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Effect of secretagogues on chromogranin A synthesis in bovine cultured chromaffin cells

Possible regulation by protein kinase C

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Chromogranin A is a major component of storage granules in many different secretory cell types. After [^{35}S]methionine labelling of proteins from cultured bovine chromaffin cells, chromogranin A was immunoprecipitated with specific antibodies, and the radioactivity incorporated into chromogranin A was determined and used as an index of its synthesis rate. Depolarization of cells with nicotine or high K^+ evoked a Ca^{2+}-dependent increase in chromogranin A synthesis, whereas muscarine, which does not evoke significant Ca^{2+} influx from bovine chromaffin cells, had no effect on chromogranin A synthesis. Forskolin, an activator of adenylate cyclase, affected neither the basal nor the nicotine-stimulated rate of chromogranin A synthesis. In contrast, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), an activator of protein kinase C, significantly enhanced the incorporation of radioactivity into chromogranin A. Sphingosine, an inhibitor of protein kinase C, abolished both nicotine-stimulated and TPA-induced chromogranin A synthesis. In addition, long-term treatment of chromaffin cells with TPA decreased protein kinase C activity and inhibited the nicotine-stimulated chromogranin A synthesis. These results suggest that protein kinase C may play an important role in the control of chromogranin A synthesis.

INTRODUCTION

Chromogranin A (CGA) is a large acidic protein which was discovered in the catecholamine-storage granules of bovine adrenal medulla [1]. In these chromaffin granules, CGA is the major polypeptide, comprising 40% of the total soluble protein [2]. The sequence of this protein has been determined [3,4], and it is a single gene product. Recent evidence indicates that CGA is not restricted to chromaffin cells of the adrenal medulla, but is widely distributed in other endocrine cells and tissues [5–14]. Although the function of this protein is not yet known, CGA-derived peptides have recently been shown to exert a negative-feedback control on the secretory activity of chromaffin cells [15]. At present, little is known about CGA synthesis and its regulation.

CGA has an apparent molecular mass of 74 kDa and a pI of approx. 4.5 (for references see [16]). Antibodies raised against the native protein have been found to cross-react with several granule proteins with lower molecular masses. Thus CGA is the major molecule of a family of secretory proteins normally found in these storage granules. It has been proposed that CGA is broken down into smaller polypeptides by endogenous proteases in chromaffin granules [17,18], but such enzymes have yet to be identified.

Here, we have examined the regulation of CGA biosynthesis in bovine chromaffin cells maintained in primary culture. By using a radiolabelled amino acid as precursor and a specific antibody to immunoprecipitate the protein, the incorporation of radioactive amino acid into CGA, employed as an index of CGA synthesis, was determined in resting cells and in cells stimulated with nicotine or high K^+ in the presence and absence of agents affecting protein kinase C activity. The results are discussed in term of the factors regulating CGA synthesis, including a possible relationship to protein kinase C activation and catecholamine secretion.

MATERIALS AND METHODS

Cultured chromaffin cells

Chromaffin cells were isolated from bovine adrenal glands, purified on Percoll gradients and plated on plastic Petri dishes as previously described [19,20]. They were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal-calf serum containing cytosine arabinoside (10 μM), fluorodeoxyuridine (10 μM), streptomycin (50 μg/ml) and penicillin (50 units/ml).

Anti-CGA antiserum

Production of anti-CGA antiserum in rabbit 'Europium' and the characterization of its specificity have been described [11,21]. This antiserum has been previously used to demonstrate the presence of CGA in extra-chromaffin tissues [11,12] and to precipitate CGA synthesized on polysomes *in vitro* [3].

Incorporation of [^{35}S]methionine in chromaffin cells

Cells were cultured on plastic dishes (50 mm diam.) at a density of 128000 cells/cm^2. After 3 days in culture, the

Abbreviations used: CGA, chromogranin A; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PBS, phosphate-buffered saline.
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medium was removed and the cells were incubated for 24 h in methionine-free medium. Cells were then incubated for the indicated times with 50 μCi of [^{35}S]-methionine (1120 Ci/mmol; Amersham France, Les Ullis, France) in 2.5 ml of methionine-free culture medium. To estimate CGA synthesis in stimulated cells, cultured chromaffin cells were first stimulated as indicated with secretagogues (20 μM-nicotine or 59 mM-K^+) in Locke's solution (140 mM-NaCl, 4.7 mM-KCl, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$, 11 mM-glucose, 0.546 mM-ascorbic acid, 15 mM-Hepes, pH 7.5) containing 2.2 mM-$CaCl_2$ and then kept for 20 h in [^{35}S]methionine-containing culture medium. During stimulations, the presence of drugs and their concentrations are indicated in the text and in Figure legends.

