solution. The assay was run in duplicate, and a boiled tissue extract was used as a blank.

The protein kinase activity of the gel filtrates from super-

Table 1. Relationship between cAMP content, increase in protein kinase activation index, and tyrosine 3-monooxygenase activity in rat adrenal medulla

Stimulus	cAMP (pmol/mg protein) 0.5 hr	cAMP (pmol/mg protein) 1 hr	Kinase activation index at 1.5 hr†	Monooxygenase (nmol/hr per gland) at 24 hr
Carbamylcholine (3.2 μmol/kg i.p.)	280 ± 25	34 ± 8	0.18 ± 0.12	5 ± 0.5
(9.2 μmol/kg i.p.)	380 ± 35*	100 ± 9*	0.42 ± 0.05*	11 ± 1.0*
Reserpine (16 μmol/kg i.p.)	145 ± 12*	70 ± 10*	0.59 ± 0.03*	10 ± 0.5*
Exposure to 4° (2 hr)	190 ± 15*	175 ± 20*	0.48 ± 0.04*	8.2 ± 0.8*
Aminophylline (200 μmol/kg i.p.)	375 ± 40*	350 ± 25*	0.45 ± 0.04*	9.2 ± 1.2*
Corticotropin (1 IU/kg i.v.)	225 ± 18*	38 ± 7	0.17 ± 0.005	6 ± 0.4
Dopamine (50 μmol/kg s.c.)	80 ± 7*	32 ± 7	0.22 ± 0.03	4.8 ± 0.5
Propranolol (40 μmol/kg i.p.)	100 ± 6*	25 ± 5	0.20 ± 0.01	5.5 ± 0.6

i.p., intraperitoneally; i.v., intravenously; s.c., subcutaneously. Corticotropin = ACTH.

* $P < 0.05$ when compared to saline-treated rats ($n = 5$).

† The kinase activation index is the ratio of the activity in 20,000 × *g* supernatant in the absence and presence of cAMP (0.7 μM). In all experiments, the phosphate acceptor was a calf thymus mixture of histones (300 μg/ml). Each point is the mean ± SE of five experiments. The concentration of cAMP in saline-treated rats was 28 ± 2 pmol/mg of protein; the kinase activation index was 0.18 ± 0.01; and the monooxygenase activity was 5 ± 5.0 nmol/hr per gland.

natant and pellet extracts was measured using either a histone mixture from calf thymus or a purified histone fraction (F_1, F_{2a}, F_{2b}, F_3) obtained from Sigma. The activation of cAMP-dependent protein kinase was measured by its capability of phosphorylating in the absence or presence of added amounts of cAMP (0.7 μM). The degree of activation was expressed as the activation index, that is, the ratio of the phosphorylating activity in the absence and presence of added amounts (0.7 μM) of cAMP. Thus, the activation index represents the fraction of the catalytic subunits of cAMP-dependent protein kinase that is dissociated and is in an active form. This index would reach a value of 1 if all the protein kinase present were activated. The cAMP content of adrenal medullae was assayed as previously described (10), the monoamine oxidase (EC 1.4.3.4) activity according to Goridis and Neff (14), the DNA and RNA content according to Karsten and Wollenberger (15), and the protein content according to Lowry *et al.* (16).

RESULTS

Relationship between increase in cAMP, activation of protein kinase, and induction of tyrosine 3-monooxygenase

Adrenal medullae of rats were treated with aminophylline, carbamylcholine, reserpine, or exposure to cold. When the cAMP content was increased for at least 1 hr, the protein kinase of cytosol was activated at 90 min (Table 1). When the activation index remained elevated for 90 min or longer, the monooxygenase was induced 24 hr later (Table 1). Although dopamine, corticotropin (ACTH), and propranolol increased the cAMP content of adrenal medulla to about the same extent as cold exposure or aminophylline injection, the cAMP increase lasted less than 1 hr (Table 1). The duration of the increase has a critical value (about 1.5 hr) for the increased index of protein kinase activation and for the subsequent induction of monooxygenase. Dopamine, corticotropin, and propranolol did not increase the index nor did they cause a subsequent induction of monooxygenase (Table 1). In contrast, reserpine, cold exposure, carbamylcholine, and aminophylline increased cAMP content for 1 hr or longer, enhanced protein kinase activation index at 1.5 hr, and induced monooxygenase 24 hr later (Table 1). These results suggest that the activation of adenylate cyclase must persist for a certain period of time in order to activate protein kinase and induce monooxygenase at a later time.

