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Receptor-activated Ca^{2+} Influx

TWO INDEPENDENTLY REGULATED MECHANISMS OF INFLUX STIMULATION COEXIST IN NEUROSECRETORY PC12 CELLS*

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Receptor-activated Ca^{2+} influx was investigated in PC12 cells clones loaded with fura-2. Cells were stimulated in a Ca^{2+}-free medium and studied after reintroduction of the cation or addition of Mn^{2+} into the medium. A first influx component, independent of receptor activation and sustained by depletion of the intracellular inositol 1,4,5-trisphosphate sensitive Ca^{2+} store (store-dependent Ca^{2+} influx, SDCI), was identified by experiments with carbachol followed by atropine and with agents that induce store discharge without polyphosphoinositide hydrolysis: thapsigargin, an inhibitor of Ca^{2+}-ATPase activity; ryanodine and caffeine, activators of the ryanodine receptor. A second component of Ca^{2+} influx, induced by carbachol and rapidly blocked by atropine, relies on receptor-effector coupling via G protein(s) different from that (those) involved in phospholipase C activation. SDCI and receptor-coupled influx are similar in their voltage dependence and insensitivity to forskolin and phorbol esters but they differ with respect to their Mn^{2+} permeability and their sensitivity to the SC 38249 imidazole blocker. The two components might play different roles. SDCI might act as a safety device to prevent Ca^{2+} store depletion whereas receptor-dependent influx might control physiological functions such as secretion and growth.

Stimulation of Ca^{2+} influx across the plasma membrane is one of the key events in transmembrane signaling. So far, two families of channels, one activated by depolarization, the other by direct binding of ligands (voltage- and receptor-operated channels, respectively), have been identified, and many of their members have been isolated, cloned, and reconstituted into membranes (see 1-3). Voltage- and receptor-operated Ca^{2+} channels are not ubiquitory but are expressed only by excitable cells. However, in almost all cells, stimulation of Ca^{2+} influx occurs after the activation of receptors coupled to the hydrolysis of polyphosphoinositides. These receptors are not channels themselves but are known to function via appropriate GTP-binding proteins and activation of polyphosphoinositide-specific phospholipase(s) C (4-6). Of the two second messengers generated by the latter enzyme one, inositol 1,4,5-trisphosphate ($Ins-P_3$)[1] releases Ca^{2+} from a cytoplasmic storage organelle via the activation of the intracellular $Ins-P_3$ receptor; the other messenger, diacylglycerol, activates protein kinase C (4, 5). These two processes do not account for the entire spectrum of events elicited by receptor activation. In fact, concomitantly or slightly after intracellular Ca^{2+} release, influx of the cation across the plasma membrane is stimulated, apparently via the activation of channel(s) (7-9). Although this receptor-induced Ca^{2+} influx has attracted a great deal of interest (recent reviews: 10-13), the present information on the channels involved, as well as on the mechanism(s) of their activation and function, is still very limited. Initially, the channels were suggested to be activated by second messengers (increased $[Ca^{2+}]_i$; $Ins-P_3$ itself, working alone or together with its phosphorylation product, inositol 1,3,4,5-tetrakisphosphate) (5, 13-16). More recently, evidence has begun to accumulate suggesting a direct, G protein-mediated coupling of these channels to the activated receptors (8, 11, 13, 17-22). At the same time, work in platelets, endothelium, hepatocytes, exocrine secretory cells, and lymphocytes has revealed an unexpected link between intracellular release and transmembrane influx of Ca^{2+}: stimulation of the latter process is sustained by the emptying of the intracellular $Ins-P_3$-sensitive store (store-dependent Ca^{2+} influx (SDCI); Ref. 23-32; for review see 12). At least two hypothetical mechanisms have been proposed to account for SDCI: (*a*) a direct interaction of the store (possibly via the $Ins-P_3$ receptor) with Ca^{2+} channels in the plasma membrane (33, 34) and (*b*) the generation of a reverse second messenger, signaling to the surface channel the state of filling of the store (12, 35). As a whole, however, SDCI regulation remains obscure, and alternative mechanisms of activation cannot be excluded. Moreover, initial evidence suggests that SDCI is not a property of all cells since it has not been observed in bovine chromaffin cells and in NG 115-401, a neuronal cell line (36, 37).

To investigate receptor-induced stimulation of Ca^{2+} influx

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The abbreviations used are: $Ins-P_3$, inositol 1,4,5-trisphosphate; $[Ca^{2+}]_i$, cytosolic concentration of free ionized calcium; NGF, nerve growth factor; SC 38249, ((±)-1-2,3-bis[(4-methoxyphenyl)-methoxy]propyl)-1H-imidazole; SDCI, store-dependent Ca^{2+} influx; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PMA, phorbol 12-myristate 13-acetate.

we have employed PC12 cells, a line originating from a rat chromaffin cell tumor which is used as a neurosecretory/neuronal cell model. The experiments were not carried out on the parent line but on a group of well characterized clones recently isolated in our laboratory (38). Careful analyses of fura-2 $[Ca^{2+}]_i$ and Mn^{2+} quenching responses in cells exposed to a variety of treatments clearly revealed the coexistence in PC12 cells of SDCI together with at least one other mechanism of influx stimulation, more directly coupled to receptor activation. A number of important properties of these two events are described.

EXPERIMENTAL PROCEDURES

Selection of the PC12 cell clones employed in this work has been described elsewhere (38). The clones were cultured at 37 °C in Dulbecco's modified Eagle's medium containing 2 mM glutamine, 10% horse serum, and 5% fetal calf serum, in a humidified atmosphere with 5% CO_2. Cells were plated weekly 1:4 in 10-cm Petri dishes. To induce a neuronal phenotype, cells were cultured for 7 days in the presence of NGF (50 ng/ml; Ref. 39). At the beginning of the experiments cell monolayers were detached from the Petri dishes by applying a gentle flow of incubation medium containing (in mmol/liter): NaCl, 125; KCl, 5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2; glucose, 6; Hepes/NaOH buffer, pH 7.4, 25. Cells were then washed in the same medium and loaded for 30 min at 37 °C with the Ca^{2+}-sensitive dye fura-2, added as acetoxymethylester at final concentrations of 2–5 μM. After rinsing once with the above medium, the loaded cell suspensions were divided into aliquots of a 0.5–1 × 10^6 cells and kept at room temperature until use (maximum, 2.5 h). Results are shown as traces and graphs. The traces are representative of results in 3–20 experiments. The number of experiments summarized in the various graphs is indicated in the figure legends.

