Muscarinic Receptor–induced Phosphoinositide Hydrolysis at Resting Cytosolic Ca^{2+} Concentration in PC12 Cells

L. M. VICENTINI,* A. AMBROSINI,*‡ F. DI VIRGILIO,§ T. POZZAN,§ and J. MELDOLESI*‡

*Department of Pharmacology, and ‡National Research Council Center of Cytopharmacology, University of Milano, Italy; §Institute of General Pathology, National Research Council Center for the Physiology of Mitochondria, University of Padova, Italy.

ABSTRACT In PC12 cells, cultured in the presence of nerve growth factor to increase their complement of muscarinic receptors, treatment with carbachol induces muscarinic receptor–dependent rises in free cytosolic Ca^{2+} as well as hydrolysis of membrane phosphoinositides. Experiments were carried out to clarify the relationship between these two receptor-triggered events. In particular, since inositol-1,4,5-trisphosphate (the hydrophilic metabolite produced by the hydrolysis of phosphatidylinositol-4,5-bisphosphate) is believed to mediate intracellularly the release of Ca^{2+} from nonmitochondrial store(s), it was important to establish whether it can be generated at resting cytoplasmic concentration of Ca^{2+} (~0.1 μM). Cells incubated in Ca^{2+}-free medium were depleted of their cytoplasmic Ca^{2+} stores by pretreatment with ionomycin. When these cells were then treated with carbachol, their cytosolic concentration of Ca^{2+} remained at the resting level, whereas inositol-1,4,5-trisphosphate generation was still markedly stimulated. Our results demonstrate that an increase in the concentration of cytosolic Ca^{2+} is not a necessary intermediate between receptor activation and phosphoinositide hydrolysis, and therefore support the second-messenger role of inositol-1,4,5-trisphosphate.

Hydrolysis of membrane phosphoinositides was initially described by Hokin and Hokin in pancreatic slices exposed to acetylcholine (17) and later shown to occur in many other systems as the consequence of the activation of a variety of receptors (muscarinic, α_1 adrenergic, H_1 histaminergic, serotonergic, and peptidergic receptors, references 4, 10, 11, 21–24, 27, 32). Recent developments in the understanding of the process of cell activation have stimulated great interest in this reaction. Two distinct mechanisms, one dependent on the concentration of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$)[1], and the other dependent on the activation of protein kinase C, have been shown to act coordinately in the control of exocytosis and other forms of secretion, smooth muscle contraction, and, possibly, cell growth (4, 22–25, 27). Both of these intracellular transduction mechanisms can be activated by the hydrolysis of one type of membrane phosphoinositide, phosphatidylinositol-4,5-bisphosphate (PIP_2). Indeed, of the metabolites generated by this reaction, diacylglycerol is believed to activate protein kinase C (4, 22, 24, 27), whereas inositol-1,4,5-trisphosphate (IP_3), once released to the cytoplasm, may trigger the release of Ca^{2+} from intracellular stores, thus causing $[Ca^{2+}]_i$ to rise (4, 7, 18, 26, 30).

Although the conclusions drawn above now appear sound, a number of important questions are still unresolved. In particular, the definition and the sequence of the events initiated by receptor activation are still debated. The problem of which event is first has not been settled, primarily because (*a*) no direct studies were reported in which $[Ca^{2+}]_i$ rise and phosphoinositide hydrolysis were resolved, and (*b*) in some systems, phosphoinositide hydrolysis has been found to be strictly dependent on the presence of Ca^{2+} in the extracellular fluid (1–3, 12–14), suggesting that a rise in $[Ca^{2+}]_i$ by increased influx might be the initial event of the transduction cascade triggered by receptor activation (1, 8, 12–14).

