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Second-messenger generation in PC12 cells

Interactions between cyclic AMP and Ca^{2+} signals

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Changes in cyclic AMP concentrations were studied in intact PC12 pheochromocytoma cells exposed to a variety of treatments. A marked increase was triggered by *N*-(L-2-phenylisopropyl)adenosine, the activator of an adenosine receptor, whereas a decrease (observed even after phosphodiesterase blockade) was induced by carbachol, working through a muscarinic receptor inhibited by the selective muscarinic blocker pirenzepine, only at high concentration (K_i 450 nM). A decrease in cyclic AMP was also induced by clonidine, an α_2-adrenergic-receptor agonist. Both the α_2-adrenergic and the muscarinic inhibitions were prevented by pretreatment of the cells with pertussis toxin, and were unaffected by the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate. The latter drug caused a decrease in the resting cyclic AMP concentrations, and a potentiation of the increase induced by adenosine-receptor activation. Except for clonidine, all these treatments were found to be effective in both growing PC12 cells and, although to a smaller degree, in cells that had stopped growing and had acquired a neuron-like phenotype after prolonged treatment with nerve growth factor (NGF). Neither forskolin (a direct activator of adenylate cyclase) nor the activation of adenosine and α-adrenergic receptors was able to modify the resting cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ in PC12 cells. Likewise, the K^+-induced $[Ca^{2+}]_i$ transients were unchanged after these treatments, whereas the transients induced by carbachol through the activation of a muscarinic receptor highly sensitive to pirenzepine were moderately potentiated by forskolin (and, to a lesser degree, by the adenosine analogue) and attenuated by clonidine. These results characterize in further detail the spectrum and the mutual interrelationships of the intracellular signals induced by receptor activation in PC12 cells, also as a function of the NGF-induced differentiation.

INTRODUCTION

An increasing body of evidence indicates that the various intracellular second messengers, generated as a result of receptor and channel activation, can play interconnected roles in the processes ultimately leading to the functional responses of the cell. Only part of the effects elicited by each messenger can therefore be envisioned as direct [i.e. mediated exclusively by the corresponding effector system(s), such as the specific protein kinases], whereas the others are, at least in part, indirect, as they involve modulations of the generation, function and/or metabolism of other messengers (see Reuter, 1984; Rasmussen & Barrett, 1984; Nishizuka, 1986; Meldolesi *et al.*, 1987). Adequate information on these problems necessarily requires a comprehensive experimental approach, in which various intracellular messengers are investigated in parallel. The cellular model that we have chosen for such studies is PC12, a line of neurosecretory cells, that was originally developed from a rat pheochromocytoma (Greene & Tischler, 1976). When PC12 cells are grown in conventional culture media ($PC12^-$ cells), they resemble chromaffin cells in both their phenotype and the ability to take up, store and release catecholamines. When, however, these cells are cultured in the presence of nerve growth factor (NGF), they stop dividing, grow in size, acquire a neuronal like-phenotype and modify extensively their surface and metabolic properties ($PC12^+$ cells) (Greene & Tischler, 1976; Greene, 1984). PC12 cells can thus be studied at two well-defined stages, and offer therefore the opportunity to investigate whether and to what extent various cellular processes are modified during differentiation. In previous reports from our laboratory the intracellular events induced in $PC12^-$ and $PC12^+$ cells by the activation of both voltage-gated Ca^{2+} channels and a muscarinic receptor highly sensitive to the selective blocker pirenzepine (changes of the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$ and the hydrolysis of polyphosphoinositides) were characterized in some detail (Vicentini *et al.*, 1985*a*, 1986; Pozzan *et al.*, 1986; DiVirgilio *et al.*, 1986). The present report is focused on the changes in cellular cyclic AMP induced by various treatments, including the activation of various receptors, and on the interactions between the cyclic AMP and the Ca^{2+} signals.

Abbreviations used: PIA, *N*-(L-2-phenylisopropyl)adenosine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; NGF, nerve growth factor; PTx, pertussis toxin; $[Ca^{2+}]_i$, free cytoplasmic Ca^{2+} concentration.

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MATERIALS AND METHODS

Materials

N-(L-2-Phenylisopropyl)adenosine (PIA), clonidine, forskolin, 8-bromo cyclic AMP and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma, St. Louis, MO, U.S.A.; the kit for cyclic AMP radioimmunoassay was from NEN, Florence, Italy; fura-2 acetoxymethyl ester was from Molecular Probes, Junction City, OR, U.S.A. Affinity-purified pertussis toxin (PTx) and 2.5 S NGF were kindly given by Dr. R. Rappuoli and Dr. P. Calissano. All other chemicals were reagent grade, purchased from the sources specified elsewhere (Pozzan *et al.*, 1986; Vicentini *et al.*, 1986).

Cells

The source of PC12 cells and the conditions for their culture and differentiation by treatment with NGF (50 ng/ml; 10–12 days) are specified elsewhere (Pozzan *et al.*, 1986). At the beginning of the experiments, cells were detached and large clumps dissociated (Pozzan *et al.*, 1986). Assays were carried out on cell suspensions. The incubation medium (KRH) contained (mM): NaCl, 125; KCl, 5; $MgSO_4$ and KH_2PO_4, 1.2; $CaCl_2$, 2; Hepes/NaOH buffer, pH 7.4, 25; glucose, 6. Drugs were added dissolved in water, ethanol or dimethyl sulphoxide. Controls received solvents only (maximal concn. 0.2%).