$[Ca^{2+}]_i$ Measurements—Cell aliquots were resuspended by gentle swirling in 1.5 ml of the incubation medium supplemented with 250 μM sulfinpyrazone to prevent dye leakage. Cell suspensions were then transferred to a thermostatted cuvette (37 °C), maintained under continuous stirring, and analyzed in a Perkin-Elmer LS-5B fluorometer as recommended by Grynkiewicz *et al.* (40). Most of our experiments were carried out according to the Ca^{2+}-free/Ca^{2+}-reintroduction protocol. To this end, cell suspensions in the above medium were supplemented with excess EGTA (usually 3 mM), and stimulants were added 1 min later. Thereafter the recording was continued for 2–6 min, *i.e.* until the end of the first $[Ca^{2+}]_i$ peak (intracellular Ca^{2+} release). Ca^{2+} (3 mM) was then reintroduced into the medium, and the ensuing second peak (Ca^{2+} influx) was recorded. In parallel experiments, the small $[Ca^{2+}]_i$ changes occurring in nonstimulated cells as a consequence of medium Ca^{2+} chelation and reintroduction were carefully evaluated, and corresponding values were subtracted graphically from the experimental traces of stimulated cells. Protocols employed to estimate the refilling of emptied Ca^{2+} stores and to reveal the effects of ryanodine on Ca^{2+} influx are described in the legends to Figs. 2 and 3, respectively. In the experiments aimed at establishing the voltage dependence of the $[Ca^{2+}]_i$ increases, parallel aliquots of cells were analyzed while suspended in the medium as above or modified by isosmotic substitution of 25, 55, or 125 mM NaCl with KCl.

Mn^{2+} Quenching of Fura-2 Fluorescence—Cells loaded with fura-2 as described above were suspended in an incubation medium from which $CaCl_2$ was omitted, KH_2PO_4 was reduced to 0.6 mM, and $MgCl_2$ was substituted for $MgSO_4$. In the experiments investigating membrane potential dependence of Mn^{2+} fluxes, KCl was substituted for NaCl as described above for $[Ca^{2+}]_i$ measurements. Two types of experiments were carried out. In the first $MnCl_2$ (25 μM, final concentration) was added first and the stimulants 2–3 min later; in the second the order of additions was inverted. Fluorescence was excited at 360 nm, *i.e.* the isosbestic wavelength at which Ca^{2+} does not affect fura-2 fluorescence and at which, therefore, changes were caused by Mn^{2+} quenching (23, 24). Emission was recorded at 500 nm. In the experiments in which the effects of La^{3+} (50 μM) were investigated, phosphate was completely removed from the medium, and gelatin (1 mg/ml) was added to prevent precipitation of the cation. To obtain results in the same range as those observed with 25 μM $MnCl_2$ in the medium without gelatin, the concentration of the cation had to be increased to 100 μM. Maximal Mn^{2+} quenching values were estimated in each preparation at the end of the recordings by permeabilization of the cells with Triton X-100 (0.1%, in the experiments with La^{2+} 30 mM Tris was also added.

Materials—Pertussis toxin was the kind gift of Dr. R. Rappuoli, Sclavo Research Institute, Siena, Italy; NGF was from Dr. A. Leon, Fidia Research Institute, Abano Terme, Italy; and SC 38249 was from Dr. H. Ruegg, Sandoz AG, Basel, Switzerland. Fura-2 and ryanodine were purchased from Calbiochem; thapsigargin from LC Services Corp., Woburn, MA; culture sera and media from GIBCO; caffeine, bradykinin, carbachol, atropine, forskolin, phorbol 12-myristate 13-acetate (PMA), cholera toxin, and the other chemicals from Sigma.

RESULTS

Effects of Muscarinic Stimulation and Thapsigargin—In the present study six recently isolated PC12 clones (38) were employed. Among these, we concentrated on clone 64, characterized by high responsiveness of the muscarinic receptor, for which classical high affinity antagonists, such as atropine, are available. As with other clones we have isolated, clone 64 does not express a functional nicotinic receptor (38). To dissociate the intracellular Ca^{2+} release and Ca^{2+} influx responses, most of our experiments were carried out according to the Ca^{2+}-free/Ca^{2+}-reintroduction protocol (38, 39): excess EGTA (3 mM; estimated $[Ca^{2+}]_o < 10^{-8}$ M) was added to cell suspensions in complete medium 1 min before application of the drug to be tested, and Ca^{2+} was reintroduced into the medium (estimated $[Ca^{2+}]_o \simeq 2$ mM) 2–6 min after the drug, a time when the initial $[Ca^{2+}]_i$ response (*i.e.* intracellular Ca^{2+} release) was over. Fig. 1 illustrates $[Ca^{2+}]_i$ results obtained in cell suspensions of PC12-64 analyzed before and after NGF differentiation (NGF^- and NGF^+ cells, respectively). As can be seen treatment with either the cholinergic agonist carbachol or the Ca^{2+} ATPase blocker thapsigargin (41) induced a $[Ca^{2+}]_i$ rise followed by a decline to or (especially with carbachol) slightly below the resting level (Fig. 1, *A*, *B*, and *D*). These $[Ca^{2+}]_i$ peaks were sharp with carbachol, much more shallow with thapsigargin (compare Fig. 1, *A*, *B*, and *C* with *D*). When optimal concentrations of the two drugs were administered together, the peaks were undistinguishable from those induced by the cholinergic agent alone (not shown). Since these $[Ca^{2+}]_i$ peaks did take place in the cells suspended in the Ca^{2+}-free medium, they can only originate from intracellular release. Reintroduction of Ca^{2+} at the end of the peak induced fluorescence to rise again to levels much higher than those observed in suspensions of unstimulated cells processed in parallel (Fig. 1*E*). The fluorescence effects of EGTA and Ca^{2+} reintroduction in unstimulated cells is caused primarily by a small fraction of the dye free in the incubation medium. They were thus subtracted graphically from the traces of stimulated cells. The results discussed so far document the stimulation of Ca^{2+} influx by both carbachol and thapsigargin (see also Ref. 38, 39).

Two important aspects of the influx process are revealed in figure 1. The first concerns the voltage dependence of the Ca^{2+}-influx responses by carbachol and thapsigargin (in the presence of 20 μM verapamil and 500 nM ω-conotoxin to block voltage-operated Ca^{2+} channels of the L and N type; 42). By themselves these drugs have no effect on the $[Ca^{2+}]_i$ responses induced by either agent (Refs. 38, 42 and results not shown). In both cases a marked decrease was observed when the cells were tested in a medium in which 30 mM NaCl had been substituted isosmotically by KCl, with almost complete inhibition at 60 and disappearance of the response at 130 mM. In contrast, intracellular Ca^{2+} release by both carbachol and thapsigargin was unaffected by these K^+-induced depolarizations (Fig. 1, *traces C* and *D*). The second aspect concerns the effects of atropine. When this high affinity muscarinic antagonist was administered before carbachol, the whole $[Ca^{2+}]_i$ response induced by the carbachol was prevented (not shown;