In the present report, we characterize the relationship between $[Ca^{2+}]_i$ and phosphoinositide hydrolysis in a line of neurosecretory cells (PC12) exposed to muscarinic stimulation, and demonstrate that the hydrolysis of PIP_2 can be stimulated even when $[Ca^{2+}]_i$ is at the level of resting cells (~0.1 μM). The cells used in our experiments were pretreated for 12–15 d with nerve growth factor to induce (in addition to a neuronal-like differentiation; see reference 16) a large increase in the number of muscarinic receptors (19) (in our

[1] *Abbreviations used in this paper:* $[Ca^{2+}]_i$, concentration of free ionized calcium in the cytosol; $[Ca^{2+}]_o$, concentration of free ionized calcium in the extracellular medium; IP_1, inositol monophosphate; IP_2, inositol bis phosphate; IP_3, inositol tris phosphate; KR, modified Krebs-Ringer incubation medium; PIP_2, phosphatidylinositol-4,5-bisphosphate.

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studies, from 2,600 to 36,000/cell) and thus greatly increase the size of the transduction processes under study.

MATERIALS AND METHODS

Cells: Monolayers of PC12 cells (initially provided by Dr. P. Calissano, Consiglio Nazionale delle Ricerche Laboratory of Cell Biology, Rome) were cultured as described by Greene and Tischler (16) using RPMI 1640 medium (Flow Laboratories, Milan) supplemented with 10% horse serum, 5% fetal calf serum, and, during the last 12–15 d, nerve growth factor (50 ng/ml). Immediately before the experiments, the cells were detached from the monolayers and then dissociated to yield single cells and small (2–5 cells) aggregates, as described elsewhere (25). Drugs were dissolved in either water or dimethyl sulfoxide. Controls received solvents only (maximal concentration, 1%).

Incubation Media: A modified Krebs-Ringer medium buffered with Hepes (complete KR) was used which contained, in millimoles per liter: 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).

Quin2 Measurement of $[Ca^{2+}]_i$: PC12 cells, suspended in RPMI 1640 medium buffered with Hepes, pH 7.4 to a density of 12×10^6 cells/ml, were mixed with a 0.5–1% vol of 10 mM quin2 acetoxymethylester in dimethylsulfoxide and incubated at 37°C for 1 h. Before use, the cells were pelleted and resuspended in either KR or Ca^{2+}-free KR (0.5–0.8×10^6 cells/ml). Assays were carried out in a thermostated cuvette equipped with magnetic stirring, in a Perkin-Elmer 650-40 spectrofluorimeter (Perkin-Elmer Corp. Eden Prairie, MN; excitation, 339 ± 2; emission, 492 ± 10 nm). Calibration of the fluorescent signal was made as described (25). Whenever necessary, the data were corrected for changes in cell autofluorescence.

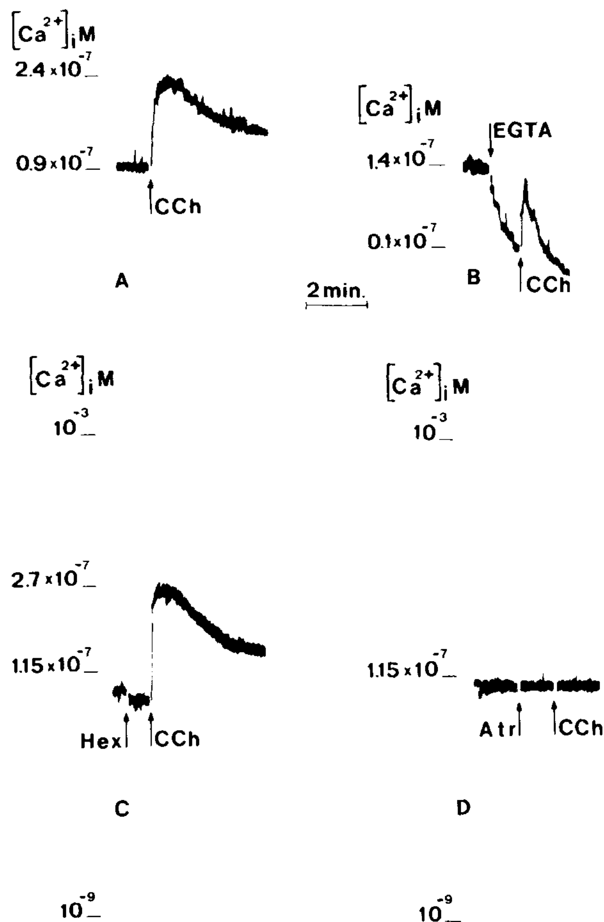

FIGURE 1 Effect of carbachol (*CCh*, 0.5 mM), and acetylcholine receptor blockers hexamethonium (*Hex*, 0.5 mM) and atropine (*Atr*, 2 μM), on $[Ca^{2+}]_i$ in PC12 cells incubated in complete KR (*A, C, D*) or Ca^{2+}-free KR (*B*). Concentration of cells, 0.6×10^6/ml; concentration of quin2, 2 nmol/10^6 cells. $[Ca^{2+}]_i$ calibration is indicated on the left of each single trace.

