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Primary Cultures of Chromaffin Cells: Molecular Mechanisms for the Induction of Tyrosine Hydroxylase Mediated by 8-Br-cyclic AMP

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SUMMARY

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Primary cultures of chromaffin cells from bovine adrenal medulla were used as a model to evaluate the ability of 8-Br cyclic AMP (8-Br cAMP) to induce tyrosine hydroxylase (TH) and to study the role of cAMP-dependent protein kinase (cAPK) in this induction. This cell preparation maintains a constant level of cyclic nucleotides, catecholamines and related enzyme activities for about four weeks. Exposure of the cells for 5 hr to 8-Br cAMP produces, 48 hr later, a dose-related increase in the TH activity; 8-Br cGMP fails to modify TH. The increase in TH activity caused by 8-Br cAMP is due to an increase of the V_{max} and is preceded by an activation of cytosol cAPK associated with a decrease of the total cytosol cAPK. A sustained increase in nuclear phosphorylation begins 8 to 12 hr after 8-Br cAMP application. The delayed increase in TH activity induced by 8-Br cAMP is blocked by actinomycin D, cycloheximide, colchicine and vinblastine. The reduction of the TH induction by colchicine and vinblastine (1 nM) is observed only when the inhibitors of the microtubular protein polymerization were added 4 to 12 hr after the incubation with 8-Br cAMP. The addition of colchicine 15 hr after 8-Br cAMP fails to inhibit TH induction. This blockade of TH induction is associated with an inhibition of the increase in nuclear phosphorylation, but is not associated with an inhibition of protein synthesis. The increase of endogenous cAMP and the induction of TH was also produced by cholera toxin. These results suggest that the increase of TH elicited by 8-Br cAMP is mediated by the translocation of cAPK subunits from cytosol to the nuclei and that this translocation requires the function of the microtubular network.

INTRODUCTION

In the rat adrenal medulla model, the *de novo* synthesis of tyrosine hydroxylase (TH)[1] that follows trans-synaptic stimulation of nicotinic receptors (1–3) has been related to a chain of events initiated by the increase in cAMP concentration (4). These include the subsequent activation of cytosol cAPK and the translocation and retention in the nucleus of cAPK catalytic subunits from cytosol (5, 6). An injection of a nicotinic receptor antagonist or the denervation of adrenal medulla reduces and shortens the cAMP elevation caused by cold exposure (1, 4, 5). The treatment also prevents the cAPK activation, inhibits the nuclear translocation of cAPK catalytic subunits and blocks the induction of TH (5–7; see also 1 for review). Though these experi-

[1] The abbreviations used are: TH, tyrosine hydroxylase; cAPK, cAMP dependent protein kinase; 8-Br cAMP, 8-Br-cyclic AMP; [^{3}H]QNB, DL-[benzilic-4.4-^{3}H(N)]-quinuclodyl benzilate; TCA, trichloroacetic acid; MAO, monoamino oxidase.

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ments demonstrate a temporal succession between the translocation of the catalytic subunits of cAPK to the nucleus, the increase in nuclear protein phosphorylation and the stimulation of gene activity, they fail to elucidate: 1) the specific regulatory mechanism involved in the nuclear uptake of catalytic subunits of cAPK; 2) the precise role of these protein kinase subunits that translocate to the nucleus in the control of gene expression in general and of the transcription of specific genes in particular; 3) the mechanisms underlying gene transcription specificity; and 4) the necessity of nuclear translocation of cAPK catalytic subunits in eliciting the trans-synaptic induction of TH.

In vivo experiments with rat adrenal medulla are not the most suitable to study trans-synaptic regulation of gene expression at molecular level because the limited amount of purified nuclear material that rat adrenal medullae yields is not enough to allow a rigorous monitoring of the fine biochemical events occurring in the nuclear chromatin. We now present evidence that the induction of TH elicited by 8-Br-cyclic AMP (8-Br cAMP) in primary culture of bovine adrenal chromaffin cells can represent an adequate model to study whether the movements of activated catalytic subunits of cAPK into various cell compartments and the increased phosphorylation of nuclear proteins can mediate the increase in nuclear RNA transcription of specific proteins.

METHODS

Cell preparation. Adrenal chromaffin cells were isolated from fresh bovine adrenal glands as described by Waymire *et al.* (8). Briefly, the procedure consists of dissection of the adrenal medullary tissue from the adrenal cortex; 2) digestion of the adrenal medulla by retrograde perfusion with 0.25% collagenase to dissociate the cells; 3) differential centrifugation and filtration; 4) differential plating procedures to remove nonchromaffin cells. The differential plating is crucial in order to obtain a pure population of chromaffin cells. During plating on glass (6 hr at 37°) the majority of the nonchromaffin cells (fibroblast, cortical cells, endothelial cells, etc.) will adhere to the glass surface while the chromaffin cells will float in the medium. Successive plating contributes to the purification of the chromaffin cells by eliminating contaminating cells.