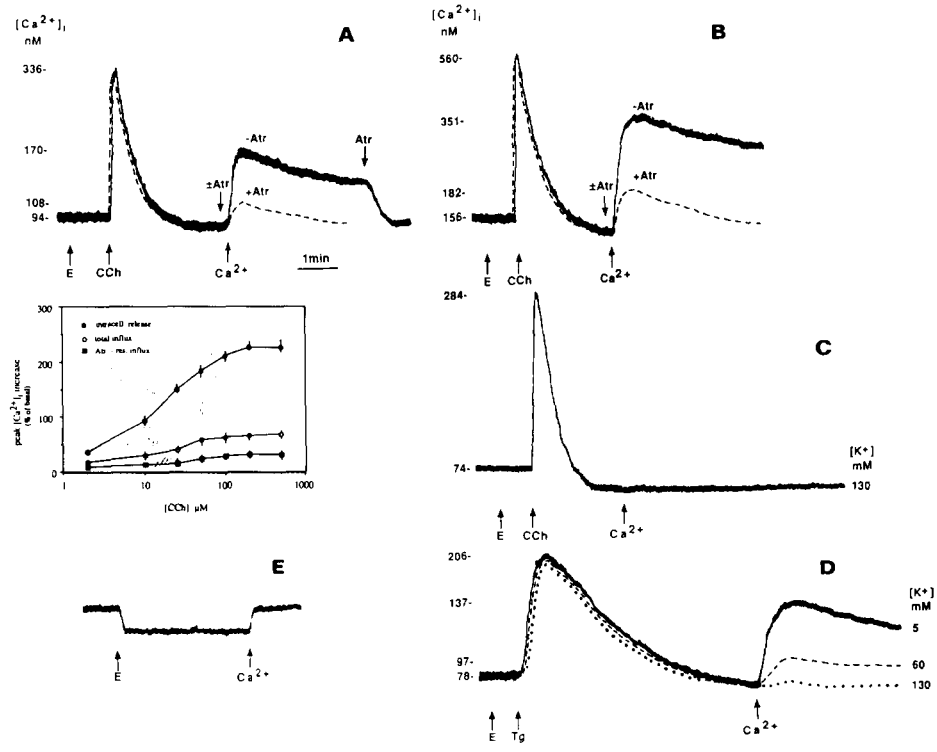

FIG. 1. **Carbachol- and thapsigargin-induced $[Ca^{2+}]_i$ increases in PC12-64 cells, revealed by the Ca^{2+} free-Ca^{2+} reintroduction protocol.** The *traces* shown illustrate the $[Ca^{2+}]_i$ changes induced by the indicated additions to the incubation medium: *E*, excess (3 mM) EGTA; *CCh*, 500 μM carbachol; Ca^{2+}, 3 mM $CaCl_2$; *Atr*, 10 μM atropine; *Tg*, 100 nM thapsigargin. *Dashed* and *dotted lines* refer to experiments carried out in parallel to the adjacent trace, under the conditions specified in the figure. *Traces A* refer to NGF^- and *B* to NGF^+ PC12 cells incubated in the standard medium; *C* and *D* refer to NGF^- PC12 incubated in media of the indicated $[K^+]$. *Trace E* illustrates the changes of fluorescence, observed in an aliquot of resting cells investigated in parallel to the others. *Numbers* to the *left* of the *traces*, here and in Figs. 3, 5, and 7, indicate $[Ca^{2+}]_i$ values. $[K^+]_o$ values are indicated to the *right* in *C* and *D*. The *graph* to the *left* shows the concentration dependence of the various carbachol-induced $[Ca^{2+}]_i$ changes; the initial peak increase in the Ca^{2+}-free medium (intracellular release) and the increases after Ca^{2+} reintroduction without and with atropine treatment (total and atropine-resistant influx). Results of the graph are averages of three experiments. *Bars* show the observed ranges.

see Ref. 39). When atropine was administered to cells 2–3 min after carbachol (*i.e.* at the end of the $[Ca^{2+}]_i$ increase sustained by release and immediately before Ca^{2+} reintroduction into the medium), the rate and extent of $[Ca^{2+}]_i$ increase were reduced considerably compared with the cells treated with carbachol alone (Fig. 1, *A* and *B*). The atropine-sensitive and -insensitive Ca^{2+} influx responses remained clearly appreciable at carbachol concentrations ranging from 10 to 500 μM (see *trace A* and the *graph* in Fig. 1) and when the time intervals between the application of the blocker and reintroduction of Ca^{2+} into the medium were varied from 2 s to 10 min (not shown in the figures). Compared with NGF^-, NGF^+ PC12-64 cells were found to show an even larger $[Ca^{2+}]_i$ increase sustained by the carbachol-induced influx, including the fraction resistant to atropine (compare *trace B* with *trace A*).

Carbachol administered to PC12 cells in the Ca^{2+}-free medium is known to induce a partial depletion of the Ins-P_3-sensitive Ca^{2+} store (38). Thus, the atropine-resistant Ca^{2+} influx could be sustained by SDCI. That this is most probably the case is indicated by the results with thapsigargin. In a variety of cells, including PC12, this drug is known to act not via receptors or polyphosphoinositide hydrolysis but rather as a blocker of the ATPase responsible for Ca^{2+} accumulation into the Ins-P_3-sensitive store (28, 30, 38, 41). This blockade causes the selective, complete, and irreversible depletion of the store by unopposed Ca^{2+} leakage. The increase of Ca^{2+} influx by this drug has therefore been attributed to SDCI (28, 30). Fig. 1*D* shows the effects of thapsigargin in PC12-64 cells analyzed according to the Ca^{2+}-free/Ca^{2+}-reintroduction protocol. As can be seen, influx was markedly stimulated, indicating that SDCI is operative in PC12 cells. Interestingly, the rate of the influx-sustained $[Ca^{2+}]_i$ increase induced by a maximal concentration of thapsigargin (100 nM) was ≃ 2-fold slower than the rate of the corresponding process stimulated by a maximal concentration (500 μM) of carbachol (initial rate of $[Ca^{2+}]_i$ increase: 4.05 ± 0.56 and 9.02 ± 1.60 nM/s, respectively). When the cells were treated with the two drugs together, the rate was the same as with carbachol (8.77 ± 1.04 nM/s).

Taken together, the atropine and thapsigargin results presented so far appear consistent with the possibility that the carbachol-induced Ca^{2+} influx responses are sustained by two components, one directly dependent on receptor activation and the second possibly identified as SDCI. Additional evidence in favor of this dual component model was obtained by experiments in which the refilling of the stores depleted by the muscarinic agonist in the Ca^{2+}-free medium was investigated. The protocol of these experiments (see the *upper panel* in Fig. 2) differed from the carbachol ± atropine, Ca^{2+}-free/Ca^{2+}-reintroduction protocol described above only because a second addition of excess (5 mM) EGTA was made at various times (5 s–10 min) after Ca^{2+} reintroduction, and thapsigargin was added 1 min later, ultimately to discharge the stores and