Phosphoinositide Hydrolysis: 24 h before the experiments, the growth medium was replaced with the basal medium Eagle's (which is free of inositol; Flow Laboratories), and supplemented with low concentrations of fetal calf serum (0.1%) and horse serum (0.2%), and with [^{3}H]myo-inositol (1 μCi/ml). The day after, the cells were detached, transferred to siliconized 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-phosphate phosphatase, reference 5), the cells were dissociated and incubated at 37°C in siliconized glass vials oscillating at 140 cycles/min in a water bath. The final cell concentration was 1–2×10^6/ml. Drugs and other agents were usually added after 2 min of equilibration at 37°C. Total phosphoinositols were extracted by trichloroacetic acid 15%, then bound to AG resin (1 × 8, 100–200 mesh, formate form, Bio-Rad Laboratories, Richmond, CA), and eluted with 0.1 M formic acid/1 M NH_4 formate (6). In order to separate the various phosphoinositols (inositol-1-phosphate, IP_1; inositol-1,4,-bisphosphate, IP_2; and IP_3), the elution protocol recommended by Berridge et al. (6) was employed. 1-ml samples were mixed with 9 ml of Atomlight (New England Nuclear, Boston, MA) and counted in a Beckman SL 30 spectrometer (Beckman Instruments, Inc., Fullerton, CA).

Materials: 2.5S nerve growth factor was the kind gift of Dr. P. Calissano, Consiglio Nazionale delle Ricerche Laboratory of Cell Biology, Rome, Italy; [^{3}H]myo-inositol was purchased from Amersham Corp., Amersham, England; carbachol, hexamethonium, atropine were from Sigma Chemical Co. (St. Louis, MO); and the source of the other chemicals is specified in reference 25.

RESULTS AND DISCUSSION

Fig. 1 illustrates changes in $[Ca^{2+}]_i$ induced in PC12 cells (differentiated by pretreatment with nerve growth factor) by an optimal concentration of carbachol (0.5 mM). These effects were due to the activation of the muscarinic receptor because they were blocked by low (1 or 2 μM) concentrations of atropine, and unaffected by the nicotinic antagonist hexamethonium. In the Ca^{2+}-free EGTA-containing medium (Fig. 1 *B*), the carbachol-induced $[Ca^{2+}]_i$ rise was fast (maximal within 10 s) and short-lived (down to the resting level within 1–2 min). In complete KR, the rise was larger, and $[Ca^{2+}]_i$ remained elevated for several minutes.[2] The same concentration of carbachol induced a large, atropine-inhibitable increase of phosphoinositide hydrolysis (Fig. 2). Also in this case, a considerable part of the response was maintained when the cells were exposed to carbachol suspended in the Ca^{2+}-free medium.

In the experiments illustrated in Fig. 2, the various inositol phosphates generated in stimulated PC12 cells were measured together, and therefore the contribution of the various phosphoinositides as substrates of the hydrolytic reaction was not established. To investigate this problem, the time-course experiments illustrated in Fig. 3 were carried out. In these experiments (*a*) incubations were brief, (*b*) cells were not pretreated with LiCl to avoid the accumulation of IP_1 uphill of the metabolic block, and (*c*) the various inositol phosphates released were separated by column chromatography. Analogous to what was observed previously in other systems (3, 6, 9, 10, 20, 28, 33), the concentration of the various [^{3}H]-inositol phosphates in resting PC12 cells was found to be $IP_1 > IP_2 > IP_3$ (inset of Fig. 3). 10 s after the application of carbachol, IP_3 showed the greatest proportional increase, continued to rise until 20 s, and then declined progressively. IP_2 rose in parallel to IP_1 during the first 10 s and then tended to level off, and IP_1 pleateaued at 20 s (Fig. 3). It should be