The chromaffin cells were identified with fluorescence microscopy (9). The purity of the chromaffin cell suspension was determined by microscopic examination of culture cell smears which were stained with Giemsa after development of specific catecholamine fluorescence (10). The cell concentration and viability in the final suspension were determined microscopically by monitoring with a hemocytometer an aliquot of the original suspension diluted 1:4 with 0.4% trypan blue solution in normal saline. When the effect of the drugs on TH activity was studied, 1 ml of cell suspension (approximately 10^7 cells) was maintained in a 0.5 cm diameter visking membrane dialysis tubing. The chromaffin cells did not form a monolayer in culture, thus, by keeping the cells in membrane tubing, one can perform time course studies of drug treatments avoiding the shock of cell transfer and centrifugation.

Stimulation of catecholamine release and catecholamine assay. The release of endogenous catecholamines from cultured cells was measured by incubating the cell suspension at 37° for 10 min in Kreb's-Ringer bicarbonate glucose buffer (pH 7.2–7.4) 0.5% bovine serum albumin with or without 2.2 mM $CaCl_2$.

Cellular and medium catecholamines (norepinephrine and epinephrine) were extracted with 0.4 N perchloric acid and analyzed fluorometrically after Al_2O_3 adsorption (11).

Assay of TH activity. Approximately 10^7 cells were homogenized in 250 μl of 50 mM Tris acetate buffer (pH 6.0) containing 0.2% Triton X-100. In 50 μl aliquots of the homogenate the TH activity was measured using carboxylabeled tyrosine coupled with a Dopa decarboxylase enzymatic reaction to measure $^{14}CO_2$ (12). In some experiments TH activity was also measured in the 10,000 × *g* supernatant or in the homogenate after dialysis against the same buffer for 6 hr or after purification on Sephadex G-25. The

results obtained with these three different procedures were similar.

Preparation of nuclei and cytosol fractions from the isolated chromaffin cells. Approximately 10^6 cells were homogenized with a glass homogenizer fitted with a Teflon pestle in 5 ml of 0.32 M sucrose, 10 mM Tris HCl (pH 8.0), 2 mM Mg $(C_2H_3O_2)_2$ 4 H_2O, 3 mM $CaCl_2$, 0.5 mM dithiothreitol and 0.1% Triton X-100. After centrifugation at 1,000 × *g* for 10 min the nuclear fraction was purified from the pellet according to the method described by Chuang *et al.* (13). The purified nuclei were resuspended in 0.32 M sucrose, 50 mM Tris HCl (pH 8.0), 5 mM Mg $(C_2H_3O_2)_2$ 4 H_2O, 1.5 mM $CaCl_2$ and 0.5 mM dithiothreitol and used for biochemical determinations. The purity of the nuclei and the lack of cytoplasmic contamination were determined by measuring DNA (6), RNA (6) and MAO activity (14). In the nuclear fraction, the ratio of RNA/DNA was 0.29 and the specific activity of monoamineoxidase was less than 0.5% of that present in crude homogenate. For the determination of protein kinase activity in cytosol and pellet the cells were homogenized in 10 mM potassium phosphate buffer (pH 6.5) containing 130 mM NaCl, 19 mM EDTA and 5 mM aminophylline (5). After centrifugation at 20,000 × *g* for 20 min the supernatant and pellet extracts were assayed for PK activity. Pellet PK activity was extracted with the same buffer containing 0.2% Triton X-100 (5).

Assay of PK activity. The PK activity in cytosol and pellet extracts was measured in a reaction mixture containing 10 mM potassium phosphate buffer (pH 6.5), 10 mM $MgCl_2$, 10 mM NaF, 5 mM aminophylline; 500 μM [^{32}P]ATP (100 Ci/mol) and 5 μM cAMP or H_2O in a final volume of 120 μl. Calf thymus histone (40 μg/120 μl) was used as substrate.

The reaction was carried out for 5 min at 30° and terminated by pipetting a 50 μl aliquot onto a filter paper disc (6).

Nuclear protein phosphorylation was measured in reaction mixture containing 50 mM Tris-HCl (pH 6.5), 10 mM Mg $(C_2H_3O_2)_2$ $4H_2O$, 10 mM NaF, and 50 μM [^{32}P]ATP (100 Ci/mol) in a final volume of 90 μl.

The reaction was carried out for 15, 30 and 60 sec at 30° and terminated as described for cytosol PK assay.

Other methods. Cyclic nucleotide content was determined by radioimmunoassay of acetylated cyclic nucleotide according to the method of Harper and Brooker (15).

Adenylate cyclase activity was determined by incubating cell homogenate (approximately 100 μg of protein) for 3 min in 500 μl of the reaction mixture prepared according to a previously described method (16).

Phosphodiesterase activity and the Ca^{++} binding protein (calmodulin) were determined in the same homogenate, using the method described by Uzunov *et al.* (17). *Alpha*-bungarotoxin binding was studied by incubating intact cells with various concentrations (1.5 nM to 15 nM) of [^{125}I]*alpha*-bungarotoxin at 20° for 1 hr. The reaction was terminated by centrifuging the cell suspension after the addition of 2 ml of ice-cold phosphate-buffered saline solution. The cells were washed twice with phosphate-buffered saline solution and then filtered on millipore (EGWP celotate; 0.2 μM). Specific *alpha*-bungarotoxin binding was determined by subtracting from total binding the binding remaining after the addition of 0.1 mM *d*-tubocurarine. Muscarinic cholinergic binding was studied by [^{3}H]QNB binding according to the method of Yamamura and Synder (18).

