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Muscarinic Acetylcholine Receptors Stimulate Ca^{2+} Influx in PC12D Cells Predominantly *via* Activation of Ca^{2+} Store–Operated Channels

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Activation of muscarinic acetylcholine receptors (mAChRs) causes the rapid release of Ca^{2+} from intracellular stores and a sustained influx of external Ca^{2+} in PC12D cells, a subline of the widely studied cell line PC12. Release of Ca^{2+} from intracellular stores and a sustained influx of Ca^{2+} are also observed following exposure to thapsigargin, a sesquiterpene lactone that depletes intracellular Ca^{2+} pools by irreversibly inhibiting the Ca^{2+} pump of the endoplasmic reticulum. In this study, we show that carbachol and thapsigargin empty the same intracellular Ca^{2+} stores, and that these stores are a subset of intracellular stores depleted by the Ca^{2+} ionophore ionomycin. Intracellular Ca^{2+} stores remain depleted during continuous stimulation of mAChR with carbachol in medium containing 2 mM extracellular Ca^{2+}, but rapidly refill following inhibition of mAChRs with atropine. Addition of atropine to carbachol-stimulated cells causes intracellular Ca^{2+} levels to return to baseline levels in two steps: a rapid decrease that correlates with the reuptake of Ca^{2+} into internal stores and a delayed decrease that correlates with the inhibition of a Mn^{2+}-permeable Ca^{2+} channel. Several lines of evidence suggest that carbachol and thapsigargin stimulate Ca^{2+} influx by a common mechanism: (i) pretreatment with thapsigargin occludes atropine-mediated inhibition of Ca^{2+} influx, (ii) carbachol and thapsigargin applied individually or together are equally efficient at stimulating the influx of Mn^{2+}, and (iii) identical rates of Ca^{2+} influx are observed when Ca^{2+} is added to cells pretreated with carbachol, thapsigargin, or both agents in the absence of extracellular Ca^{2+}. Taken together, these data suggest that the sustained influx of extracellular Ca^{2+} observed following activation of mAChRs in PC12D cells is mediated primarily by activation of a Mn^{2+}-permeable, Ca^{2+} store–operated Ca^{2+} channel.

Key words: calcium, carbachol, muscarinic acetylcholine receptor, PC12D, thapsigargin.

Abbreviations: DMSO, dimethylsulfoxide; fura-2, 1-[2-(carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxly]-2-(2′-amino-5′-methoxlphenoxy)-ethane-*N,N,N,N*-tetraacetic acid; KRH, Krebs Ringer-HEPES; mAChR, muscarinic acetylcholine receptor; SOCC, store-operated Ca^{2+} channel.

M1 muscarinic acetylcholine receptors (mAChRs) are expressed at high levels in neurons in the cortex and hippocampus, where they are proposed to play essential roles in attention, cognition and memory (*1*–*3*). Consistent with these functions, drugs that activate M1 mAChRs by increasing levels of acetylcholine in the brain improve cognition and memory in Alzheimer's disease (*4*). Although much has been learned about M1 mAChR–mediated cell signaling, many important aspects are not understood. In particular, the molecular mechanisms by which M1 mAChRs regulate the influx of extracellular Ca^{2+} remain to be elucidated.

The goal of the experiments described in this and the accompanying paper (Zhang, Guo, Kim and Saffen, 2006) is to elucidate pathways of M1 mAChR–regulated Ca^{2+} influx in neuronal PC12D cells. PC12D cells are a spontaneously arising derivative of the widely studied PC12 cell line. Unlike the parental PC12 cells, PC12D cells express M1 mAChRs (*5*). We have previously used these cells to analyze M1 mAChR signaling pathways, including the

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stimulation of Ca^{2+} influx, activation of protein kinases and induction of immediate-early genes *c-fos* and *zif268* (*5–8*). These studies revealed that influx of extracellular Ca^{2+} plays an important role in the induction of immediate-early genes. The present study was designed to elucidate the pathways for this Ca^{2+} influx.

In many cell lines and tissues, mAChR-mediated increases in intracellular Ca^{2+} can be separated into two components: (i) a sharp initial rise that is primarily the result of the release of Ca^{2+} from internal stores and (ii) a prolonged plateau phase that results from an increase in Ca^{2+} influx from the extracellular medium (*5*, *9–12*). It is now well documented that the emptying of intracellular Ca^{2+} stores often stimulates the influx of extracellular Ca^{2+}, which functions to refill the stores (*13–16*). The molecular identities and mechanisms of activation of the Ca^{2+} channels responsible for this "capacitative" or "store-operated" Ca^{2+} influx is currently the subject of debate (*17*, *18*). The fact that Ca^{2+} influx can be induced independently of membrane receptors, for example by thapsigargin, which empties intracellular Ca^{2+} store by irreversibly inhibiting the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pump (*19*, *20*), suggests that Ca^{2+} channels mediating this influx can be activated in the absence of the generation of intracellular second messengers.

Early studies using PC12-64 cells (*21*) suggested that mAChR-stimulated Ca^{2+} influx comprised at least two components: (i) influx via channels that open in response to the depletion of intracellular calcium stores, later designated store-operated Ca^{2+} channels (SOCCs), and (ii) influx via channels that are activated independently of the internal Ca^{2+} stores. Studies using A9 fibroblasts exogenously expressing M3 mAChR also showed that mAChR-stimulated Ca^{2+} channel activation can occur independently of the release of Ca^{2+} from intracellular stores (*10*).

