Activation of muscarinic receptors in PC12 cells

Stimulation of Ca^{2+} influx and redistribution

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Ca^{2+} homeostasis was investigated in pheochromocytoma neurosecretory (PC12) cells both before and after treatment with nerve growth factor, which induces a neuronal-like differentiation accompanied by a large increase in the number of muscarinic receptors. The resting concentration of free cytosolic Ca^{2+} , $[\text{Ca}^{2+}]_i$, measured by the quin2 technique, was found to be higher and more variable in differentiated cells. Moreover, the $[\text{Ca}^{2+}]_i$ rises induced by the Ca^{2+} ionophore ionomycin and by depolarizing concentrations of KCl were greater and more transient. Exposure to carbachol induced modest, but long-lasting, $[\text{Ca}^{2+}]_i$ rises, which were faster and greater in differentiated than in non-differentiated cells. These effects were due to the activation of the muscarinic receptor, because they were unaffected by nicotinic blockers (hexamethonium and D-tubocurarine) and completely eliminated by low concentrations of the muscarinic antagonists atropine and pirenzepine [IC_{50} (concn. causing 50% inhibition) = 2 and 60 nM respectively]. The muscarinic-receptor-dependent $[\text{Ca}^{2+}]_i$ rises were the result of two concomitant processes: (1) redistribution of Ca^{2+} from cytoplasmic stores to the cytosol, possibly mediated by generation of inositol 1,4,5-trisphosphate as a consequence of the muscarinic-receptor-coupled hydrolysis of polyphosphoinositides, and (2) increased Ca^{2+} influx through a pathway of the plasmalemma insensitive to verapamil and thus different from the voltage-dependent Ca^{2+} channel. The existence of this second process was documented: (a) by the difference of the $[\text{Ca}^{2+}]_i$ responses brought about by carbachol in Ca^{2+} -containing and Ca^{2+} -free media; (b) by the occurrence of $[\text{Ca}^{2+}]_i$ rise and increased ^{45}Ca accumulation in cells exposed to 1 mM- CaCl_2 after having been treated for 2 min with carbachol in Ca^{2+} -free medium; (c) by typical differences in the quin2 signal kinetics observed in parallel samples of PC12 cells loaded with different concentrations of the dye.

During the last few years, a large and apparently heterogeneous group of receptors (e.g. muscarinic, α_1 -adrenergic, H_1 -histaminergic, serotonergic and many peptidergic receptors) has attracted a great deal of interest. Activation of these receptors is believed to be transduced intracellularly by a phosphodiesterase-mediated hydrolysis of membrane (poly)phosphoinositides and by the rise of the concentration of free cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) [for reviews, see Michell (1983), Michell *et al.* (1981), Berridge (1984) and Berridge & Irvine (1984)].

Stimulation of phosphoinositide turnover was discovered over 30 years ago in pancreatic slices exposed to acetylcholine (Hokin & Hokin, 1953), and later shown to be coupled to receptor activation in a variety of different cell systems (Michell *et al.*, 1981; Berridge, 1984; Berridge & Irvine, 1984). The two classes of metabolites generated by this reaction, diacylglycerols and inositol phosphates, have both been proposed to play the role of intracellular messenger. Hydrophobic diacylglycerols are the physiological activators of protein kinase C (PKC) (Michell, 1983; Berridge & Irvine, 1984; Nishizuka, 1984), whereas IP_3 (the hydrophilic metabolite of PIP_2), when applied to permeabilized cells, was found to trigger the release of Ca^{2+} from intracellular store(s), and may thus cause $[\text{Ca}^{2+}]_i$ to rise (Berridge, 1984; Berridge & Irvine, 1984).

Extensive evidence demonstrates that both $[\text{Ca}^{2+}]_i$ rise and activation of PKC (by either diacylglycerols or their potent analogues, the tumor promoters such as *o*-tetradecanoyl phorbol acetate) can separately mimic responses elicited by receptor activation. It has therefore been proposed that many cellular functions are regulated by two interconnected mechanisms, one $[\text{Ca}^{2+}]_i$ -dependent, the other PKC-dependent (for reviews, see Michell (1983), Berridge (1984), Berridge & Irvine (1984) and Nishizuka (1984)]. Knowledge about the relationships between these two mechanisms, in particular about the sequence of events in the transduction cascade initiated by receptor activation, is still a matter of dispute. On the basis of indirect evidence obtained in several cell systems, phosphoinositide hydrolysis was suggested to be a secondary event, preceded by a fast $[\text{Ca}^{2+}]_i$ rise, possibly mediated by increased Ca^{2+} influx at the plasmalemma (Cockcroft, 1981; Fischer & Agranoff, 1980; Fischer *et al.*, 1981; Prpic *et al.*, 1982). Results of our recent direct experiments (Vicentini *et al.*, 1985a) demonstrated, however, that hydrolysis of PIP_2 can occur even at resting $[\text{Ca}^{2+}]_i$. Together with additional evidence from other laboratories, these results indicate that phosphoinositide hydrolysis is a reaction closely coupled across the plasma membrane to receptor activation, and that $[\text{Ca}^{2+}]_i$ rise is

Abbreviations used: NGF, nerve growth factor; $[\text{Ca}^{2+}]_i$, concentration of free cytosolic Ca^{2+} ; IP_3 , inositol 1,4,5-trisphosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; DMSO, dimethyl sulphoxide; KR, Krebs–Ringer medium; QNB, quinuclidinyl benzylate; IC_{50} , concentration causing 50% inhibition; PC12[−] and PC12⁺, PC12 cells before and after differentiation.