Cyclic AMP assay

Cyclic AMP was measured in 400 μl portions of cell suspensions [cell concn. (2–2.5) $\times 10^6$ and (1.2–1.5) $\times 10^6$/ml, corresponding to 0.3–0.375 and 3.6–4.5 mg of protein/ml for $PC12^-$ and $PC12^+$ cells respectively] incubated at 37 °C (for the times indicated and with the various concentrations of different agents) in a controlled-temperature water bath. At the end of the incubations, the samples were usually treated with ice-cold 7% trichloroacetic acid. Other samples were chilled in ice and then centrifuged (9000 *g*, 10 s) to separate the cells from the incubation media, which were then separately treated with trichloroacetic acid. The acid-treated samples were kept at 4 °C for 30 min and then centrifuged at 9000 *g* for 10 min to sediment proteins. The supernatants were washed with diethyl ether to remove the acid, and used for measuring cyclic AMP by radioimmunoassay, with the RIANEN ^{125}I-cyclic AMP kit. The amount of protein in each sample was determined by the bicinchoninic acid procedure (Pierce & Suelter, 1977), and the cyclic AMP content was normalized to protein.

$[Ca^{2+}]_i$ assay

$[Ca^{2+}]_i$ was measured in suspensions of cells [(0.5–1) $\times 10^6$/ml] loaded with the fluorescent indicator fura-2 (Grynkiewicz *et al.*, 1985), by the procedure described in detail elsewhere (Malgaroli *et al.*, 1987).

RESULTS

Cyclic AMP content in $PC12^-$ and $PC12^+$ cells

The total cyclic AMP content of freshly suspended $PC12^-$ and $PC12^+$ cells was found to be 227 ± 78 and 137 ± 10 pmol/mg of protein respectively (mean $\pm$ S.D.; $n = 11$ and 9). No major change in the cyclic AMP content was observed after 10 min incubation of the cell suspensions in KRH. After this incubation, over 90% of the cyclic AMP was recovered with the cells, and the rest in the medium (Fig. 1).

Effects of receptor agonists

Figs. 1–3 illustrate the changes in cyclic AMP content induced by the application of various receptor stimulants. In agreement with previous data (Guroff *et al.*, 1981; Rabe & McGee, 1983; Baizer & Weiner, 1985*a*; Race &

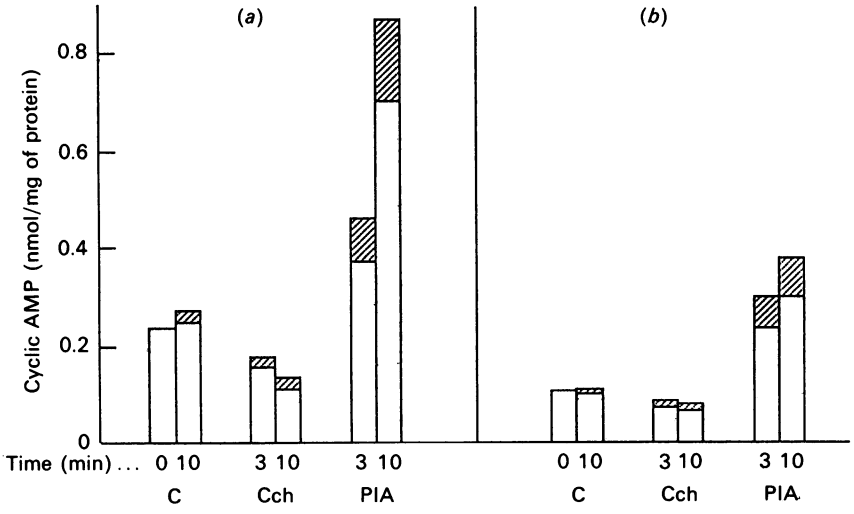

Fig. 1. Distribution of cyclic AMP in cells and medium after incubation of $PC12^-$ (*a*) and $PC12^+$ (*b*) cell suspensions: effects of carbachol and PIA treatment

Parallel samples of cell suspensions were incubated at 37 °C without drugs (C), with carbachol (Cch; 100 μM) or with PIA (10 μM) for the times indicated on the abscissa, after which the cells were separated from the medium by centrifugation. The cyclic AMP recovered in the cell pellets and supernatants is indicated by □ and ▨ respectively. Results are averages of two experiments.