Activation of protein kinase in supernatant (20,000 × *g*) of adrenal medulla

Fig. 1 depicts the effects of reserpine on the chromatographic profile of the protein kinase present in the 20,000 × *g* supernate prepared from adrenal medulla homogenates. The protein kinase activity, measured in the absence of the calf thymus histone mixture as phosphate acceptor (Fig. 1), was negligible; this histone mixture was used to express the activity (Fig. 1). Two distinct peaks of enzyme activity were found in the chromatogram of the supernatant fraction of homogenates prepared with medullae of saline-treated rats (Fig. 1, upper panel). A high-molecular-weight protein kinase was eluted by buffer fractions 5 to 8, which consisted of a cAMP-dependent holoenzyme, whereas the low-molecular-weight protein peak consisted of an active cAMP-independent enzyme. In the chromatogram of the 20,000 × *g* supernate from adrenal medulla of reserpine-treated rats, the activity of the cAMP dependent enzyme was considerably reduced, while that of the activated, low-molecular-weight kinase was markedly increased (Fig. 1, lower panel). Similar differences were obtained in rats either exposed to 4° or injected with doses of carbamylcholine that induced monooxygenase (3, 4).

Translocation of activated kinase from cytosol into particulate fractions of adrenal medulla.

Reserpine elicited a decrease in the amount of cAMP-dependent kinase activity in the supernate of adrenal medulla (Fig. 2). This decrease was maximal at 2–4 hr; after this time the activity progressively increased and reached the control

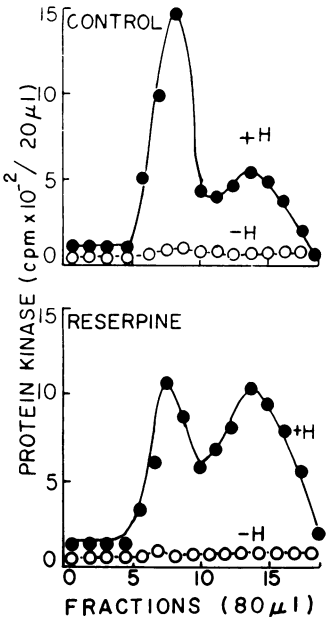

FIG. 1. Sephadex G-200 elution profiles of protein kinase activity in the 20,000 × *g* supernatant of adrenal medulla homogenate from control and reserpine-injected rats. Adrenal medulla homogenate was prepared with 3 volumes of 0.5 M NaCl–10 mM potassium phosphate buffer (pH 6.5)–10 mM EDTA–5 mM aminophylline, at 2 hr after the intraperitoneal injection of saline or 16 µmol/kg i.p. of reserpine. The homogenate was centrifuged for 20 min at 20,000 × *g*, and the supernatant (900 µg of protein) was chromatographed on a Sephadex G-200 column (0.2 × 50 cm) with 0.5 M NaCl–10 mM potassium phosphate buffer (pH 6.5)–10 mM EDTA. Protein kinase activity was measured in 20 µl of each fraction in the presence of 0.7 µM cAMP with (+H) or without (−H) calf thymus histone mixture (300 µg/ml) as substrate.