As described above, the aim of the experiments described in this paper was to determine the properties of the

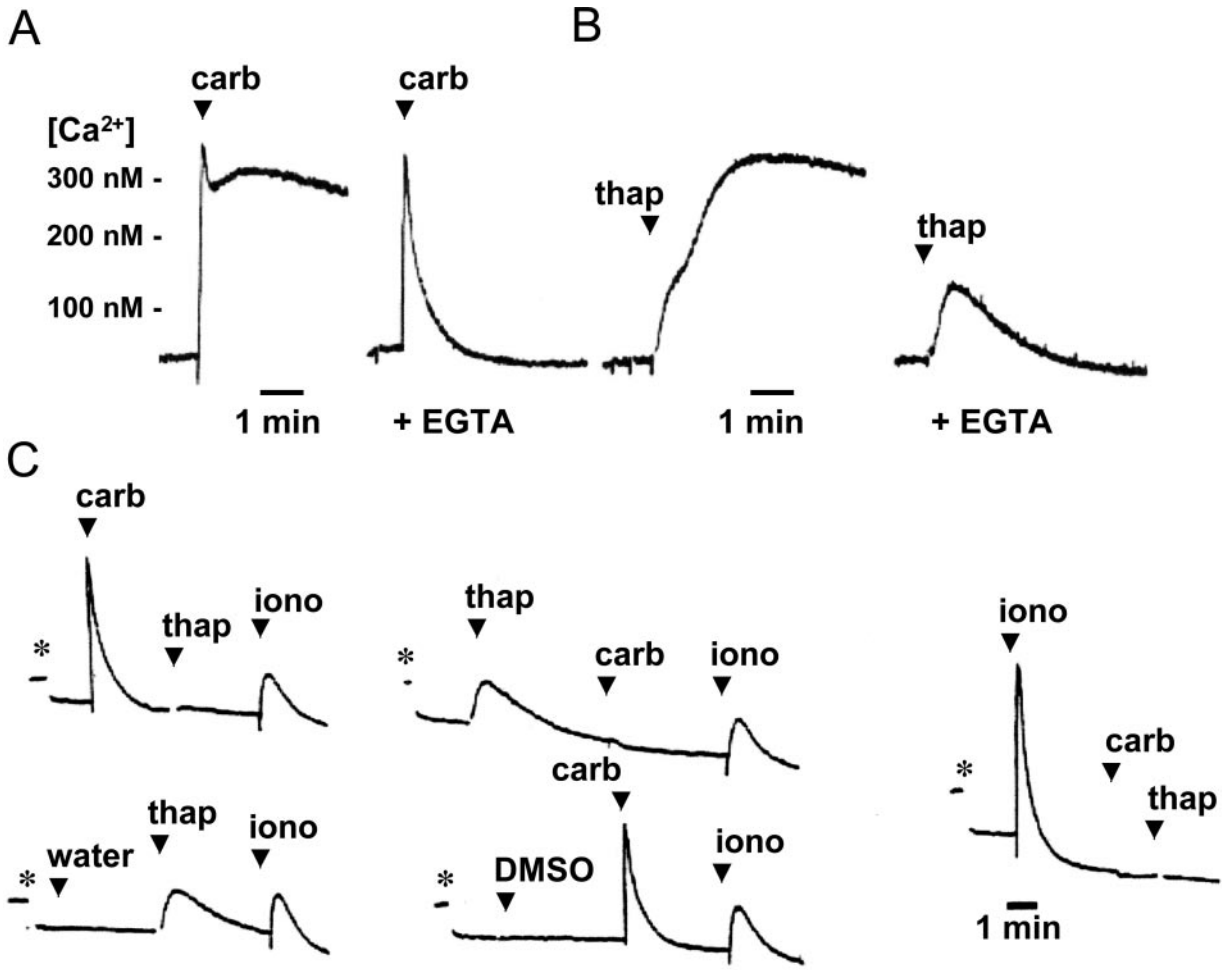

Fig. 1. **Carbachol and thapsigargin stimulate the influx of extracellular Ca^{2+} and induce the release of Ca^{2+} from common intracellular stores.** A–C: Changes in intracellular Ca^{2+} were measured in stirred suspensions of fura-2 loaded PC12D cells in KRH buffer using a Nippon Bunko/Jasco FP-770 spectrofluorometer. Cells were pretreated with 50 μM tubocurarine to block nicotinic acetylcholine receptors. A: Cells in KRH (left) or KRH containing 3 mM EGTA (right) were stimulated with 500 μM carbachol at the times indicated by the arrowheads. B: Cells in KRH (left) or KRH containing 3 mM EGTA (right) were exposed to 100 nM thapsigargin at the indicated times. C: PC12D cells in KRH containing 3 mM EGTA were exposed to 500 μM carbachol, 100 nM thapsigargin, and 1 μM ionomycin at the indicated times. The initial drop in fluorescence associated with the addition of EGTA (asterisks) is due to the chelation of Ca^{2+} bound to extracellular fura-2 that had leaked from the cells. The results shown are representative of 3 independent experiments.

mAChR-stimulated Ca^{2+} influx in PC12D cells (*22*). Specifically, we wanted to determine if mAChR-stimulated Ca^{2+} influx is mediated by a store-operated pathway, and if so, whether this pathway accounts for all, or only a portion, of the Ca^{2+} influx.

MATERIALS AND METHODS

Reagents—Carbachol (carbamylcholine), atropine, D-tubocurarine, nifedipine, verapamil, were obtained from Wako Chemicals Industries, Ltd. Thapsigargin, ionomycin were obtained from Sigma, and ω-conotoxin was from Peptide Institute, Inc. (Osaka).

Cell Culture—PC12D cells were a generous gift from Dr. M. Sano (Kyoto Prefectural University of Medicine) and were cultured as described previously (*5*). Nondifferentiated cells were used in all of the experiments.