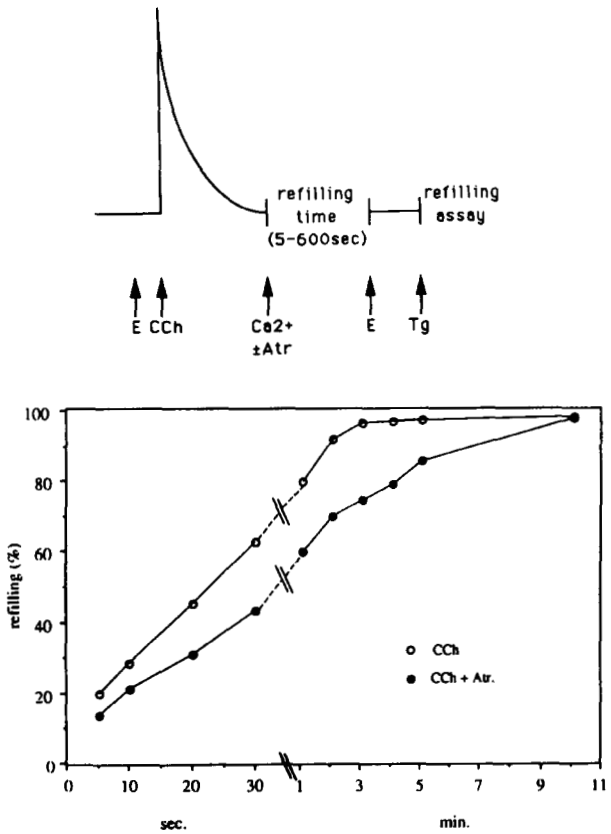

FIG. 2. **Refilling of the Ins-P_3-sensitive store in PC12-64 cells after carbachol treatment in the Ca^{2+}-free medium: effect of muscarinic receptor blockade with atropine.** The *scheme* shown at the *top* illustrates the experimental protocol. Cells suspended in the standard medium are first exposed to excess EGTA and then stimulated with carbachol. Two min later Ca^{2+} is reintroduced into the medium with or without atropine for a time variable from 5 s to 10 min, after which excess EGTA is added 1 min before thapsigargin. The maximum $[Ca^{2+}]_i$ increase triggered by the last agent is compared with that observed in a parallel aliquot of unstimulated cells and expressed as percent of the latter (percent refilling). The *graph* at the *bottom* illustrates the results in batches of cells whose refilling time was as indicated in abscissa. Values given are averages of four experiments. Concentrations of agents are as in Fig. 1.

thus permit the estimation of their refilling. The timing of the thapsigargin addition after EGTA was apparently not important since identical results were obtained when EGTA and thapsigargin were applied together (data not shown). The rationale of these experiments is that, if indeed atropine administration inactivates one of the influx components, less Ca^{2+} is expected to become available for reaccumulation within the Ins-P_3-sensitive store, and thus refilling would be delayed. However, if the difference in the rate of influx-sustained $[Ca^{2+}]_i$ increase observed in the cells treated with carbachol ± atropine was caused not by a difference of Ca^{2+} influx but rather by a more rapid and efficient Ca^{2+} uptake into the Ins-P_3-sensitive store in the presence of the inhibitor, then a faster refilling was to be expected in atropine-treated cells. Results obtained with both NGF^- (*lower panel* in Fig. 2) and NGF^+ (not shown) cells clearly demonstrate that atropine delays refilling of carbachol-treated cells. During the first 30 s after reintroduction of Ca^{2+} into the medium rates were linear and over 60% faster without than with the blocker. Moreover, 100% refilling was achieved after ≃ 3 min in the first and after >5 min in the second condition (Fig. 2). In NGF^+ cells refilling slopes were slightly but consistently steeper than in NGF^- cells (not shown).

Characterization of SDCI—If indeed SDCI is operative in PC12 cells, its stimulation should be dependent on the degree of filling of the stores, independently of the nature and mechanism of action of the agents employed to stimulate Ca^{2+} release. In recent studies of PC12 cells (38) we have reported individual clones to be sensitive not only to receptor agonists and thapsigargin but also to ryanodine (a plant alkaloid that induces the irreversible activation of an intracellular Ca^{2+} channel, the ryanodine receptor, a molecule different from the Ins-P_3 receptor) and to caffeine (another activator of the ryanodine receptor). In these clones, activation of either the Ins-P_3 or ryanodine receptor, or the inhibition of the corresponding Ca^{2+} ATPase by thapsigargin, induces discharge of Ca^{2+} from the same rapidly exchanging intracellular store, suggesting a colocalization of the two receptors and of the pump within the cell (38). Both ryanodine and caffeine should thus be expected to stimulate SDCI. Because of the ryanodine-caffeine insensitivity of the PC12-64 clone, this series of experiments was carried out with the clone 15 (Ref. 38 and see below). As can be seen in Fig. 3, treatment with thapsigargin, caffeine, or ryanodine resulted in a marked stimulation of Ca^{2+} influx, revealed by Ca^{2+} reintroduction (*traces A–C*). Interestingly, the degree of influx stimulation by thapsigargin and ryanodine was concentration dependent and appeared to be inversely correlated to the degree of filling of the Ins-P_3-sensitive stores. Indirect estimation of the latter parameter was obtained from the intracellular release responses triggered by bradykinin (the most effective agonist in the PC12-15 clone, see Table I) administered to parallel aliquots of cells pretreated with either thapsigargin or ryanodine (*panels D* and *E*).

Mn^{2+} Quenching—Mn^{2+} quenching of the fura-2 signal has already been employed in a variety of cell types to reveal specific aspects of the receptor-induced cation influx response (23–26, 29, 31, 32). In these experiments Mn^{2+} is assumed to be transported via the same plasma membrane channels that are permeable to Ca^{2+}. Compared with these other cell types (23–26, 29, 31, 32), PC12 cells (NGF^-) were found in preliminary experiments to be characterized by a much faster quench under resting conditions, apparently because of a more rapid transport of Mn^{2+} across the plasmalemma. To minimize this resting quench, low concentrations of Mn^{2+} (25–100 μM) were employed, administered to the cells either before or at precise times after the stimulatory treatments. Similar results were obtained by these two types of experiments. Fig. 4 illustrates those obtained with the second protocol. As can be seen, exposure of the cells to maximal concentrations of either thapsigargin or carbachol 3 min before Mn^{2+} addition induced marked increases of the quenching rates (≃3- and 2-fold; compare *traces B* and *C* with *A*). Interestingly, the carbachol-induced rate was unaffected by atropine administered at various times (2–120 s) before Mn^{2+} (Fig. 4, *trace C, dashed line*) whereas administration of the blocker either before or together with the agonist prevented any changes of the Mn^{2+} quenching rate (not shown). These results suggest that the effects of carbachol on Mn^{2+} quenching are entirely caused by SDCI and that the Ca^{2+} influx pathway directly dependent on the receptor activation is impermeable to Mn^{2+}.