values in 18–20 hr. Conversely, the amount of activated kinase extracted from the particulate fraction of the adrenal homogenate increased at 2 hr after reserpine, reached a peak at 4 hr, and returned to control values in about 20 hr (Fig. 2). In both fractions the rate of ATP degradation, the K_a of cAMP-dependent (10^{-7} M) protein kinase, and the histone phosphatase activity remained unchanged after reserpine. Moreover, appropriate recombination experiments failed to demonstrate that the increased kinase activity in the particulate fraction was due to an increase in an activator or to a decrease in an inhibitor of protein kinase. It may be concluded, therefore, that the changes in the kinase of supernate and pellet extract shown in Fig. 2 were due to a migration of activated kinase from cytosol to particulate fraction. A similar transfer has been described in other tissues and has been termed translocation (17). When extracts from the particulate fraction of adrenal medullae of rats injected 7 hr earlier with saline or reserpine were chromatographed on a Sephadex G-200 column (Fig. 3), the elution profile of the protein kinase activity consisted of two peaks (peaks A and B of Fig. 3). The protein included in the low-molecular-weight fraction could not function as phosphate acceptor, but the protein of the high-molecular-weight fraction could act as a phosphate acceptor. In reserpine-treated rats, the kinase activity associated with the high-molecular-weight protein (peaks A and A′ of Fig. 3) was slightly greater than that of saline-treated rats (see inset of Fig. 3); however, in reserpine-treated rats the low molecular weight protein peak (peaks B and B′ of Fig. 3) contained three times as much kinase activity than the correspondent peak from saline-treated rats (see inset of Fig. 3).

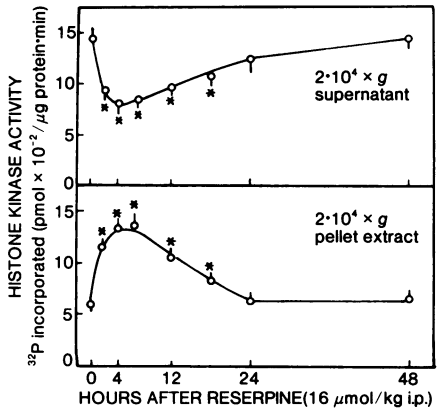

FIG. 2. Time course of histone kinase activity in the 20,000 × *g* supernatant and pellet extract of adrenal medulla homogenate from reserpine-injected rats. Groups of six pairs of animals were sacrificed at various times after the administration of 16 µmol/kg i.p. of reserpine. The 20,000 × *g* supernatant of adrenal medulla homogenate was prepared as described in *Materials and Methods*. The pellet was extracted with 120 µl of 0.5 M NaCl, 10 mM potassium phosphate buffer (pH 6.5), 10 mM EDTA, 5 mM aminophylline, 0.2% Triton X-100. The assay was performed in the presence of 0.7 µmol of cAMP and Triton mixture. Each value is the mean ±SE of six determinations.

* $P < 0.05$ when compared with control animals.

Substrate affinity of translocated protein kinase

In order to characterize the translocated kinase, we studied how various histones functioned as phosphate acceptors for the activated kinase (peak B of Fig. 3) extracted from particulate fraction of reserpine- or saline-treated rats. As shown in Table 2, the kinase in the low-molecular-weight protein in the particulate fraction of saline-treated rats phosphorylated histone F_2b at the highest rate. The kinase activity was lower when histones F_1, F_{2a}, or F_3 or alpha-casein were the phos-

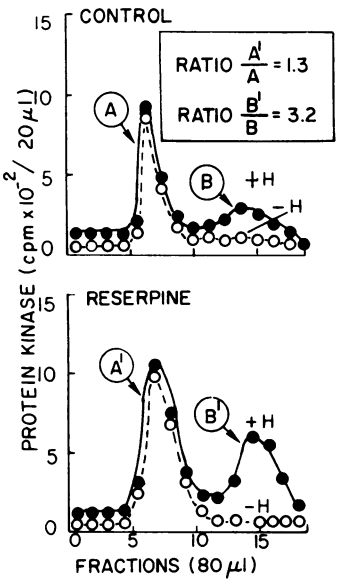

FIG. 3. Sephadex G-200 elution profiles of protein kinase activity in the pellet extract of adrenal medulla homogenate from normal and reserpine-injected rats. Experimental conditions for preparation of extracts were those described in the legend of Fig. 2; chromatographic conditions were as described in *Materials and Methods*. The protein kinase assay was carried out in the presence (+H) or in the absence (−H) of calf thymus histone mixture (300 µg/ml).