Measurements of Intracellular Ca^{2+} Using Fura-2—Measurements of Ca^{2+}-dependent changes in intracellular Fura-2 fluorescence of PC12D cells in stirred suspensions were carried out in Krebs Ringer-HEPES (KRH) in quartz cuvettes with an excitation wavelength (λ_{ex}) of 340 nm using a Nippon Bunko/Jasko FP770 spectrofluorometer, as described in Ref. *5*. Measurements of Mn^{2+} quenching of fura-2 fluorescence were carried out using an excitation wavelength of 360 nm, the isosbestic absorption wavelength for fura-2. Fluorescence emissions ($F_{340\ nm}$ and $F_{360\ nm}$) were measured at 510 nm, which is near the emission maximum for both Ca^{2+}-dependent and -independent fura-2 fluorescence. All measurements were carried out at 22°C. Differences in baseline values of $F_{340\ nm}$ and $F_{360\ nm}$ reflect differences in the: (i) number of cells in the cuvette, (ii) efficiency of fura-2 loading, and/or (iii) spectrofluorometer settings. Maximal Ca^{2+}-dependent fluorescence signals were typically 50–60% of the maximal fura-2 fluorescence signal measured by lysing the cells with 0.1% Triton X-100.

The output from the Nippon Bunko/Jasco FP770 comprised analog traces inscribed on heat-sensitive graph paper. Traces were photocopied on acid-free paper for long-term preservation of the data. Composite traces were constructed manually by photocopying one of the traces on an overhead projector (OHP) transparency and carefully aligning the OHP sheet on top of a second trace copied on regular paper. The combined traces were then photocopied. Composites containing three or more traces were produced by sequentially adding traces copied on OHP transparencies. All traces were digitally scanned and stored as TIFF files for the construction of figures. Artifacts related to the opening and closing of the cuvette chamber were omitted from the fluorescence traces shown.

RESULTS

Carbachol and Thapsigargin Discharge the Same Intracellular Pools of Ca^{2+}—As shown in Fig. 1A (left), PC12D cells in Krebs Ringer-HEPES (KRH) containing 2 mM calcium chloride undergo a two-phase increase in intracellular Ca^{2+} following exposure to the acetylcholine analog carbachol. Pretreatment of the cells with the nicotinic receptor antagonists D-tubocurarine (50 μM) had almost no effect on carbachol-induced changes in intracellular Ca^{2+}, while pretreatment of the cells with the mAChR antagonists atropine (2 μM) completely blocked carbachol-mediated increases in intracellular Ca^{2+} (Ebihara and Saffen, data not shown). These results indicate that carbachol increases intracellular Ca^{2+} in these cells by stimulating mAChRs. Chelation of extracellular Ca^{2+} with 3 mM EGTA completely eliminated the plateau phase without affecting the initial sharp rise in intracellular Ca^{2+} (Fig. 1A, right). This result suggests that the initial sharp peak corresponds to the release of Ca^{2+} from internal stores and the plateau phase to the influx of extracellular Ca^{2+}. Figure 1B shows that thapsigargin also induces a two-phase increase in intracellular

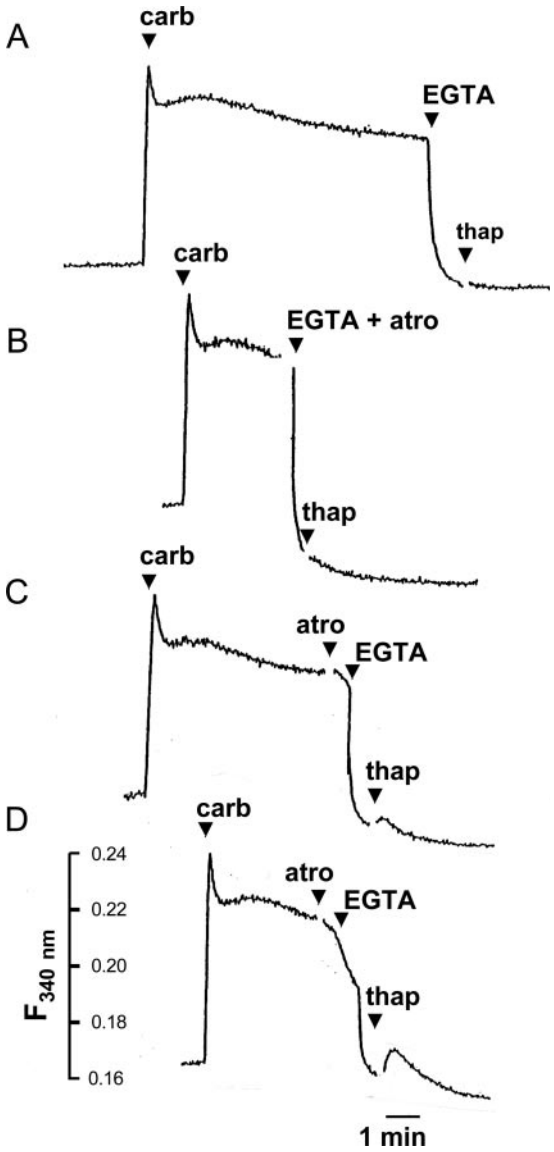

Fig. 2. **Intracellular Ca^{2+} stores remain empty during the continuous activation of mAChRs.** Changes in intracellular fura-2 fluorescence (F_{340}) were measured in PC12D cells exposed to 500 μM carbachol, 3 mM EGTA, 2 μM atropine and 100 nM thapsigargin at the times indicated by arrowheads. Traces A: no atropine added; B: atropine and EGTA were added simultaneously; C: atropine was added 30 s before addition of EGTA; D: atropine was added 60 s before addition of EGTA. The fluorescence and time calibrations apply to traces A–D.

Ca^{2+}, which also reflects the sequential release of Ca^{2+} from intracellular Ca^{2+} stores and the influx of extracellular Ca^{2+}. Differences in the shape of the traces representing mAChR- and thapsigargin-mediated release of Ca^{2+} from internal stores reflect differences in the kinetics of Ca^{2+} release induced by these agents.