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possibly a later event, inasmuch as it can be mediated by IP_3 -induced redistribution from the stores to the cytosol (Berridge, 1984; Berridge & Irvine, 1984; Vicentini *et al.*, 1985). It is not clear, however, whether redistribution is the only process responsible for receptor-triggered $[Ca^{2+}]_i$ rise, or whether increased influx through the plasma-membrane is also involved; and, if this second possibility holds, what is the relative importance of the two processes, influx and redistribution, and which is the trans-membrane route of the influx. Another open question concerns the physiological importance of phosphoinositide hydrolysis in the various cell types. In some cells the evidence is now compelling. Others, however, namely neurons and neurosecretory cells, seem to depend primarily on extracellular Ca^{2+} for their activation. In these and other cells the role of phosphoinositide hydrolysis has been questioned (Fischer & Agranoff, 1980; Fischer *et al.*, 1981; Hawthorne, 1982; Ambler *et al.*, 1984).

PC12 is a cell line derived from a rat pheochromocytoma (Greene & Tischler, 1976). Our previous studies demonstrated that these neurosecretory cells are endowed with a muscarinic receptor coupled to phosphoinositide hydrolysis (Vicentini *et al.*, 1985) and that their process of neurotransmitter release is regulated by the dual ($[Ca^{2+}]_i$ - and PKC-dependent) control mechanism (Pozzan *et al.*, 1984). PC12 cells cultured in conventional media express a chromaffin-like phenotype, with a low number of muscarinic receptors (PC12⁻ cells). When PC12⁻ cells are treated with NGF they stop dividing, enlarge, acquire a neuronal-like phenotype (Greene & Tischler, 1976) and greatly increase their complement of muscarinic receptors (Jumblatt & Tischler, 1982). The results obtained by studying the intracellular events triggered in these cells by the activation of the muscarinic receptors are reported in this and the following paper (Vicentini *et al.*, 1986). The present paper focuses on Ca^{2+} homeostasis, with reference to $[Ca^{2+}]_i$, Ca^{2+} influx and redistribution; the following paper (Vicentini *et al.*, 1986) deals with the correlation between receptor-coupled phosphoinositide and Ca^{2+} responses.

MATERIALS AND METHODS

Cells

PC12⁻ cells (initially provided by Dr. P. Calissano and Dr. S. Biocca, CNR Laboratory of Cell Biology, Rome, Italy) were cultured as monolayers in polystyrene dishes as described by Greene & Tischler (1976), with RPMI 1640 medium (Flow Laboratories, Milan, Italy) supplemented with 10% (v/v) horse serum and 5% (v/v) foetal-calf serum. PC12⁺ cells were obtained by culturing PC12⁻ cells for 12–18 days in the above medium supplemented with NGF (50 ng/ml). Immediately before the experiments, PC12⁻ and PC12⁺ cells were detached by gently streaming the culture medium on to the surface of the monolayers and then dissociated to yield single cells and small (two to five cells) aggregates as described elsewhere (Meldolesi *et al.*, 1983). Drugs were added dissolved in either water or DMSO. Controls received solvents only (maximal concn. 0.5%).

Incubation media

A modified Krebs–Ringer medium buffered with Hepes (complete KR) was used, which contained (in mmol/litre): NaCl, 125; KCl, 5; KH_2PO_4 and $MgSO_4$, 1.2; $CaCl_2$, 2;

Hepes/NaOH buffer, pH 7.4, 25; glucose, 6. Ca^{2+} -free KR medium differed from the complete KR in having no Ca^{2+} added and in containing twice as much $MgSO_4$ (2.4 mM) and EGTA (1 mM). Additional changes of the media used are specified in the text and figure legends.

Radioreceptor binding

In order to measure binding of $[^3H]QNB$, homogenates of PC12⁺ and PC12⁻ cells in complete KR medium (0.25 ml) were exposed to the radioactive ligand (concentrations ranging between 0.1 and 1 nM) for 60 min at 37°, then centrifuged (10 500 rev./min; $r = 6$ cm; 60 s). The pellets were rinsed once with 0.4 ml of ice-cold KR. Unspecific binding was measured in the presence of an excess (10 μ M) of atropine and subtracted from total binding to yield the specific binding.

Membrane potential

Plasma-membrane-potential changes were qualitatively indicated by bis(oxonol) (Meldolesi *et al.*, 1984), which responds to depolarization with an increase in fluorescence (excitation, 540 ± 2 nm; emission, 580 ± 5 nm). No attempt was made to convert the dye signal into absolute readings.

