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Second-messenger control of catecholamine release from PC12 cells

Role of muscarinic receptors and nerve-growth-factor-induced cell differentiation

Jacopo MELDOLESI,*‡ Giuliana GATTI,* Anna AMBROSINI,* Tullio POZZAN†§ and Edward W. WESTHEAD*‖

* Department of Pharmacology, CNR Center of Cytopharmacology and Scientific Institute S. Raffaele, University of Milano, via Olgettina 60, 20132 Milano, Italy, and † Institute of General Pathology, CNR Center of Biomembranes, University of Padova, 35100 Padova, Italy

The role of various intracellular signals and of their possible interactions in the control of neurotransmitter release was investigated in PC12 cells. To this purpose, agents that affect primarily the cytosolic concentration of Ca^{2+}, $[Ca^{2+}]_i$ (ionomycin, high K^+), agents that affect cyclic AMP concentrations (forskolin; the adenosine analogue phenylisopropyladenosine; clonidine) and activators of protein kinase C (phorbol esters) were applied alone or in combination to either growing chromaffin-like $PC12^-$ cells, or to neuron-like $PC12^+$ cells differentiated by treatment with NGF (nerve growth factor). In addition, the release effects of muscarinic-receptor stimulation (which causes increase in $[Ca^{2+}]_i$, activation of protein kinase C and decrease in cyclic AMP) were investigated. Two techniques were employed to measure catecholamine release: static incubation of [^{3}H]dopamine-loaded cells, and perfusion incubation of unlabelled cells coupled to highly sensitive electrochemical detection of released catecholamines. The results obtained demonstrate that: (1) release from PC12 cells can be elicited by both raising $[Ca^{2+}]_i$ and activating protein kinases (protein kinase C and, although to a much smaller extent, cyclic AMP-dependent protein kinase); and (2) these various control pathways interact extensively. Activation of muscarinic receptors by carbachol induced appreciable release responses, which appeared to be due to a synergistic interplay between $[Ca^{2+}]_i$ and protein kinase C activation. The muscarinic-induced release responses tended to become inactivated rapidly, possibly by feedback desensitization of the receptor mediated by protein kinase C. Muscarinic inactivation was prevented (or reversed) by agents that increase, and accelerated by agents that decrease, cyclic AMP. Agents that stimulate release primarily through the Ca^{2+} pathway (ionomycin and high K^+) were found to be equipotent in both $PC12^-$ and $PC12^+$ cells, whereas the protein kinase C activator 12-*O*-tetradecanoylphorbol 13-acetate was approx. 10-fold less potent in $PC12^+$ cells, when administered either alone or in combination with ionomycin. In contrast, the cell binding of phorbol esters was not greatly modified by NGF treatment. Thus control of neurotransmitter release from PC12 cells is changed by differentiation, with a diminished role of the mechanism mediated by protein kinase C.

INTRODUCTION

PC12 cells, a cultured line developed from a rat pheochromocytoma (Greene & Tischler, 1976), are a popular model of neurosecretory cell that has been extensively investigated at two defined stages of differentiation: growing $PC12^-$ cells, which express a chromaffin-like phenotype; and $PC12^+$ cells, which have stopped growing and have acquired a neuron-like phenotype owing to prolonged (10–12 days) treatment with nerve growth factor (NGF). Previous studies by us and others have dealt with the intracellular signals generated in response to a variety of treatments, in particular those that induce activation of receptors and channels (Vicentini *et al.*, 1985*a*, 1986; Baizer & Weiner, 1985; Pozzan *et al.*, 1986; Di Virgilio *et al.*, 1986; Harris *et al.*, 1986; Gatti *et al.*, 1988), whereas other studies have dealt with the control of neurotransmitter release (Greene & Rein, 1977; Rabe *et al.*, 1982; Rabe & McGee, 1983; Pozzan *et al.*, 1984; Meldolesi *et al.*, 1984; Baizer & Weiner, 1985; Kongsamut & Miller, 1986). On the whole, however, the information accumulated on release was less detailed than that on transmembrane signalling, as it was limited to treatments that activate (or mimic) one single intracellular signal, and to a few combined treatments. Other treatment combinations were not tested for their effects on release, nor was the activation of physiological receptors coupled to the hydrolysis of polyphosphoinositides (Berridge, 1987). Finally, the possible effect(s) of differentiation on the second-messenger control of the release process was investigated only in part (Kongsamut & Miller, 1986). All these

Abbreviations used: $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; NGF, nerve growth factor; PIA, *N*-(L-2-phenylsopropyl)adenosine; PDBu, phorbol 12,13-dibutyrate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

‡ To whom reprint requests should be addressed.
§ Present address: Institute of General Pathology, University of Ferrara, 44100 Ferrara, Italy.
‖ Present address: Department of Biochemistry, University of Massachusetts, Amherst, MA 01003, U.S.A.

problems are systematically dealt with in the present paper.

MATERIALS AND METHODS

Information on the sources of PC12 cells and most of the materials used, the conditions for cell culture, cell differentiation with NGF, detachment and dissociation of cell clumps, protein measurement, the incubation medium (Krebs–Ringer buffered with Hepes; KRH) and the drugs used is given in the preceding paper (Gatti *et al.*, 1988). [^{3}H]Dopamine and [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) (sp. radioactivity 50 and 23 Ci/mmol respectively) were purchased from Amersham International, Amersham, Bucks., U.K. PDBu, phenylmethanesulphonyl fluoride, benzamidine and aprotinin were from Sigma, St. Louis, MO, U.S.A. $[Ca^{2+}]_i$ was measured by the fura-2 technique, as described elsewhere (Malgaroli *et al.*, 1987).

Catecholamine release

Two techniques were used. Static incubations were carried out as described by Meldolesi *et al.* (1984). In brief, 0.2 ml volumes of cell suspension [$(1–2)\times10^6$ and $(0.4–0.75)\times10^6$ cells/ml for PC12$^-$ and PC12$^+$ respectively], preloaded with [^{3}H]dopamine (15000–25000 d.p.m./sample) by a 30 min incubation with the tracer in the presence of ascorbic acid and pargyline, were transferred to plastic Eppendorf tubes and incubated at 37 °C in the presence of desmethylimipramine, with or without the other agents to be tested. At the end of the incubations (3–10 min, depending on the time course of the release response elicited by the stimulatory agent used), the samples were chilled in ice, centrifuged (10000 ***g***, 10 s) and washed once with cold incubation medium. The cell pellet and, in many experiments, the first supernatant, were mixed with Atomlight (NEN, Florence, Italy) and counted for radioactivity in a Beckman LS 7500 liquid-scintillation spectrometer.

For the perfusion technique, cell suspensions (approx. 20×10^6/sample) were washed with incubation medium, mixed with Sephadex G-15 resin and poured into an Uptight short column (20 mm × 4 mm; Upchurch Scientific, Oak Harbor, WA, U.S.A.) which served as the cell chamber. One side of the column was connected via a pump to a reservoir, the other side to an electrochemical detector (Bioanalytical System, Lafayette, IN, U.S.A.), which permitted the continuous read-out of catecholamines in the perfusate. Perfusion was carried out at 37 °C and 1 ml/min flow speed. Short (1.2 s) pulse administrations of agents dissolved in the incubation buffer were delivered by means of a manual injector valve. Longer administrations were given by perfusing the cells with a drug-containing medium. At the end of the experiments the cells were lysed by perfusing medium containing 1% Triton X-100, and the catecholamine content remaining within the cells was measured. The contribution to the electrode responses of the drugs used as such was estimated systematically by injections into the system without cells. The values thus obtained were subtracted in all the experiments from the experimental traces. Calibration of the traces was obtained by running through the system known amounts of dopamine (the major catecholamine of PC12 cells; Greene & Tischler, 1976). Further details can be found in Herrera *et al.* (1985).

[^{3}H]PDBu binding

The technique for equilibrium binding of [^{3}H]PDBu was essentially as described by Shoyab & Todaro (1980). In brief, triplicate tubes containing either 2×10^6 or 0.8×10^6 cells (PC12$^-$ and PC12$^+$ respectively) in 0.5 ml of KRH medium were incubated for 40 min at 27 °C with 5–100 nM-[^{3}H]PDBu with or without 10 μM non-radioactive PDBu. After incubation the samples were filtered through glass-fibre filters (Whatman GF/C) in a Millipore vacuum system, washed with 5×0.5 ml of cold KRH, then transferred to scintillation vials containing 5 ml of Atomlight and counted for radioactivity as described above. In a further series of experiments, cell suspensions in KRH (1.1×10^6 and 0.7×10^6 for PC12$^-$ and PC12$^+$ respectively, in 0.2 ml) were given [^{3}H]PDBu (20 nM, with or without 10 μM unlabelled PDBu) for 10 min at 37 °C, after which they were diluted with 1 ml of ice-cold KRH and centrifuged (10000 ***g***, 10 s). The pellets were resuspended and washed once with 1 ml of KRH; then the cells were resuspended in 1 ml of 0.17 M-sucrose buffered at pH 7.4 with 5 mM-Hepes/NaOH containing a mixture of proteinase inhibitors (phenylmethanesulphonyl fluoride, 0.5 mM; benzamidine, 1 mM; aprotinin, 5 munits/ml). The cell were homogenized by 30 up-and-down strokes in a hand-operated Dounce homogenizer, and membrane pellets separated from the supernatants by centrifugation at 150000 ***g*** for 20 min in a Spinco L7 refrigerated ultracentrifuge. Samples of the resuspended pellets and of the supernatants were mixed with Atomlight and counted for radioactivity. Specific binding was defined by the difference of labelling between samples incubated without and with excess of unlabelled PDBu (10 μM).

RESULTS

The two techniques used in parallel for the study of catecholamine release from PC12 cells were preferentially employed for different purposes. Static incubations of [^{3}H]dopamine-loaded cells were employed when dealing with relatively large release responses. Such a technique is advantageous when a large number of samples are analysed in parallel. When, on the other hand, small responses were investigated, the perfusion technique coupled with the electrochemical on-line measurement of catecholamines was employed. In addition to its greater sensitivity, this technique offers advantages when studying time courses of either a single or multiple events, the latter triggered by successive administrations of one or several agents.

Effect of depolarizing agents and Ca^{2+} ionophores

Previous studies demonstrated that release of [^{3}H]-dopamine induced from PC12 cells by agents that act primarily by increasing $[Ca^{2+}]_i$, i.e. high K^+ and Ca^{2+} ionophores, is composed of two subsequent phases: a brisk, intense but short-lived peak, followed by a much smaller and longer plateau phase (Greene & Rein, 1977; Meldolesi *et al.*, 1984; Pozzan *et al.*, 1986; Di Virgilio *et al.*, 1987). Our present results demonstrate that these release responses are unchanged by PC12-cell differentiation with NGF. In fact, not only the time course (not shown), but also the concentration-dependence of the release induced by K^+ and ionomycin were essentially the same in PC12$^-$ and PC12$^+$ cells (Figs. 1*a* and 1*b*).

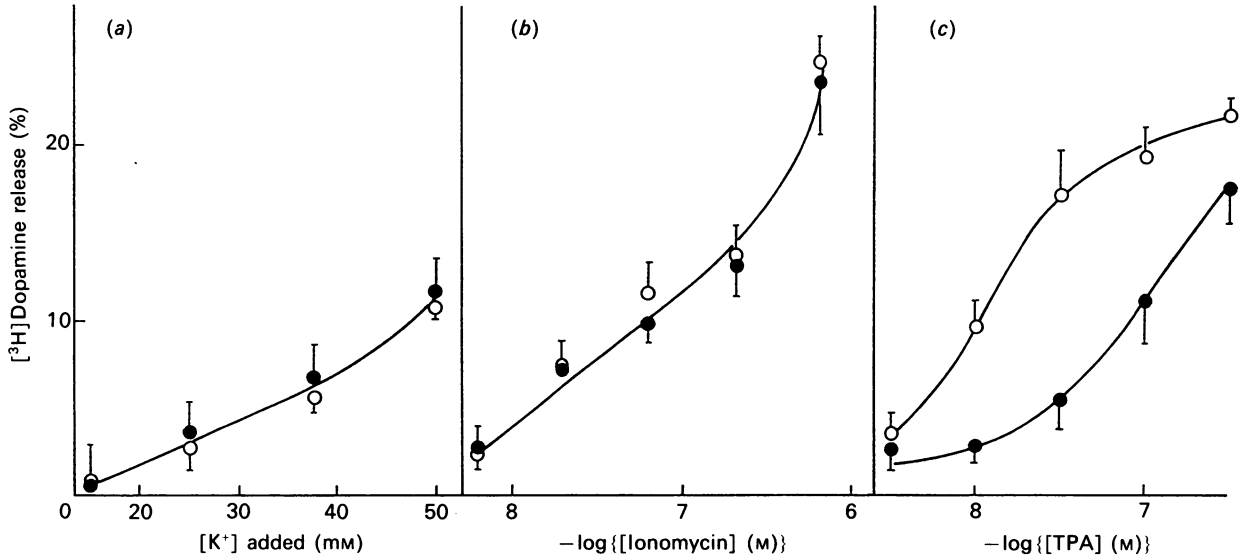

Fig. 1. Concentration-dependence of [^{3}H]dopamine release from PC12$^-$ and PC12$^+$ cells stimulated with various agents

Portions (0.2 ml) of either PC12$^-$ (○) or PC12$^+$ (●) cell suspensions [final concns. (1–2) × 10^6 and (0.4–0.75) × 10^6 cells/ml respectively] that had been preloaded with [^{3}H]dopamine were mixed with the stimulants at the final concentrations indicated on the abscissa, and incubated at 37 °C for either 3 (*a*, *b*) or 10 (*c*) min. The results shown are averages of 10–15 experiments ± S.D., expressed as percentages of the radioactivity recovered in the controls incubated in parallel. Ionomycin and TPA were dissolved in dimethyl sulphoxide. The corresponding controls received the solvent only (concn. 0.2%).

Effect of phorbol esters

Catecholamine release induced from PC12 cells by phorbol esters was already known to differ substantially from those induced by the agents discussed so far, in both its much lower $[Ca^{2+}]_i$-dependency and its slow time course (Pozzan *et al.*, 1984). Comparison of the effects of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in PC12$^-$ and PC12$^+$ cells now revealed a marked change in the potency of this agent, depending on differentiation. As shown in Fig. 1*c*, the half-maximal effect of TPA in PC12$^-$ cells was observed at a concentration (15 nM) almost ineffective in PC12$^+$ cells. Higher concentrations of TPA, however, were considerably effective even in PC12$^+$ cells (half-maximal effect at 80 nM). Similar results were obtained when another phorbol ester, PDBu, was used instead of TPA (half-maximal effects at 110 nM and 600 nM in PC12$^-$ and PC12$^+$ cells respectively; results not shown). The marked difference in potency of the phorbol esters suggested the possibility that the specific binding of these drugs (known to be accounted for by the protein-phosphorylating enzyme protein kinase C; Nishizuka, 1986) was modified at the two differentiation stages of PC12 cells. However, direct experiments failed to substantiate this possibility. In fact, equilibrium binding of [^{3}H]PDBu to PC12$^+$ cells was found to be not smaller, but rather slightly greater, than that to PC12$^-$ cells (Fig. 2). Likewise, no substantial difference between PC12$^-$ and PC12$^+$ cells was observed in another series of experiments, in which binding of [^{3}H]PDBu (20 nM) was carried out under the conditions used for the release experiments (37 °C, 10 min incubation), after which cells were homogenized and centrifuged at high speed to separate membranes (which contained 18.12 ± 2.2 and 17.15 ± 2.5% of the specifically bound [^{3}H]PDBu in PC12$^-$ and PC12$^+$ cells respectively; $n = 4$) from the soluble cytoplasm (results not shown).

Effects of agents that modify cyclic AMP concentrations: phenylisopropyladenosine (PIA), clonidine, and the activator of adenylate cyclase, forskolin

Even when the very sensitive perfusion technique was employed, no detectable effect on catecholamine release was observed after application to either PC12$^+$ or PC12$^-$ cells of receptor agonists that increase or decrease cyclic AMP (PIA and clonidine respectively; Gatti *et al.*, 1988). In contrast, the direct activator of adenylate cyclase, forskolin (0.1–2 μM), induced a small but persistent stimulation, which was revealed when the cells were perfused for many minutes with the drug (Fig. 5). Such an effect, which subsided slowly when forskolin was removed from the perfusion medium, could not be due either to released cyclic AMP, which had no effect when perfused through the electrode, or to cytotoxicity, because treatment for up to 2 h with the indicated concentrations of the drug, carried out in parallel under identical conditions, failed to increase significantly the proportion of Trypan Blue-positive PC12$^-$ or PC12$^+$ cells in the analysed preparations.

Effects of combinations of agents

The interconnection of the various mechanisms that regulate the process of catecholamine release was investigated by studying the effects of appropriate agent combinations. A moderate potentiation by forskolin (1 μM), previously noticed in PC12 cells by Rabe & McGee (1983) and by Baizer & Weiner (1985), was found here to occur also in PC12$^+$ cells not only with high K^+, but also with the Ca^{2+} ionophore ionomycin (results not shown). In contrast, the adenosine analogue PIA and the α_2-agonist clonidine failed to modify appreciably the release responses induced by these two agents.

Another group of combinations investigated included

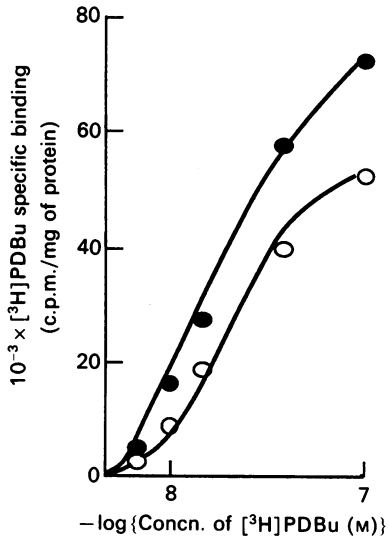

Fig. 2. Specific binding of [^{3}H]PDBu to intact PC12$^-$ (○) and PC12$^+$ cells (●)

Triplicate samples of cells (2×10^6 and 0.8×10^6 cells for PC12$^-$ and PC12$^+$ respectively), suspended in the KRH incubation medium, were mixed with the tracer at the final concentrations shown on the abscissa, and incubated at 27 °C for 40 min. Bound and free [^{3}H]PDBu were separated by filtration. The data shown (averages of two experiments) were corrected by subtraction of the unspecific binding, measured in parallel samples treated with the tracer together with an excess (10 μM) of non-radioactive PDBu.

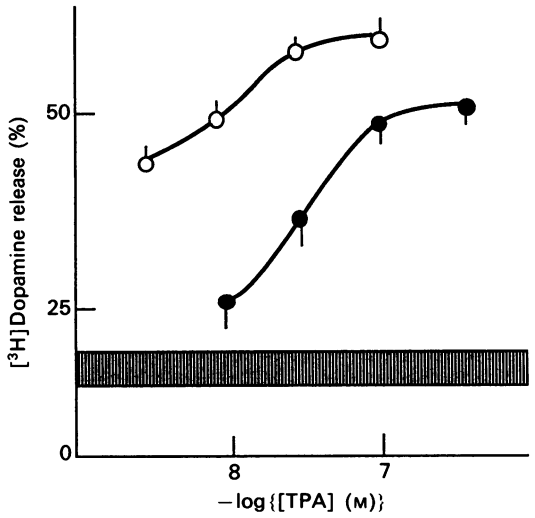

Fig. 3. Synergistic [^{3}H]dopamine release induced by the TPA–ionomycin combination in PC12$^-$ (○) and PC12$^+$ (●) cells

Portions of [^{3}H]dopamine-loaded cell suspensions were mixed with ionomycin (0.2 μM) together with TPA at the concentrations indicated on the abscissa, and then incubated for 10 min at 37 °C. The shadowed area shows the release response (±S.D.) elicited by 0.2 μM-ionomycin alone. Controls received the solvent (dimethyl sulphoxide; 0.2%). Results (expressed as in Fig. 1) are averages ± S.D. of eight experiments.

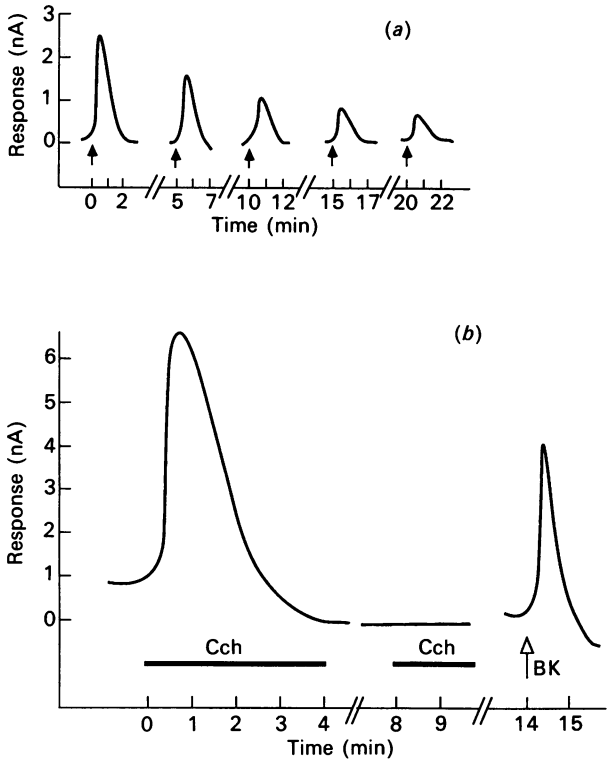

Fig. 4. Carbachol-induced catecholamine release from perfused PC12$^-$ cells: effect of multiple pulses (*a*) and administration by prolonged perfusion (*b*)

The cells illustrated in (*a*) (20.5×10^6, containing a total of 11.3 μmol of catecholamine), were continuously perfused with KRH as described in the Materials and methods section. Carbachol (40 μM) pulses were administered every 5 min (arrows). The catecholamine peak elicited by the first carbachol peak was estimated to correspond to 0.57% of total cell catecholamine. The cells in (*b*) (22×10^6; 13.5 μmol of catecholamine) were perfused as in (*a*), but carbachol administration (Cch) was made by switching the perfusion from KRH to the same medium containing 40 μM-carbachol as indicated by the bars below the trace. The open arrow marks the administration of a pulse of bradykinin (BK; 50 nM).

TPA. Fig. 3 confirms the marked synergism of the release responses elicited by the concomitant administration of low concentrations of this phorbol ester and ionomycin in PC12$^-$ cells (Pozzan *et al.*, 1984). This effect was observed also in PC12$^+$ cells, but the concentrations of TPA had to be considerably increased (10–100 nM range). Moreover, the maximal response elicited by the TPA–ionomycin combination in PC12$^+$ cells, although quite large, was still smaller than those attainable in PC12$^-$ cells. In contrast, the combination of TPA with high K^+ failed to induce any synergistic release responses (results not shown).

Effects of muscarinic activation with carbachol

The effects of carbachol on catecholamine release were given special attention because of the complexity of the signal array elicited by such an agent, through the activation of muscarinic receptors. Such an array includes the hydrolysis of polyphosphoinositides, increase in [Ca^{2+}] and decrease in cyclic AMP (Vicentini *et al.*,

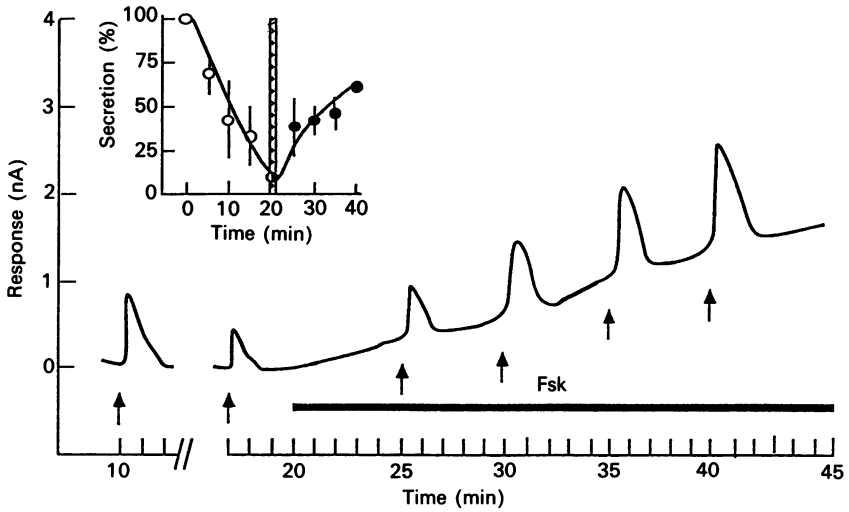

Fig. 5. Catecholamine release from perfused PC12⁻ cells: effects of carbachol pulses and forskolin perfusion

Carbachol pulses were administered every 5 min (arrows). Forskolin (Fsk) perfusion (1.2 μM) was performed and is illustrated as in Fig. 4. The inset illustrates the inactivation of the muscarinic release (○) and the partial reversal of the inactivation during a subsequent forskolin perfusion (●). Each point is the response to successive carbachol pulses, expressed as % of the initial response (0.51 ± 0.12 % of the total cell catecholamine content). Values shown are averages ± S.D. of five (○) and three (●) experiments.

1985*a*, 1986; Pozzan *et al.*, 1986; Gatti *et al.*, 1988). When the static incubation technique was used to measure catecholamine release, a stimulation by carbachol was difficult to reproduce consistently, in both $PC12^+$ and $PC12^-$ cells (results not shown). With the more sensitive perfusion technique, however, the results were quite clear, and essentially identical in both $PC12^-$ (Fig. 4) and $PC12^+$ cells (results not shown). Bolus administrations (1.2 s) of carbachol caused small (< 1 % of the total catacholamine store), dose-dependent (visible at 10 μM) and highly reproducible release responses (Fig. 4*a*), which were fully inhibited by atropine (0.1 μM). With repeated boluses given at 5 min intervals, the responses became progressively smaller (Figs. 4*a* and 5 inset). On the other hand, perfusion of the drug (10–50 μM) induced release responses that lasted 1–3 min, after which release could no longer be stimulated by carbachol even after a short period of cell washing (Fig. 4*b*). Under the same conditions, however, release remained stimulatable by bradykinin, another receptor stimulant of polyphosphoinositide hydrolysis (Fig. 4*b*). These results demonstrate that release, although too small to be properly appreciated by the static incubation technique, is triggered in PC12 cells by muscarinic activation. The rapid fading-out of the muscarinic responses seems to be due to receptor desensitization, and not to inhibition of the process of release, as demonstrated by the result with bradykinin.

In additional experiments, carbachol was administered in combination with other drugs. With forskolin (Figs. 5 and 6), a potentiation was observed that consisted of a slowly developing reversal of the inactivation of the muscarinic responses. Withdrawal of forskolin caused inactivation to re-appear, although at a rate slower than that observed in the cells not treated with the drug (cf. insets in Figs. 5 and 6). An effect similar to that of forskolin, although smaller and slower, was observed with the adenosine agonist PIA (results not shown). Conversely, inactivation of the muscarinic responses was greatly accelerated if clonidine, which causes cyclic AMP to decrease, was perfused after forskolin (Fig. 6).

Other combinations of carbachol investigated were those with high K^+ and ionomycin. As shown in Fig. 7(*a*), an increase in the K^+-induced release, detectable even by the static incubation technique, was induced by carbachol and inhibited by atropine. Parallel $[Ca^{2+}]_i$ measurements revealed, however, that, under the conditions of the experiment, the increases induced by the carbachol–K^+ combination were greater (by approx. 30 %) than those induced by K^+ alone (results not shown). A small, but reproducible and atropine-inhibitable, potentiation was observed also when carbachol was administered together with ionomycin (Fig. 7*b*), a condition in which the $[Ca^{2+}]_i$ increase induced by the ionophore was not modified appreciably by the cholinergic agent (results not shown). The synergism of the carbachol–ionomycin combination just mentioned was limited to $PC12^-$ cells. In fact, in $PC12^+$ cells the release elicited by the two drugs was not appreciably different from that induced by the ionophore alone. Another combination that failed to induce synergism was that of carbachol + TPA, where the release observed was not different from that induced by TPA alone, in both $PC12^-$ and $PC12^+$ cells (results not shown).

DISCUSSION

In the present work we have investigated the effects of a variety of treatments, given alone or in combination, on catecholamine release from PC12 cells. The effects of these treatments on second messengers were already known in this cell type. The results of the present work concur with those of previous studies (Greene & Rein, 1977; Rabe *et al.*, 1982; Rabe & McGee, 1983; Pozzan *et al.*, 1984; Baizer & Weiner, 1985; Di Virgilio *et al.*, 1987) to emphasize the dominant role of $[Ca^{2+}]_i$ in the

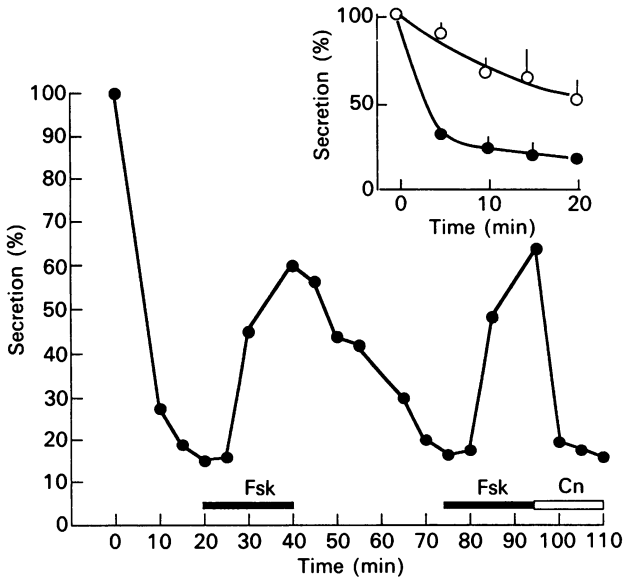

Fig. 6. Time course of catecholamine release induced by successive pulse administrations of carbachol in PC12⁻ cells: effect of forskolin and clonidine perfusion

The main panel illustrates a typical experiment. Each point shows the response to a pulse administration of carbachol (40 μM), administered at the times indicated on the abscissa. Perfusions with forskolin (Fsk, 1.2 μM) and clonidine (Cn, 10 μM) are indicated by the bars below the graph. The inset shows the rate of muscarinic-response inactivation occurring after the perfusion with forskolin was interrupted, with (●) and without (○) clonidine in the perfusion buffer. Data (averages of two experiments) are expressed as in Fig. 5, but 100% is the response to the last carbachol pulse before forskolin withdrawal.

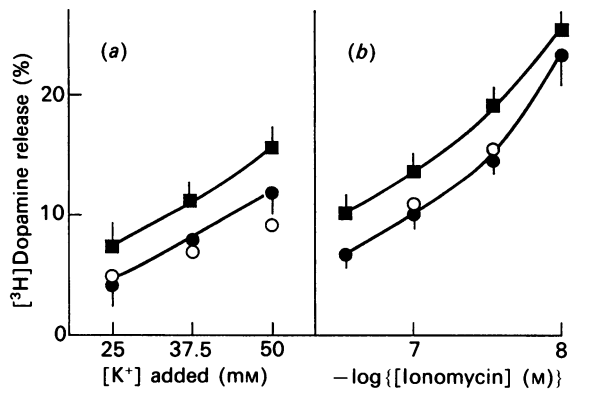

Fig. 7. Effects of the co-administration of carbachol with or without atropine on [³H]dopamine release induced by either K^+ (*a*) or ionomycin (*b*) from PC12⁻ cells

Samples of cell suspensions were mixed with either K^+ or ionomycin alone (●), in combination with carbachol (100 μM; ■), or with carbachol together with atropine (0.1 μM; ○). Incubations were carried out at 37 °C for 3 min. The release induced by carbachol alone, measured in parallel, was < 1%, statistically not significant. Results are expressed as in Fig. 1; n = 14 (*a*) and 18 (*b*).

control of catecholamine release from PC12 cells. In particular, the good correlation between the data obtained with high K^+ and ionomycin (two agents that affect $[Ca^{2+}]_i$ by different means) confirms that the increase in $[Ca^{2+}]_i$ above a threshold (estimated in PC12⁻ cells to be around 0.4 μM; Di Virgilio *et al.*, 1987) is a sufficient signal to trigger a release response. Such an increase, however, is not necessary, inasmuch as release can be stimulated even at resting $[Ca^{2+}]_i$ when protein kinase activation (with TPA for protein kinase C; with forskolin for cyclic AMP-dependent protein kinase) takes place (see also Pozzan *et al.*, 1984; Baizer & Weiner, 1985). The simplest explanation for these data, supported also by the synergistic responses obtained with various agent combinations (TPA + ionomycin; forskolin + either ionomycin or high K^+), is that phosphorylation(s) by either protein kinase shifts to the left the concentration-dependence curve of catecholamine release to the point that even resting $[Ca^{2+}]_i$ becomes above threshold. The initial evidence supporting this interpretation for protein kinase C was obtained some years ago in permeabilized chromaffin cells by Knight & Baker (1983) [see also Baker (1984) and the more recent report by Matthies *et al.* (1987)]. The failure of another combination (TPA with high K^+) to trigger any synergistic release might be explained by an additional effect of phorbol esters in PC12 cells, i.e. the inhibition of voltage-gated Ca^{2+} influx (Di Virgilio *et al.*, 1986; Harris *et al.*, 1986).

Muscarinic stimulation of catecholamine release

At least two types of muscarinic receptors exist in the PC12 subclone used in the present studies. The first, which is highly sensitive to the blocker pirenzepine (K_i 16 nM) is coupled stimulatorily to polyphosphoinositide hydrolysis, and thus to increased $[Ca^{2+}]_i$ and diacylglycerol generation (Pozzan *et al.*, 1986; Vicentini *et al.*, 1986); the second, which is less sensitive to pirenzepine (K_i 450 nM), appears to couple inhibitorily to adenylate cyclase (Gatti *et al.*, 1988). The study of the release responses induced by carbachol at concentrations known to activate both these responses could thus provide information on the physiological relevance of the interplay among the various intracellular signals, previously revealed in PC12 cells by the use of combinations of non-physiological drugs, such as phorbol esters and Ca^{2+} ionophores. The role of muscarinic receptors in secretion had previously been investigated in chromaffin cells, the non-tumoral counterpart cells of PC12, but with variable results. In the cat and guinea pig, muscarinic stimulation was found to be responsible for up to 50% of the stimulation induced by acetylcholine, the rest being nicotinic (Role & Perlman, 1983), whereas in bovine chromaffin cells, the cellular system most extensively investigated, inhibition (DeRome *et al.*, 1981; Swillem *et al.*, 1983), modest stimulation (Kirkpatrick *et al.*, 1980) and simple potentiation of the nicotinic response (Forsberg *et al.*, 1986) were reported. The $[Ca^{2+}]_i$ increased by carbachol is known to be much (approx. 2-fold) greater in PC12⁺ than in PC12⁻ cells (Pozzan *et al.*, 1986; Gatti *et al.*, 1988). In the latter cells, therefore, it would be expected to remain sub-threshold with respect to catecholamine release (Di Virgilio *et al.*, 1987). Yet release responses were consistently observed not only in PC12⁺ but also in PC12⁻ cells perfused with carbachol. Such results in PC12⁻ cells can only be explained by a synergism of the two signals generated by polyphosphoinositide hydrolysis, the increase of $[Ca^{2+}]_i$

and the activation of protein kinase C. This conclusion is also supported by the synergistic responses obtained only in PC12$^-$ cells by the combination of carbachol with ionomycin, without a concomitant greater increase of $[Ca^{2+}]_i$ with respect to the effects of the ionophore alone.

Another interesting aspect of the release responses induced by carbachol was their rapid inactivation observed when cells were perfused or exposed to multiple pulse administrations of the drug. Our previous results with phorbol esters (Vicentini *et al.*, 1985*b*) demonstrate that in PC12 cells (as in other cell types: Orellana *et al.*, 1985; Nishizuka, 1986) the muscarinic receptors coupled to polyphosphoinositide hydrolysis are desensitized after activation of protein kinase C. The rapid inactivation of the muscarinic-induced responses in PC12 cells might thus be due to feedback desensitization of the receptor. Interestingly, the inhibition of muscarinic-induced release was reversed by agents that increase cyclic AMP (forskolin, PIA) and accelerated by clonidine, which causes cyclic AMP to decrease. Moreover, Gatti *et al.* (1988) found that pretreatment with forskolin causes a slight enhancement, and clonidine a slight inhibition of the $[Ca^{2+}]_i$ increases induced by carbachol. These effects appear receptor-specific, because they did not occur when the increase in $[Ca^{2+}]_i$ was induced by depolarization with high K^+. Taken together, these data indicate that the muscarinic receptor coupled to polyphosphoinositide hydrolysis is under the dual, antagonistic, control of at least two protein kinases, one inducing and the other protecting from desensitization. In this scenario the cyclic AMP-decreasing effect induced by carbachol through the muscarinic receptor less sensitive to pirenzepine (Gatti *et al.*, 1988) might co-operate with protein kinase C to cause densensitization of the polyphosphoinositide-hydrolysis-coupled receptor.

Effects of cell differentiation

Previous results from our laboratories, obtained by the use of the first-generation fluorescent dye quin-2, suggested that the $[Ca^{2+}]_i$ increases induced by high K^+ and ionomycin were distinctly greater in PC12$^+$ than in PC12$^-$ cells (Pozzan *et al.*, 1986). However, when this problem was re-investigated by the use of fura-2, a dye which, because of its higher fluorescence, can be loaded intracellularly at lower concentration, and which is thus less prone to the artifacts caused by the increase in the cytoplasmic buffering capacity, the difference between differentiated and undifferentiated cells was found to be only minor (Gatti *et al.*, 1988). The close similarity of the transmitter release responses induced by high K^+ and ionomycin in PC12$^-$ and PC12$^+$ cells indicates therefore that the sensitivity of the release process to $[Ca^{2+}]_i$ is not greatly modified by differentiation. Kongsamut & Miller (1986), working on PC12$^-$ and PC12$^+$ monolayers, also reported similar release at 15 mM- and 30 mM-K^+, but noticed a greater release for PC12$^-$ cells at concentrations of 50 mM or higher. The reasons for this discrepancy are not clear.

In contrast with the similar results with high K^+ and ionomycin, strikingly different results were obtained when phorbol esters were used to stimulate release from PC12$^-$ and PC12$^+$ cells. In fact, almost 10-fold greater concentrations of either TPA or PDBu were needed to elicit release responses in PC12$^+$ cells similar to those in PC12$^-$ cells. The decrease of phorbol ester potency was not paralleled by a decrease, or a change in the membrane/soluble distribution, of [^{3}H]PDBu binding. These latter results suggest that differentiation of PC12 cells does not modify substantially the expression of protein kinase C, in agreement with the enzyme-activity measurements by Matthies *et al.* (1987). Recent independent results (K. Ase & Y. Nishizuka, personal communication) have revealed that the protein kinase C activity of PC12$^-$ cells is due mostly to the α, and in small part to the $\beta_1+\beta_2$, isoenzymes (Ono *et al.*, 1987), and that this pattern remains unchanged after differentiation. These results make us believe that in the differentiated sympathoblast-like PC12$^+$ cells the control of catecholamine release mediated by protein kinase C-induced phosphorylations could only play a marginal physiological role, if any.

The differences in secretion control which we have revealed by comparing PC12 cells at two stages of differentiation might not be unique to this cellular system. Chromaffin cells can be envisioned as a counterpart of PC12$^+$ cells, differentiated, however, along a programme different from that activated by NGF. In chromaffin cells, various groups (Burgoyne & Norman, 1984; Pocotte *et al.*, 1985; Brocklehurst *et al.*, 1985) have reported phorbol esters to be less potent and efficacious on catecholamine release than in PC12$^-$ cells (Pozzan *et al.*, 1984; the present work). It therefore seems possible that changes in sensitivity to second messengers during differentation are widespread and might account for phenomena of profound physiological importance.

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