We have previously shown that PC12D cells express mRNA encoding M_1 and M_4 subtypes of mAChR (*5*), which couple to pertussis toxin–insensitive and –sensitive G proteins, respectively. Since pretreatment of the cells with pertussis toxin (100 ng/ml 18 h) has no effect on the ability of carbachol to activate Ca^{2+} influx (Ebihara and Saffen, unpublished observations), we presume that carbachol-stimulated Ca^{2+} release and influx are mediated by the M_1 mAChR subtype, which couples to PLCβ *via* the $G_q/_{11}$ subtypes of G protein (*23*). We have previously shown that exposure of PC12D cells to carbachol results in the robust increase in levels of IP_3 (*5*), a result consistent with the expression of functional M_1 mAChR in these cells. The rapid release of Ca^{2+} from internal stores seen following exposure to carbachol is therefore likely to be the result of the activation of the IP_3 receptor/Ca^{2+} channels in the endoplasmic reticulum.

In contrast to carbachol, thapsigargin stimulates the release of Ca^{2+} from internal stores by irreversibly inhibiting the SERCA pump, which normally functions to refill the intracellular Ca^{2+} stores (*19*). To determine whether the Ca^{2+} stores depleted following stimulation of mAChR are equivalent to the stores depleted by thapsigargin, we examined the effects of sequential exposure to carbachol and thapsigargin on the release of Ca^{2+}. Figure 1C shows that pretreatment of PC12D cells with carbachol is sufficient to block the release of intracellular Ca^{2+} by thapsigargin. Conversely, pretreatment with thapsigargin blocks the release of Ca^{2+} by carbachol. These data suggest that both agents discharge the same intracellular Ca^{2+} stores. By contrast, the ionophore ionomycin is able to

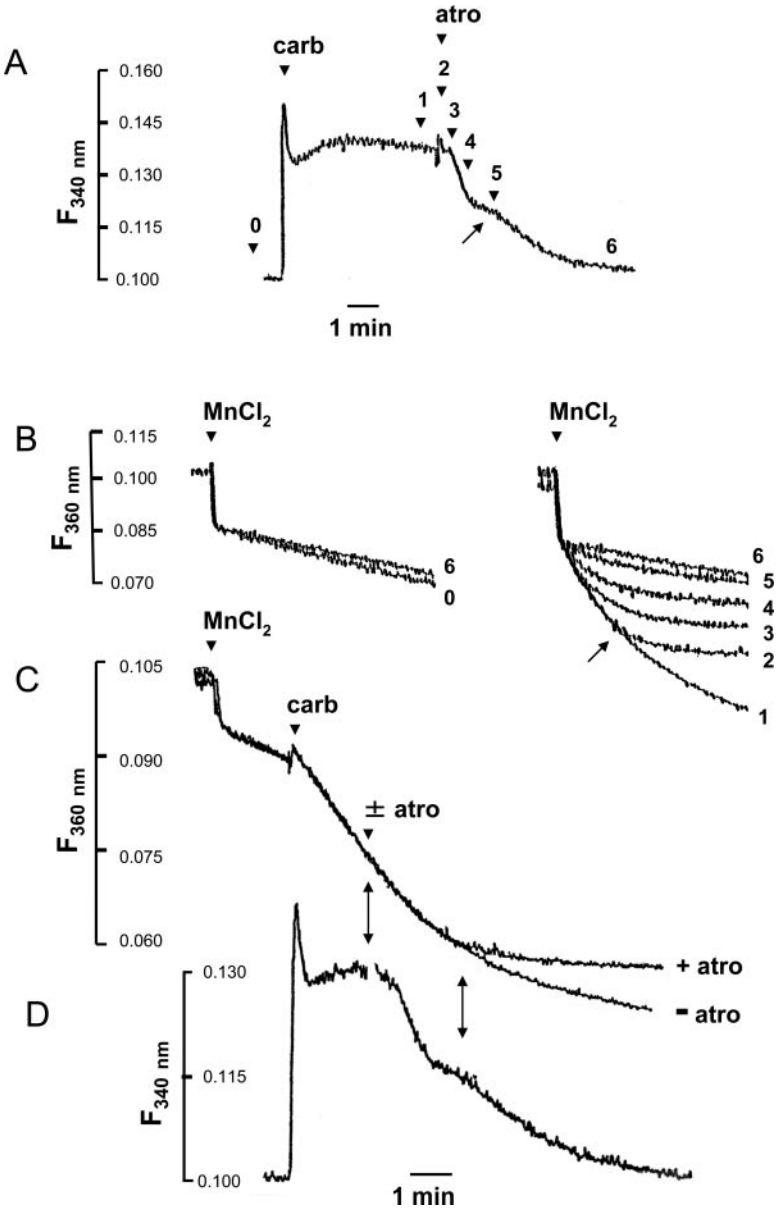

Fig. 3. **Addition of atropine to carbachol-stimulated PC12D cells causes intracellular Ca^{2+} to return to baseline levels in two steps; the second step is correlated with the closing of a Mn^{2+}-permeable channel.** A: Top: PC12D cells in standard KRH were pretreated with 50 μM tubocurarine prior to exposure to 500 μM carbachol and 2 μM atropine at the times indicated by the arrowheads. Ca^{2+}-dependent changes in intracellular fura-2 fluorescence ($F_{340\ nm}$) were measured at excitation wavelength (λ_{ex}) = 340 nm. Numbers (1–6) indicate the times at which Mn^{2+} influx was measured in a parallel suspension of cells. The arrow indicates the time at which the second plateau phase begins to decline to baseline. B: (Left and right sets): Quenching of intracellular fura-2 in parallel cultures was initiated by addition of 50 μM $MnCl_2$ at the times indicated by the numbers in the above trace. Mn^{2+}-dependent changes in fluorescence ($F_{360\ nm}$) were measured at λ_{ex} = 360. The immediate decrease in fluorescence observed at the time of addition of $MnCl_2$ is caused by the quenching of extracellular fura-2. Traces were aligned to compensate for small differences in the amounts of extracellular fura-2 in each aliquot of cells. The arrow indicates the time at which Mn^{2+} influx begins to slow in cells treated with atropine at time 2. C: Quenching of intracellular fura-2 fluorescence ($F_{360\ nm}$) was measured in cells exposed to 50 μM $MnCl_2$, 500 μM carbachol and 2 μM atropine (+atro) or water (–atro) at the indicated times. D: Changes in Ca^{2+}-dependent intracellular fura-2 fluorescence ($F_{340\ nm}$) measured in a parallel cell suspension. The traces in C and D have been aligned to show the correlation between changes in Mn^{2+} influx and changes in cytoplasmic levels of Ca^{2+} (double-headed arrows). The results shown are representative of 5 independent experiments.