[2] The different size of the carbachol-induced responses suggests that different Ca^{2+} sources were involved: redistribution from the intracellular stores in the Ca^{2+}-free medium; and redistribution plus increased influx across the plasmalemma in complete KR. Additional evidence supporting this interpretation will be reported elsewhere (Pozzan, T., F. Di Virgilio, L. M. Vicentini, and J. Meldolesi, manuscript in preparation).

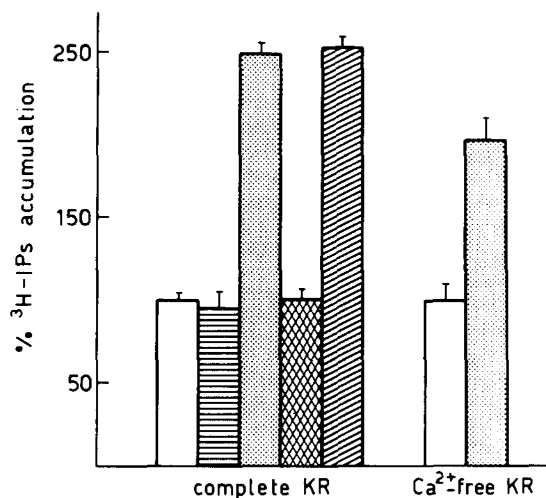

FIGURE 2 Effect of carbachol and/or acetylcholine receptor blockers on the accumulation of labeled inositol phosphates (^{3}H-IPs) in PC12 cells incubated in KR medium containing LiCl or Ca^{2+}-free KR medium containing 1 mM EGTA for 5 min at 37°C. □, control; ▤, atropine alone (2 μM); ▨, carbachol alone (0.5 mM); ▩, 0.5 mM carbachol + 2 μM atropine; ▧, 0.5 mM carbachol + 50 μM d-tubocurarine. Values shown are averages of 4–6 determinations ± SE. Proteins in the analyzed samples ranged from 200 to 250 μg. Basal radioactivities were 2,260 ± 120 cpm/mg protein in complete KR medium; 1,670 ± 107 cpm/mg protein in Ca^{++}-free-KR medium.

emphasized that in the experimental conditions used, dephosphorylation of inositol phosphates is expected to occur at high rates. The faster timecourse of IP_3 appearance might therefore be interpreted as an indication that the phosphodiesteratic hydrolysis of PIP_2 is the event most proximal to receptor activation and that the accumulation of IP_2 and IP_1 results from the stepwise degradation of IP_3 (see references 4, 6, 9, 15, 20, 28, and 33 for similar observations in other systems). However, the possibility that PIP and PI are hydrolyzed as well cannot be excluded.

From the data of Figs. 1 and 3, it appears that in PC12 cells, Ca^{2+} rise and phosphoinositide hydrolysis occur rapidly and, as far as we can judge, concomitantly after stimulation of the muscarinic receptor. In order to establish whether or not phosphoinositide hydrolysis requires a rise in $[Ca^{2+}]_i$ to occur, as has been suggested in other systems (1, 8, 12–14), attempts were made to dissociate the two processes. Quin2 experiments (Fig. 4) revealed that, when PC12 cells were incubated in Ca^{2+}-free, EGTA-containing medium, and then treated with the Ca^{2+} ionophore ionomycin, no rise in $[Ca^{2+}]_i$ occurred upon application of carbachol. This result was expected because the experimental conditions used preclude both Ca^{2+} influx (due to the very low concentration of free ionized calcium in the extracellular medium $[Ca^{2+}]_o$) and Ca^{2+} redistribution (due to the ionomycin-induced depletion of the stores, which in our experiments was documented by the transient increase in $[Ca^{2+}]_i$ following the application of the ionophore). The experiments of phosphoinositide hydrolysis (without LiCl) carried out on a parallel aliquot of the same batch of quin2-loaded cells (Fig. 4) revealed that ionomycin alone was without effect. In contrast, in the cells depleted of Ca^{2+} by EGTA + ionomycin, the application of carbachol was still able to induce considerable responses. Compared with the results obtained in complete KR (Fig. 3), a larger accumulation of IP_3 and a smaller accumulation of IP_1 was noted, suggesting that the lack of $[Ca^{2+}]_i$ rise impairs dephosphorylation of IP_3.