Protein synthesis in the isolated cells was determined by the incubation of the cells in the culture medium containing 10 μCi/ml of [^{14}C]arginine (specific activity 60 mCi/nmol) for 3 hr at 37° in a final volume of 1 ml.

At the end of the incubation the reaction was stopped by adding 2 ml of 10% TCA, and the radioactivity incorporated into the TCA precipitate was determined. MAO activity and Dopa decarboxylase activity were measured with the method of Goridis and Neff (14) and of Waymire *et al.* (19) respectively.

Protein was measured by the method of Lowry *et al.* (20).

MATERIALS

Fresh bovine adrenal glands were obtained from a local slaughter house. Mini-

mum essential medium (catalog no. 410-1200) fetal calf serum and penicillin-streptomycin ((10,000 units penicillin and 10,000 mcg streptomycin/ml) were obtained from Grand Island Biological Company, New York.

L-1-^{14}C-tyrosine (51 mCi/nmol), [^{32}P]-adenosine triphosphate tetra (triethylammonium) salt (20 Ci/mmol), [^{125}I] *alpha*-bungarotoxin (20 μCi/μg), DL-[benzilic-4.4-^{3}H (N)]-Quinuclidyl benzilate (29 Ci/mmol) and [^{125}I]-2-o-succinyl (iodothyrosine methyl ester)-adenosine or -guanosine 3',5' cyclic phosphoric acid (186 Ci/mmol) were obtained from New England Nuclear, Massachusetts. Collagenase Type 1, 8-Br cAMP, 8-Br cGMP, nicotine, colchicine, vinblastine and cycloheximide were from Sigma Chemical Co., St. Louis, Missouri. Cytochalasin B and Actinomycin D were from Calbiochem., California. Lumicolchicine was prepared by ultraviolet irradiation (21). Conversion of colchicine to lumicolchine was measured by monitoring absorbance at 267 and 350 nm.

RESULTS

Characteristics of adrenal chromaffin cells in culture. After isolation and purification, chromaffin cells were maintained in culture (in HAMs F-12 medium containing 10% calf fetal serum and 1% penicillin-streptomycin) for different time periods. When examined with a light or phase-contrast microscope, the cells appeared aggregated in clusters. The majority of clusters were formed during the second and third day, the number and size of the clusters were constant for at least three weeks in culture. Approximately 98% of the cells failed to take up trypan blue intravitally. The purity of the chromaffin cells was tested by determining histologically in the same smear the number of catecholamine fluorescent cells and of cells stained with Giemsa. Most of the cells detected by Giemsa staining showed a positive fluorescent reaction (see Fig. 1 A and B). The extent of contamination by non-fluorescent cells, as detected in a large number of samples was approximately 5% and although the total number of cells decreased by approximately 30% in one month, the ratio of fluorescent versus non-fluorescent cells remained constant during this time. With this procedure a large number of chromaffin cells could be purified. Approximately 10^8 cells were obtained from a single bovine adrenal medulla.

During culture the chromaffin cells maintained a rather constant content of cyclic nucleotides; the cAMP-phosphodiesterase, PK and TH activities were also constant. Table 1 shows the values from chromaffin cells maintained for 7 days in culture. Phosphodiesterase showed two K_m values for cAMP (K_{m_1} = 8 μM and K_{m_2} = 125 μM). The K_m value of TH for pteridine cofactor was 0.5 mM.

The catecholamine content of the cultured cells remained constant for at least 9 to 10 days. However, after 21 days, the catecholamine content decreased from 20 to 4 nmol/10^6 cells; also, the TH activity failed to decline during 21 days of culture. Intact adrenal chromaffin cells display a specific high affinity binding for alpha-bungarotoxin (Table 1). Also the B_{max} of this binding remained relatively constant for 18 days of culture. No significant specific [^{3}H]QNB binding was detected.

The chromaffin cells in culture maintained the ability of releasing endogenous catecholamines in response to nicotinic receptor stimulation (Fig. 2). The release of catecholamines induced by nicotine was Ca^{++}-dependent and was inhibited by hexamethonium (see Fig. 2). Carbamyl-*beta*-methylcholine (a muscarinic receptor agonist), 8-Br cAMP and 8-Br cGMP failed to elicit a release of catecholamines (Fig. 2). The ability of nicotinic receptor stimulation to release catecholamines from isolated chromaffin cells was maintained up to 10 days in culture. However, in cells kept for 21 days in culture, the secretion of catecholamines in response to nicotinic receptor stimulation was greatly reduced.