discharge additional stores, probably including Ca^{2+} stored in mitochondria and heterogeneous, non-acidic compartments (*24*). Prior treatment with ionomycin completely blocks release by carbachol and thapsigargin, suggesting that carbachol and thapsigargin-sensitive pools are a subset of the ionomycin-sensitive pools. Exposure to 20 mM caffeine failed to produce a detectable release of Ca^{2+}, suggesting that ryanodine receptor–releasable Ca^{2+} stores are absent from this subline of PC12 cells (Ebihara and Saffen, data not shown). These data are consistent with previous reports showing the identity of IP_3- and thapsigargin-releasable pools (*24*, *25*) and the heterogeneity among PC12 sublines with respect to the types of Ca^{2+} stores present (*21*).

Intracellular Ca^{2+} Stores Remain Depleted in the Continuous Presence of Carbachol—In the experiments depicted in Fig. 2, the state of the intracellular Ca^{2+} stores was assessed by measuring the ability of thapsigargin to release Ca^{2+}. Visualization of thapsigargin-stimulated release was facilitated by the addition of EGTA to the cell suspension to chelate extracellular Ca^{2+}. Chelation of extracellular Ca^{2+} immediately blocks the influx of extracellular Ca^{2+} and causes intracellular Ca^{2+} to rapidly fall due to the highly efficient efflux of Ca^{2+} via the Na^+/Ca^{2+} exchanger and Ca^{2+} ATP pump. Figure 2A shows that thapsigargin-inducible release of Ca^{2+} is not observed in cells maintained for 10 min in the continuous presence of carbachol. Figure 2, B–D, shows that intracellular Ca^{2+} stores rapidly refill following inactivation of mAChR with atropine, as shown by the progressive increase in the amount of Ca^{2+} released by thapsigargin with time following the addition of atropine. These data reveal that intracellular Ca^{2+} stores remain empty during continuous activation of mAChRs, even though cytoplasmic levels are high, and suggest that mAChR-mediated Ca^{2+} influx may be "capacitative influx" linked to the empty state of the intracellular Ca^{2+} stores.

Carbachol Stimulates and Atropine Inhibits the Influx of Ca^{2+} and Mn^{2+}—Previous studies have shown that in many cell lines, increases in Ca^{2+} influx stimulated by inositol trisphosphate (IP_3) production or by exposure to thapsigargin are accompanied by an increase in permeability to Mn^{2+} (*26*, *27*). The experiment depicted in Fig. 3 shows that carbachol also stimulates the influx of Mn^{2+} into PC12D cells.

Addition of the mAChR antagonist atropine causes intracellular Ca^{2+} levels to rapidly return to baseline levels (Fig. 3A). The time-course of the decline in intracellular Ca^{2+} typically exhibits the following components: (i) a short lag of approximately 15–30 s followed by (ii) a rapid decrease, (iii) another lag of 15–30 s, and finally (iv) a slow decline to baseline levels lasting several minutes. (The beginning of the slow decline to baseline is marked by the arrow.) As shown in Fig. 3B, atropine-induced decline in intracellular Ca^{2+} levels is correlated with a decrease in the rate of Mn^{2+} influx in a parallel suspension of cells. (Compare traces 1 and 6.) Thus, Mn^{2+} influx is stimulated by carbachol and inhibited by atropine in a manner that parallels changes in steady-state levels of intracellular Ca^{2+}. Taken together, these data suggest that atropine reduces steady-state levels of Ca^{2+} by inhibiting the influx of extracellular Ca^{2+} through a Mn^{2+}-permeable channel.

A closer examination of traces 2–6 in Fig. 3B reveals that the rate of Mn^{2+} influx does not decrease immediately