A wide consensus exists at the present time on the fact that both $[Ca^{2+}]_i$ rise and PIP_2 hydrolysis are crucial events in the signal cascade triggered by the activation of a variety of receptors, and leading to cell activation (4, 9, 10, 11, 20, 22, 24, 27, 28, 30, 32, 33). Data obtained primarily on permeabilized cells and/or isolated subcellular fractions (7, 18, 26, 30) have provided evidence for a second-messenger role of IP_3 in causing release of Ca^{2+} from nonmitochondrial store(s). According to this interpretation, PIP_2 hydrolysis would be a reaction closely coupled across the membrane to receptor activation (4, 9, 15, 20, 22, 23). However, the kinetic competence of the two processes (i.e., whether IP_3 generation precedes $[Ca^{2+}]_i$ rise) has never been resolved in any systems so far investigated. In some systems, phosphoinositide hydrolysis has been shown to depend on $[Ca^{2+}]_o$ and/or to be triggered by Ca^{2+} ionophores (1, 8, 12–14), whereas in other systems ionophores were inefficient, and a large part of the response was maintained in Ca^{2+}-free medium (4, 9, 15, 22, 23). It can be argued that the lack of effect of ionophores in the absence of receptor activation does not prove the Ca^{2+}-independence of the reaction, but only that high $[Ca^{2+}]_i$ alone is not sufficient to trigger it. Conversely, the observation that the receptor activation-triggered reaction takes place in some systems independently of $[Ca^{2+}]_o$ does not prove that changes of $[Ca^{2+}]_i$ are not involved. On the one hand, $[Ca^{2+}]_o$ could be needed not to raise $[Ca^{2+}]_i$ but to couple individual recep-

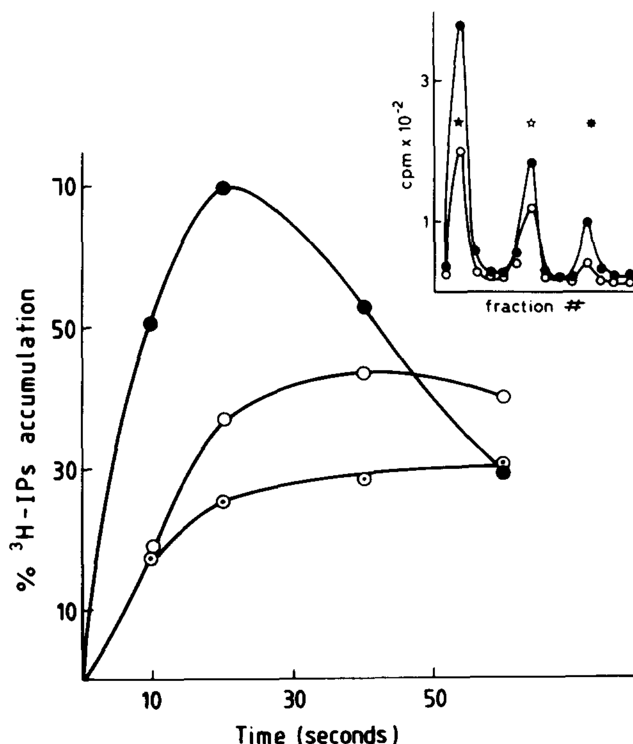

FIGURE 3 Time course of the effect of carbachol (0.5 mM) on the accumulation of labeled IP_1 (○), IP_2 (⊙), and IP_3 (●) in PC12 cells (500 μg protein/sample) incubated in complete KR without LiCl. The data show a typical experiment which was repeated three times. Basal radioactivities in counts per minute per sample for IP_1, IP_2, and IP_3 were 872 ± 30, 441 ± 32, and 210 ± 20, respectively. The inset illustrates the separation of IP_1 (★), IP_2 (☆), and IP_3 (✱) accumulated in PC12 cells incubated for 30 s with (●) or without (○) carbachol in LiCl-containing KR medium.