Quin2 measurement of $[Ca^{2+}]_i$

PC12 cells, suspended in RPMI 1640 medium buffered with Hepes, pH 7.4 (12×10^6 cells/ml), were mixed with a 0.5–1% vol. of 10 mM-quin2 acetoxymethyl ester in DMSO, and incubated at 37 °C for 1 h. Before use the cells were pelleted, resuspended in either KR or Ca^{2+} -free KR and dissociated. Because of the different size of PC12⁺ and PC12⁻ cells, the concentrations used were different: (0.5–0.8) and $(1–2) \times 10^6$ /ml respectively. Assays were carried out, in a thermostatically controlled cuvette equipped for magnetic stirring, with a Perkin–Elmer (Eden Prairie, MN, U.S.A.) 650–40 spectrofluorimeter (excitation: 339 ± 2 ; emission: 492 ± 10 nm). Calibration of the fluorescent signal was done exactly as described elsewhere (Meldolesi *et al.*, 1984). Whenever necessary the data were corrected for changes in cell auto-fluorescence.

Ca^{2+} transport

Cell suspensions in Ca^{2+} -free KR medium ($[EGTA] = 0.2$ mM) $\pm$ carbachol (0.5 mM) were incubated at 37 °C for 2 min, after which a mixture of $CaCl_2$ (final free concn: 1 mM), ^{45}Ca (1 μ Ci/ml) and $[^3H]$ sucrose (an extracellular-space marker; 3 μ Ci/ml) were added. Aliquots were withdrawn at the time points indicated in the Figure legends, mixed with 2.5 mM-EGTA/5 μ M-Ruthenium Red (final concns.; to remove superficial Ca^{2+}) and immediately centrifuged through a layer of oil [dibutyl phthalate/dinonyl phthalate (7:3, v/v)]. The supernatants remaining above the oil layer were carefully removed, and the surface of the layer was rinsed. Finally the oil layer was discarded and the resuspended pellets were mixed with 4 ml of Atomlight (New England Nuclear, Dreirich, Germany) and counted for radioactivity. Values shown are corrected for the contribution of extracellular ^{45}Ca , estimated from the recovery of $[^3H]$ sucrose in the pellets (Nicholls *et al.*, 1982).

Materials

2.5S NGF was kindly given by Dr. P. Calissano, bis(oxonol) by Dr. R. Y. Tsien, and ionomycin by Dr.

Fig. 1. $[\text{Ca}^{2+}]_i$ changes in PC12^+ (a, b) and PC12^- (c, d) cells exposed to ionomycin (Iono, $0.2 \mu\text{M}$) and KCl (50 mM) in complete KR

Concentration of cells: 0.8 and $2 \times 10^6/\text{ml}$ for PC12^+ and PC12^- respectively; concentration of quin2: 1 and $0.5 \text{ nmol}/10^6$ cells in PC12^+ and PC12^- respectively. The $[\text{Ca}^{2+}]_i$ calibration is indicated on the left of each single trace.

C. M. Liu. Verapamil hydrochloride was kindly provided by Knoll A. G., Ludwigshafen/Rhein, Germany. Radiochemicals were purchased from Amersham International; hexamethonium bromide, D-tubocurarine chloride, atropine sulphate, pirenzepine and carbachol chloride were purchased from Sigma. quin2 acetoxy-methyl ester was from Calbiochem-Behring.

RESULTS

The experiments illustrated in Fig. 1 were carried out with the aim of characterizing the homeostasis of Ca^{2+} in PC12 cells, investigated before and after differentiation by NGF, i.e. PC12^- and PC12^+ cells. The $[\text{Ca}^{2+}]_i$ of resting PC12^- cells (measured by the quin2 technique) was $90 \pm 3 \text{ nM}$ (average of seven measurements $\pm \text{S.E.M.}$), in good agreement with our previous results (Meldolesi *et al.*, 1984). In PC12^+ cells the resting $[\text{Ca}^{2+}]_i$ level was found to be more variable. In some batches of cells it was only slightly higher than in PC12^- cells; in others it was of the order of 200 nM (average 161 ± 17 ; $n = 35$). In order to induce changes of $[\text{Ca}^{2+}]_i$, we used (i) high concentrations of KCl, which depolarize the plasmalemma and thus cause the opening of the voltage-dependent Ca^{2+} channel, and (ii) ionomycin, an ionophore that exchanges Ca^{2+} with 2H^+ across membrane lipid bilayers. Application of ionomycin induced considerable increases in both types of cells, which were greater, more variable and more transient in PC12^+ (Figs. 1b and 1d). Thus with $0.1 \mu\text{M}$ -ionomycin the maximal values obtained in PC12^- cells remained always below $1 \mu\text{M}$, whereas in PC12^+ the values approached, and in some cases even exceeded, that concentration. Similar results were obtained with KCl (Figs. 1a and 1c). The averages of the maximal $[\text{Ca}^{2+}]_i$ rises induced by 50 mM -KCl were 635 ± 91 and 430 ± 40 in PC12^+ and PC12^- cells respectively ($n = 10$ and 15). In addition, a difference in time course appeared. In PC12^- cells the raised $[\text{Ca}^{2+}]_i$ declined very slowly over several minutes; by contrast, in PC12^+ it decreased to

approx. 60% within 2–3 min and then declined at a slow rate (Figs. 1a and 1c).

The cholinergic agonist carbachol ($5\text{--}500 \mu\text{M}$) induced distinct changes of $[\text{Ca}^{2+}]_i$ in PC12 cells (Fig. 2), whether applied in complete Ca^{2+} -containing medium, or in EGTA-containing Ca^{2+} -free medium. The $[\text{Ca}^{2+}]_i$ transients induced under these two conditions differed not only in amplitude, but also in their time course. In Ca^{2+} -free KR the $[\text{Ca}^{2+}]_i$ rises were fast (maximal within 10 s) and short-lived (down to the resting level within 1–2 min) (Figs. 2b and 2d). In complete KR, on the other hand, the maxima were reached in 30–180 s (depending on the intracellular concentration of quin2; see below), and $[\text{Ca}^{2+}]_i$ remained elevated above the resting values for several (more than 5) min (Figs. 2a and 2c). These rises of $[\text{Ca}^{2+}]_i$ were observed in both PC12^+ and PC12^- cells. However, in the differentiated cells they were always greater (Fig. 2) (average of maximal $[\text{Ca}^{2+}]_i$ values obtained with 0.5 mM -carbachol in PC12^+ and PC12^- cells respectively: 328 ± 28 and $136 \pm 10 \text{ nM}$; $n = 11$ and 6 ; $P < 0.005$).

Carbachol is a mixed cholinergic agonist that is known to be able to activate both muscarinic and nicotinic receptors. Muscarinic receptors were previously reported to increase in number as a consequence of NGF-induced differentiation (Jumblatt & Tischler, 1982). In the present work we confirmed this finding by using $[^3\text{H}]\text{QNB}$ as the ligand. The number of sites per mg of protein rose 4–5-fold, whereas the affinity of $[^3\text{H}]\text{QNB}$ binding remained unchanged (K_d : 0.15 nM) (not shown in the Figures). When recalculated on a per-cell basis, the data indicated an increase of almost 15-fold during differentiation: from 2600 to 36000 sites/cell. Previous reports from other laboratories indicated that PC12 cells possess nicotinic receptors whose activation induces depolarization of the plasma membrane, generation of action

Fig. 2. Effect of carbachol (CCh, 0.5 mM) on $[\text{Ca}^{2+}]_i$ in PC12^+ (a, b) and PC12^- (c, d) cells suspended in complete KR (a, c) or Ca^{2+} -free KR (b, d)

Concentration of cells: 0.6 and $1 \times 10^6/\text{ml}$ for PC12^+ and PC12^- respectively; concentration of quin2: 2 and $1 \text{ nmol}/10^6$ cells in PC12^+ and PC12^- respectively. The calibration was as for Fig. 1.

Fig. 3. Effects of atropine (Atr), verapamil (Vp), hexamethonium (Hex) and pirenzepine (Pz) on carbachol (CCh, 0.5 mM), KCl (50 mM) and ionomycin (Iono, 0.3 μ M)-induced changes of $[Ca^{2+}]_i$ and membrane potential in PC12⁺ cells

(a, b) quin2 measurements of $[Ca^{2+}]_i$. Atropine (0.1 μ M) when administered before carbachol blocked its effect (c) and caused a return of $[Ca^{2+}]_i$ to the resting level when administered afterwards (a). In contrast, atropine was without effect on the KCl-induced $[Ca^{2+}]_i$ rise (c). Verapamil (20 μ M) inhibited the latter, and was without effect with carbachol and ionomycin. Concentration of cells: 0.55×10^6 /ml; concentration of quin2: 1.15 nmol/ 10^6 cells. Inset graph: concentration-dependence of atropine (■) and pirenzepine (●) inhibition of the maximal $[Ca^{2+}]_i$ rise induced by carbachol. Experimental conditions were as in (a–c). (d, e) Effect of carbachol and KCl $\pm$ hexamethonium (0.5 mM) on membrane potential, revealed by bis(oxonol). Concentration of cells: 1×10^5 /ml; concentration of bis(oxonol) added: 0.1 μ M. Maximal (50 nM) concentration of atropine on the carbachol-induced increase of $[Ca^{2+}]_i$. (c, d) Effect of verapamil (25 μ M) and atropine (2 μ M) on the carbachol- and KCl-induced $[Ca^{2+}]_i$ rises. Concentration of cells: 0.5×10^6 /ml; concentration of quin2: 1.15 nmol/ 10^6 cells. The calibration was as in Fig. 1. (e, inset) Effect of carbachol and KCl $\pm$ hexamethonium (0.5 mM) on membrane potential. Concentration of cells: 1×10^5 /ml; concentration of bis(oxonol) added: 0.1 μ M.

potentials, increased Ca^{2+} influx and stimulation of transmitter release (Dichter *et al.*, 1977; Ritchie, 1979; Stellcup, 1979; Rudy *et al.*, 1982). Our recent studies, however, demonstrated that, in PC12 cells available in our laboratory, the effects of carbachol on Ca^{2+} homeostasis are entirely due to the activation of the muscarinic receptor, without detectable effect attributable to the nicotinic receptor (Vicentini *et al.*, 1985). Additional evidence along these lines has now been obtained. Nicotinic blockers, hexamethonium and D-tubocurarine, were without effect on carbachol-induced $[Ca^{2+}]_i$ rises, even when these drugs were used at concentrations (up to 0.5 and 0.05 mM) reported to inhibit nicotinic responses completely (results not shown). Measurements of the plasma-membrane potential carried out with the use of the fluorescent indicator bis(oxonol) failed to reveal any depolarization after application of carbachol (Figs. 3d and 3e). Verapamil, a blocker of the voltage-dependent Ca^{2+} channel, neither prevented $[Ca^{2+}]_i$ rising when applied before carbachol, nor induced a return towards the resting level when applied after carbachol, although it showed the expected inhibitory effect with respect to the $[Ca^{2+}]_i$ changes induced by depolarizing agents such as high KCl (Fig. 3b). In contrast, the carbachol-induced Ca^{2+} rises were readily blocked by atropine. The IC_{50} of atropine was 2 nM, and complete blockade occurred at concentrations above 20 nM (Figs. 3a, 3c and the inset). The effective concentrations of atropine were therefore in the range specific for muscarinic receptors. Moreover, application of atropine to PC12⁺ cells whose $[Ca^{2+}]_i$ had been raised by the previous application of carbachol resulted in a rapid change of the $[Ca^{2+}]_i$ transient kinetics, with