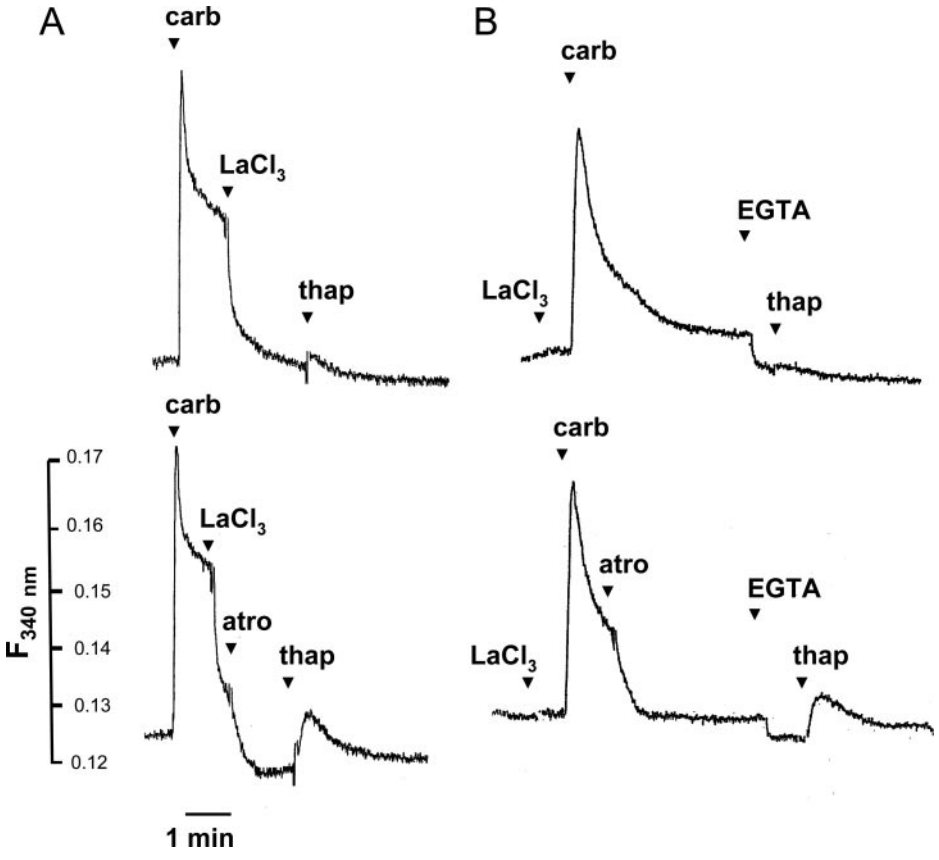

Fig. 4. **Atropine stimulates the refilling of intracellular stores in the absence of influx of extracellular Ca^{2+}.** A: Top: Fura-2 loaded PC12D cells in phosphate-free KRH (+0.1% gelatin) were stimulated with 500 μM carbachol prior to addition of 100 μM $LaCl_3$ and 100 nM thapsigargin at the times indicated by the arrowheads. Bottom: PC12D cells were treated as above, except that 2 μM atropine was added prior to addition of thapsigargin. B: Top: Fura-2 loaded PC12D cells in phosphate-free KRH (+0.1% gelatin) were exposed to 100 μM $LaCl_3$ prior to addition of 500 μM carbachol and 100 nM thapsigargin. Bottom: Cells were treated as above, except that 2 μM atropine was added prior to addition of thapsigargin. The fluorescence ($F_{340\ nm}$) and time scales apply to each of the traces in the figure. These results are representative of 3 independent experiments.

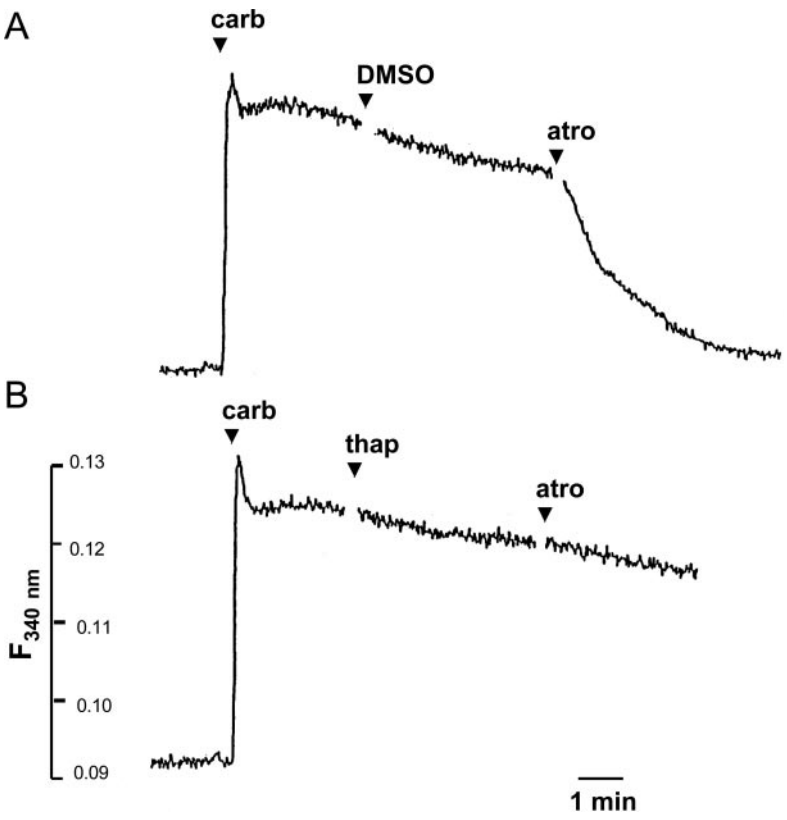

Fig. 5. **Depletion of intracellular stores with thapsigargin blocks mAChR regulation of Ca^{2+} influx.** Fura-2 loaded PC12D cells in KRH were exposed to 500 μM carbachol, 0.1% DMSO (top) or 400 nM thapsigargin (bottom), and 2 μM atropine at the times indicated by the arrowheads. The fluorescence ($F_{340\ nm}$) and time scales apply to both of the traces in the figure. These results are representative of 4 independent experiments.

following inhibition of mAChR. Rather, the rate of Mn^{2+} entry remains unchanged for about 60 to 90 s after the addition of atropine. (The arrow in Fig. 3B marks the time at which Mn^{2+} influx begins to slow.) Aligning Mn^{2+} quenching traces (Fig. 3C) with traces depicting Ca^{2+} dependent changes in intracellular fura-2 fluorescence (Fig. 3D) shows that the slowing of Mn^{2+} entry correlates with the second drop in intracellular Ca^{2+} levels. These results suggest that the second step in the two-step return to baseline is caused by the closing of a Mn^{2+}-permeable channel.