Immunoprecipitation of CGA

The procedure for immunoprecipitating CGA was adapted from a previously described technique [22]. Cultured cells were washed three times with phosphate-buffered saline (PBS: 25 mM-potassium phosphate buffer containing 0.15 M-NaCl, pH 7.4). For each culture dish, cells were scraped off the plate in 1 ml of extraction buffer [0.15 M-NaCl, 1 mM-EDTA, 1% Nonidet P40, 20 mM-Tris/HCl, pH 7.2, containing 0.1 mg of soya-bean trypsin inhibitor/ml (Sigma, St Louis, MO, U.S.A.), 10 μM-leupeptin (Sigma), 1 mM-phenylmethanesulphonyl fluoride (Sigma), 10 μM-pepstatin (Sigma) and 0.1 mg of aprotinin/ml (Boehringer, Mannheim, Germany)]. The cell suspension was then homogenized, frozen and thawed, and cleared by centrifugation (100000 *g*, 30 min, at 4 °C in a R-40 Beckman rotor).

To decrease non-specific background radioactivity, normal rabbit serum (100 μl) and Protein A–Sepharose (0.2 ml, at a concentration of 0.3 mg/ml in the extraction buffer; Pharmacia, Bois d'Arcy, France) were added to the 100000 *g* supernatant (1 ml), and the mixture was incubated for 20 min at 4 °C. After centrifugation at 10000 *g* for 10 min, the supernatant was recovered and again incubated with Protein A–Sepharose in the presence of normal rabbit serum. This step was repeated a third time, except that the supernatant was incubated with Protein A–Sepharose in the absence of normal rabbit serum. Then 100 μl of anti-CGA antiserum was added to the resulting supernatant, and the mixture was incubated for 2 h at 20 °C. Then 200 μl of Protein A–Sepharose was added, and the suspension was incubated for a further 1 h with gentle agitation. Sepharose beads were then collected by centrifugation at 10000 *g* for 10 min. The pellet, composed of antibody–antigen complexed with Protein A–Sepharose, was then washed three times with the extraction buffer.

The Protein A–Sepharose–CGA–anti-CGA complex was resuspended and solubilized in 60 μl of SDS-containing buffer [10 mM-Tris/HCl, pH 8.0, containing 1 mM-EDTA, 3% (w/v) SDS, 10% (v/v) β-mercaptoethanol and 20% (v/v) glycerol]. After heating at 100 °C for 3 min, Protein A-coupled Sepharose beads were removed by centrifugation. To measure the incorporated radioactivity, a 10 μl sample was taken, added to 5 ml of Biofluor (New England Nuclear, Dreiech, Germany) and counted in an Intertechnique SL-4000 scintillation counter.

The remaining 50 μl sample was analysed by electrophoresis on 8–15%-polyacrylamide gels. After electrophoresis, the gel was fixed, dried and exposed at −70 °C to Amersham Hyperfilm MP with an intensifying screen. Autoradiographs were quantified by scanning densitometry with a LKB 2202 Ultroscan laser densitometer at 633 nm. The radioactivity incorporated into CGA precursor (molecular mass > 74 kDa), native CGA (74 kDa) and CGA breakdown products (< 74 kDa) was estimated by determining surface areas of individual peaks relative to the total radiactivity.

Measurement of protein kinase C activity in cultured chromaffin cells

Chromaffin cells in culture (3×10^6 cells) were scraped off and homogenized with a Polytron PCU (Kinematica, Luzern, Switzerland) in 0.6 ml of ice-cold buffer, composed of 20 mM-Tris/HCl, 2 mM-EDTA, 0.5 mM-EGTA, 2 mM-dithiothreitol, 2 mM-phenylmethanesulphonyl fluoride, 20 μg of leupeptin/ml, 20 μg of aprotinin/ml, 20 μg of pepstatin/ml, 0.3 M-sucrose and 0.1% Triton X-100. After incubation on ice for 15 min, the cell homogenate was clarified by centrifugation (100000 *g*, 60 min). A 40 μl sample of this homogenate was added to the protein kinase C assay medium [23] containing 100 nM-TPA (12-*O*-tetradecanoylphorbol 13-acetate), 1.75 mM-Ca^{2+} and 0.16 mg of phosphatidylserine/ml. Protein kinase C was assayed by the protocol described by Castagna *et al.* [24].

[^{3}H]Noradrenaline-release assay

Cells were grown on 24 multiple 16 mm-well Costar plates (Costar, Data Packaging Corp., Cambridge, MA, U.S.A.) at a density of 5.0×10^5 cells/well. After 3 days in culture, the medium was removed and the cells were incubated for 24 h in a methionine-free medium. Cells were then loaded with [7-^{3}H]noradrenaline (16 Ci/mmol; Amersham France) for 45 min [20]. They were then washed six times (10 min each wash) with Locke's solution and stimulated for 5 min with 20 μM-nicotine in Locke's solution or 59 mM-K^+ (53.1 mM-NaCl replaced by KCl). [^{3}H]Noradrenaline release after stimulation was determined by measuring the radioactivity present in the incubation media after centrifugation for 10 min at 12000 *g* and in cells after precipitation with 10% (w/v) trichloroacetic acid. The amount of released [^{3}H]noradrenaline was calculated as the percentage of total radioactivity present in the cells before stimulation, assessed from the amount released plus the amount remaining in the cells.