Table 2. Substrate specificity of the protein kinase present in the low-molecular-weight protein peak (peak B) obtained by gel filtration of pellet extract on Sephadex G-200

Substrate	Protein kinase activity (pmol/mg protein per min)	
	Saline	Reserpine
F_1	70 ± 10	395 ± 63*
F_2a	31 ± 5	185 ± 30*
F_2b	246 ± 31	251 ± 15
F_3	29 ± 4	80 ± 15*
α-Casein	55 ± 4	61 ± 7

Rats were sacrificed at 7 hr after the i.p. injection of saline or 16 μmol/kg of reserpine. The 20,000 × *g* pellet extract of adrenal medulla homogenate was prepared as described in *Materials and Methods*. The pellet extract was chromatographed on a Sephadex G-200 column as shown in Fig. 3, and the fractions corresponding to the second peak were collected. Protein kinase activity was assayed in the presence of 0.7 μM cAMP with 300 μg/ml of substrate. Each value represents the mean ± SEM of triplicate experiments.
* $P < 0.05$.

Table 3. Translocation of protein kinase from cytosol to nuclei in adrenal medulla of rats injected with reserpine†

	Protein kinase activity	
	Unit/mg of protein	Unit/adrenal
Cytosol		
Saline	190 ± 18	9.5 ± 0.8
Reserpine	135 ± 4*	7.5 ± 0.2*
Nuclei		
Saline	65 ± 11 (52 ± 3)	0.5 ± 0.03 (0.4 ± 0.02)
Reserpine	144 ± 26* (127 ± 8*)	1.0 ± 0.05* (0.9 ± 0.02)*

Eighty animals were used in each experiment to obtain one cytosol and nuclear fraction. Each value in the table refers to the mean ± SEM of three experiments. The protein kinase activity was determined using calf thymus histone mixture (300 μg/ml) and cAMP (0.7 μM). The numbers in parentheses are the values without cAMP. One unit of protein kinase corresponds to the incorporation of 1 pmol of ^{32}P/min.
* $P < 0.05$.
† 16 μmol/kg i.p. 7 hr before.

phate acceptors (Table 2). The homologous enzyme from reserpine-treated rats phosphorylated histone F_1 and F_{2a} (Table 2) at a rate 5- to 6-fold greater than that of the corresponding peak from saline-treated rats. Since this difference persisted even after acid hydrolysis (18), it could not be attributed to NH_2-terminal phosphorylation. Also, histone F_3 but not histone F_{2b} was phosphorylated at higher rates by the low-molecular-weight kinase from the particulate fraction of reserpine-treated rats (Table 2). These results indicate that the translocated kinase could be differentiated from the kinase normally associated with the low-molecular-weight enzyme of the particulate fraction.

Nuclear location of the translocated protein kinase

To determine whether the kinase was translocated into the nucleus, we compared the kinase activity extracted from the nuclear fractions prepared from adrenal medullae of reserpine- and saline-injected rats. The degree of purification achieved in the nuclear fractions used in our studies was characterized by measuring monoamine oxidase and 5′-nucleotidase activity, which are indices of mitochondria and membrane contamination, respectively. The monoamine oxidase and 5′-nucleotidase activities per DNA content were reduced by 50-fold; the RNA/DNA ratio was reduced by about 15-fold. These ratios remained unchanged when the preparations were washed with 0.2% Triton X-100.

With histone mixture as phosphate acceptor, the kinase activity in the nuclear fraction of rats injected with reserpine was twice that of rats injected with saline. An equal increase of kinase activity was present when the data were calculated per one adrenal medulla (Table 3). Similar results were obtained 7 hr after cold exposure or after doses of carbamylcholine which induced adrenal tyrosine 3-monooxygenase (3).