Stimulation of quenching by both thapsigargin and carbachol was completely prevented by the addition of La^{3+} (50 μM) to the incubation medium (*traces B* and *C*). In contrast, quenching of unstimulated cells was unaffected (*trace A*). Likewise, depolarization by substitution of the Na^+ with K^+ in the incubation medium (in the presence of 20 μM verapamil + 500 nM ω-conotoxin, to prevent a possible contribution of

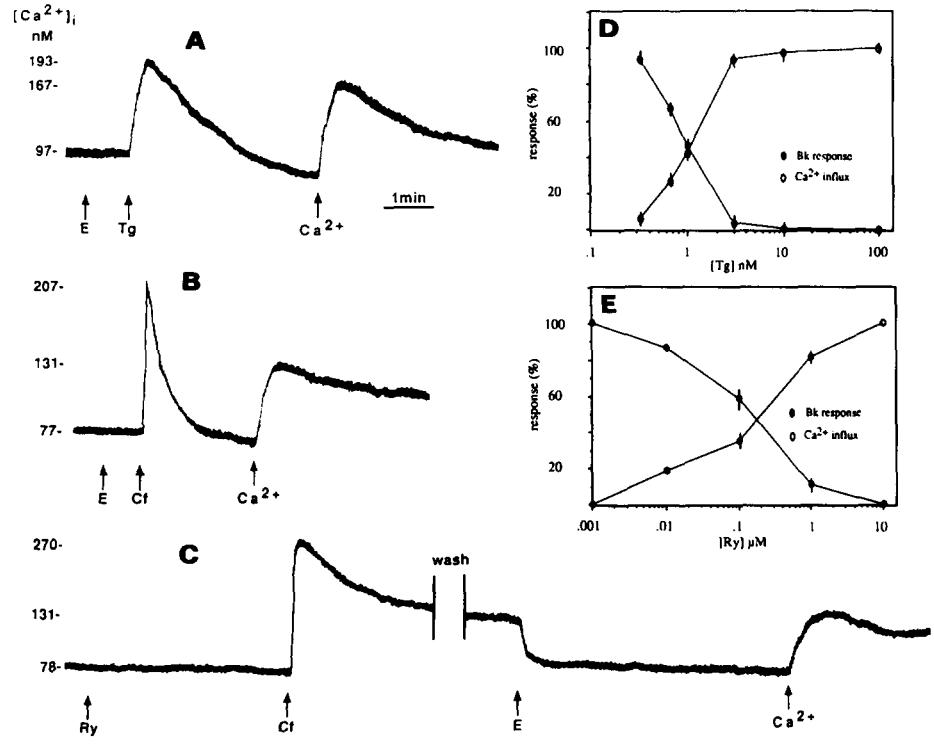

FIG. 3. **SDCI in PC12-15 cells stimulated with thapsigargin, caffeine, and ryanodine.** Thapsigargin (*Tg*) (100 nM) and caffeine (9 mM) experiments were carried out according to the Ca^{2+}-free/Ca^{2+}-reintroduction protocol as in Fig. 1. The cells treated with ryanodine (*Ry*) (10 µM) for 3 min in the standard medium were first exposed to 9 mM caffeine (to trigger the use-dependent activation of the ryanodine channel, 37) and then washed to remove unbound drugs. The *interruption* in the *trace* corresponds to 6 min. After resuspension, the cells were exposed to excess EGTA (3 mM). Finally Ca^{2+} was reintroduced, and the ensuing $[Ca^{2+}]_i$ increase was measured. The *graphs* in *D* and *E* correlate the size of the influx stimulations observed after the administration of increasing concentrations of either thapsigargin or ryanodine with the size of the $[Ca^{2+}]_i$ release responses triggered by the administration of 100 nM bradykinin to aliquots of cells processed in parallel up to the stage preceding Ca^{2+} reintroduction into the medium. Values shown are averages of three experiments. *Bars* indicate ranges.

TABLE I

Intracellular Ca^{2+} release and Ca^{2+} influx in PC12 clones treated with either carbachol (CCh, 500 µM), bradykinin (Bk, 100 nM), caffeine (Cf, 9 mM), or thapsigargin (Tg, 100 nM) according to the Ca^{2+}-free/Ca^{2+} reintroduction protocol (see Fig. 1)

Values shown are averages of 5–10 experiments ± S.D. They represent maximal $[Ca^{2+}]_i$ increases above resting values, measured after administration of the agents in Ca^{2+}-free medium (release) and reintroduction of Ca^{2+} into the medium (influx).

Clone N° :		7	14	15	16a	27	64
CCh:	*release*	20.00±1.00	69.50±2.12	70.00±1.41	76.40±2.30	63.75±9.84	378.2±31.9
	influx	17.33±1.53	38.00±2.83	38.00±1.41	40.40±8.88	41.75±6.55	137.2±24.4
	ratio						2.76±0.72
Bk:	*release*	20.67±2.31	625.5±27.6	418.2±36.7	429.5±27.2	60.67±13.3	372.0±61.9
	influx	16.50±1.32	132.5±0.71	177.0±10.8	163.7±24.9	36.00±2.65	53.00±6.08
	ratio		4.72±0.18	2.41±0.16	2.66±0.36		7.20±2.49
Cf:	*release*	205.7±1.53	229.0±10.1	266.5±2.12	250.3±34.5	.....	.....
	influx	81.67±14.4	94.33±3.51	110.0±2.83	154.6±25.3	.....	.....
	ratio	2.57±0.49	2.43±0.45	2.42±0.04	1.64±0.28	.....	.....
Tg:	*release*	88.33±0.58	116.7±7.27	105.6±2.52	125.7±30.7	101.6±17.6	122.2±31.3
	influx	61.33±4.16	159.0±1.41	118.6±2.52	198.1±40.4	80.60±3.06	160.7±10.6
	ratio	1.44±0.08	0.73±0.17	0.89±0.04	0.65±0.17	1.26±0.30	0.75±0.17

N and L type voltage-gated Ca^{2+} channels) induced a parallel inhibition, up to the complete blockade, of the responses by thapsigargin and carbachol, with hardly any effect on the resting quench (*graph* in Fig. 4). This latter result suggests that in resting cells Mn^{2+} influx occurs not via a channel but by via an electroneutral transport reaction which seems to require no other extracellular ions since it remained unchanged in a lightly buffered sucrose medium (0.3 M sucrose, 5 mM HEPES/Tris, pH 7.4) containing no Na^+, Cl^-, and PO_4^{2-} (results not shown).

Pharmacology of Ca^{2+} Influx—In previous studies carried out with the parent PC12 cell line (43) we reported the entire $[Ca^{2+}]_i$ response to carbachol (intracellular release and influx) to be markedly inhibited by pretreatment with the activator of protein kinase C, PMA. When the effect of PMA (100 nM) was investigated in the PC12-64 (NGF^-) cells according to the Ca^{2+}-free/Ca^{2+}-reintroduction protocol, an inhibition was found, which affected the release more than the influx process (Fig. 5*A*). The inhibition of influx became unappreciable when PMA was administered before Ca^{2+} reintroduction into the medium, *i.e.* after the carbachol-induced release was completed (Fig. 5*B*).