Other assays

Proteins were measured by the method of Bradford [25], with bovine serum albumin as standard. Radioactivity incorporated into proteins was determined after precipitation with ice-cold 15% trichloroacetic acid. Precipitated material was collected by centrifugation for 15 min at 12000 *g*, and radioactivity in pellets was determined by liquid-scintillation counting.

RESULTS

Quantitative analysis of CGA synthesis

In preliminary experiments the uptake of [^{35}S]-methionine in 3-day-old cultured chromaffin cells and the incorporation of radioactive amino acid into proteins were measured as a function of time. As shown in Fig. 1, the uptake rate of [^{35}S]methionine in cultured cells was very rapid for the first 5 h and then decreased to become

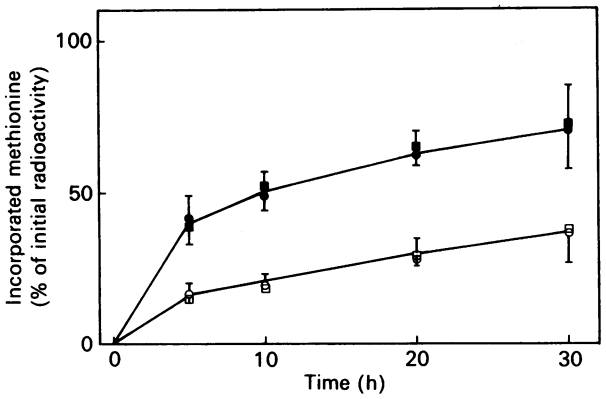

Fig. 1. Time-dependent uptake of [^{35}S]methionine in chromaffin cells and incorporation of radioactivity into total trichloroacetic acid-precipitable proteins

Cultured chromaffin cells (3 days old) were deprived of methionine for 24 h. Cells were then incubated for 4 × 5 min in the absence (○, ●) or presence (□, ■) of 20 μM-nicotine (a 30 min washing period separating each stimulation) and then exposed to [^{35}S]methionine. At the indicated times, radioactivity taken up by cells was measured (●, ■). Proteins were precipitated with trichloroacetic acid and incorporated radioactivity was measured (○, □). Each point represents the determination (±S.D.) on three different chromaffin-cell cultures. Error bars not drawn are smaller than the symbol.

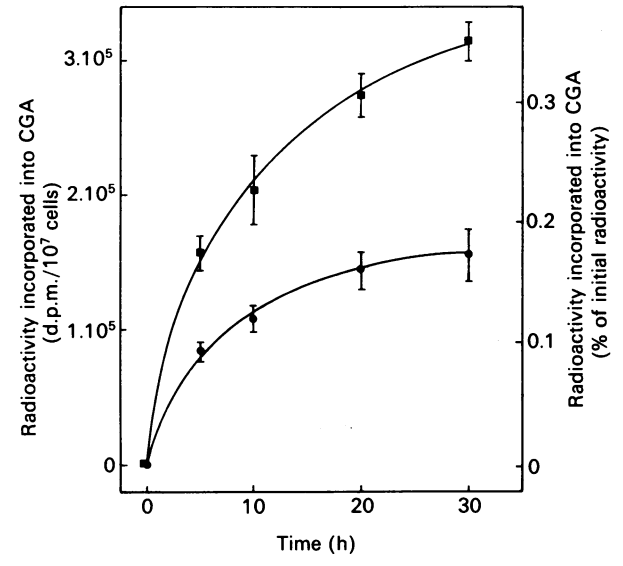

Fig. 2. Time-dependent incorporation of [^{35}S]methionine into CGA

Methionine-deprived cultured chromaffin cells were incubated in the absence (●) or presence (■) of nicotine as indicated in Fig. 1 and then cultured in the presence of [^{35}S]methionine. At the indicated times, cells were lysed and CGA was immunoprecipitated with anti-CGA antiserum. Radioactivity recovered in the immunoprecipitate was measured, and is expressed in d.p.m or in % of total radioactivity initially added to the culture medium. Each point represents the determination (±S.D.) on five different cultures for non-stimulated cells, or on three different cultures for stimulated cells.

linear from 5 h until 30 h. After incubation for 20 h, 63.0±4.5% of the initial radioactivity was incorporated into the cell interior.

Similarly, the rate of incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable proteins was rapid during the first 5 h and then became linear from 5 to 30 h (Fig. 1). After incubation for 20 h with [^{35}S]-methionine, 28.1±1.5% of initial radioactivity was recovered in acid-precipitable proteins, the difference from total radioactivity taken up by cells consisting mostly of free methionine.

As shown in Fig. 2, specific incorporation of radioactivity into CGA was rapid for the first 5 h, during which 50% of maximum radioactivity was reached, and then continued at a steadily decreasing rate during the next 25 h. The amount of radioactivity incorporated after 5 h represented 0.09% of the radioactivity initially present in the medium, and reached 0.16% by 20 h.