DISCUSSION

Our data show that in intact adrenal medulla the administration reserpine and other conditions that increase the cAMP content for a time period of about 1 hr cause an increase in the protein kinase activity 1.5 hr after the stimulus and an induction of tyrosine 3-monooxygenase 18–24 hr later. In the supernate of adrenal medullae of rats injected 1.5 hr previously with reserpine, the activation of protein kinase is increased (Table 1) (3). This enhanced activity persists after the increase of medullary cAMP has disappeared. Probably in terms of chromaffin cell function, the activation of protein kinase is a temporal and spatial amplification of the second messenger signal. If the activation of protein kinase is an intracellular amplifier of the *in vivo* stimulation of adenylate cyclase (10, 18), one can measure protein kinase activation instead of facing the difficulties of defining the extent and duration of the cAMP increase that must be attained in order to induce medullary tyrosine 3-monooxygenase (20, 21).

Since we do not understand the molecular mechanisms that link the release of acetylcholine, the stimulation of adenylate cyclase, and the activation and long-lasting translocation of protein kinase, the measurement of the protein kinase activation and translocation may be the appropriate method to evaluate whether cAMP mediates the transsynaptic induction of tyrosine monooxygenase (7). It is important that the protein kinase activation index is measured in the presence of high NaCl concentration to prevent artifactual aggregation of catalytic subunits with the particulate fraction (22).

The increase in the activation index of supernate protein kinase is due to a decrease in the kinase activity measured in the presence of exogenous cAMP and to an increase in the kinase activity measured without cAMP. Since 90 min after stimulus application the addition of regulatory subunits prepared from calf brain protein kinase abolishes the increase in cAMP-independent phosphorylating activity (this laboratory, unpublished), it may be inferred that at 90 min catalytic subunits of protein kinase are dissociated from the holoenzyme. The data of Fig. 2 show that at 4 hr these catalytic subunits have migrated to the particulate fraction. This redistribution of the enzyme is termed translocation, in agreement with current terminology (17, 23). The translocated protein kinase has a low molecular weight (Fig. 3) and loses its phosphorylating activity in the absence of exogenous cAMP when purified regulatory subunits of calf brain

(cAMP-dependent) protein kinase are added (this laboratory, unpublished). To provide additional characterization of the translocated enzyme, we have studied its ability to phosphorylate various histones. We found that the enzyme translocated in the particulate fraction of reserpine-treated rats phosphorylates lysine-rich histones F_1 and F_{2a} at a rate 5- and 8-fold greater than that of the enzyme extracted from the particulate fraction of saline-treated rats. However, the activity of the enzyme in particulate fraction of reserpine- and saline-treated rats is the same if histone F_{2b} or alpha-casein are the phosphate acceptors. This suggests that before the synthesis of mRNA (9) or that of tyrosine 3-monooxygenase (3) is increased, a specific class of protein kinases has been transferred from the cytosol to the particulate fraction of chromaffin cells. Although one might speculate that phosphorylation of F_1 histones plays a role in gene activation, DNA replication, and mitosis (24–28), it must be emphasized that our experiments do not suggest that the translocated enzyme has as a natural substrate histone F_1.

Since we have shown that the phosphorylating activity in the nuclei of adrenal medulla of rats injected with reserpine is increased when mRNA synthesis is also increased (9), we can infer that translocation of protein kinase regulates the synthesis of mRNA (9, 29) for tyrosine 3-monooxygenase.

The data presented in Table 3 show that nuclear protein kinase is not regulated by cAMP directly, but through translocation of catalytic subunits of protein kinase. This phosphorylation may have a role in the amplification of template activity, thus contributing to the increase of the production of certain types of mRNA. It is possible that a change in nuclear phosphorylation elicited by kinase translocation is a specific mechanism in regulating the expression of the metabolic code during transsynaptically elicited induction of tyrosine 3-monooxygenase in chromaffin cells. This may be only one of a number of regulatory mechanisms mediated by transsynaptic processes. The translocation of protein kinases may mediate hormonal effects in liver (17), ovary, and uterus (30, 31). Our report adds the transsynaptic induction of tyrosine 3-monooxygenase in adrenal medulla to this list.

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