The Initial Decrease in Intracellular Ca^{2+} That Occurs Following Application of Atropine Is Caused by the Refilling of Intracellular Ca^{2+} Stores—The data in Fig. 3 show that the rate of Mn^{2+} entry does not change during the first 60 to 90 s after the addition of atropine to PC12D cells stimulated with carbachol. By contrast, levels of intracellular Ca^{2+} rapidly decrease during this period. To determine if the refilling of the intracellular Ca^{2+} could account for this decrease in intracellular Ca^{2+}, we examined the effects of atropine on intracellular Ca^{2+} levels under conditions where Ca^{2+} entry into the cells was inhibited. In these experiments we blocked Ca^{2+} influx using La^{3+}, which has previously been shown to block mAChR-regulated Ca^{2+} influx in PC12 cells (*28*). As shown in Fig. 4A (top trace), addition of 100 μM $LaCl_3$ to the cells pretreated with carbachol causes a rapid decrease in intracellular (more precisely, cytoplasmic) Ca^{2+} levels due to the combined effects of inhibition of Ca^{2+} influx and rapid expulsion of Ca^{2+} from the cells. Addition of 2 μM atropine increases the rate at which cytoplasmic Ca^{2+} levels decrease (Fig. 4A, bottom). Figure 4B shows that similar results are obtained in cells pretreated with $LaCl_3$ prior to stimulation with carbachol. Here, the initial increase in cytoplasmic Ca^{2+} levels is due exclusively to the release of Ca^{2+} from intracellular stores. Again, addition of 2 μM atropine increases the rate at which these levels decrease. The rate at which cytoplasmic Ca^{2+} levels decrease reflects the rates of export from the cell and sequestration into intracellular stores. A plausible explanation of these data is that atropine accelerates the rate at which cytoplasmic Ca^{2+} levels decrease by stopping the production of IP_3, thereby allowing the Ca^{2+} stores to refill. The observation that thapsigargin releases Ca^{2+} only from cells treated with atropine (Fig. 4, A and B, bottom traces) is consistent with this idea. Taken together, the results shown in Figs. 3 and 4 suggest that the two-stage return to baseline Ca^{2+} levels observed following addition of atropine to carbachol-stimulated cells may be explained by an initial decrease in intracellular Ca^{2+} due to the refilling of intracellular Ca^{2+} stores and the subsequent closing of a Mn^{2+}-permeable Ca^{2+} channel.

Carbachol and Thapsigargin Stimulate Calcium Influx via a Common Mechanism—If activation and inhibition of mAChRs in PC12D cells controls intracellular Ca^{2+} levels primarily through the activation and inhibition of a "capacitative influx" Ca^{2+} channel (*i.e.*, SOCC), the effects of carbachol on Ca^{2+} influx should be similar to those of thapsigargin. To determine if carbachol and thapsigargin activate the same or different Ca^{2+} influx pathways in PC12D cells, we examined the effects of these agents independently and in combination on: (i) steady-state levels of intracellular Ca^{2+}, (ii) Mn^{2+} influx, and (iii) Ca^{2+} influx. Figure 5 shows that addition of thapsigargin

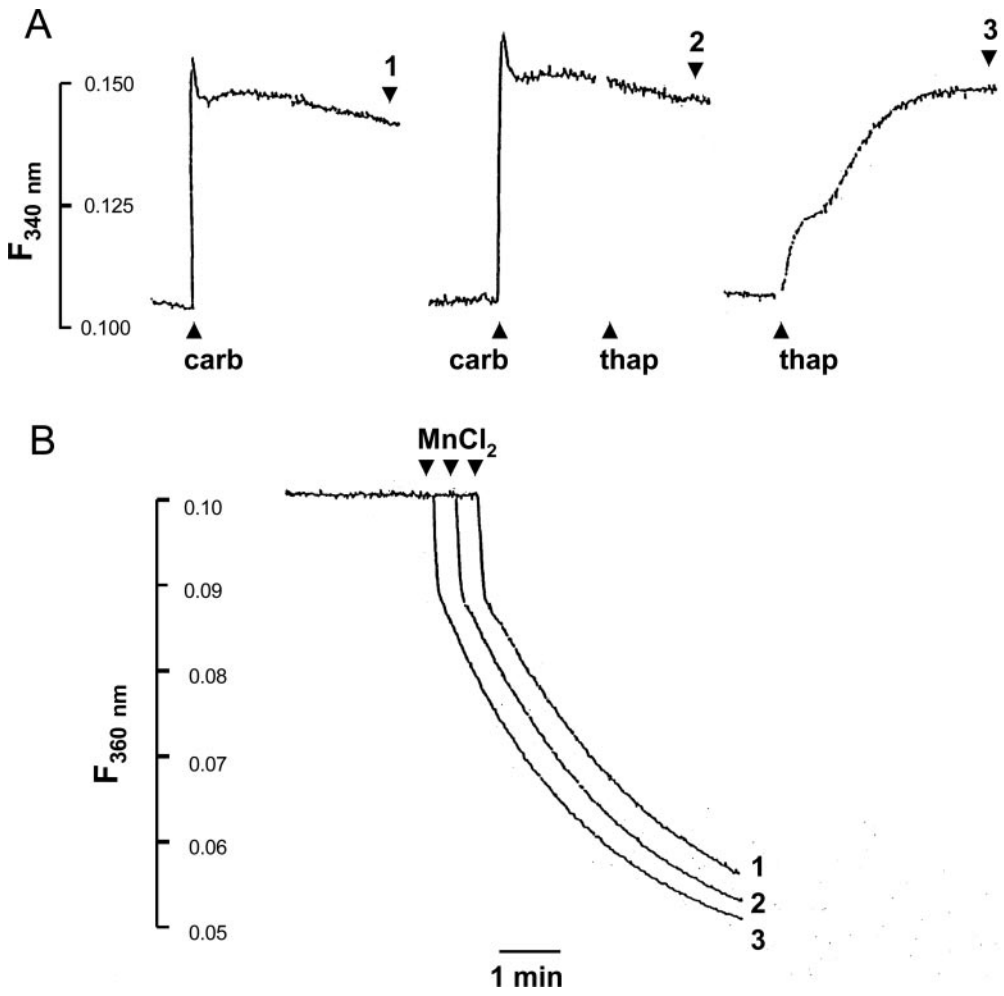

Fig. 6. **Carbachol and thapsigargin stimulate nearly identical maximal rates of Mn^{2+} influx and these rates do not sum when the cells are exposed to both agents.** Top: Ca^{2+}-dependent changes in fura-2 fluorescence induced by 500 μM carbachol (left), 1 μM thapsigargin plus 500 μM carbachol (middle), or 1 μM thapsigargin (right). Bottom: Mn^{2+} quenching curves obtained by adding 25 μM $MnCl_2$ to parallel cultures of fura-2 loaded cells at the times indicated by the numbered arrowheads in the traces above. The time scale applies to the traces in A and B. These results are representative of 4 independent experiments.