In additional experiments, carbachol was administered to cells suspended in the Ca^{2+}-containing medium. In this case the $[Ca^{2+}]_i$ increases sustained by release and influx are not separated, but an initial peak (primarily release) evolves into

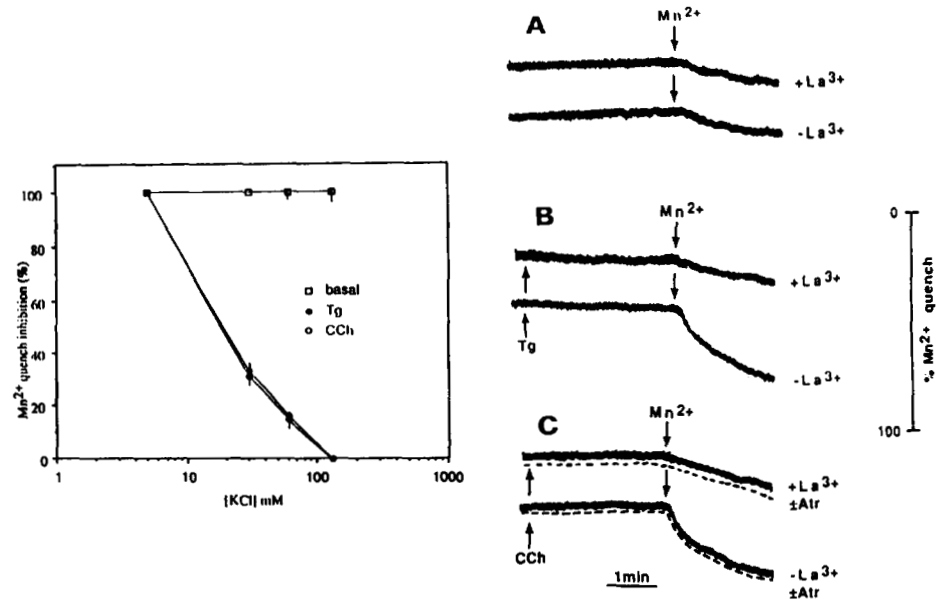

FIG. 4. **Mn^{2+} quench of the fura-2 fluorescence in PC12-64 cells.** The effect of Mn^{2+} addition to fura-2-loaded cells incubated without stimulation (*A*) or with thapsigargin (*Tg*) (100 nM) and carbachol (*CCh*) (500 μM), with and without La^{3+} (50 μM) into the medium is illustrated. The incubation medium contained in all cases 1 mg/ml gelatin, and the [Mn^{2+}] was 100 μM (see "Experimental Procedures"). The *dashed lines* shown in *C* illustrate the lack of effect of atropine (*Atr*) (10 μM) added 30 s before Mn^{2+} in cells pretreated with carbachol. The *vertical bar* to the *right* specifies the extent of percent Mn^{2+} quench in the experimental conditions employed. The *graph* to the *left* shows the effects of KCl depolarization on the basal and stimulated Mn^{2+} quenches. Results shown are averages of three experiments. *Bars* represent ranges.

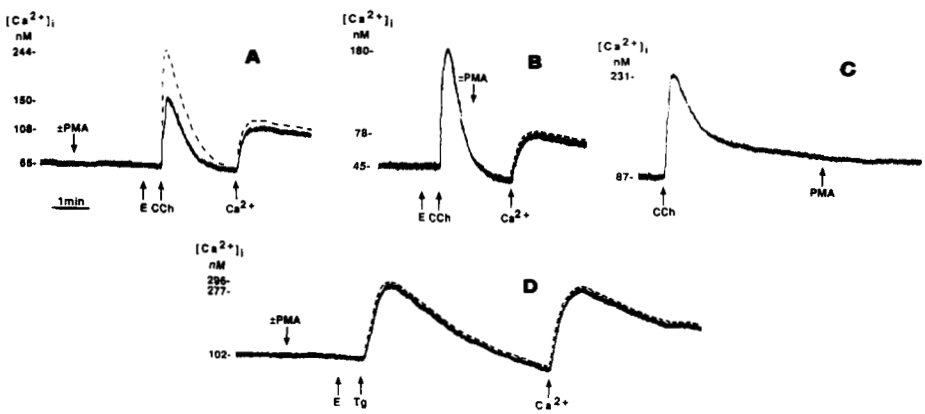

FIG. 5. **Effects of PMA on stimulated $[Ca^{2+}]_i$ responses in PC12$^-$-64 cells.** *Traces A–C* show the effects of PMA (100 nM) administered either before (*A*) or after (*B*, *C*) carbachol (*CCh*) (500 μM) to cells processed either according to the Ca^{2+}-free/Ca^{2+}-reintroduction protocol (*A*, *B*) or by incubation in the standard medium (*C*). *D* illustrates the lack of effect of PMA on the $[Ca^{2+}]_i$ effects induced by thapsigargin (*Tg*). The *dotted lines* in *A*, *B*, and *D* show results in cell aliquots exposed only to the PMA solvent (dimethyl sulfoxide, 1.5 μl), investigated in parallel.

a slowly declining plateau (influx) (Fig. 5*C*; Refs. 8 and 39). Also in these conditions PMA was inhibitory when administered before carbachol (not shown) but showed no effect when given during the plateau phase (Fig. 5*C*). These results suggest that in contrast to intracellular Ca^{2+} release both the receptor-dependent and the SDCI components of the stimulated influx are insensitive to inhibition by phorbol esters. This conclusion regarding SDCI is reinforced by the lack of effect of PMA on the $[Ca^{2+}]_i$ response induced by thapsigargin (Fig. 5*D*). Similarly, forskolin, the direct activator of adenylylcyclase, administered either before the stimulants or at the end of the release response (*i.e.* before Ca^{2+} reintroduction), had no effect on Ca^{2+} influx (data not shown).

Further studies were carried out using SC 38249, a member of an imidazole drug family known for the blockade of various Ca^{2+} channels, including those activated by receptors (11, 13, 32, 44, 45). When this drug was administered during the plateau $[Ca^{2+}]_i$ phase of cells stimulated in the Ca^{2+}-containing medium, a rapid dissipation of the influx-sustained $[Ca^{2+}]_i$ increase was observed. Fig. 6*A* shows that this effect occurred at lower blocker concentrations with carbachol than with thapsigargin (apparent $IC_{50} \sim 3$ and 13 μM, respectively). This result suggests that the SDCI process is less sensitive to the imidazole drug than the influx directly sustained by receptor activation. Additional evidence in favor of this conclusion was obtained by experiments in which SC 38249 was administered in the Ca^{2+}-free medium after the stimulants, shortly before the reintroduction of Ca^{2+}. In these conditions, the concentrations of the drug necessary for blockade were greater than in the complete medium, and the receptor-controlled component was more severely affected than the SDCI component of the carbachol or thapsigargin Ca^{2+} influx responses. (Fig. 6*A*, *inset*). Finally, SC 38249 was employed to investigate Mn^{2+} influx in cells at rest and stimulated by thapsigargin. SC 38249 inhibited the thapsigargin quenching response at concentrations lower than those active on Ca^{2+} influx ($IC_{50} \sim$ 0.6 μM; compare Fig. 6*B* with 6*A*). Also the resting quench was inhibited at concentrations $\simeq$ 1 order of magnitude higher (Fig. 6*B*).

Bacterial toxins (pertussis toxin and cholera toxin), which ADP-ribosylate the G protein subunits of the G_i-G_o and G_s families, respectively, were employed next. Pretreatment of PC12-64 (NGF^-) cells with pertussis toxin (12 h; 300 ng/ml) failed to modify the responses induced by thapsigargin and bradykinin (data not shown) but induced complex effects on the carbachol response. These effects (which varied quantitatively depending on the carbachol concentration used and were best visible at 10 μM, Fig. 7, *A* and *B*) consisted in a large decrease (up to 70%) of the intracellular Ca^{2+} release phase, with no parallel decrease of the Ca^{2+} influx. In these pertussis toxin-pretreated cells carbachol-stimulated influx remained largely inhibitable by atropine (not shown). In contrast, cholera toxin administered for 12 h at 1 ng/ml induced no change of the carbachol-induced $[Ca^{2+}]_i$ response of PC12-64 cells (not shown).

Studies in Other PC12 Clones—The PC12 cell line is known

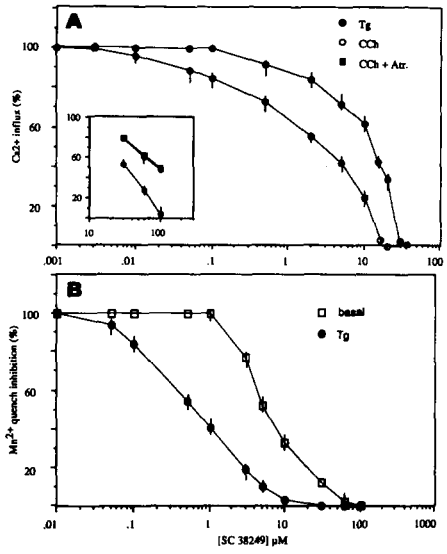

FIG. 6. **Effects of the imidazole drug, SC 38249, on the $[Ca^{2+}]_i$ increase induced by Ca^{2+} reintroduction into the medium (Ca^{2+} influx) and the Mn^{2+} quench in PC12-64 cells treated with carbachol (*CCh*) (+atropine (*Atr*)) and thapsigargin (*Tg*).** $[Ca^{2+}]_i$ experiments (*A*) in the *large panel A* were carried out in the Ca^{2+}-containing incubation medium, those in the *inset* by the Ca^{2+}-free/Ca^{2+}-reintroduction protocol, at the same drug concentrations as in Fig. 1, and Mn^{2+} quench experiments by the protocol of Fig. 4 in the gelatin-free medium, measuring the percent changes of the initial fluorescence decrease rates in the various samples. $[Mn^{2+}] = 25$ μM. Values shown are the averages of three experiments. *Bars* indicate ranges.

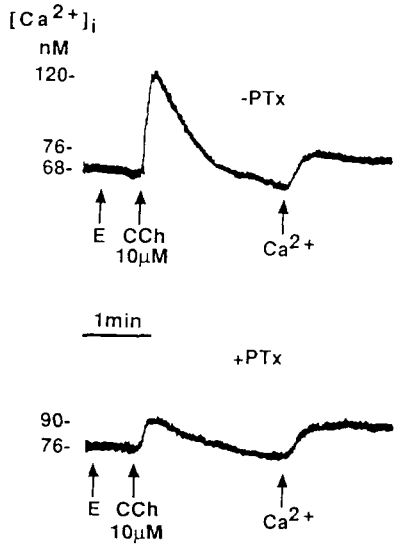

FIG. 7. **Effect of pertussis toxin (*PTx*) pretreatment on the $[Ca^{2+}]_i$ responses induced by a low concentration of carbachol (CCh) in PC12-64 cells.** Cells were preincubated for 12 h with and without pertussis toxin (300 ng/ml), and then $[Ca^{2+}]_i$ responses were measured according to the Ca^{2+}-free/Ca^{2+}-reintroduction protocol.

to be extremely heterogeneous, with marked differences among the various preparations employed in different laboratories and among recently isolated clones (38, 46–49). A cogent example of this heterogeneity is given in Table I, in which quantitative data on peak Ca^{2+} release and influx responses as revealed by Ca^{2+} free-Ca^{2+} reintroduction experiments in the two already discussed PC12 clones, PC12-64 and 15, are compared with those of four additional clones. Notice that clone 64 is the only one highly responsive to carbachol whereas four clones responded well to bradykinin and also four to caffeine (and ryanodine; see Ref. 38). In contrast, all clones responded to thapsigargin. Comparison of the release and influx values in individual clones also revealed interesting results, documented by the corresponding ratio values. The latter values are reported in the table only for those clones exhibiting clear responses and statistically consistent results. As can be seen, ratios were lower with thapsigargin (0.65–1.44) than with the other agents, most probably because with the ATPase blocker Ca^{2+} release is more sluggish, and therefore peak $[Ca^{2+}]_i$ increases are blunted. Interestingly, clone PC12-64 showed a ratio value of 2.7 when treated with carbachol. In contrast, with bradykinin the influx was much smaller, and therefore the release/influx ratio value was much higher (7.2). This result suggests that at least one of the influx processes activated by carbachol (presumably the component directly dependent on receptor activation) is insensitive to bradykinin in this PC12 clone. An apparently analogous, yet less extreme situation occurred in another clone exposed to bradykinin (PC12-14; ratio value 4.72). However, since this clone is poorly sensitive to carbachol, a direct comparison of the two agents could not be made. Table I therefore indicates that heterogeneity of PC12 cells does not affect equally the various mechanisms involved in transmembrane signaling. SDCI (as revealed by the influx response to thapsigargin) was found in all the clones. In contrast the clones appear heterogeneous in the expression not only of surface and ryanodine receptors, as demonstrated previously (38, 46–48), but also with respect to the channels responsible for the influx directly coupled to receptor activation.

DISCUSSION

Recent evidence has clearly documented that stimulation of Ca^{2+} influx is involved in the regulation of important intracellular events triggered by the activation of receptors coupled to polyphosphoinositide hydrolysis. Thus, secretion of catecholamines from bovine chromaffin cells stimulated with histamine and angiotensin II is apparently sustained by influx but not by intracellular Ca^{2+} release (50, 51). Likewise, the growth response observed in fibroblasts exposed to either epidermal growth factor or bombesin requires influx to be fully operative (52, 53). In spite of its important physiological role, influx remains incompletely known in its underlying mechanisms. Intense studies during the last couple of years have shown SDCI to be the predominant, if not the unique, mechanism of Ca^{2+} influx in endothelia, exocrine cells of the pancreas, lacrimal and salivary glands (25, 27–31). In contrast, in bovine chromaffin cells and the neuronal cells, NG 115-401, SDCI was not observed, and influx appeared to be sustained by one (or more) mechanism(s) dependent on receptor activation (36, 37). Finally, in platelets both SDCI and the receptor-dependent mechanism(s) were revealed (23, 24).

To investigate this problem, we have focused on PC12, a cell line often employed for transmembrane signaling studies. PC12 cells are a useful model because they can be investigated both before and after treatment with NGF, *i.e.* when they express either a neurosecretory or a neuronal phenotype. Moreover, by the use of recently isolated PC12 clones available in the laboratory we could escape one of the major drawbacks of the parent line, namely its marked heterogeneity (38, 46–48). In this respect it is worth emphasizing that although each clone appears homogeneous (48) the various clones are markedly different from each other (38, this work). It is therefore not surprising that part of the results obtained now with individual clones are at variance with those with other clones or the parent cell line. Rather than experimental inconsistencies, these variable results should be considered as

intellectual hints, potentially useful for the understanding of the mechanisms underlying the process of Ca^{2+} influx. The results that we have obtained demonstrate the coexpression in the clone 64 (before and after NGF differentiation) and in another five PC12 clones of at least two mechanisms of Ca^{2+} influx, one (SDCI) independent, the other directly dependent on receptor activity. From this point of view PC12 cells resemble platelets rather than the other cells of similar phenotype investigated previously, *i.e.* bovine chromaffin and NG 115-401 cells (36, 37). Thus, the lack of expression of SDCI in these latter cells should not be interpreted as a property of neurosecretory and neuronal cells in general, as hypothesized previously (12).

Properties of SDCI—Evidence documenting the existence of this process in PC12 cells was obtained by various treatments: with carbachol followed by atropine, administered in Ca^{2+}-free medium; with the Ca^{2+} ATPase blocker, thapsigargin; and (only in the clones sensitive to these drugs) with the activators of the ryanodine receptor, ryanodine itself, and caffeine. Although different in their mechanism of action, all these treatments share the ability to deplete the Ins-P_3-sensitive Ca^{2+} store of PC12 cells. With each individual drug employed the degree of stimulation varied depending on the concentration. Actually, with the irreversibly acting drugs, thapsigargin and ryanodine, a good correlation could be established between SDCI stimulation and Ins-P_3-sensitive store depletion. The degree of stimulation observed with maximal concentrations of the various drugs was not the same (thapsigargin > ryanodine > carbachol). This can be explained because, on the one hand, store depletion by receptor agonists is not complete and affects most, but not all cells, even in the clones (48) whereas those by thapsigargin and ryanodine are complete and general; on the other hand, the protocol of ryanodine treatment was more complex and long lasting than that of thapsigargin, thus some degree of SDCI desensitization (visible also during prolonged incubations with thapsigargin; not shown) occurred with the first and not with the second drug.

Of the various properties of SDCI now revealed in PC12 cells, a few have already been described, however, only in nonneuronal cells: voltage dependence (26, 32, 54, 55); permeability to Mn^{2+} (in lymphocytes and acinar cells of the pancreas (31, 32) but not observed in cells of the lacrimal gland; Ref. 29); and blockade by La^{2+} (29, 30, 32). In contrast the insensitivity of SDCI to phorbol esters, forskolin, and cholera toxin has not been reported in any cell type. Particularly interesting results were obtained with the imidazole drug SC 38249. Considerable differences in apparent potency were observed when SC 38249 was applied in Ca^{2+}-containing *versus* Ca^{2+}-free medium and when the drug was employed to block Mn^{2+} quenching. Moreover, in parallel experimental conditions, SDCI required almost 5-fold higher concentrations of the drug for blockade compared with the influx directly dependent on receptor activation. To our knowledge, a detailed analysis of the effects of imidazole blockers on the various components of Ca^{2+} influx had never been reported. The different concentration dependence with respect to Ca^{2+} and Mn^{2+} influx suggests the mechanism of action of these drugs to be complex.

Because of its permeability to Mn^{2+}, SDCI could be compared with, and found to be completely different from, the unstimulated uptake of the latter cation, a process occurring in all other cell types so far investigated (23–26, 29, 31, 32), which is particularly active in PC12 cells. Unstimulated Mn^{2+} uptake was in fact insensitive to depolarization and La^{3+}, unaffected by removal of Cl^- and Na^+ from the medium, and blocked by SC 38249 only at concentrations ≃ 10-fold higher than those active on SDCI. These properties seem to exclude a channel and suggest the involvement of a nonelectrogenic transporter (see also 26, 32) requiring influx of neither Na^+ nor Cl^-.

Properties of Receptor-dependent Influx—Except for its voltage dependence, insensitivity to phorbol esters, and cAMP increase, which were similar, the other properties of this influx component triggered by carbachol were different from those of SDCI: impermeability to Mn^{2+} (as shown previously in platelets, Ref. 23) and higher sensitivity to SC 38249 blockade. Of particular interest was the lack of major direct inhibitory effect of phorbol esters on the muscarinic receptor-dependent influx. In fact, in our previous studies with bradykinin in PC12 cells (parent line) these drugs were found to induce considerable influx inhibition (8). In the PC12-64 clone we have now observed that, when carbachol and bradykinin were used at concentrations yielding identical Ca^{2+} release responses, the influx responses were much (almost 3-fold) greater with the first than with the second agent. Taken together, the results of these experiments and those with phorbol esters strongly suggest that the influx mechanisms activated by the muscarinic and bradykinin (B_2; Ref. 8) receptors in PC12 cells are not the same. In particular, bradykinin could work through channel(s) inhibited by phorbol esters which are not expressed in the PC12-64 clone, carbachol (at least in PC12-64 cells) through channel(s)/G protein(s) insensitive to those agents. The hypothesis about G proteins is reinforced by our present results with pertussis toxin in PC12-64 cells. Compared with controls, both phases of the $[Ca^{2+}]_i$ response to bradykinin were in fact unchanged in the toxin-pretreated cells whereas with carbachol the intracellular release was clearly, although partially, inhibited, while influx was maintained. A pertussis toxin sensitivity of the muscarinic Ca^{2+} response, not observed in our previous studies on the parent line (56), had been reported sometime ago by Inoue and Kenimer (57). The release-influx dissociation that we have now observed strongly suggests that these two events are not strictly coupled but could be triggered independently by the receptor via the interaction with different G proteins, some sensitive, the other insensitive to the toxin. This conclusion is in line with a previous hypothesis (8) and with scattered experimental evidence in other systems (17–22).

The coexistence in many PC12 cell clones of at least two distinct mechanisms of influx stimulation opens the problem of their physiological importance, which is probably quite different. In fact, the experimental conditions we have employed to stimulate SDCI markedly (stimulation of cells incubated in Ca^{2+}-free media; blockade of Ca^{2+} pumps and channels) are somewhat extreme. In normal cell life SDCI might thus rarely be fully activated. Its role could be more that of a safeguard process, destined to help prevent complete depletion of the rapidly exchanging Ca^{2+} stores, rather than that of a process with distinct physiological functions. The important functions (*e.g.* secretion, cell growth) recently attributed to Ca^{2+} influx (50–53) might in contrast be effected by the receptor-dependent component. The level of expression of the channels responsible for the two processes appears to be low, and this has so far hampered electrophysiological (8, 9, 21), pharmacological (11, 13, 44, 45), and precluded molecular studies. The precise characterization of the two processes in various cell types promises that these developments will be possible in the near future, as is occurring with other types of channels operated by voltage or by direct ligand binding (1–3).

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