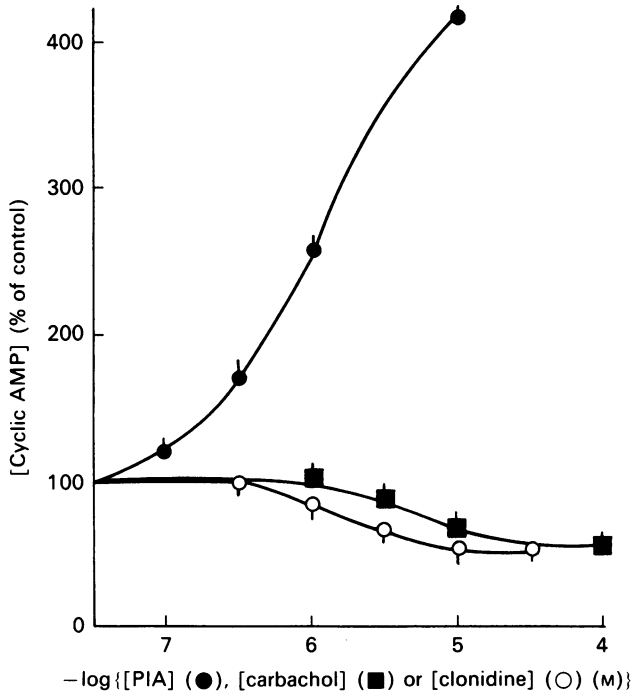

Fig. 2. Concentration-dependence of the changes in cyclic AMP content induced by PIA (●), carbachol (■) or clonidine (○) in $PC12^-$ cell suspensions

Parallel suspensions of cells were incubated at 37 °C for 10 min with the concentrations of drugs indicated on the abscissa. Results are averages ± S.D. for three to five experiments. Cyclic AMP content in control samples, incubated without drugs, was 225 ± 70 pmol/mg of protein.

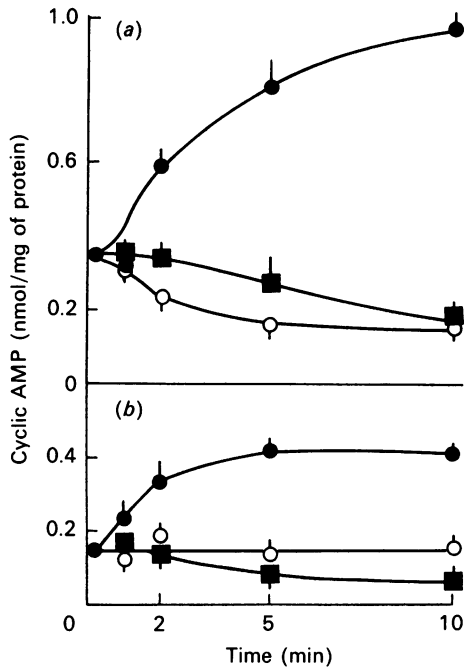

Fig. 3. Time course of the changes in cyclic AMP content induced by PIA (●), carbachol (■) or clonidine (○) in $PC12^-$ (*a*) and $PC12^+$ (*b*) cell suspensions

Parallel suspensions of cells were incubated at 37 °C with the drugs. Samples were taken and processed at the indicated time points. Concentrations of drugs were: PIA, 10 μM; carbachol, 100 μM; clonidine, 10 μM. Results are averages ± S.D. for three or four experiments.

Wagner, 1985), the synthetic adenosine analogue PIA was found to increase cyclic AMP up to 4-fold in $PC12^-$ cells (half-maximal effect at 1 μM) (Figs. 1*a*, 2 and 3*a*). The PIA-induced increase was visible within 2 min of incubation (Fig. 3*a*). It was mostly accounted for by intracellular cyclic AMP, because, even after 10 min incubation with a 10 μM concentration of the stimulant, only about 20 % of the cyclic nucleotide was recovered in the medium (Fig. 1*a*). The cholinergic agonist carbachol, on the other hand, induced decreases of up to 50 % of the resting cyclic AMP concentrations (half-maximal effect at 6 μM), again with no major change of the distribution of the nucleotide between cells and medium (Figs. 1*a*, 2 and 3*a*). Carbachol effects were completely blocked by the muscarinic antagonist atropine, administered at 0.1 μM (results not shown). A decrease in the cyclic AMP content of $PC12^-$ cells was also induced by the α_2-adrenergic agonist clonidine (half-maximal effect at 2 μM). The effect of this drug was found to be faster than that of carbachol, but the maximal decrease in cyclic AMP was similar for the two compounds (Figs. 2 and 3*a*).

In $PC12^+$ cells the effects of the adenosine and cholinergic agonists, PIA and carbachol, resembled those in $PC12^-$ cells in cell/medium distribution (Fig. 1*b*), time course (Fig. 3*b*), concentration-dependence and inhibition by atropine (results not shown). However, the stimulatory response by PIA was always smaller in $PC12^+$ than in $PC12^-$ cells [on average, +252 ± 61 and +410 ± 98 pmol/mg of protein respectively (means ± S.D., $n = 9$ and 11), after 10 min incubation with 10 μM-PIA]. In addition, an inhibition by clonidine (0.1–10 μM) could not be detected reproducibly in $PC12^+$ cells (Fig. 3*b*).

In a further series of experiments, the ability of clonidine and carbachol to antagonize the rise in cyclic AMP induced by a 5 min pretreatment with PIA was investigated (Fig. 4). Even in the continuous presence of the stimulator, the two drugs caused in $PC12^-$ cells a decrease in cyclic AMP that reached 50 % of the PIA-induced value within 5 min. In $PC12^+$ cells carbachol was similarly effective, whereas with clonidine no appreciable inhibition was observed (Fig. 4*b*). When cells were pretreated for 18 h with PTx (1 μg/ml), the inhibition by both clonidine and carbachol disappeared (Fig. 4), as expected for effects occurring though the involvement of a toxin-sensitive G coupling protein, possibly G_i (Gilman, 1987).

Previous studies demonstrated that application of carbachol to PC12 cells triggers the hydrolysis of polyphosphoinositides and the consequent increase in $[Ca^{2+}]_i$ through the activation of a muscarinic receptor highly sensitive to the selective blocker pirenzepine (Vicentini *et al.*, 1985*a*, 1986; Pozzan *et al.*, 1986). High $[Ca^{2+}]_i$ could then activate phosphodiesterase cleavage of cyclic AMP, and thus be responsible for the decline of the cellular nucleotide concentrations that we have now observed. In order to investigate this possibility, the experiments illustrated in Fig. 5(*a*) were carried out, in which cells pretreated for 5 min with PIA together with the potent phosphodiesterase inhibitor RO 20-1724 were

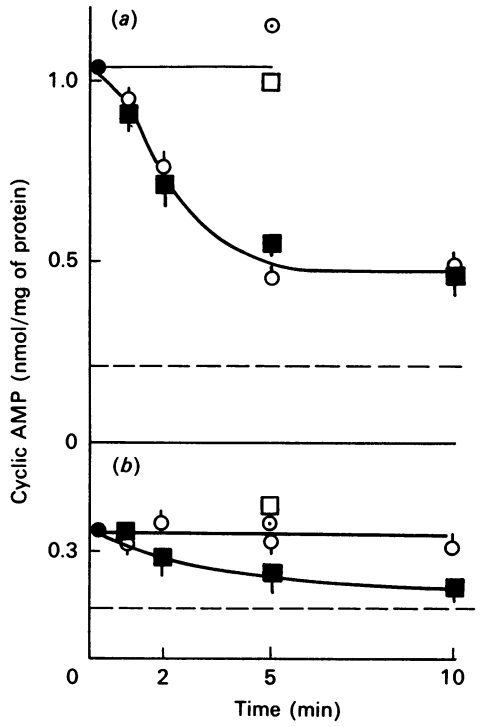

Fig. 4. Time course of the changes in cyclic AMP content induced by PC12⁻ (*a*) and PC12⁺ (*b*) cell suspensions by carbachol (■) or clonidine (○) administered after a 5 min preincubation with PIA

Experiments were carried out as in Fig. 3, but the cell suspensions received PIA (10 μM) at −5 min, and then carbachol (100 μM) or clonidine (10 μM) at zero time: □ and ⊙ refer to the effect of carbachol and clonidine, respectively, in cell suspensions pretreated with PTx (1 μg/ml) for 18 h. Results are averages ± S.D. for three experiments. Broken lines indicate the resting cyclic AMP concentration, measured in parallel suspensions of control PC12⁻ and PC12⁺ cells.

further incubated for 10 min in the continuous presence of these drugs and of various concentrations of carbachol. Inclusion of RO 20-1724 caused a greater increase of cyclic AMP compared with PIA alone (2087 and 1176 pmol/mg of protein), but had no effect on the percentage inhibition by carbachol. Thus stimulation of polyphosphoinositide hydrolysis and $[Ca^{2+}]_i$ increase seem to play no major role in the cyclic AMP-decreasing effect of carbachol. This conclusion was reinforced by results with pirenzepine. As shown in Fig. 5(*b*), this blocker was able to inhibit partially the effect on cyclic AMP of 100 μM-carbachol only when administered at concentrations $\geqslant 1$ μM. This corresponds to a K_i (calculated as described by Cheng & Prusoff, 1973) of 450 nM.

Effects of other drugs

Various other drugs affected the cyclic AMP content in PC12⁻ cells (Table 1). The Ca^{2+} ionophore ionomycin (10–300 nM) caused a decrease in cyclic AMP. This effect was probably mediated by the activation of phosphodiesterase, as it was abolished by RO 20-1724 (Table 1). The phorbol ester TPA, on the other hand, had complex effects. When applied alone (10–100 nM) it induced moderate decreases in cyclic AMP (Table 1), which were

Table 1. Changes in cyclic AMP concentration induced by various treatments in PC12⁻ cells

Incubations with the indicated agent were carried out for 10 min at 37 °C. Values shown are averages of two to four experiments carried out in triplicate.

Agent	Concn. (M)	Cyclic AMP (% of controls)
Ionomycin*	10^{-8}	73
	6×10^{-8}	69
	3×10^{-7}	64
Ionomycin + RO 20-1724	3×10^{-7} and 5×10^{-4}	93
TPA*	10^{-9}	100
	10^{-8}	78
	10^{-7}	73
TPA‡ in PIA-treated cells	10^{-9}	161
	10^{-8}	182
	10^{-7}	208
Forskolin†	10^{-5}	5410

* Controls received equal volumes of the drug solvent (dimethyl sulphoxide).
† Also, but solvent was ethanol.
‡ Cells were treated with PIA (10^{-5} M) from −5 min, then with either dimethyl sulphoxide (controls) or TPA from 0 min.

further decreased by the application of carbachol (results not shown). In contrast, TPA applied together with PIA caused a potentiation (up to 2-fold) of the stimulation brought about by the adenosine analogue (Table 1). Finally, the direct activator of adenylate cyclase, forskolin (10 μM), caused an increase in cyclic AMP of approx. 50-fold. In PC12⁺ cells the changes induced by these agents were similar, although usually smaller (especially after forskolin and PIA + TPA) than those observed in PC12⁻ cells (results not shown).

Effects on $[Ca^{2+}]_i$

Ca^{2+} homoeostasis in PC12⁺ and PC12⁻ cells has previously been investigated by use of quin-2 (Pozzan *et al.*, 1986). The results indicated that PC12⁺ cells are characterized by a higher resting $[Ca^{2+}]_i$, and by greater $[Ca^{2+}]_i$ transients induced by depolarizing concentrations of K^+, ionomycin and carbachol (the last through the activation of a muscarinic receptor highly sensitive to pirenzepine; Pozzan *et al.*, 1986). The present results were obtained by the use of fura-2, a newer dye which is advantageous with respect to quin-2 because it is more fluorescent and can therefore be loaded intracellularly at lower concentration, with correspondingly smaller increase in the cytosolic buffering capacity (Grynkiewicz *et al.*, 1985). The results obtained confirm the higher resting $[Ca^{2+}]_i$ and the larger transients induced by carbachol in PC12⁺ than in PC12⁻ cells [189 ± 37 versus 110 ± 12 nM ($n = 14$ and 25) and 456 ± 82 versus 246 ± 37 nmol at 100 μM-carbachol ($n = 3$ and 9); see also Fig. 6]. In contrast, the differences of the transients induced by K^+ and ionomycin in differentiated and non-differentiated PC12 cells were smaller than previously reported (10–20 % instead of 40–50 % as in Pozzan *et al.*, 1986). Whether this different result is due to technical

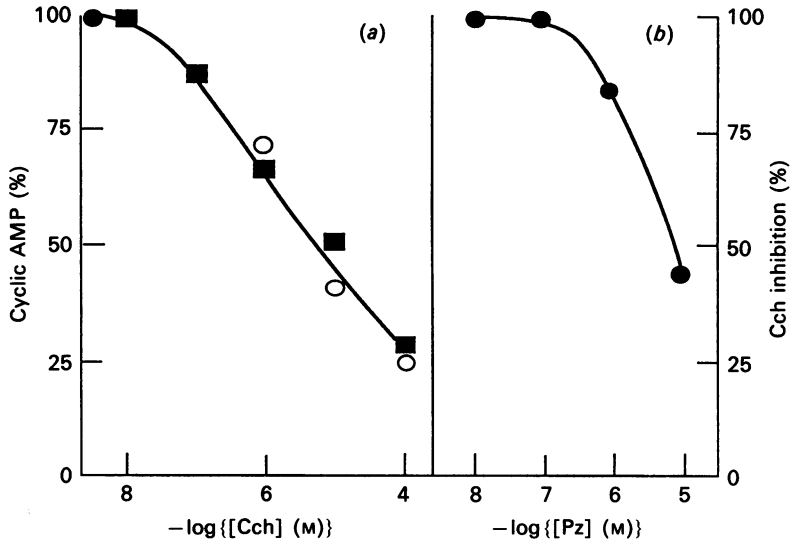

Fig. 5. Effects of carbachol on the cyclic AMP content of $PC12^-$ cell suspensions pretreated with either PIA + RO 20-1724 (*a*) or pirenzepine (*b*)

In the experiments shown in (*a*), the cell suspensions received first (−5 min) PIA (10 μM) without (■) or with (○) RO 20-1724 (0.5 mM), then (0 min) carbachol at the concentrations indicated on the abscissa, and the incubation was carried out for 10 min. Results are averages of two duplicate experiments. The 100% cyclic AMP value is that measured in the cells treated with PIA or PIA + RO 20-1724 only: 1.176 and 2.087 μmol/mg of protein respectively. In the experiments shown in (*b*), the cells were preincubated for 2 min with pirenzepine (Pz) at the concentrations shown on the abscissa, after which carbachol (100 μM) was added, and the incubation continued for 10 min. The 100% carbachol inhibition was a 45% lowering of the cyclic AMP content observed with respect to the suspensions incubated with or without pirenzepine alone. Results are averages of two duplicate experiments.

reasons, or to a modified responsiveness of the cells in culture, was not systematically investigated.

All the treatments found to affect cyclic AMP concentrations were tested for their possible effects on $[Ca^{2+}]_i$. The simple increase or decrease in the cyclic nucleotide concentrations failed to induce any detectable changes in the resting $[Ca^{2+}]_i$ in both $PC12^-$ and $PC12^+$ cells, as observed with forskolin (10 μM; see also Vicentini *et al.*, 1986), PIA (10 μM), 8-bromo cyclic AMP (0.5 mM) and clonidine (10 μM) (results not shown). Moreover, pretreatment with these agents was unable to modify significantly the size and kinetics of the $[Ca^{2+}]_i$ increases induced by K^+ depolarization in both $PC12^-$ (Figs. 6*a* and 6*b*) and $PC12^+$ cells (results not shown). The only positive interaction between cyclic AMP and $[Ca^{2+}]_i$ signals that we were able to identify concerned the effects of carbachol. Pretreatment with either forskolin (10 μM; cf. Figs. 6*d* and 6*f* with 6*c* and 6*e*) or, to a lesser extent, PIA (10 μM; results not shown) caused a slight but reproducible increase which affected the initial $[Ca^{2+}]_i$ peak less than the subsequent declining plateau phase of the transient in both $PC12^+$ and $PC12^-$ cells [averages with forskolin at 1 min after the application of the cholinergic agonist (100 μM) were $+19 \pm 4$ and $+12 \pm 5\%$ ($n = 3$ and 2)]. Pretreatment with the α_2-agonist clonidine (10 μM), on the other hand, caused a moderate inhibition of the carbachol-induced $[Ca^{2+}]_i$ transients of $PC12^-$ cells, that concerned both the peak ($-30 \pm 5.9\%$, $n = 3$) and the plateau phases (cf. Figs. 6*h* and 6*g*). The effects of forskolin and clonidine were seen also when carbachol was applied to the cells in a KRH medium without Ca^{2+} and containing 1 mM-EGTA (results not shown).

DISCUSSION

Although cell stimulation with a variety of agonists may trigger initially the generation of one single second messenger, the ultimate physiological response is often the result of a complex network of interactions in which the primary messenger is involved together with other signals (Rasmussen & Barrett, 1984; Nishizuka, 1986; Meldolesi *et al.*, 1987). In order to dissect out the various components of such a network, the different signals need to be identified and studied in parallel under carefully controlled experimental conditions. This is being done in our laboratory with PC12, a cell line extensively employed as a model system of neuroendocrine and neuronal cells (Greene, 1984). The present paper is focused primarily on cyclic AMP and the interactions of this nucleotide with $[Ca^{2+}]_i$. Cyclic AMP is believed to play important roles in PC12 cells, concerning both the regulation of neurotransmitter release (Rabe *et al.*, 1982; Rabe & McGee, 1983; Baizer & Weiner, 1985*a*,*b*) and the acquisition of neuronal properties during differentiation (Guroff *et al.*, 1981; Race & Wagner, 1985).

Changes in cyclic AMP: effects on $[Ca^{2+}]_i$

Two receptor agonists, PIA and clonidine (addressed to adenosine and α_2-adrenergic receptors respectively), and the direct adenylate cyclase activator, forskolin, were used. The existence in PC12 cells of an adenosine receptor coupled to adenylate cyclase stimulation was already known (Guroff *et al.*, 1981; Rabe & McGee, 1983; Baizer & Weiner, 1985*a*; Race & Wagner, 1985), whereas an α_2 receptor negatively coupled to the enzyme had never been reported. The number of α_2 receptors

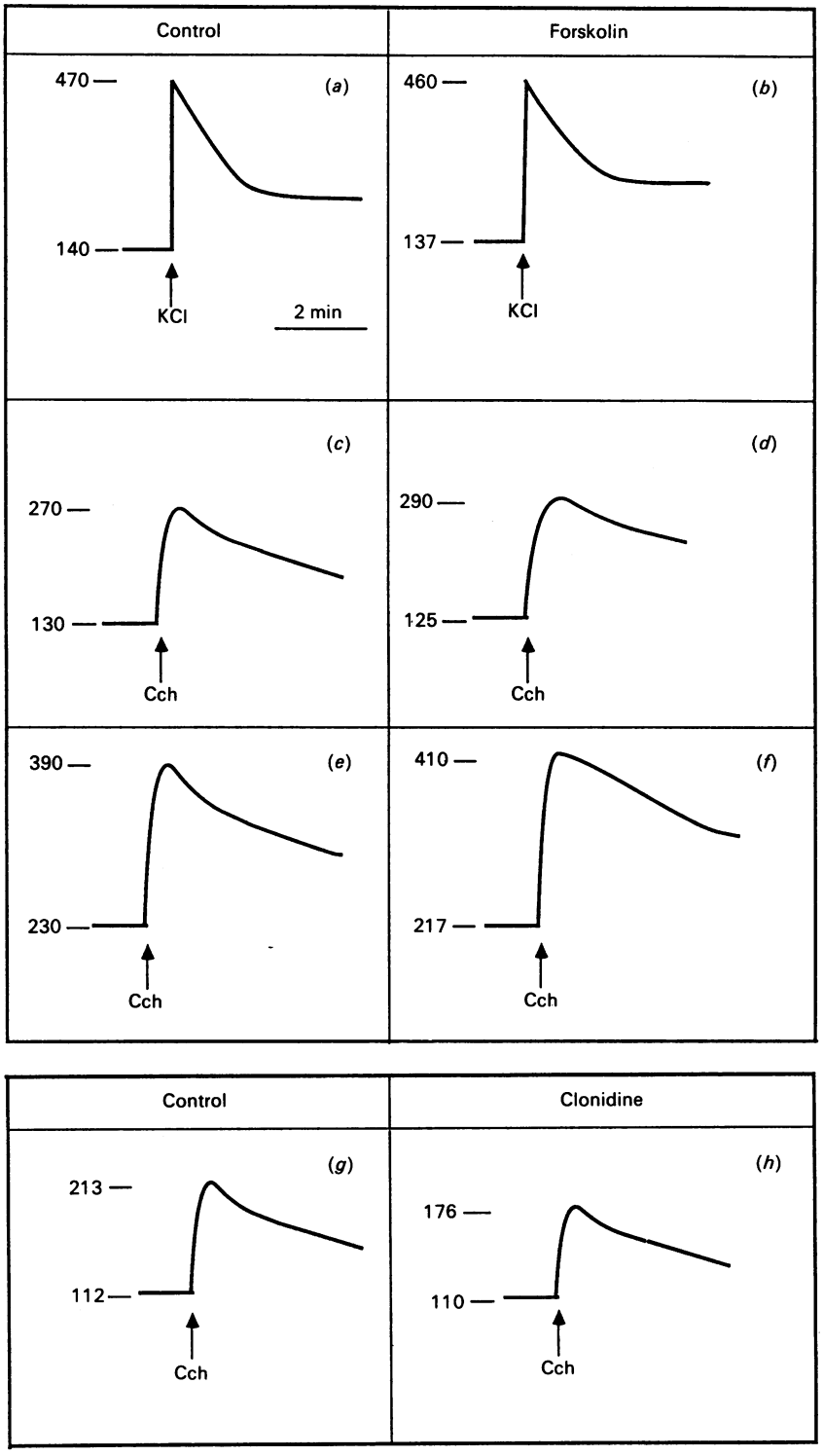

Fig. 6. $[Ca^{2+}]_i$ transients induced by various treatments in $PC12^-$ (*a–d*, *g* and *h*) and $PC12^+$ (*e*, *f*) cells

Each pair of traces illustrates transients induced in parallel samples of the same cell suspension loaded with fura-2, which were preincubated at 37 °C for 15 min either without (controls) or with forskolin (10 μM) or clonidine (10 μM) as indicated, before treatment with either KCl (50 μM) or carbachol (CCh; 100 μM) (arrows). Concentrations of cells were $(0.6–0.9) \times 10^6$/ml; concentrations of fura-2 were 0.2 and 0.07 nmol/10^6 $PC12^-$ and $PC12^+$ cells respectively. The numbers to the left of traces are calibrated $[Ca^{2+}]_i$ values (nM).

expressed by PC12 cells is probably small, because binding experiments with [^{3}H]clonidine yielded inconsistent results (not shown). None of the above drugs was able to modify resting $[Ca^{2+}]_i$, and none affected the $[Ca^{2+}]_i$ transients induced by the application of a depolarizing stimulus (high K^+). Thus PC12 cells seem to differ in this respect from other cellular systems, such as the heart and various types of neurons, in which cyclic

AMP has been reported to play a major role in the regulation of $[Ca^{2+}]_i$ homoeostasis by increasing the activation probability of voltage-gated Ca^{2+} channels (for review see Reuter, 1984).

Effects of Ca^{2+} ionophores and phorbol esters

In previous studies, both ionomycin and TPA were found to stimulate catecholamine release when applied to PC12 cells (Pozzan *et al.*, 1984). Whether these effects were mediated only by the corresponding intracellular signals ($[Ca^{2+}]_i$ rise and protein kinase C activation respectively) or involved cyclic AMP was not established. Our present data rule out this latter possibility, because cyclic AMP was not increased, but rather decreased, by both these agents. The effect of TPA was, however, complex because, when administered with PIA, it caused a potentiation of the cyclic AMP increase induced by the latter drug, as previously reported by Hollingsworth *et al.* (1986). In other cell types, evidence suggesting the existence of multiple interaction mechanisms between the two signalling systems has been reported (Kadata *et al.*, 1985; Bouvier *et al.*, 1987; Yoshimasa *et al.*, 1987). No similar evidence is yet available in PC12 cells.

Effects of carbachol

A decrease in cellular cyclic AMP, with respect both to resting values and to the increases induced by treatment with PIA, was observed not only after treatment with clonidine, but also with carbachol. The PC12 subclone used for the present studies does not express a functioning nicotinic receptor (Vicentini *et al.*, 1985*a*, 1986; Pozzan *et al.*, 1986); thus the cyclic AMP-decreasing effect observed with carbachol could only be due to muscarinic-receptor activation, as indicated also by its inhibition by low concentrations of atropine. In addition, the effect of carbachol (1) was relatively resistant to pirenzepine (K_i 450 nM), a muscarinic blocker partially selective for some muscarinic receptor subtypes (Peralta *et al.*, 1988), (2) was unaffected by short pretreatment of the cells with TPA, and (3) was no longer seen after cell treatment with PTx, indicating that the receptor involved is coupled to adenylate cyclase via a toxin-sensitive G protein, possibly G_i (Gilman, 1987). Moreover, the cyclic AMP-decreasing effect of carbachol was unaffected by phosphodiesterase blockade with RO 20-1724, demonstrating that it is not the consequence of the activation of the enzyme which might be triggered by the carbachol-induced increase of $[Ca^{2+}]_i$. All these properties demonstrate that the effect of carbachol on cyclic AMP is due to the activation of a muscarinic receptor different from the muscarinic receptor coupled to the hydrolysis of polyphosphoinositides in PC12 cells. The latter is in fact blocked by low concentrations of pirenzepine (K_i 16 nM), rapidly desensitized by TPA, and unaffected by PTx (Vicentini *et al.*, 1985*b*, 1986). Carbachol induced half-maximal inhibition of cyclic AMP generation at 6 μM concentration, and half-maximal stimulation of polyphosphoinositide hydrolysis at 12 μM (Vicentini *et al.*, 1986). Therefore in the 1–100 μM concentration range the two sets of signals, each mediated by a distinct receptor subtype, are elicited in parallel by carbachol in PC12 cells. Another interesting result with carbachol was the slight but reproducible potentiation and inhibition, respectively, of the $[Ca^{2+}]_i$ transients observed in cells pretreated with drugs that increase or decrease cyclic AMP, i.e. PIA and a forskolin on the one hand, and clonidine on the other. In our previous studies with quin-2 (Vicentini *et al.*, 1986), the potentiation by forskolin of the carbachol transients was not detected, probably because of the less sensitive experimental conditions employed (different dye, greater dye loading, less accurate matching of the samples etc.). The difference of the present results with carbachol from the results with high K^+ suggests that the potentiation effect occurs at the level of the muscarinic receptor coupled to polyphosphoinositide hydrolysis, possibly by a protection towards desensitization, as suggested by the results reported in the following paper (Meldolesi *et al.*, 1988).

NGF-induced cell differentiation

Compared with undifferentiated $PC12^-$ cells, cells treated long-term with NGF ($PC12^+$) exhibited a lower resting concentration of cyclic AMP, and smaller changes induced by the various treatments employed. The meaning of these observations, which are at variance with those previously reported by Baizer & Weiner (1985*a*), remains to be clarified. Another difference observed was the lack of an appreciable cyclic AMP response of $PC12^+$ cells to clonidine, which suggests a lower (or absent) expression of the α_2-adrenergic receptor, to be added to the list of receptor changes already known to occur in PC12 cells during differentiation (Greene, 1984).

In conclusion, the results reported in the present paper characterize in further detail the degree of interconnection among the various intracellular second messengers in PC12 cells. Together with previous reports from our and other laboratories, the information herewith presented is employed in the following paper (Meldolesi *et al.*, 1988) to identify the intracellular mechanisms reponsible for the control of catecholamine release in PC12 cells.

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REFERENCES

Baizer, L. & Weiner, N. (1985*a*) J. Neurosci, **5**, 1176–1179
Baizer, L. & Weiner, N. (1985*b*) J. Neurochem. **44**, 495–501
Bouvier, M., Leeb-Lundberg, L. M. F., Benovic, J. L., Caron, M. G. & Lefkovitz, R. J. (1987) J. Biol. Chem. **262**, 3106–3113
Cheng, Y. C. & Prusoff, W. H. (1973) Biochem. Pharmacol. **22**, 3099–3108
DiVirgilio, F., Pozzan, T., Wollheim, C. B., Vicentini, L. M. & Meldolesi, J. (1986) J. Biol. Chem. **261**, 32–36
Gilman, A. G. (1987). Trends Pharmacol. Sci. **8**, 467–472
Greene, L. A. (1984) Trends Neurosci. **7**, 91–94
Greene, L. A. & Tischler, A. S. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 2424–2428
Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
Guroff, G., Dickens, G., End, D. & Londos, C. (1981) J. Neurochem. **37**, 1431–1437
Hollingsworth, E. B., Ukena, D. & Daly, J. M. (1986) FEBS Lett. **196**, 1431–1437
Kadata, T., Gilman, A. G., Watanabe, Y., Bauer, S. & Jakobs, K. H. (1985) Eur. J. Biochem. **151**, 431–437

Malgaroli, A., Milani, D., Meldolesi, J. & Pozzan, T. (1987) J. Cell Biol. **105**, 2145–2153
Meldolesi, J., Pozzan, T. & Ceccarelli, B. (1987) Handb. Exp. Pharmacol. **83**, 339–359
Meldolesi, J., Gatti, G., Ambrosini, A., Pozzan, T. & Westhead, E. W. (1988) Biochem. J. **255**, 761–768
Nishizuka, Y. (1986) Science **233**, 305–312
Peralta, E. G., Winslow, J. W., Ashkenazi, A., Smith, D. H., Rachmandran, J. & Capon, D. J. (1988) Trends Pharmacol. Sci. **9**, Suppl. 12–18
Pierce, J. & Suelter, C. H. (1977) Anal. Biochem. **82**, 478–480
Pozzan, T., Gatti, G., Dozio, N., Vicentini, L. M. & Meldolesi, J. (1984) J. Cell Biol. **9**, 628–638
Pozzan, T., DiVirgilio, F., Vicentini, L. M. & Meldolesi, J. (1986) Biochem. J. **234**, 547–553
Rabe, C. S. & McGee, R. (1983) J. Neurochem. **41**, 1623–1634
Rabe, C. S., Schneider, J. & McGee, R. (1982) J. Cyclic Nucleotide Res. **8**, 371–384
Race, H. M. & Wagner, J. A. (1985) J. Neurochem. **44**, 1588–1592
Rasmussen, H. & Barrett, P. Q. (1984) Physiol. Rev. **44**, 938–984
Reuter, H. (1984) Nature (London) **301**, 569–576
Vicentini, L. M., Ambrosini, A., DiVirgilio, F., Meldolesi, J. & Pozzan, T. (1985*a*) J. Cell Biol. **100**, 1330–1333
Vicentini, L. M., DiVirgilio, F., Ambrosini, A., Pozzan, T. & Meldolesi, J. (1985*b*) Biochem. Biophys. Res. Commun. **127**, 310–317
Vicentini, L. M., Ambrosini, A., DiVirgilio, F., Meldolesi, J. & Pozzan, T. (1986) Biochem. J. **234**, 555–562
Yoshimasa, T., Sibley, D. R., Bouvier, M., Lefkovitz, R. J. & Caron, M. G. (1987) Nature (London) **327**, 67–70

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