to cells previously stimulated with carbachol does not cause additional increases in intracellular levels of Ca^{2+}, suggesting that channels mediating "capacitive influx" may have already been maximally stimulated by carbachol. Pretreatment with thapsigargin, however, blocks the ability of atropine to decrease carbachol-stimulated increases in steady-state levels of intracellular Ca^{2+}. A plausible interpretation of this result is that thapsigargin prevents the refilling of intracellular Ca^{2+} stores that usually takes place following mAChR inhibition by atropine. Thus, the intracellular stores remain empty and capacitive influx of calcium continues in the presence of atropine. Figure 6 shows that both carbachol and thapsigargin stimulate the same maximal rates of Mn^{2+} influx. Identical results were obtained in experiments using different concentrations of Mn^{2+} (1 to 200 μM; Ebihara and Saffen, data not shown). The fact that there is no summation of the Mn^{2+} influx rates following simultaneous exposure to both agents is consistent with the idea that carbachol and thapsigargin activate the same influx pathways. Finally, Fig. 7 shows that the rates of Ca^{2+} influx are the same following stimulation with carbachol, thapsigargin, or a combination of both. Together these data imply that carbachol and thapsigargin increase intracellular Ca^{2+} levels by activating the same "capacitative" influx Ca^{2+} channels.

DISCUSSION

In this study, we show that activation of mAChRs in PC12D cells rapidly increases intracellular Ca^{2+} levels by stimulating the release of Ca^{2+} from internal stores and the influx of extracellular Ca^{2+}. These observations are consistent with the results of previous studies using PC12 (*21*) and other cells (*10*, *11*). As has been observed in many cell lines and tissues (*19*, *20*, *25*), treatment of PC12D cells with the SERCA pump inhibitor thapsigargin also causes the release of Ca^{2+} from internal stores and a sustained influx of extracellular Ca^{2+}. We also showed that the internal Ca^{2+} stores released by carbachol are identical to those released by thapsigargin (Fig. 1). These data suggest that carbachol and thapsigargin may stimulate Ca^{2+} influx by a common mechanism.

A model to explain mAChR-stimulated Ca^{2+} influx in PC12D cells is depicted in Fig. 8. First, stimulation of M_1 mAChRs activates PLCβ, thereby stimulating production of the second messengers diacylglycerol and IP_3. IP_3 binds to IP_3-receptor/channels on the surface of the endoplasmic reticulum, causing the channels to open and allow Ca^{2+} to diffuse into the cytoplasm. The empty Ca^{2+} stores then activate Ca^{2+} channels in the plasma membrane. These channels are proposed to be identical to the channels activated following depletion of intracellular

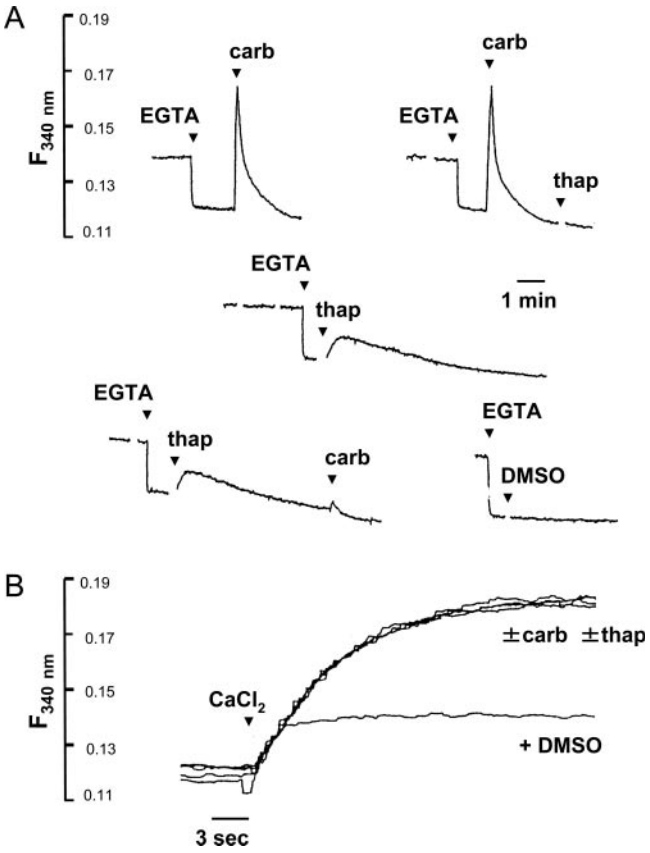

Fig. 7. **Ca^{2+} influxes stimulated by carbachol and thapsigargin are not additive.** A: Following addition of 3 mM EGTA to chelate extracellular calcium, fura-2 loaded PC12D cells in KRH were exposed to carbachol, thapsigargin, both agents or DMSO. B: At the times indicated by the termination of traces in shown in A, the chart-recorder (originally set at 1 cm/min) was stopped and restarted at a higher speed (20 cm/min). $CaCl_2$ (3 mM) was then added back to the cultures at the time indicated by the arrowhead. The off-settings of the baseline fluorescence reflect adjustments to the alignments of the curves that were introduced to compensate for small increases in the amounts of extracellular fura-2 that leaked from the cells during the course of the measurements. The initial increase in fluorescence following the reintroduction of Ca^{2+} to DMSO-treated cells is due to the rebinding of Ca^{2+} to extracellular fura-2. