Activation of muscarinic receptors in PC12 cells

Correlation between cytosolic Ca^{2+} rise and phosphoinositide hydrolysis

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The intracellular signals generated by carbachol activation of the muscarinic receptor [release of inositol phosphates as a consequence of phosphoinositide hydrolysis and rise of the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$, measured by quin2)] were studied in intact PC12 pheochromocytoma cells that had been differentiated by treatment with nerve growth factor. When measured in parallel samples of the same cell preparation 30 s after receptor activation, the release of inositol trisphosphate and of its possible metabolites, inositol bis- and mono-phosphate, and the $[\text{Ca}^{2+}]_i$ rise were found to occur with almost superimposable carbachol concentration curves. At the same time carbachol caused a decrease in the radioactivity of preloaded phosphatidylinositol 4,5-bisphosphate, the precursor of inositol trisphosphate. Neither the inositol phosphate nor the $[\text{Ca}^{2+}]_i$ signal was modified by preincubation of the cells with either purified *Bordetella pertussis* toxin or forskolin, the direct activator of adenylate cyclase. Both signals were partially inhibited by dibutyryl cyclic AMP, especially when the nucleotide analogue was applied in combination with the phosphodiesterase inhibitors RO 201724 and theophylline. The latter drug alone profoundly inhibited the carbachol-induced $[\text{Ca}^{2+}]_i$ rise, with only minimal effect on phosphoinositide hydrolysis. Because of the diverging results obtained with forskolin on the one hand, dibutyryl cyclic AMP and phosphodiesterase inhibitors on the other, the effects of the latter drugs are considered to be pharmacological, independent of the intracellular cyclic AMP concentration. Two further drugs tested, mepacrine and MY5445, inhibited phosphoinositide hydrolysis at the same time as the $^{45}\text{Ca}^{2+}$ influx stimulated by carbachol. Taken together, our results concur with previous evidence obtained with permeabilized cells and cell fractions to indicate phosphatidylinositol 4,5-bisphosphate hydrolysis and $[\text{Ca}^{2+}]_i$ rise as two successive events in the intracellular transduction cascade initiated by receptor activation. The strict correlation between the carbachol concentration curves for inositol trisphosphate generation and $[\text{Ca}^{2+}]_i$ rise, and the inhibition by theophylline of the Ca^{2+} signal without major effects on inositol phosphate generation, satisfy important requirements of the abovementioned interpretation.

INTRODUCTION

In the preceding paper (Pozzan *et al.*, 1986) we described the changes of Ca^{2+} homeostasis brought about by the activation of muscarinic receptors in pheochromocytoma PC12 cells. Both stimulation of Ca^{2+} influx through a pathway different from the voltage-dependent Ca^{2+} channel, and redistribution of Ca^{2+} from bound, probably membrane-delimited, store(s) to the cytosol, was demonstrated. As a consequence, the concentration of free cytosolic Ca^{2+} , $[\text{Ca}^{2+}]_i$, rose 2–3-fold with respect to the resting level in PC12⁺ cells. This increase was prevented or rapidly reversed ($t_{1/2}$ 15–30 s) by low concentrations of muscarinic blockers added before or after carbachol respectively.

Changes in Ca^{2+} homeostasis represent only a part of the intracellular events coupled to the activation of muscarinic as well as other receptors which share common transducing mechanism(s). The $[\text{Ca}^{2+}]_i$ effects

occur together with a metabolic reaction, the hydrolysis of membrane (poly)phosphoinositides, and these two processes appear to be functionally interconnected. In a variety of cells permeabilized by mild detergent or other treatments, application of IP_3 , a hydrophilic metabolite generated by hydrolysis of PIP_2 , was found to cause release of Ca^{2+} from intracellular membrane-bound store(s) that, upon subcellular fractionation, is recovered in the microsomal fraction [for reviews, see Berridge & Irvine (1984) and Hokin (1985)]. The generation of IP_3 has been proposed, therefore, as the intermediate, second-messenger step, linking the activation of receptors to the redistribution of Ca^{2+} from the 'microsomal' store to the cytosol. Although the results with fractions and permeabilized cells appear very convincing, direct proof of this interpretation in intact cells remains to be given. IP_3 generation and $[\text{Ca}^{2+}]_i$ rise were found to occur very rapidly after receptor activation, and their kinetic competence has yet to be established. Moreover, parallel

Abbreviations used: dbcAMP, dibutyryl cyclic AMP; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; IAP, islet-activating protein, the active principle of *Bordetella pertussis* toxin; IP_1 , IP_2 and IP_3 , inositol 1-phosphate, 1,4-bisphosphate and 1,4,5-trisphosphate; NGF, nerve growth factor; PC12⁺ and PC12⁻, PC12 cells before and after differentiation by NGF; PI, PIP and PIP_2 , phosphatidylinositol, phosphatidylinositol 4-phosphate and 4,5-bisphosphate; PMSF, phenylmethanesulphonyl fluoride.

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studies of the two processes in the same cell system have yielded conflicting results (Ambler *et al.*, 1984; Beaven *et al.*, 1984; Brown Masters *et al.*, 1984; Thomas *et al.*, 1984; Volpi *et al.*, 1984; Charest *et al.*, 1985; Cockcroft & Gomperts, 1985; Rossi *et al.*, 1985).

In a previous paper (Vicentini *et al.*, 1985) we have shown that, in PC12 cells, the activation of the muscarinic receptor induced both the hydrolysis of polyphosphoinositides and the rise of $[Ca^{2+}]_i$, and that the first signal is maintained even under conditions in which the second is completely abolished. Here we report additional studies aimed to establish the degree of correlation of the two intracellular signals. Our experimental approach was twofold. On the one hand we carried out thorough analyses of phosphoinositide hydrolysis and $[Ca^{2+}]_i$ responses induced by muscarinic-receptor activation in parallel cell samples, processed under identical experimental conditions. On the other hand we studied the effects of a number of inhibitors and other drugs that were expected to interfere at various levels with the processes initiated by receptor activation.

MATERIALS AND METHODS

Cells and incubation media

The conditions used for culturing monolayers of undifferentiated PC12 cells (PC12⁻) and for inducing their differentiation with NGF (PC12⁺ cells) are described in the preceding paper (Pozzan *et al.*, 1986). At the beginning of the experiments the cells were detached from the dishes and used in suspension. Drugs were added dissolved in either water or dimethyl sulphoxide. Controls received solvents only, at the maximal final concentration of 0.5%.

The composition of the media employed (complete KR and Ca^{2+} -free KR; the latter supplemented with EGTA) is given in the preceding paper (Pozzan *et al.*, 1986).

Phosphoinositide hydrolysis

At 24 or 48 h before the experiments the growth medium was replaced with another medium (BME from Flow Laboratories, Milano, Italy, which is inositol-free), supplemented with low concentrations of foetal-calf serum (0.1%) and horse serum (0.2%), and with *myo*- $[^3H]$ inositol (1–2 μ Ci/ml). At the beginning of the experiments the cells were detached, transferred to silicone-treated glass tubes and washed three times with 10 ml of complete KR medium. After resuspension in appropriate volumes of incubation media (which were often supplemented with 10 mM-LiCl to block inositol-1-phosphatase; Berridge *et al.*, 1982), the cells were dissociated and incubated at 37 °C in silicone-treated glass vials oscillating at 140 cycles/min in a water bath. Because of the larger size of PC12⁺ with respect to PC12⁻, the final cell concentrations used were different: (1–2) and (3–6) $\times 10^6$ /ml respectively. Drugs and other agents were usually added after 2 min of equilibration at 37 °C. The incubations were terminated by precipitation with trichloroacetic acid (15%, w/v). Inositol phosphates, recovered in the supernatants, were bound to AG resin (1X8, 100–200 mesh, formate form; Bio-Rad, Richmond, CA, U.S.A.), and eluted with 0.1 M-formic acid/1 M-ammonium formate (Berridge *et al.*, 1983). In order to separate the various inositol phosphates, the ion-exchange columns were eluted sequentially as recommended by Berridge *et al.* (1983). Fractions (1 ml each) were

collected. In order to measure radioactivity in phosphoinositides, trichloroacetic-acid pellets were covered with 2 ml of chloroform/methanol (1:2, v/v) and resuspended by means of an Ultra-Turrax grinder operated at medium speed for 30 s. After addition of 0.6 ml of chloroform and 0.3 ml of water, the samples were centrifuged. The organic phase was collected and dried under N_2 . Phospholipids were separated on high-performance t.l.c. plates impregnated with potassium oxalate (1%). The solvent system consisted of chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.; Imai *et al.*, 1983). Labelled phospholipids were detected by using a thin-layer scanner (Berthold, Wiesbaden, Germany). The plate areas corresponding to the individual inositol lipids were scraped into vials containing 1 ml of water. All samples were mixed with 9 ml of Atomlight (New England Nuclear, Dreirich, Germany) and counted for radioactivity in a Beckman SL 30 spectrometer.

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

The technique used for loading the cells with the probe, and for measuring and calibrating the fluorescent signal, were described in the preceding paper (Pozzan *et al.*, 1986).

$^{45}Ca^{2+}$ influx

The accumulation of $^{45}Ca^{2+}$ within cells was measured under non-steady-state conditions exactly as described in the preceding paper (Pozzan *et al.*, 1986), using suspensions of PC12⁺ cells that had been preincubated $\pm$ inhibitors for 2 min before addition of carbachol. Duplicate samples of the incubated suspensions were withdrawn 25 and 65 s after the addition of $^{45}Ca^{2+}$, and immediately separated from the medium by centrifugation through a layer of mineral oil.

IP_3 -induced release of Ca^{2+} from permeabilized PC12 cells

Cells were detached, washed in 'intracellular' medium and permeabilized with digitonin (10 μ M, 4 min, 37 °C). They were then resuspended (at a concentration of 10^8 /ml) in the 'intracellular' medium supplemented with ATP and an ATP-generating system (Biden *et al.*, 1984). The $[Ca^{2+}]$ of the suspension was monitored continuously by a specific minielectrode, as described by Biden *et al.* (1984).

Materials

In addition to the materials specified in the preceding paper (Pozzan *et al.*, 1986), we used: *myo*[2- 3H]inositol (sp. radioactivity 10–20 Ci/mmol) and the kit for cyclic AMP radioimmunoassay, purchased from Amersham International; mepacrine (quinacrine hydrochloride) from ICN Pharmaceuticals, Plainview, NJ, U.S.A.; dbcAMP, theophylline and PMSF from Sigma, St. Louis, MO, U.S.A.; forskolin from Calbiochem—Behring, San Diego, CA, U.S.A.; RO 201724 from Hoffman-La Roche, Basel, Switzerland. MY5445 was kindly given by Dr. Y. Kobayashi, Mitsubishi-Yuka Co., Tokyo, Japan; IAP, the pure active component of *Bordetella pertussis* toxin, was kindly given by Dr. R. Rappuoli, Sclavo Research Laboratory, Siena, Italy; IP_3 from Dr. R. Irvine, AFRC Institute for Animal Physiology, Babraham, Cambridge, U.K.; α -latrotoxin from Dr. L. Madeddu, Department of Pharmacology, University of Milan, Milan, Italy.

Fig. 1. Concentration-dependence of carbachol-induced accumulation of labelled inositol phosphates (^3H)IPs in PC12⁺ (●) and PC12⁻ (○) cells

Cell suspensions (210 and 230 μg of protein/sample of PC12⁺ and PC12⁻) in complete KR containing 10 mM-LiCl were treated with carbachol at the concentrations specified on the abscissa for 5 min at 37 °C. Values (averages $\pm$ S.E.M. of three determinations) are expressed as percentage increases over controls, which contained 1530 and 2440 d.p.m./sample of PC12⁺ and PC12⁻ respectively.

RESULTS

IP_3 -induced redistribution of Ca^{2+} in permeabilized PC12 cells

Previous studies in a variety of cell types (reviewed by Berridge & Irvine, 1984) demonstrated that after permeabilization the release of Ca^{2+} from a non-mitochondrial pool can be induced by IP_3 , whereas IP_2 and IP_1 are inactive. This result has now been obtained for PC12 cells (not shown in the Figures). Suspensions of cells permeabilized by digitonin were found to buffer Ca^{2+} towards the physiological intracellular resting concentration when supplemented with ATP and an ATP-generating system. Application of 1 μM - IP_3 induced a rapid $[\text{Ca}^{2+}]_i$ rise of approx. 200 nM that lasted ~ 1 min and reappeared, although attenuated, on a subsequent addition of the messenger. Under the conditions of the experiment, IP_2 and IP_1 were completely ineffective.

Dependence of $[\text{Ca}^{2+}]_i$ and inositol phosphates on agonist concentration

Fig. 1 illustrates the concentration-dependence of the carbachol-induced phosphoinositide hydrolysis, measured as accumulation of labelled inositol phosphates. Carbachol was applied for 5 min to ^3H inositol-labelled PC12 cells incubated in KR medium supplemented with LiCl (to block inositol 1-phosphatase: Berridge *et al.* 1982). Considerable stimulation was observed at 60 μM -carbachol, and further increases occurred up to 150–200 μM . The effect was clearly appreciable in PC12⁻ cells, and much greater in PC12⁺ cells, as expected from the different complement of muscarinic receptors (Jumblatt & Tischler, 1982; Pozzan *et al.*, 1986). Because of the greater signals they generate, PC12⁺ cells were used in all subsequent experiments.

Fig. 2. Concentration-dependence of the $[\text{Ca}^{2+}]_i$ and phosphoinositide signals induced by carbachol in parallel samples of PC12⁺ cells

PC12⁺ monolayers were labelled overnight with ^3H -inositol, then washed and detached from the dishes. Suspended cells were loaded with quin2 and then divided in two parallel samples (final concn: 1.6×10^6 cells/ml; concentration of quin2: 0.8 nmol/ 10^6 cells), one of which was used for measuring $[\text{Ca}^{2+}]_i$ (●), the other for measuring the accumulation of the various inositol phosphates. (○, IP_1 ; ▽, IP_2 ; □, IP_3). Measurements were made 30 s after the application of carbachol at the concentrations specified on the abscissa. Values (averages of two experiments) are expressed as a percentage of the increase observed with 500 μM -carbachol. Basal and 100%-stimulated values were: $[\text{Ca}^{2+}]_i$ (nM), 105 and 265; inositol phosphates (d.p.m./sample): IP_1 , 2070 and 3020; IP_2 , 1020 and 1554; IP_3 , 413 and 826.

The concentrations of carbachol used in the experiments of Fig. 1 are in the same range as those inducing $[\text{Ca}^{2+}]_i$ rises, as revealed by quin2 experiments (Vicentini *et al.*, 1985; the preceding paper, Pozzan *et al.*, 1986). In order to investigate more closely the correlation between Ca^{2+} and inositol phosphate signals, experiments were carried out using samples of PC12⁺ cells processed exactly in parallel, i.e. labelled overnight with ^3H inositol, and then loaded with quin2. The incubation medium used was in all cases KR medium without LiCl. Measurements were made at 30 s after various concentrations of carbachol were added, i.e. at the time when both the $[\text{Ca}^{2+}]_i$ and the IP_3 signals are at peak (Vicentini *et al.*, 1985; Pozzan *et al.*, 1986). Moreover, the radioactivity recovered in IP_1 , IP_2 and IP_3 was assayed separately. The results obtained are shown in Fig. 2. At carbachol concentrations in the 5–500 μM range the measured percentage $[\text{Ca}^{2+}]_i$ rises were almost superimposable on the percentage rises of IP_3 . Analogous behaviour was observed with the other two metabolites, IP_1 and IP_2 . Only at very low carbachol concentration was a small, but reproducible, rise of IP_3 observed without detectable change of the resting $[\text{Ca}^{2+}]_i$ (Fig. 2).

Effects of muscarinic receptor activation on phosphoinositide radioactivity

In order to investigate the origin of the released inositol phosphates, the distribution of radioactivity in the various phosphoinositides (PI, PIP and PIP_2) was measured in cells prelabelled with ^3H inositol for 48 h and then

Table 1. Effect of carbachol (CCh) and α -latrotoxin (α LTx) on the radioactivity of the various phosphoinositides and total inositol phosphates (IPs) in PC12⁺ cells

Monolayers of PC12⁺ cells were labelled with [³H]inositol for 48 h. Aliquots of suspended cells (300 μ g of protein) were incubated at 37 °C without or with carbachol or α -latrotoxin. Values shown are averages $\pm$ S.E.M. for three to five determinations.

Addition	Radioactivity (c.p.m.)			
	PI	PIP	PIP ₂	IPs
None (control)	24035 $\pm$ 1700	1192 $\pm$ 113	205 $\pm$ 25	1300 $\pm$ 37
CCh (100 μ M)				
30 s	25900 $\pm$ 320	1510 $\pm$ 120	118 $\pm$ 39	1682 $\pm$ 15
8 min	22486 $\pm$ 430	1295 $\pm$ 144	179 $\pm$ 76	2509 $\pm$ 140
α LTx (3 nM)				
8 min	19192 $\pm$ 2500	762 $\pm$ 170	248 $\pm$ 60	5179 $\pm$ 61

treated with either carbachol (30 s and 8 min) or the presynaptic neurotoxin α -latrotoxin. The latter agent was used as a reference because it induces a phosphoinositide hydrolytic reaction much greater than that elicited by carbachol (Vicentini & Meldolesi, 1984). The results obtained are illustrated in Table 1. In control cells almost 95% of the labelled phosphoinositides was accounted for by PI, 4.5% by PIP and only 0.8% by PIP₂. After 30 s treatment with carbachol the level of labelled PI and PIP was not changed, whereas a considerable (~40%) decrease was observed in the radioactivity of PIP₂. A decrease of radioactivity in PI was found with carbachol and, especially, α -latrotoxin treatment for 8 min. The latter agent also induced a decrease of PIP. The hydrolytic responses observed, especially those by carbachol, were small. Indeed, the increases of [³H]inositol phosphates observed after 30 s with carbachol was only 1.5% of the counts present in total phosphoinositides at the beginning of the experiment. The corresponding values for 8 min carbachol and α -latrotoxin were almost 5 and 15.3% respectively.

Effects of inhibitors and other drugs

Various types of inhibitors were used that were expected to interfere with either receptor activation or transmembrane signalling. Fig. 3 illustrates the effects of two muscarinic antagonists, atropine and pirenzepine, on the phosphoinositide hydrolytic response induced by carbachol in PC12⁺ cells. Both drugs blocked the response with an IC₅₀ (concn. causing 50% inhibition) of about 5.5 and 100 nM respectively. The apparent K_i of the two inhibitors, calculated by the method of Cheng & Prusoff (1973), was 1.5 nM for atropine and 16 nM for pirenzepine. These results compare with those obtained with the same inhibitors on the carbachol-induced Ca²⁺ signal (the preceding paper, Pozzan *et al.*, 1986).

The effect of IAP, the active principle of *Bordetella pertussis* toxin, was investigated next. In mast cells, polymorphonuclear leucocytes and HL60 cells exposed to various agonists, IAP was found previously to block both the inositol phosphate and Ca²⁺ signals (Bokoch & Gilman, 1984; Moltski *et al.*, 1984; Bradford & Rubin,

Fig. 3. Inhibition by atropine (Atr, ■) and pirenzepine (Pz, ●) of the accumulation of inositol phosphates (IPs) induced by 500 μ M-carbachol in PC12⁺ cells

Cell suspensions (150 μ g of protein/sample) were pre-incubated with the muscarinic blockers for 2 min and then exposed to carbachol. Other experimental conditions were as for Fig. 1. The results shown are averages for three experiments. Basal and 100%-stimulated radioactivities in inositol phosphates were 747 and 1512 d.p.m./sample respectively.

1985; Brandt *et al.*, 1985; Nakamura & Ui, 1985; Verghese *et al.*, 1985). However, in PC12 cells pre-incubated with IAP (at concentrations varying from 5 to 1000 ng/ml) for periods up to 18 h, the [Ca²⁺]_i rise and the accumulation of inositol phosphates induced by carbachol were unchanged with respect to controls (Table 2). Under the same experimental conditions, IAP effectively blocked another response caused by carbachol in PC12 cells, namely the inhibition of cyclic AMP accumulation induced by phenylisopropyladenosine treatment (not shown in the figures).

An additional series of experiments was carried out by using drugs that increase the intracellular level of cyclic AMP (Table 2). Forskolin, a direct activator of adenylate cyclase that induces massive intracellular accumulation of cyclic AMP, was without detectable effect on both the inositol phosphate and Ca²⁺ signals induced by carbachol in PC12⁺ cells, whereas dbcAMP induced slight, but appreciable, inhibition of either processes. The inhibitory effect of dbcAMP was increased by the combination with the phosphodiesterase inhibitors RO 201724 and, especially, theophylline. Together with dbcAMP the latter drug inhibited inositol phosphate accumulation stimulated by carbachol by almost 50% and the [Ca²⁺]_i rise almost completely. Even when given alone, theophylline had profound inhibitory effects on the [Ca²⁺]_i signal that were already appreciable at concentrations as low as 0.25 mM. By contrast, theophylline was almost inactive on phosphoinositide hydrolysis (Table 2).

A number of other drugs were then considered, in view of their reported or presumed inhibitory effect on the phosphoinositide hydrolytic reaction. PMSF, a serine-proteinase inhibitor, was reported previously to inhibit the generation of phosphatidic acid in platelets (Walenga *et al.*, 1980). In PC12⁺ cells, however, PMSF had effect neither on the [Ca²⁺]_i rise nor on inositol phosphate accumulation induced by carbachol (Table 2). More interesting results were obtained with two other drugs, namely mepacrine, known as an inhibitor of phospho-

Table 2. Effect of various agents on the hydrolysis of phosphoinositides and rise of $[\text{Ca}^{2+}]_i$ induced by carbachol (0.5 mM) and cyclic AMP content of PC12⁺ cells

Results shown are averages from two to four experiments. Basal radioactivity in $[\text{^3H}]\text{IPs}$ (inositol phosphates) ranged between 500 and 2000 d.p.m./sample, and percentage carbachol stimulation between 60 and 100. The 100% carbachol-induced $[\text{Ca}^{2+}]_i$ rise was from 100–150 to 280–340 nM. The basal cyclic AMP level was 61 pmol/mg of protein.

Agent	Conditions	Inhibition (%) of:		
		Accumulation of $[\text{^3H}]\text{IPs}$	$[\text{Ca}^{2+}]_i$ rise	Increase in cyclic AMP (%)
IAP	5–1000 ng/ml; 2–18 h	0	0	–
Forskolin	1–50 mM (10 min)	0	0	39 10
dbcAMP	1 mM (5 min)	15	26	–
dbcAMP + RO201724	1 mM + 0.5 mM (5 min)	23	–	–
dbcAMP + theophylline	1 mM + 2 mM (5 min)	48	90	–
Theophylline (5 min)	0.25 mM	0	15	–
	0.50 mM	0	34	38
	1 mM	0	55	–
	2 mM	15	82	54

Fig. 4. Inhibition by mepacrine (■) and MY5445 (●) of the accumulation of inositol phosphates (IPs) induced by 500 μM -carbachol in PC12⁺ cells

Experimental conditions were as for Fig. 3; the amount of protein/sample was 90 μg . Basal and 100%-stimulated radioactivities in inositol phosphates were 360 and 740 d.p.m./sample respectively.

lipase A_2 and that at high concentration was reported also to be active on platelet phospholipase C (the enzyme presumably responsible for phosphoinositide hydrolysis) (Hoffman *et al.*, 1982), and MY5445, a recently developed inhibitor of cyclic GMP-dependent phosphodiesterase (Hagiwara *et al.*, 1984). Both these drugs inhibited phosphoinositide hydrolysis, but the inhibition by mepacrine was at the concentrations active on phospholipase A_2 , whereas that by MY5445 required concentrations two orders of magnitude greater than for phosphodiesterase inhibition and never exceeded 50–60% of the carbachol-induced response (Fig. 4). The effects of

Table 3. Effect of atropine and MY5445 on the carbachol-induced stimulation of $^{45}\text{Ca}^{2+}$ influx in PC12⁺ cells

The time in seconds is given with respect to the addition of $^{45}\text{Ca}^{2+}$ (see the Materials and methods section and the preceding paper, Pozzan *et al.*, 1986). Samples contained 4.5×10^5 cells. Data are shown as d.p.m. of intracellular $^{45}\text{Ca}^{2+}$, with the contamination by extracellular radioactivity subtracted, and are averages $\pm$ S.E.M. of three to four determinations.

Treatments		Intracellular $^{45}\text{Ca}^{2+}$ (d.p.m./sample)	
– 240 s	– 120 s	25 s	65 s
–	–	405 $\pm$ 15	445 $\pm$ 36
–	Carbachol 500 μM	740 $\pm$ 35	727 $\pm$ 24
Atropine (0.1 μM)	Carbachol (500 μM)	448 $\pm$ 33	493 $\pm$ 42
Mepacrine (100 μM)	Carbachol (500 μM)	410 $\pm$ 25	376 $\pm$ 12
MY5445 (100 μM)	Carbachol (500 μM)	398 $\pm$ 42	629 $\pm$ 16

the two drugs on $[\text{Ca}^{2+}]_i$ could not be investigated, because they are coloured and markedly interfere with quin2 measurements. Table 3 reports the results of $^{45}\text{Ca}^{2+}$ -influx experiments carried out as described in the preceding paper (Pozzan *et al.*, 1986) on PC12⁺ cells pretreated with either one of the two drugs. As can be seen, mepacrine caused complete inhibition, whereas with MY5445 the carbachol-induced response was delayed.

DISCUSSION

Activation of a large class of receptors is known to be transduced intracellularly by two interconnected processes: phosphoinositide hydrolysis and $[\text{Ca}^{2+}]_i$ rise [for

reviews, see Berridge (1983) and Berridge & Irvine (1984)]. During the last few years, there has been considerable progress in our understanding of these processes, primarily due to results obtained with permeabilized cells and subcellular fractions. As shown first by Streb *et al.* (1983) in permeabilized pancreatic acinar cells, and then rapidly confirmed in a number of other permeabilized systems (for a review, see Berridge & Irvine, 1984), IP_3 , at concentrations about $1 \mu\text{M}$, causes the release of Ca^{2+} from an intracellular store that, upon cell fractionation, is mainly recovered in the heavy-microsome fraction. The Ca^{2+} -release effect of IP_3 has now also been shown in permeabilized PC12 cells. IP_3 is the metabolite generated by phosphodiesterase hydrolysis of PIP_2 . Thus these results strongly suggest that this hydrolytic reaction is the process closely coupled with receptor activation, i.e. the initial intracellular event, whereas the $[\text{Ca}^{2+}]_i$ rise is a subsequent event in the ensuing signal cascade. In order for this scheme to be accepted as the sequence of events taking place in intact cells, a number of predictions should be met. Among these are the following: (a) PIP_2 hydrolysis should occur immediately after receptor activation and precede $[\text{Ca}^{2+}]_i$ rise; (b) the two processes should show similar dependence on the concentration of receptor agonists; and (c) PIP_2 hydrolysis should be maintained even under conditions in which $[\text{Ca}^{2+}]_i$ rise is inhibited, whereas the reverse should not occur. The fast rate of development of receptor-activated intracellular events has so far precluded the study of their kinetic competence, as demanded by prediction (a). In contrast, results have been obtained concerning predictions (b) and (c).

As far as the dependence on agonist concentration is concerned, the existing data of the literature appear very contradictory. Apart from the complete dissociation (mobilization of Ca^{2+} without concomitant phosphoinositide hydrolysis) observed by two groups in neutrophil leucocytes (Volpi *et al.*, 1984; Rossi *et al.*, 1985), a finding that is incompatible with prediction (c), variable extents of correlation have been found in different cell systems exposed to a variety of receptor agonists. Almost exact coincidence of the concentration-dependence curves of the two responses were reported in 2H3 basophil cells exposed to antigen (Beaven *et al.*, 1984) and rat hepatocytes exposed to vasopressin (Thomas *et al.*, 1984). However, in 2H3 cells the rise of $[\text{Ca}^{2+}]_i$ was shown to be due to stimulated influx and not to redistribution from the stores (Beaven *et al.*, 1984); in hepatocytes, the results of Thomas *et al.* (1984) were not confirmed by Charest *et al.* (1985), who found that generation of IP_3 was 20–30-fold less sensitive to vasopressin and adrenaline than was $[\text{Ca}^{2+}]_i$ rise. This last result has also been observed in other cell systems (Volpi *et al.*, 1984; Cockcroft & Gomperts, 1985; Powers *et al.*, 1985a,b). Additional studies have attempted to correlate phosphoinositide hydrolysis with $^{45}\text{Ca}^{2+}$ fluxes, again with conflicting results. Dissociation between the concentration dependence of the two responses has been reported in BH6 muscle cells exposed to α_1 -agonists (Ambler *et al.*, 1984) and a close correlation in astrocytes exposed to muscarinic agonists (Brown Masters *et al.*, 1984).

The differences in the concentration-dependence curves for interconnected intracellular messengers could be due to different degrees of saturability of their generation processes. In other words, the lower agonist sensitivity of IP_3 generation versus $[\text{Ca}^{2+}]_i$ rise could be

explained if a small fraction of the IP_3 generated by full receptor activation would be able to cause maximal Ca^{2+} redistribution from the stores to the cytosol (Charest *et al.*, 1985). Were this explanation valid, in the cells where receptor activation yields small hydrolytic responses, a good correlation would be expected. This expectation has been borne out in the present study. PC12 cells that, compared with other cells, generate small amounts of IP_3 when exposed to muscarinic agonists, showed almost superimposable carbachol concentration curves for IP_3 generation and Ca^{2+} rise. Two technical aspects of our experiments need to be emphasized. Quin2 and inositol phosphate measurements were carried out on parallel samples of cells processed under identical conditions; incubations lasted 30 s only. The brevity of the incubation makes highly unlikely a significant contamination of the IP_3 peak with inositol 1,3,4-trisphosphate, an isomer devoid of Ca^{2+} -releasing activity that in other cells has been shown to accumulate on prolonged incubation (Irvine *et al.*, 1984). Concomitant with inositol phosphate release, a decrease of PIP_2 radioactivity was observed after a 30 s treatment with carbachol. Thus the data on the phosphoinositide parallel those on IP_3 and indicate PIP_2 to be a substrate of the receptor-triggered hydrolytic reaction. Besides IP_3 , IP_1 and IP_2 were also generated in PC12 cells stimulated for 30 s with carbachol, whereas radioactivity did not decrease in their putative precursors, PI and PIP. It is therefore possible that IP_1 and IP_2 originate, at least in part, by dephosphorylation of IP_3 . Whether PI and PIP are also hydrolysed cannot be established with certainty. These phosphoinositides are much more abundant than PIP_2 in PC12 cells, and a hydrolysis of the size indicated by the inositol phosphate recoveries could have been easily undetected under our experimental conditions.

Indirect evidence that phosphoinositide hydrolysis can occur even under conditions in which $[\text{Ca}^{2+}]_i$ rise is inhibited, as predicted in (c), had been obtained in several systems (Beaven *et al.*, 1984; Dougherty *et al.*, 1984; Simon *et al.*, 1984). Recently we, in PC12 cells (Vicentini *et al.*, 1985) and in granulocytes (Di Virgilio *et al.*, 1985) and others in a T-cell leukaemia line (Sasaki & Hagesawa-Sasaki, 1985) have provided a direct demonstration of this dissociation in cells depleted of their stored Ca^{2+} by treatment with Ca^{2+} ionophores. In the present study this problem has been further investigated by the use of several inhibitors. IAP of *Bordetella pertussis* toxin, known as a blocker of the inhibitory GTP-binding protein of receptor adenylate cyclase complexes (see Gilman, 1984), has been recently found to cause inhibition of phosphoinositide hydrolysis and $[\text{Ca}^{2+}]_i$ rise in mast cells (Nakamura & Ui, 1985), neutrophils (Moltski *et al.*, 1984; Verghese *et al.*, 1985; Bradford & Rubin, 1985; T. Pozzan & F. Di Virgilio, unpublished work) and HL60 leukaemic cells (Brandt *et al.*, 1985). A GTP-binding protein is believed to play a role in the coupling of muscarinic receptors to phosphoinositide hydrolysis (Florio & Sternweis, 1985), yet IAP proved to be ineffective in astrocytoma (Brown Masters *et al.*, 1985) and PC12 cells (the present work; see also Schlegel *et al.*, 1985). Heterogeneity of GTP-binding proteins at the various receptors coupled to phosphoinositide hydrolysis, or in various cellular systems, could account for these results.

Also, cyclic AMP seems to have a variable role in the regulation of phosphoinositide hydrolysis. It causes

inhibition in some systems, such as platelets and lymphocytes (Tou & Maier, 1976; Billah *et al.*, 1979; Kaibuchi *et al.*, 1982; Taylor *et al.*, 1984), and is without effect in others, such as hepatocytes (Kaibuchi *et al.*, 1982). In our experiments we found that preincubation with forskolin, a direct activator of adenylate cyclase that causes massive accumulation of cyclic AMP, was without effect on the muscarinic-receptor-stimulated phosphoinositide hydrolysis and $[\text{Ca}^{2+}]_i$ rise. The inhibitions brought about by dbcAMP and phosphodiesterase inhibitors should therefore be regarded as pharmacological effects, probably unrelated to the rise of cyclic AMP these drugs induce within the cells. In this respect the results with theophylline appear interesting, because at 2 mM this drug caused a modest cyclic AMP increase with an almost complete block of the carbachol-induced $[\text{Ca}^{2+}]_i$ rise, and only minimal effect on phosphoinositide hydrolysis. The mechanism of this theophylline effect could be the inhibition of the IP_3 -induced Ca^{2+} release at the 'microsomal' stores. This result confirms that phosphoinositide hydrolysis can be triggered by receptor activation at resting $[\text{Ca}^{2+}]_i$ (see also Vicentini *et al.*, 1985; Di Virgilio *et al.*, 1985; Sasaki & Hagesawa-Sasaki, 1985).

A final group of drugs was studied as possible direct inhibitors of phosphoinositide hydrolysis. Two such drugs, mepacrine and MY5445, were indeed found to block the carbachol-induced hydrolytic response. These two inhibitors are not ideal for our purposes because they are not specific for the reaction under study and interfere with quin2 fluorimetric measurement of $[\text{Ca}^{2+}]_i$. We were, however, able to demonstrate that mepacrine and MY5445 inhibit the muscarinic-receptor-activated Ca^{2+} influx approximately in proportion to their effect on phosphoinositide hydrolysis.

In conclusion, our results on the generation of intracellular messengers induced in intact cells by the activation of the muscarinic receptor concur with the extensive evidence obtained with permeabilized cells and subcellular fractions (reviewed by Berridge & Irvine, 1984) to indicate PIP_2 hydrolysis and $[\text{Ca}^{2+}]_i$ rise as two successive events of the intracellular transduction cascade. In particular, the almost exact coincidence of the carbachol concentration curves for IP_3 generation and $[\text{Ca}^{2+}]_i$ rise and the inhibition by theophylline of one carbachol-induced signal ($[\text{Ca}^{2+}]_i$ rise) with only minimal effect on the other (phosphoinositide hydrolysis) satisfy important requirements of the abovementioned interpretation.

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