accelerated return to the resting level ($t_{1/2}$ of the decline = 15–30 s) (Fig. 3a). These latter results suggest that the prolonged duration of the carbachol-induced $[Ca^{2+}]_i$ rise requires the occupancy of the muscarinic receptor by the agonist. Pirenzepine, a blocker preferentially addressed to a subclass of muscarinic receptors (Hammer & Giachetti, 1984), inhibited the carbachol-induced $[Ca^{2+}]_i$ rise at low concentrations (IC_{50} 60 nM; Fig. 3, inset).

The marked difference between the effects of carbachol applied to PC12 cells in either Ca^{2+} -containing or Ca^{2+} -free media (Fig. 2) suggested that the rises of $[Ca^{2+}]_i$ originate from a dual source: (i) redistribution from intracellular stores, which accounts for the part of the response remaining in the Ca^{2+} -free KR medium, and (ii) increased Ca^{2+} influx through a pathway in the plasmalemma not inhibitable by verapamil and therefore different from the voltage-dependent Ca^{2+} channel. Alternatively, this second component of the $[Ca^{2+}]_i$ rise could have been due to a carbachol-induced decreased efflux of Ca^{2+} . The dual origin of carbachol-induced Ca^{2+} rises was further indicated by the results of experiments in which Ca^{2+} redistribution and increased Ca^{2+} influx (or decreased efflux) were temporally dissociated. Cells were first resuspended in Ca^{2+} -free medium and exposed to carbachol. Under these conditions the expected transient rise of $[Ca^{2+}]_i$ due to redistribution took place (Fig. 4a). When $[Ca^{2+}]_i$ was back to the resting level (2 min), $CaCl_2$ was added to the medium. This addition caused a second, slower, $[Ca^{2+}]_i$ rise, up to the levels observed when carbachol additions were made directly in complete Ca^{2+} -containing KR medium (cf. Figs. 4a and 4b). The second rise depended entirely on the presence of

Fig. 4. Temporal dissociation of the carbachol-induced $[\text{Ca}^{2+}]_i$ rises due to redistribution from intracellular stores and influx from the incubation medium

PC12⁺ cells suspended in KR medium without CaCl_2 added were treated with EGTA (1 mM), carbachol (CCh, 0.5 mM), CaCl_2 (Ca^{2+} , 1 mM final free concn.) and atropine (Atr, 2 μM) where indicated. Concentration of cells: $0.5 \times 10^6/\text{ml}$; concentration of quin2: 0.5 nmol/ 10^6 cells. The calibration was as in Fig. 1.

Fig. 5. Effect of carbachol (0.5 mM) on $^{45}\text{Ca}^{2+}$ accumulation in PC12⁺ cells

Aliquots of PC12⁺ cells [(4–5) and (1.7–2) $\times 10^6$ cells/ml] were suspended in KR medium without CaCl_2 added and containing EGTA, 0.2 mM, 2 min before the addition of a mixture of ^{45}Ca (1 $\mu\text{Ci}/\text{ml}$; final concn of free Ca^{2+} : 1 mM) and [^3H]sucrose (3 $\mu\text{Ci}/\text{ml}$); one out of two paired samples of cells received carbachol (●) and the other served as control (○). Values given in (a) are averages $\pm$ S.E.M. of the results obtained in three experiments carried out in duplicate. They represent recoveries of $^{45}\text{Ca}^{2+}$ in cell pellets, with contaminating extracellular $^{45}\text{Ca}^{2+}$ subtracted. In (b), the data from (a) are recalculated as d.p.m./s per mg of protein.

carbachol (Fig. 4b) and was prevented by atropine, added either before carbachol (not shown) or even before CaCl_2 (Fig. 4c).

In order to distinguish between increased influx and inhibition of Ca^{2+} extrusion as to the origin of the second $[\text{Ca}^{2+}]_i$ rise, we measured $^{45}\text{Ca}^{2+}$ transport by a protocol similar to that of the quin2 experiments illustrated in Fig. 4. PC12⁺ cells were first resuspended in a Ca^{2+} -free KR medium $\pm$ carbachol for 2 min, and then given $^{45}\text{Ca}^{2+}$. As Fig. 5 shows, carbachol-treated cells showed a greater accumulation of the tracer with respect to the controls at the earliest time points investigated (10–25 s). This was followed by an equilibration during the next 1 min of

incubation. Similar, but less pronounced, stimulation of ^{45}Ca transport was observed with PC12[–] cells (not shown in the Figures). Both in PC12⁺ and in PC12[–] cells the effects of carbachol on $^{45}\text{Ca}^{2+}$ influx were completely inhibited by pretreatment with atropine and unaffected by verapamil (0.1 and 20 μM ; not shown in the Figures). The fact that the increased $^{45}\text{Ca}^{2+}$ accumulation was visible within seconds from the application of the tracer, and tended to equilibrate rapidly, strongly supports increased influx as the mechanism underlying the second rise of $[\text{Ca}^{2+}]_i$ induced by the activation of the muscarinic receptor (illustrated in Fig. 4).