Long and short term effects of 8-Br cAMP on TH and cAPK activities in isolated chromaffin cells. In these experiments, we used cells cultured for 4 to 10 days. The addition of 0.1 mM of 8-Br cAMP to the culture produced no significant immediate increase of TH activity; the activity of this enzyme began to increase 36 hr

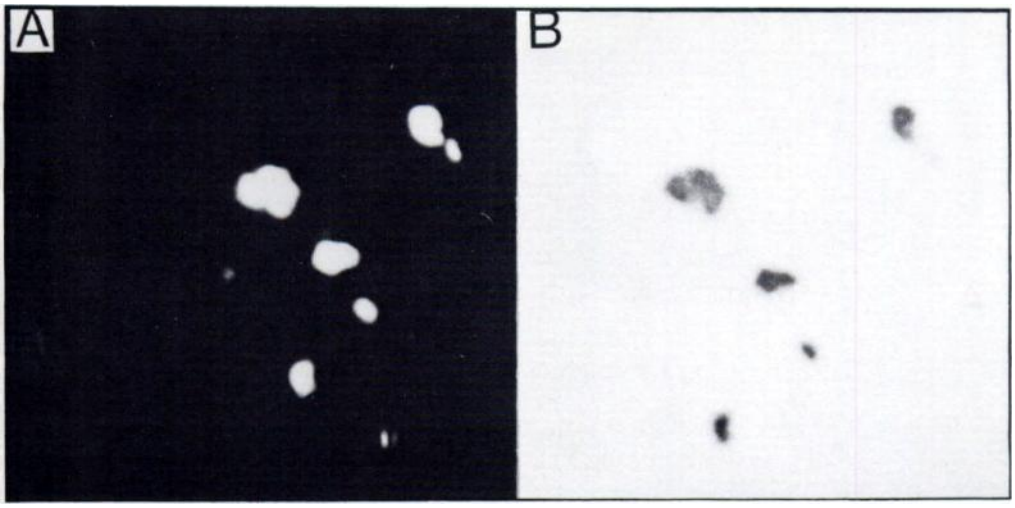

FIG. 1. *Photomicrographs of primary cultures of adrenal chromaffin cells kept in culture for 7 days*
A) Field of cells at × 400 original magnification after the smear was treated with formaldehyde vapors according to Falck and Owman (9). B) The same field at × 200 original magnification after the smear was stained with Giemsa stain (10).

TABLE 1

Biochemical properties of primary culture[a] of chromaffin cells from bovine adrenal

For the procedures used to measure the different parameters see METHODS. PK = protein kinase.

Adenylate cyclase	2.5 pmoles/10^6 cells min
Phosphodiesterase	0.5 nmoles/10^6 cells × min (K_{m_1} = 8 μM, K_{m_2} = 125 μM)
Calmodulin	107 ng/mg protein
cAMP	2 pmoles/10^6 cells
cGMP	0.4 pmoles/10^6 cells
Cytosol cAMP dependent PK	200 pmoles/mg protein × min
Nuclear PK	130 pmoles/mg protein × min
Tyrosine hydroxylase	2 nmoles/hr × 10^6 cells (K_m for $DMPH_4$ = 0.5 mM)
Catecholamines	20 nmoles/10^6 cells
α-Bungarotoxin binding	B_{max} = 5 fmoles/10^6 cells (K_D = 2.5 nM)

[a] 7 Days in culture

later and this increase peaked between 48 and 72 hr. 8-Br cAMP (0.01, 0.1, and 1 mM) produced a 35, 55 and 100% increase in TH activity, respectively. The extent of the TH increase induced by 1 mM of 8-Br cAMP was dependent on the time of incubation, five hours or longer induced TH. Treatment with 1 mM of 8-Br cGMP was without effect on TH. The increase of TH activity elicited by 8-Br cAMP was not paralleled by changes of MAO or Dopa decarboxylase activities. The protein synthesis was determined by measuring the incorporation of [^{14}C]arginine into 10% TCA precipitate 12, 24, 36 and 48 hr following 5 hr of treatment with 8-Br cAMP. No significant changes of total protein synthesis were detected after incubation with 8-Br cAMP.

When the cells were cultivated in a membrane tubing which was immersed in a medium enriched with 0.8 mM 8-Br cAMP, the concentration of 8-Br cAMP inside the tubing approached equilibrium within 2 hr (Fig. 3). The concentration of 8-Br cAMP inside the tubing became undetectable 2 hr after the tubing was transferred to an 8-Br cAMP free medium. Figure 3 shows TH activity measured 48 hr after an exposure for different time periods to 8-Br cAMP. Exposure of the cells to 8-Br cAMP for less than 4 hr failed to elicit an increase in the TH activity measured 48 hr later (upper trace of Fig. 3). In contrast, exposure of the cells to 8-Br cAMP for 5 hr or longer caused a significant increase in the TH activity measured 48 hr after the application of the 8-Br cAMP stimulus. The extent of the TH increase was proportional to the time of exposure to 8-Br cAMP up to 12 hr of