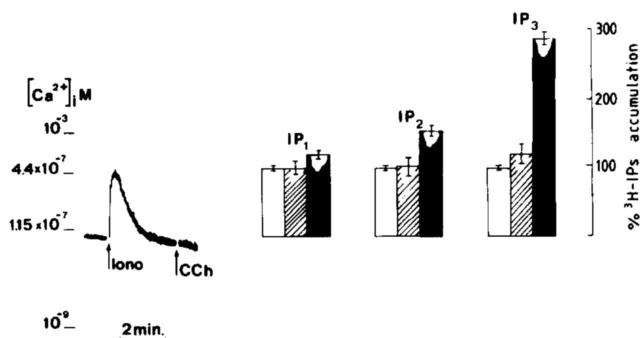

FIGURE 4 Effect of carbachol on $[Ca^{2+}]_i$ and accumulation of labeled IP_1, IP_2, and IP_3 in PC12 cells incubated in Ca^{2+}-free KR and treated with ionomycin. Suspensions of PC12 cells, prepared from monolayers labeled for 24 h with [^{3}H]inositol, were loaded with quin2 (concentration, 0.8 nmol/10^6 cells). Parallel aliquots of these suspensions were used for measuring $[Ca^{2+}]_i$ and accumulation of labeled inositol phosphates. (*Left*) Quin2 fluorimetric trace; additions of ionomycin (*Iono*, 0.2 μM) and carbachol (*CCh*, 0.5 mM) were made when indicated. (*Right*) Recoveries (averages of three determinations ± SE) of labeled IP_1, IP_2, and IP_3 (separated as illustrated in Fig. 3) in cells (600 μg protein/sample) incubated in Ca^{2+}-free KR medium and treated with ionomycin for 3 min followed by ± carbachol for 1 min. □, control; ▨, ionomycin alone; ■, ionomycin and then carbachol (*Iono* + *CCh*). Basal radioactivities in counts per minute per milligram protein for IP_1, IP_2, and IP_3 were 1095 ± 141, 316 ± 39, and 140 ± 12, respectively.

tors to the phosphoinositide hydrolyzing process;[3] on the other hand, $[Ca^{2+}]_i$ can rise even in cells incubated in Ca^{2+}-free media, due to redistribution from intracellular stores. Until now, it has therefore been impossible to exclude the possibility that a rise in $[Ca^{2+}]_i$ (due to stimulated influx and/or IP_3-independent redistribution), could precede and cause PIP_2 hydrolysis. Even two recent studies (2, 3, 29) which specifically addressed the problem, did not go beyond the demonstration of a lack of quantitative correlation between $[Ca^{2+}]_i$ rise and phosphoinositide hydrolysis. The answer provided by our results now seems unambiguous. In intact PC12 cells, Ca^{2+} influx and redistribution were prevented by incubation in Ca^{2+}-free media and by depletion of cellular stores with ionomycin. Under these conditions, $[Ca^{2+}]_i$ remained unchanged at the resting level after carbachol application, yet IP_3 generation was markedly stimulated. To our knowledge, this is the first demonstration that receptor-coupled phosphoinositide hydrolysis can occur at resting $[Ca^{2+}]_i$. This excludes the possibility that a rise in $[Ca^{2+}]_i$ is a necessary intermediate between receptor activation and phosphoinositide hydrolysis, therefore strongly supports the hypotheses of both a close coupling between the two processes, and the second-messenger role of IP_3 in causing Ca^{2+} redistribution.

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[3] Our work on PC12 cells seems to be in agreement with this possibility. Here we demonstrate that the muscarinic receptor–triggered phosphoinositide hydrolysis is independent of $[Ca^{2+}]$ ($[Ca^{2+}]_i$ as well as $[Ca^{2+}]_o$); however, the same response triggered by the activation of another receptor (the receptor for the neurotoxin αLTx) disappeared when $[Ca^{2+}]_o$ was decreased to a very low level by the addition of EGTA to the medium (31).

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