The data obtained from autoradiography of immunoprecipitated material separated by polyacrylamide-gel electrophoresis showed two radioactive bands (Fig. 3). The faster component, with an apparent molecular mass of 74 kDa, is the native CGA molecule. The component with an apparent molecular mass of 80 kDa, which is systematically immunoprecipated with the anti-CGA antiserum, represents 10% of immunoprecipitated material. As the antiserum used in this study has been described to be specific for CGA and CGA-related

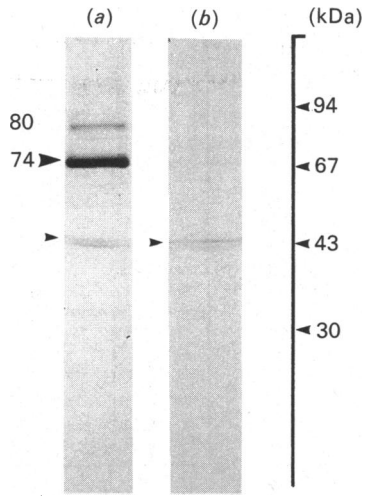

Fig. 3. Autoradiography of immunoprecipitated material from cultured chromaffin cells

Cultured cells were labelled with [^{35}S]methionine for 20 h. At this time, CGA was immunoprecipitated with anti-CGA antiserum, and the CGA–anti-CGA complex was solubilized with SDS and separated by electrophoresis on polyacrylamide gels. Positions of marker proteins of known molecular masses (kDa) are indicated. (*a*) Proteins immunoprecipitated with anti-CGA antiserum. Two radioactive bands are visible: native CGA protein, with an apparent molecular mass of 74 kDa, and the 80 kDa CGA-related component, which might be CGA precursor. (*b*) Proteins immunoprecipitated with normal rabbit serum (control), showing a radioactive band at 43 kDa. This faint radioactive band is also visible in (*a*) and is an artefact owing to migration of background material at the front of the bulk of immunoglobulins.

polypeptides [3,11,12,15,21], we suggest the 80 kDa component to be the precursor of the 74 kDa CGA, although this remains to be clearly demonstrated. After 20 or 30 h incubation with [^{35}S]methionine, no CGA-derived degradation products of less than 74 kDa were detectable on autoradiographs. For practical reasons, a 20 h incubation period was used throughout the present study, and the quantity of radioactivity incorporated at that time into the immunoprecipitated CGA was used as a measure of the rate of CGA synthesis.

Effect of secretagogues on CGA synthesis

Exposure of cells to methionine-free medium for 24 h did not alter their secretory ability in response to nicotine or to direct depolarization with high K^+. This is shown in Table 1: stimulation of cells with either 20 μM-nicotine or 59 mM-K^+ produced a similar release of catecholamine from cells incubated for 24 h either in normal culture medium or in methionine-free culture medium. As shown in Fig. 1, there was also no detectable modification of the total amount of [^{35}S]methionine taken up by stimulated cells compared with resting cells. Furthermore, the incorporation of [^{35}S]methionine into total trichloroacetic acid-precipitable proteins extracted from resting and stimulated cells was similar. Therefore, stimulation of chromaffin cells in culture, which is not altered after a 24 h incubation in methionine-free medium, did not affect the rate of methionine uptake or methionine incorporation into total precipitable proteins.

In contrast, the incorporation of radioactivity into CGA was modified by the secretory activity of chromaffin cells. In these experiments cultured chromaffin cells were

Table 1. Effect of secretagogues on catecholamine release from chromaffin cells cultured for 24 h in normal or methionine-free medium

Cells were loaded with [^{3}H]noradrenaline, washed, and then stimulated for 5 min with secretagogues. The amount of [^{3}H]noradrenaline released from cells preincubated with methionine-free medium ($n = 3$; mean ± S.D.) 24 h before stimulation was similar to that released from control cells ($n = 6$; ± S.D.)

	[^{3}H]Noradrenaline release (%)	
	Normal medium	Methionine-free medium
Basal	4.8 ± 1.3	5.3 ± 0.7
Nicotine (20 μM)	20.6 ± 3.7	21.9 ± 5.4
K^+ (59 mM)	18.6 ± 2.9	19.2 ± 1.3