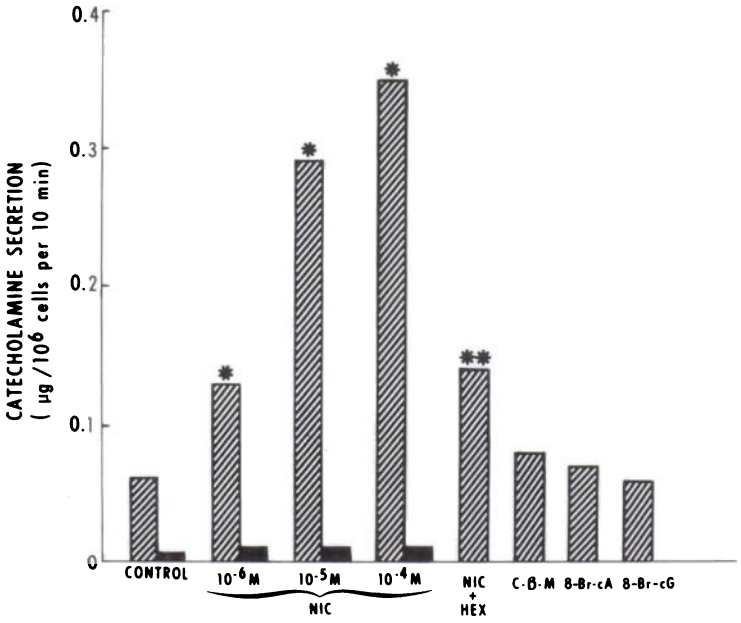

FIG. 2. *Effect of ACh receptor agonists, ACh receptor antagonists, 8-Br-cyclic nucleotides and Ca^{++} on the release of catecholamines from chromaffin cells of a primary culture of bovine adrenal medulla*

Each bar represents the mean value of 3 to 5 experiments. The S.E. was consistently less than 10% of the mean values. NIC = nicotine; Hex = 0.01 mM hexamethonium; C-β-M = 0.01 mM carbamyl-β-methylcholine chloride; 8-Br-cA = 0.5 mM 8-Br cAMP; 8-Br-cG = 0.5 mM 8-Br cGMP. $^{*}p < 0.05$ when compared with controls, $^{**}p < 0.05$ when the release of catecholamines after 0.1 mM Nic + 0.1 mM Hex was compared with the release after 0.1 mM Nic alone. ▨ incubation in normal Krebs-Ringer containing 2.2 mM $CaCl_2$; ■ incubation in $CaCl_2$-free Krebs-Ringer.

incubation; a longer lasting exposure failed to produce a greater increase in the TH activity at 48 hr (Fig. 3).

Kinetic studies demonstrate that this increase in TH activity elicited by 8-Br cAMP was due to an increase in the V_{max} but not to a change in the K_m for pteridine cofactor. The K_m value of TH for $DMPH_4$ in the cells cultured in the absence or in the presence of 8-Br cAMP was 0.51 mM and 0.55 mM, respectively. On the other hand, the exposure to 8-Br cAMP for longer than 4 to 5 hr increased the V_{max} of TH from 4 to 7.5 nmoles Dopa/hr/10^6 cells at 48 hr. Pretreatment of the cells with cycloheximide blocked the increase in the V_{max} of the TH elicited by 8-Br cAMP (Table 2). Actinomycin D, in a dose of 5 μg/ml (a dose that blocks mRNA synthesis) (22), completely blocked the TH increase elicited by 8-Br cAMP. Smaller doses (0.05 to 0.5 μg/ml) of actinomycin D failed to prevent the TH induction, though it is known (22) that these doses of actinomycin D can inhibit ribosomal RNA synthesis. Exposure of the cells to 8-Br cGMP failed to change the K_m or V_{max} of TH measured 48 hr later. Furthermore, TH was not induced by adding NGF (1 or 3 units/ml) or dexamethasone (0.01 mM) to culture medium. On the other hand, Table 3 shows that doses of cholera toxin, which produced a 10-fold increase in the cAMP content of the chromaffin cells within 6 hr, also produced an increase in the TH activity measured 48 hr later.

To establish whether the intracellular effects of 8-Br cAMP were mediated through an activation of cAPK, we measured the activity of this enzyme in the cytosol and nuclear fraction of cultured cells. As shown in Table 4, in cultured chromaffin cells the PK activity of cytosol measured in the presence of 5 μM cAMP was 203 ± 11 pmol ^{32}P/mg protein/min. The activity ratio between the ^{32}P incorporated into the histone in the absence and in the presence of 5 μM cAMP