Additional evidence of the existence of a Ca^{2+} pathway operated by the muscarinic receptor in the plasmalemma of PC12 cells was obtained by experiments carried out on PC12 cells loaded with different concentrations of quin2. As has been discussed previously (Tsien *et al.*, 1982; Pozzan *et al.*, 1982), if the attainment of a $[\text{Ca}^{2+}]_i$ steady-state level in response to an agonist is due to the establishment of a new pump-and-leak equilibrium at the plasma membrane, the rate, but not the magnitude, of the $[\text{Ca}^{2+}]_i$ change would depend on the Ca^{2+} buffer capacity of the cytosol. In contrast, if the $[\text{Ca}^{2+}]_i$ change is due to redistribution of Ca^{2+} from stores that have limited capacity (e.g., mitochondria and/or other cytoplasmic organelles), then the magnitude should clearly decrease when the buffer capacity is increased. Quin2 is a substance particularly suited for these experiments because, once trapped inside the cells, it works at the same time as a fluorescent probe, monitoring $[\text{Ca}^{2+}]_i$, and as a high-affinity Ca^{2+} chelator, which buffers Ca^{2+} within the cytosol without causing major damage to cell functions. Figs. 6(a) and 6(c) compare $[\text{Ca}^{2+}]_i$ changes induced by the application of carbachol in parallel aliquots of PC12⁺ cells loaded with quin2 at 0.5 and 3.3 nmol/ 10^6 cells, and suspended in complete KR medium. As predicted by the pump-and-leak mechanism, the time required to reach the maximum $[\text{Ca}^{2+}]_i$ level after carbachol addition was much longer (about 6-fold) in the heavily than in the

Fig. 6. Effect of intracellular quin2 concentration on $[\text{Ca}^{2+}]_i$ rises induced by carbachol (CCh, 0.5 mM) in PC12⁺ cells incubated in complete KR (a, c) or Ca^{2+} -free KR (b, d)

Concentration of cells: $0.7 \times 10^6/\text{ml}$; concentration of quin2: 0.5 (a, b) and 3.3 (c, d) nmol/ 10^6 cells. The calibration was as in Fig. 1.

lightly loaded cells, but the final levels were hardly different. In contrast, when the experiments were carried out in the Ca^{2+} -free medium, the size of the measured $[\text{Ca}^{2+}]_i$ rises was greatly decreased at high intracellular concentrations of quin2 (Figs. 6b and 6d). Data similar to those of Fig. 6 were obtained with PC12⁻ cells as well (results not shown).

DISCUSSION

PC12 is a line of neurosecretory cells originally developed by Greene & Tischler (1976) and later extensively characterized in a series of elegant studies (Jumblatt & Tischler, 1982; Dichter *et al.*, 1977; Ritchie, 1979; Stellcup, 1979; Greene, 1984). Work carried out in many laboratories demonstrates that PC12 cells are a useful model for investigating various aspects of neurosecretion. Here we report about the changes of intracellular Ca^{2+} homeostasis induced by the activation of the muscarinic acetylcholine receptor.

Two features of PC12 cells should be emphasized here because they were important for the studies reported in this and the following article (Vicentini *et al.*, 1986): their homogeneity and ability to differentiate when treated with NGF. Heterogeneous cell populations can be endowed with different classes of muscarinic receptors, transduced intracellularly by different mechanisms. Results obtained with these experimental systems can therefore be very complex and difficult to interpret (Jacobson *et al.*, 1985; Brown & Brown, 1984a,b). On the other hand, differentiated PC12⁺ cells, which possess a large number of muscarinic receptors (Jumblatt & Tischler, 1982), responded to the application of carbachol with intracellular signals that were greater than those of undifferentiated PC12⁻ cells. This was a powerful tool to investigate the relationship between the various processes triggered by muscarinic receptor activation.

Previous studies by others indicated that PC12 cells possess two types of acetylcholine receptors: not only muscarinic, but also nicotinic. The activation of the latter receptors was reported to cause increased Na^+ and Ca^{2+} influx, plasma-membrane depolarization and transmitter release (Dichter *et al.*, 1977; Ritchie, 1979; Stellcup, 1979; Rudy *et al.*, 1982). However, when the cells used in our work were exposed to the mixed cholinergic agonist carbachol, no effect attributable to the activation of the nicotinic receptor was observed. Indeed, the cells were not depolarized, and the increased $[\text{Ca}^{2+}]_i$ and $^{45}\text{Ca}^{2+}$ influx were unaffected by nicotinic blockers, even when used at high concentration. We conclude, therefore, that our cells are devoid of a functioning nicotinic receptor. Differences in PC12 subclones available in different laboratories can account for the discrepancy between the present data and those reported previously by others.

All changes of the $[\text{Ca}^{2+}]_i$ homeostasis induced by carbachol were inhibited by atropine at the low concentrations (1–10 nM) specific for the muscarinic receptors. Our results clearly demonstrate that these changes are due to a dual mechanism: (i) redistribution of Ca^{2+} from the stores to the cytosol, and (ii) increased influx through the plasmalemma. Carbachol-induced redistribution of Ca^{2+} from cellular stores to the cytosol was clearly documented by the persistence of some atropine-inhibitable $[\text{Ca}^{2+}]_i$ rise even in cells treated in the Ca^{2+} -free medium. In such a medium the concentration of the Ca^{2+} is calculated to be lower than 10 nM. We have

no direct information as to the mechanisms responsible for such a redistribution effect. Elsewhere (Vicentini *et al.*, 1985, 1986), however, we demonstrated that, concomitantly with the $[\text{Ca}^{2+}]_i$ rise, IP_3 is generated by muscarinic-receptor-coupled hydrolysis of PIP_2 . This process could be responsible for the Ca^{2+} -redistribution effect we observed, since in a variety of cell types IP_3 has been recently proposed as the intracellular messenger that triggers release of Ca^{2+} from non-mitochondrial vesicular pool(s) to the cytosol [for reviews, see Berridge (1984) and Berridge & Irvine (1984)]. Muscarinic-receptor-coupled Ca^{2+} redistribution has been shown previously to occur in glandular and smooth-muscle cells as well as in astrocytes (Putney, 1978; Bolton, 1979; Putney *et al.*, 1981; Schulz & Stolze, 1980; Brown Masters *et al.*, 1984; Sekar & Roufogalis, 1984). In neurosecretory cells, such an effect had never been shown (see McKinney & Richelson, 1984), except for a recent note by Koo & Schneider (1985) concerning adrenal chromaffin cells.

Our $^{45}\text{Ca}^{2+}$ experiments, which demonstrated a larger accumulation of the tracer occurring within seconds after Ca^{2+} addition to carbachol-treated cells, document the existence of a muscarinic-receptor-triggered increased Ca^{2+} influx in PC12 cells. This conclusion is supported also: (a) by a difference in both rate and extent of the $[\text{Ca}^{2+}]_i$ rise responses induced by carbachol in complete and Ca^{2+} -free KR media; (b) by the experiments in which $[\text{Ca}^{2+}]_i$ rises by redistribution and increased influx were dissociated in time by first applying carbachol in Ca^{2+} -free medium and then reintroducing Ca^{2+} into the medium; (c) by the analysis of the $[\text{Ca}^{2+}]_i$ rises induced by carbachol in parallel samples of PC12⁺ cells loaded with different concentrations of quin2. Two features of the muscarinic-receptor-coupled increases of Ca^{2+} influx are worth emphasizing: (1) they were small; indeed, the increased $^{45}\text{Ca}^{2+}$ influx was appreciable only under the non-steady-state conditions described in Fig. 6, whereas the greater influx induced in PC12 cells by depolarizing concentrations of K^+ is easily demonstrable under steady-state conditions; (2) they were due to the activation of a transmembrane pathway different from the voltage-dependent Ca^{2+} channel, as shown by the insensitivity to verapamil, which, in contrast, inhibited the effects of high K^+ (Madeddu *et al.*, 1985).

The existence of Ca^{2+} channels operated by the activation of various receptors (receptor-operated channels) was suggested repeatedly in the past in a variety of cell systems based on the results of $^{45}\text{Ca}^{2+}$ flux experiments (Putney, 1978; Bolton, 1979; Schulz & Stolze, 1980; Janis & Triggle, 1983). The early results, however, can be questioned, since the measurements of $^{45}\text{Ca}^{2+}$ accumulation were usually made a long time (several minutes to hours) after the application of the stimulants. Under these conditions, a discrimination between increased influx, redistribution and decreased efflux becomes problematical.

Recent studies in various cells systems [hepatocytes (α_1 -adrenergic, vasopressin and angiotensin receptors; Mauger *et al.*, 1984); neuroblastoma × glioma hybrids (bradykinin receptors; Yano *et al.*, 1984) cultured muscle cells (α_1 -adrenergic receptor; Brown *et al.*, 1984; Reynolds & Dubyak, 1985); astrocytes (muscarinic receptor; Brown Masters *et al.*, 1984)], in which $^{45}\text{Ca}^{2+}$ fluxes were measured within a few seconds from the application of the stimulants, have provided fresh evidence for the existence of receptor operated Ca^{2+}

channel(s). It should be noted that all the receptors investigated in the studies mentioned above are coupled to phosphoinositide hydrolysis. It has been previously suggested that hydrolysis of PIP₂ (Downes & Michell, 1982; Sandler *et al.*, 1984), possibly through the generation of metabolites such as IP₃ (Berridge, 1984), could activate a Ca²⁺ channel in the plasmalemma. Alternatively, metabolites of the phosphoinositide cycle, in particular phosphatidic acid, which results from the phosphorylation of diacylglycerol, could play the role of Ca²⁺ ionophores (Michell *et al.*, 1981; Putney *et al.*, 1981; see, however, Holmes & Yoss, 1983). The problem of the correlation between Ca²⁺ and phosphoinositide responses to muscarinic-receptor activation is specifically addressed in the following paper (Vicentini *et al.*, 1986), where results also obtained by the use of several inhibitors are reported.

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REFERENCES

- Ambler, K. S., Brown, R. D. & Taylor, P. (1984) *Mol. Pharmacol.* **26**, 405–413
- Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Bolton, T. B. (1979) *Physiol. Rev.* **59**, 606–662
- Brown, J. H. & Brown, S. L. (1984a) *J. Biol. Chem.* **259**, 3777–3781
- Brown, J. H. & Brown, S. L. (1984b) *Trends Pharmacol. Sci.* **5**, 417–420
- Brown, D. A., Berger, K. D. & Taylor, P. (1984) *J. Biol. Chem.* **259**, 7554–7562
- Brown Masters, S. L., Harden, T. K. & Brown, J. H. (1984) *Mol. Pharmacol.* **26**, 149–155
- Cockcroft, S. (1981) *Trends Pharmacol. Sci.* **2**, 340–342
- Dichter, M. C., Tischler, A. S. & Greene, L. A. (1977) *Nature (London)* **268**, 501–504
- Downes, P. & Michell, R. H. (1982) *Cell Calcium* **3**, 467–502
- Fischer, S. & Agranoff, B. W. (1980) *J. Neurochem.* **34**, 1231–1240
- Fischer, S. K., Holz, K. W. & Agranoff, B. W. (1981) *J. Neurochem.* **37**, 491–499
- Greene, L. A. (1984) *Trends Neurosci.* **9**, 91–94
- Greene, L. A. & Tischler, A. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2424–2428
- Hammer, R. & Giachetti, A. (1984) *Trends Pharmacol. Sci.* **5**, 18–20
- Hawthorne, J. N. (1982) *Nature (London)* **295**, 281–282
- Hokin, M. R. & Hokin, L. E. (1953) *J. Biol. Chem.* **203**, 967–977
- Holmes, R. P. & Yoss, N. L. (1983) *Nature (London)* **305**, 637–638
- Jacobson, M. D., Wusterman, M. & Downes, C. P. (1985) *J. Neurochem.* **44**, 465–472
- Janis, R. A. & Triggle, D. J. (1983) *J. Med. Chem.* **26**, 775–785
- Jumblatt, J. E. & Tischler, A. S. (1982) *Nature (London)* **297**, 152–154
- Koo, L. S. & Schneider, A. S. (1985) *J. Biol. Chem.* **260**, 2019–2020
- Madeddu, L., Pozzan, T., Robello, M., Rolandi, R., Hsiao, T. H. & Meldolesi, J. (1985) *J. Neurochem.* **45**, 1708–1718
- Mauger, J. P., Poggioli, J., Guesdon, F. & Claret, M. (1984) *Biochem. J.* **221**, 121–127
- McKinney, M. & Richelson, E. (1984) *Annu. Rev. Pharmacol. Toxicol.* **24**, 121–146
- Meldolesi, J., Madeddu, L., Torda, M., Gatti, G. & Niutta, E. (1983) *Neurosci.* **10**, 997–1009
- Meldolesi, J., Huttner, W. B., Tsien, R. Y. & Pozzan, T. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 620–624
- Michell, R. H. (1983) *Trends Biochem. Sci.* **8**, 263–265
- Michell, R. H., Kirk, C. J., Jones, L. M., Downes, C. P. & Creba, J. A. (1981) *Philos. Trans. R. Soc. London Ser. B* **296**, 123–136
- Nicholls, D. G., Rugolo, M., Scott, I. G. & Meldolesi, J. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7924–7928
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- Pozzan, T., Arslan, P., Tsien, R. Y. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 335–340
- Pozzan, T., Gatti, G., Dozio, N., Vicentini, L. M. & Meldolesi, J. (1984) *J. Cell Biol.* **99**, 628–638
- Prpic, V., Blackmore, P. F. & Exton, J. H. (1982) *J. Biol. Chem.* **257**, 11323–11331
- Putney, J. W. (1978) *Pharmacol. Rev.* **30**, 209–245
- Putney, J. W., Poggioli, J. & Weiss, S. J. (1981) *Philos. Trans. R. Soc. London Ser. B* **296**, 37–45
- Reynolds, E. E. & Dubyak, G. R. (1985) *Biochem. Biophys. Res. Commun.* **130**, 627–632
- Ritchie, A. (1979) *J. Physiol. (London)* **286**, 541–561
- Rudy, B., Kirschenbaum, B. & Greene, L. A. (1982) *J. Neurosci.* **2**, 1405–1402
- Sandler, K., Litosch, I. & Fain, J. N. (1984) *Biochem. J.* **222**, 327–334
- Schultz, I. & Stolze, H. H. (1980) *Annu. Rev. Physiol.* **42**, 127–156
- Sekar, M. C. & Roufogalis, B. D. (1984) *Biochem. J.* **223**, 527–531
- Stellcup, W. B. (1979) *J. Physiol. (London)* **286**, 525–540
- Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 325–334
- Vicentini, L. M., Ambrosini, A., Di Virgilio, F., Pozzan, T. & Meldolesi, J. (1985) *J. Cell Biol.* **100**, 1330–1333
- Vicentini, L. M., Di Virgilio, F., Ambrosini, A., Meldolesi, J. & Pozzan, T. (1986) *Biochem. J.* **234**, 555–562
- Yano, K., Higashida, H., Inone, R., Nozawa, Y. (1984) *J. Biol. Chem.* **259**, 1021–1027

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