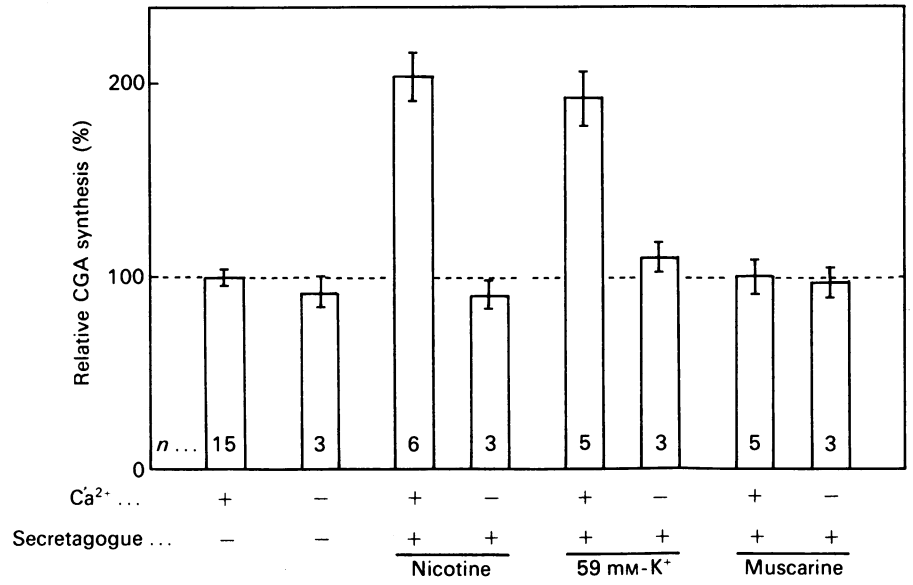

Fig. 4. Effect of secretagogues on CGA synthesis rate in cultured chromaffin cells

Cells were stimulated (four pulses of 5 min each) with either 20 μM-nicotine or 59 mM-K^+; a 30 min washing period separated each stimulation (six changes). Successive stimulations with nicotine provoked net [^{3}H]noradrenaline release of respectively 16.6 ± 5.45 % (first stimulation), 12.0 ± 3.45 % (second stimulation), 7.9 ± 2.1 % (third stimulation) and 6.1 ± 2.0 % (fourth stimulation). Successive direct depolarizations with 59 mM-K^+ evoked net [^{3}H]noradrenaline release of respectively 13.9 ± 1.47 % (first depolarization), 11.9 ± 0.86 % (second depolarization), 10.0 ± 0.045 % (third depolarization) and 8.9 ± 0.30 % (fourth depolarization). Where indicated, Ca^{2+} was omitted from Locke's solution in both the washing and stimulating media. Cells were then incubated for 20 h with [^{35}S]methionine, and radioactivity was measured in the CGA immunoprecipitate. The radioactivity (d.p.m.) found associated with the 80 kDa CGA component and the 74 kDa native CGA from non-stimulated cells was taken as 100 % (control). Experimental values are expressed as a percentage of control values. In three experiments, cells were first washed with Ca^{2+}-free Locke's solution and stimulated with nicotine in the absence of Ca^{2+} from the external medium. In three experiments, cells were incubated with 0.1 mM-muscarine (four incubations of 5 min each) in the absence or presence of calcium in the external medium. Data are given as means (± S.E.M.) of n determinations each performed on a different culture preparation.

first stimulated, then incubated with [^{35}S]methionine, and finally the incorporated radioactivity in immunoprecipitated CGA was determined. As shown in Fig. 2, the incorporation of [^{35}S]methionine into CGA into nicotine-stimulated cells was significantly increased. A single exposure of cells to 20 μM-nicotine resulted in a 30% increase in incorporated radioactivity relative to non-stimulated controls (results not shown), and four successive stimulations with 20 μM-nicotine produced a 104% increase (Figs. 2 and 4). Similar modifications of incorporation of radioactivity into CGA were also observed when cells were stimulated with 59 mM-K^+. Four successive depolarizations with high K^+ provoked a 93% increase (Fig. 4). These data indicate that in cultured chromaffin cells CGA synthesis, estimated from the incorporation of [^{35}S]methionine into CGA, can be modified by agents known to evoke secretion.

Dependence of CGA synthesis on external Ca^{2+}

When bovine chromaffin cells are stimulated, there is an influx of Ca^{2+} into the cell interior [26], where it acts as a second messenger [27]. In order to determine whether CGA synthesis is controlled by the intracellular Ca^{2+} concentration, cells were stimulated in the absence of external Ca^{2+}. Cells were thoroughly washed with Ca^{2+}-free EGTA-containing medium: under these conditions they remained firmly attached to the substratum. In unstimulated cells extensive washing and incubation in Ca^{2+}-free Locke's solution did not change the incorporation of radioactivity into CGA (basal rate), as shown in Fig. 4.

Cells were then stimulated with four successive pulses of 20 μM-nicotine or 59 mM-K^+ in Ca^{2+}-free medium. Under these conditions, neither K^+ nor nicotine caused catecholamine release ([26]; results not shown). As shown in Fig. 4, the rate of CGA synthesis was not modified, suggesting that the nicotine- or high-K^+-stimulated increase in CGA synthesis, like that of nicotine- or high-K^+-stimulated secretion, utilizes an influx of extracellular Ca^{2+}.

In bovine chromaffin cells, muscarinic cholinergic stimulation causes an increase in cytosolic free Ca^{2+} to a value which is below the threshold necessary for activation of the secretory response [27–30]. The effect of muscarine on CGA synthesis was also examined: as shown in Fig. 4, incubation of cells with 0.1 mM-muscarine, a concentration known to evoke only a small increase in free cytosolic Ca^{2+} [29], did not induce any modifications in CGA synthesis, in the presence or absence of Ca^{2+} in the extracellular medium. It is possible that CGA synthesis requires a greater increase in cytosolic Ca^{2+} than the 100 nM increase evoked by muscarine. Nicotine, on the other hand, can raise cytosolic Ca^{2+} to micromolar concentrations [30].

Role of adenylate cyclase in the regulation of CGA synthesis

It has been shown that the synthesis of chromaffin-granule peptides, enkephalin and vasoactive intestinal polypeptide is regulated by cyclic AMP [31–33]. To examine whether the synthesis of CGA is controlled in a similar manner, adenylate cyclase was activated by preincubating cultured chromaffin cells in the presence of 100 μM-forskolin in Locke's solution for 30 min. Under these conditions the basal incorporation of radioactivity into CGA in forskolin-treated cells was not altered relative to control cells, being respectively 110.5±9.5% (±S.E.M.; $n = 3$) and 100.0±4.5% (±S.E.M.; $n = 15$). Forskolin-treated cells were then stimulated with 20 μM-nicotine (four pulses) in the presence of forskolin. After

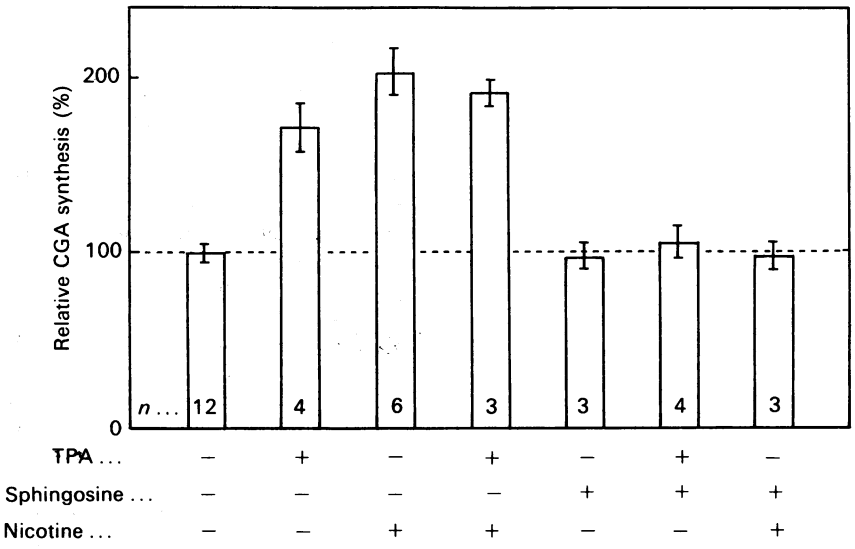

Fig. 5. Effect of TPA on CGA synthesis in cultured chromaffin cells

Cells were incubated with either 0.2 μM-TPA for 30 min or 100 μM-sphingosine for 10 min and then stimulated with nicotine (as described in Fig. 4) in the presence of TPA or sphingosine. Unstimulated cells were either incubated with 0.2 μM-TPA for 30 min or 100 μM-sphingosine for 40 min, or first preincubated with 100 μM-sphingosine for 10 min and then incubated with 0.2 μM-TPA and 100 μM-sphingosine for 30 min. Cells were labelled with [^{35}S]methionine as described for Fig. 4. A 73% increase in radioactivity found in immunoprecipitated CGA was observed when cells were incubated with TPA alone in the absence of nicotine stimulation. This effect of TPA is totally abolished by sphingosine. Sphingosine also blocks the nicotine-induced stimulation of CGA synthesis. Stimulation of TPA-pretreated cells with 20 μM-nicotine resulted in a 92% increase in CGA synthesis rate. Data are given as means (±S.E.M.) of n determinations each performed on a different cell-culture preparation.

stimulation, the incorporation of radioactivity into CGA in forskolin-treated cells was very close to that in control cells, being respectively 193.0±17.0% (±S.E.M.; $n = 3$) and 204.0±12.5% (±S.E.M.; $n = 6$).

The observation of no changes in the incorporation of radioactivity into CGA in either non-stimulated or nicotine-stimulated cells after treatment with forskolin seems to exclude adenylate cyclase as a regulator of CGA synthesis.

Role of protein kinase C in the regulation of CGA synthesis

In chromaffin cells, nicotine stimulation activates the Ca^{2+} phospholipid/diacylglycerol-dependent kinase, protein kinase C [34]. Activation of protein kinase C requires micromolar concentrations of Ca^{2+}, a concentration which is compatible with the intracellular Ca^{2+} concentration measured in stimulated cells [30]. The possibility that the rate of CGA synthesis might be controlled by protein kinase C activation induced by Ca^{2+} was therefore examined. Protein kinase C in many secretory systems is known to be activated by TPA [24] and to be inhibited by sphingosine [35,36]. The rate of CGA synthesis was measured in chromaffin cells treated with TPA and/or with sphingosine.

When resting chromaffin cells were incubated for 30 min with 200 nM-TPA, a 73% increase in the basal rate of CGA synthesis was observed (Fig. 5). Stimulation of TPA-treated cells with nicotine resulted in 92% increase of radioactivity incorporated into CGA. This increase is comparable with the increase resulting from nicotine stimulation in untreated cells. Thus, under the experimental conditions described, the effect of TPA does not seem to be additive to that resulting from nicotine stimulation. Further evidence for a role of protein kinase C in the control of CGA synthesis derives from experiments where chromaffin cells were treated with 100 μM-sphingosine (Fig. 5). Incubation of resting cells with sphingosine did not modify the incorporation of radioactivity into CGA. However, sphingosine completely abolished the effect of TPA and nicotine on the CGA-synthesis rate. In both of these cases, the incorporated radioactivity remained the same as that observed in non-stimulated control cells.

Long-term treatment of cells with TPA decreases protein kinase C activity in many cell types [37]. A similar decrease in protein kinase C activity was found in cultured chromaffin cells incubated with TPA: as shown in Fig. 6(*a*), incubation for 24 and 48 h with 200 nM-TPA induced respectively 35% and 50% decreases in protein kinase C activity. Such a treatment with TPA did not modify cell viability, since (i) the quantity of total proteins recovered from 10^7 cells, either untreated or treated with 200 nM-TPA and then incubated for 24 h in methionine-free medium, was not altered, being respectively 2.33±0.22 mg and 2.43±0.13 mg (two different cultures; three dishes per culture), and (ii) the number of chromogranin A-positive cells counted after labelling with anti-CGA antiserum and fluorescein-coupled goat anti-rabbit immunoglobulins was identical in control and TPA-treated cultures. In addition, the uptake of [^{35}S]-methionine into chromaffin cells was not altered. In contrast, the 20 h incorporation of [^{35}S]methionine into total trichloroacetic acid-precipitable proteins was slightly decreased by 18% and 30% in two different cultures, and a similar inhibition was also found in nicotine-stimulated cells.

When CGA synthesis was measured after prolonged treatment of cells with TPA, no modification was detectable in resting cells. However, the stimulatory effect of nicotine on the incorporation of radioactivity into CGA was inhibited (Fig. 6*b*). Although the inhibition of CGA synthesis was greater than that of protein kinase C activity, the quantitative relation between the two is not at present understood. All these observations indicate a correlation between the activation of CGA synthesis and protein kinase C activity, and suggest a possible role for protein kinase C in CGA synthesis.

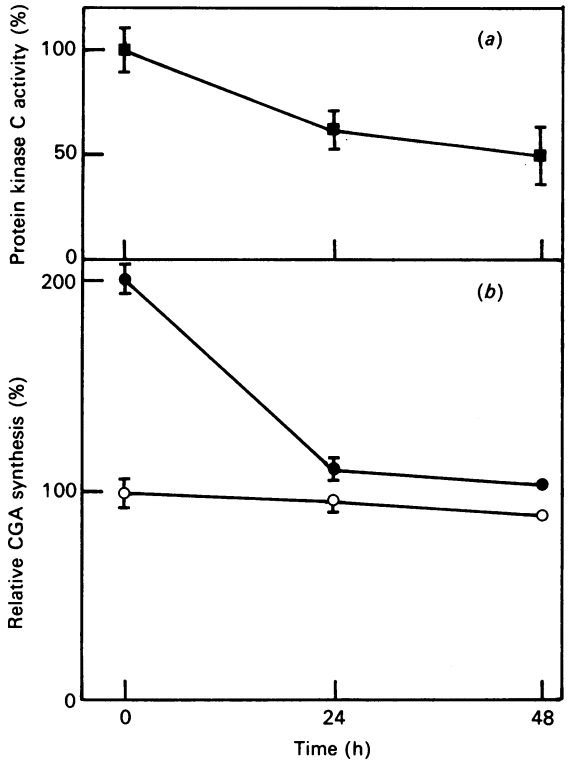

Fig. 6. Effect of long-term treatment of cultured chromaffin cells with TPA on protein kinase C activity and nicotine-induced stimulation of CGA synthesis

In (*a*), protein kinase C was determined in cultured chromaffin cells incubated with 200 nM-TPA for 0, 24 and 48 h. In (*b*), the rate of CGA synthesis was measured in 24 h-TPA- and 48 h-TPA-treated cells either not stimulated (○) or stimulated with 20 μM-nicotine (●). Cells were stimulated with nicotine and labelled with [^{35}S]methionine as described in Fig. 4 legend. Results at 24 h and 48 h are expressed relative to control values, taken as 100% at zero time. In (*a*), each point is given as the mean (±S.E.M.) of four determinations, each performed on a different culture preparation. In (*b*), data at zero time and 24 h are given as means (±S.E.M.) of respectively six and three determinations, each performed on a different culture preparation. At 48 h, data are from one experiment.

DISCUSSION

The present study is the first characterization and quantification of CGA synthesis in chromaffin cells maintained in primary culture. When chromaffin cells

were cultured in the presence of [^{35}S]methionine, the amino acid was taken up and incorporated into proteins, and CGA was labelled. With specific anti-CGA antiserum [15], native CGA and CGA-related proteins were precipitated and the incorporation of [^{35}S]methionine was quantified. We postulated that the radioactivity recovered in CGA proteins reflected the rate of synthesis, although some loss of radioactive CGA might have occurred, owing to the continuous basal release. However, our assumption is likely to be correct, since (i) CGA synthesis is a slow process, (ii) basal release represents only a small percentage of total cell granules and (iii) methionine deprivation for 24 h does not alter cell viability.

In rat adrenal medulla, it has been recently reported that levels of different constituents of chromaffin granules can be differentially affected by pharmacological agents [38]. Insulin and reserpine were found to increase dramatically the levels of enkephalins, dopamine β-hydroxylase, the amine carrier and chromogranin-B-immunoreactive components, but had no effect on chromogranin-A-immunoreactive species. Similarly, we have previously shown that nicotine-induced secretion in cultured chromaffin cells was followed by a compensatory increase in enkaphalin, but not in CGA [21]. Subsequently we observed that released CGA is very sensitive to proteolytic degradation in the external medium [15]. As our antibody does not recognize CGA-related products with a molecular mass below 43 kDa, the radioimmunoassay used in the previous study [21] may have underestimated the actual amount of CGA in the external medium, and thus the apparent absence of compensatory CGA synthesis may be misleading. The purpose of the present study was to investigate the effect of cholinergic agents on the rate of CGA synthesis rather than on CGA levels.

A major finding in the present work is the ability of secretagogues known to stimulate secretion in chromaffin cells to stimulate CGA synthesis also. Stimulation of cholinergic receptors with nicotine provoked an increase in the rate of CGA synthesis. The decrease in intracellular CGA levels owing to secretion is compensated by increasing CGA synthesis, thereby maintaining intracellular CGA content at a constant value, as previously reported [39]. The observation that direct depolarization of the cell membrane with high K^+ evoked a similar increase in the incorporation of [^{35}S]methionine into CGA demonstrates that CGA synthesis is dependent on an influx of extracellular Ca^{2+} into cells and does not depend on cholinergic receptors. This dependence on extracellular Ca^{2+} of CGA-synthesis regulation has also been shown by using stimulation in Ca^{2+}-free medium. Thus, what are the possible mechanisms involved in the regulation of CGA synthesis?

In a series of experiments Eiden and colleagues have shown that biosynthesis of both enkephalin and vasoactive intestinal peptide in cultured chromaffin cells is regulated by cyclic AMP and by nicotinic-receptor stimulation at a pretranslational site [31–33]. Forskolin, which enhances synthesis of enkephalin, vasoactive intestinal peptide and enkephalin mRNA [39], did not affect the rate of CGA synthesis. In contrast, the rate of CGA synthesis was dramatically affected by phorbol esters which activate protein kinase C. TPA itself has been described to have no direct effect on catecholamine release from freshly isolated chromaffin cells [40] and to provoke only a slight increase in the basal release of catecholamines from cultured cells [41]. Since the TPA-induced increase in CGA synthesis did not occur in parallel with secretion, its effect on CGA synthesis probably results from a direct activation of protein kinase C. It is possible that protein kinase C activation is sufficient for CGA synthesis, but not in itself sufficient for catecholamine secretion.

It has been established that the nicotinic stimulation of bovine cultured chromaffin cells causes protein kinase C activation and a concomitant shift of the enzyme from a soluble state in the cytosol to a bound state [34]. Therefore the activation of protein kinase C, caused indirectly by nicotine stimulation via the rise in cytosolic Ca^{2+} concentration or directly by phorbol esters, might result in the activation of CGA synthesis. This conclusion is strengthened by the observation that sphingosine, an inhibitor of protein kinase C, blocks the nicotine-induced increase in CGA-synthesis rate. Although several reports have shown that protein kinase C-dependent processes are inhibited by sphingosine [35,36], the selectivity of this compound in living cells has not been fully established. However, our observation that TPA-induced activation of CGA synthesis is also abolished by sphingosine demonstrates that the effect of sphingosine is antagonistic to that of phorbol esters, and argues in favour a role of protein kinase C in the control of CGA synthesis. Further support for this hypothesis comes from our data on cells which have been preincubated with TPA for 24 and 48 h. Such a treatment causes both a decrease in protein kinase C activity and an inhibition of the CGA synthesis induced by nicotine.

In conclusion, it is worthwhile emphasizing that different compounds, present in the same secretory granule and co-released from this compartment by secretagogues [20,42,43], are not regulated in the same way. Syntheses of enkephalin and catecholamine seem to be controlled by multiple kinases [44–48]. In contrast, CGA synthesis can be regulated by protein kinase C, but not by cyclic AMP-dependent protein kinase. Although different regulation pathways are thought to be involved in modulating the ratios of individual intragranular components as a function of cell secretory activity, their precise role is still unclear.

Whereas a co-regulation of CGA secretion and CGA mRNA has recently been described in a tumour cell line derived from human lung cancer [49], CGA mRNA levels in a cell line derived from a human medullary thyroid carcinoma [49] and in bovine chromaffin cells maintained in primary culture [50] do not seem to be modified. Thus the question of whether protein kinase C regulates CGA synthesis in normal cells at a translational step is still unresolved and merits further investigation.

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