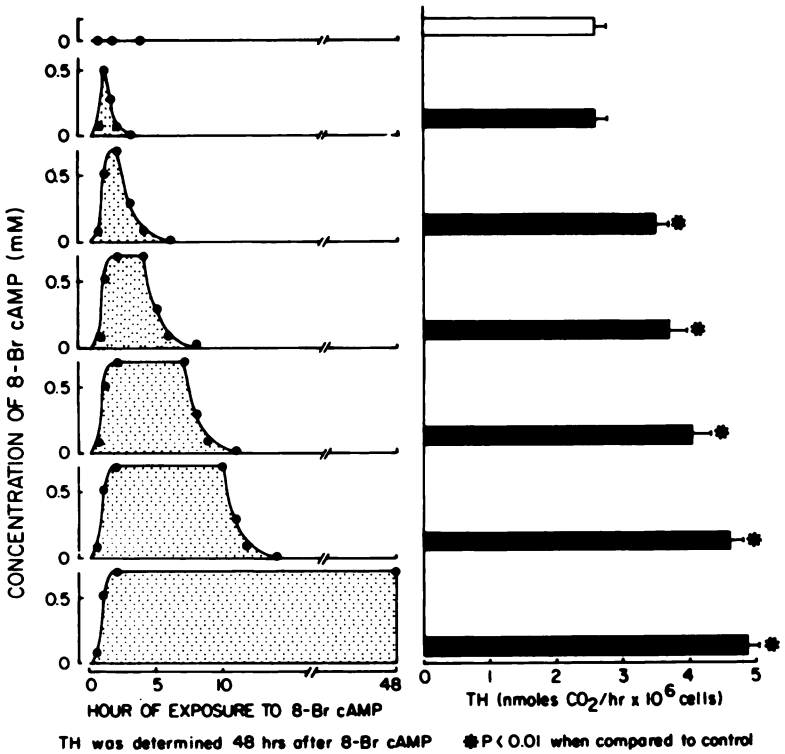

FIG. 3. *Relationship between incubation time with 8-Br cAMP and the delayed increase of TH activity*
The cells were incubated in membrane dialysis tubing. The concentration of 8-Br cAMP inside the tube was determined by UV absorption. Each value is the mean ± SE of 5 experiments.

TABLE 2

Effect of actinomycin D, cycloheximide and 8 Br-cGMP on tyrosine hydroxylase induction

8-Br cAMP (1 mM) was added for 5 hr and TH activity was measured 48 hr later. Cycloheximide, actinomycin D and 8-Br cGMP were maintained in the medium for 48 hr. Each value is the mean ± S.E. of 3 separate experiments.

Addition of 8-Br cAMP	TH activity (*nmoles CO_2/hr × 10^6 cells*)					
	None	Actinomycin D			Cycloheximide	8-Br cGMP
		0.05	0.5	5	20	
		μg/ml			*μg/ml*	*1 mM*
No	2.0 ± 0.11	1.9 ± 0.15	2.2 ± 0.20	1.8 ± 0.12	2.1 ± 0.12	1.8 ± 0.05
Yes	3.5 ± 0.16*	3.3 ± 0.25*	3.2 ± 0.38*	2.1 ± 0.12	1.9 ± 0.10	3.1 ± 0.10*

* $p < 0.05$ when the treated samples were compared with the control group.

was 0.53. This activity ratio measured 3 hrs after a 5 hr exposure to 8-Br cAMP was unaltered (0.57) but the total cytosol PK activity measured in the presence of an excess of cAMP decreased from 203 ± 11 to 132 ± 12 pmol ^{32}P/mg protein/min (see Table 4). Similarly the cytosol PK activity measured in the presence of an excess cAMP decreased if the cells were maintained for 8 hr in the presence of the 8-Br cAMP (Table 4). However in this case, due to presence of 8-Br cAMP in the assay mixture, the activity ratio of ^{32}P incorporated into histone in absence and presence

of 5 μM cAMP shifted from 0.53 (controls) to 0.91. Table 4 also shows that in the total pellet and in purified nuclei the PK activity was increased following 8-Br cAMP treatment. This increased PK activity, similar to the basal activity, failed to be stimulated by the addition of 5 μM cAMP to the reaction mixture.

Simultaneous inhibition by colchicine of the TH induction and of the nuclear translocation of PK elicited by 8-Br cAMP. In order to study whether the temporal sequence of the cascade of biochemical events preceding the TH induction elicited by 8-Br cAMP required the presence of an efficient assembly of microtubular protein, we treated the chromaffin cells with drugs that prevent this process. Colchicine and vinblastine blocked the TH induction elicited by cAMP at doses as small as 1 nM. These drug concentrations failed to change the basal TH activity (Table 5). In contrast, lumicolchicine, an isomer of colchicine, which fails to interfere with the assembly of microtubular proteins (21), and cytochlolasin B, which is known to interfere with the microfilament function (23), failed to block the TH induction elicited by 8-Br cAMP. The incorporation of [^{14}C]arginine into cell protein was not affected by colchicine (10 μM) (controls: 1.3×10^4 cpm/10^6 cells and colchicine treated: 1.3×10^4 cpm/10^6 cells). In contrast, treatment with 0.1 mM of cycloheximide for 3 hr decreased the protein synthesis by approximately 50%.

Figure 4 shows experiments directed to establish the time factor in the colchicine

TABLE 3

Effects of cholera toxin on cAMP content and tyrosine hydroxylase activity of primary cultures of bovine chromaffin cells

Cholera toxin 100 ng/ml was added for 6 hr and cAMP was measured at 6 hr. TH activity was measured 42 hr later in a separate experiment. Each value is the mean ± S.E. of 3 separate experiments.

	Cellular cAMP	TH activity
	pmoles/10^6 cells	*nmoles CO_2/hr × 10^6 cells*
Control	1.6 ± 0.10	2.0 ± 0.11
Cholera toxin	19.2 ± 2.1*	3.9 ± 0.19*

* $p < 0.05$.

TABLE 5

Effect of colchicine, vinblastine, lumicolchicine and cytochalasin B on the tyrosine hydroxylase induction elicited by 8-Br cAMP

Each value is the mean ± S.E. of three experiments.

Additions (exposure for 8 hr)	Conc.	8-Br cAMP (5 hr)	TH activity
	M		*nmoles CO_2/hr × 10^6 cells*
None	—	−	2.2 ± 0.10*
None	—	+	4.0 ± 0.16
Colchicine	10^{-8}	+	2.9 ± 0.11*
Lumicolchicine	10^{-7}	+	4.7 ± 0.11
Vinblastine	10^{-8}	+	3.1 ± 0.16*
Cytochalasin B	10^{-6}	+	4.8 ± 0.13

* $p < 0.01$ when compared to cells incubated with 8-Br cAMP.

TABLE 4

Protein kinase activity[a] in cytosol, pellet and nuclei prepared from primary cultures of chromaffin cells

Cell treatment	Protein kinase activity[a]					
	Cytosol		Total pellet		Purified nuclei	
	−cAMP	+cAMP	−cAMP	+cAMP	−cAMP	+cAMP
None	117 ± 8	203 ± 11	194 ± 18	190 ± 14	129 ± 8	124 ± 11
8-Br cAMP[b] × 5 hrs	75 ± 1.6*	132 ± 13*	248 ± 15*	258 ± 20*	——	——
8-Br cAMP[c] × 8 hrs	116 ± 16	128 ± 14*	254 ± 9*	232 ± 15*	198 ± 20*	199 ± 20*

* $p < 0.05$ when compared to control cells.

[a] PK activity was expressed as [^{32}P]pmol/mg protein × min.

[b] Cells in membrane tubing were maintained for 2 hr in a medium containing 50 mM 8-Br cAMP, and protein kinase activity was determined 6 hr after 8-Br cAMP removal. As can be seen in Fig. 3 (third panel from top) two hr exposure to a medium containing 8-Br cAMP resulted in an increase of 8-Br cAMP inside the membrane tubing for approximately 5 hr.

[c] The cells were maintained for 8 hr in the presence of 50 mM of 8-Br cAMP and protein kinase activity was determined at this time.

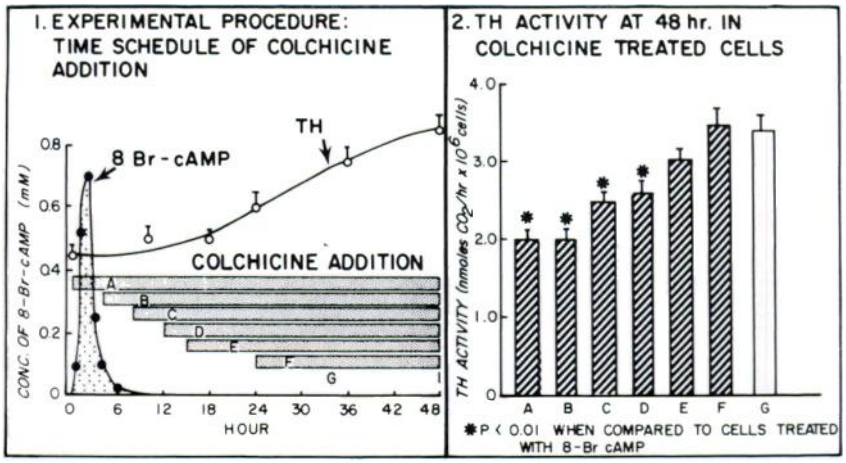

FIG. 4. *Colchicine inhibition of TH induction by 8-Br cAMP: Time factor*

Panel 1) Experimental procedure: time schedule of colchicine addition. Colchicine 10 nM was added at different times after the addition of 8-Br cAMP. Concentration of 8-Br cAMP is the concentration inside the membrane dialysis tubing (see METHODS). TH was determined at different times after the addition of 8-Br cAMP in cells incubated without colchicine. The units of TH activity are the same as those in panel 2. *Panel 2)* TH activity measured at 48 hr after 8-Br cAMP in colchicine treated cells. ▨ colchicine addition at different time intervals (see Panel 1); □ no colchicine addition. Each value is the mean ± SE of 3–5 experiments.

inhibition of TH induction elicited by 8-Br cAMP. Colchicine added within 4 hr after the addition of 8-Br cAMP blocked the TH induction. This drug could still reduce the extent of TH induction when it was added during the first 12 hr after 8-Br cAMP but it failed to act if added at a later time. We then studied whether colchicine could prevent the increase of nuclear phosphorylation observed as a result of 8-Br cAMP treatment. As shown in Figure 5, the addition of 8-Br cAMP in the presnce of 10 nM colchicine failed to increase phosphorylation of endogenous nuclear proteins.

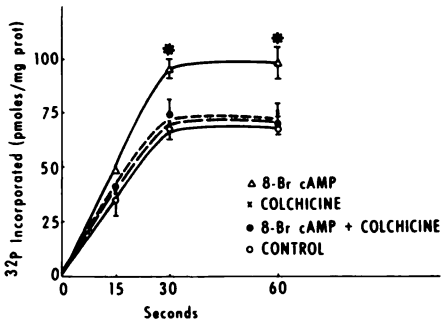

FIG. 5. *Nuclear phosphorylation of chromaffin cells incubated with 8-Br cAMP with or without colchicine*

The cells are treated with 0.5 mM 8-Br cAMP for 8 hr in the presence and the absence of 10 nM colchicine. $^*p < 0.05$ when compared to control. Vertical bars represent SE of the mean. Each value is the mean of 3–5 experiments.

DISCUSSION

In this paper a number of functional and metabolic properties of a primary culture of chromaffin cells from bovine adrenal medulla have been highlighted. These cells can survive for a long time (more than four weeks), they maintain constant levels of several enzymes related to cyclic nucleotides and catecholamine metabolism, and considering the release of catecholamine by nicotine as an index of their function, they function for at least 2 weeks. In agreement with other reports (10, 24, 25) we could prove that chromaffin cells in culture display a Ca^{2+}-dependent release of endogenous catecholamines in response to the application of nicotinic receptor agonists. In addition, we have shown that these cells also possess a specific high affinity binding site for *alpha*-bungarotoxin. The culture of bovine medullary cells differs from that of

chick adrenal medulla (26) because muscarinic agonists fail to release catecholamines from the bovine cells. This observation agrees with the lack of a specific QNB binding in the bovine medullary cell preparation. Interestingly, the release of catecholamines elicited by nicotinic receptor agonists and the number of *alpha*-bungarotoxin binding sites are constant during the first 1–2 weeks of culture, thereafter, these two parameters decline in parallel.

In good agreement with the data reported by Waymire *et al.* (8), the addition of 8-Br cAMP to the chromaffin cell cultures causes a delayed increase in the V_{max} of TH. This increase is not associated with a change in affinity of TH for the pteridine cofactor. In our studies we could determine the specificity of the incubation time and the dose requirement for the TH induction elicited by 8-Br cAMP. Concentrations of 8-Br cAMP as small as 0.01 mM can elicit a delayed increase (48 hr) in the V_{max} of TH present in chromaffin cells. The incubation time for the TH induction elicited by 8-Br cAMP appears to be around 5 hr. After such a time, the 8-Br cAMP can be washed out but the TH is induced 48 hr later. Treatment of the chromaffin cells with cholera toxin, a specific adenylate cyclase activator (27), caused a 12-fold increase in the cAMP content and a delayed induction of TH (Table 3). At variance with adrenal chromaffin cells isolated from newborn rats (28) but in agreement with a clonal cell line of pheocromocytoma (29, 30), primary culture of bovine chromaffin cells failed to respond to nerve growth factor with a delayed increase in TH activity. This finding and the lack of TH induction following incubation with 8-Br cGMP strongly suggests that in bovine adrenal medulla a transient accumulation of cAMP plays a specific role and transmits a precise metabolic message to the nucleus, leading to a delayed increase in TH activity. Because this increase is abolished when protein synthesis is inhibited, we have inferred that it corresponds to the trans-synaptic induction of TH, described *in vivo* (1–7). In rat adrenal medulla the increase in TH molecule biosynthesis (1) is preceded by an increase in nuclear phosphorylation due to an activation and nuclear translocation of catalytic subunits of cytosol cAPK (1, 6). Although the *in vivo* experiments with rats had shown the sequence of these events (1), they failed to indicate that the increase in nuclear phosphorylation was an essential requirement for the TH induction elicited by the accumulation of cAMP. The data reported here show that in the isolated chromaffin cells not only is the TH induction preceded by an activation of cytosol cAPK and by an increase in nuclear protein phosphorylation, but both the induction of TH and the increase of nuclear phosphorylation require an operative assembly of microtubular proteins. Antimicrotubular drugs such as colchicine and vinblastine (1 nM) are able to block the TH induction elicited by 8-Br cAMP, when the drugs are added less than 15 hr after 8-Br cAMP (Table 5 and Fig. 4). Thus, the microtubular network seems to play a role in the mediation of the increase of cAMP-independent phosphoryltion of nuclear proteins produced by 8-Br cAMP and leading to a delayed induction of TH. Because colchicine, when added with 8-Br cAMP for 8 hr, also prevents the increase in nuclear phosphorylation, these data suggest that the assembly of microtubular protein might be operative in the intracellular translocation and nuclear uptake of catalytic subunits of cAPK. In addition, these data support the view that an increase in nuclear protein phosphorylation is an essential step in the mediation of the acceleration of TH and mRNA synthesis elilcited by 8-Br cAMP. Elucidation of a precise role for the selective phosphorylation of a nuclear protein in the regulation of TH biosynthesis is currently under investigation. Hence, we believe that the primary cultures of adrenal chromaffin cells are an excellent model system for studying the cAMP mediated regulation of gene expression.

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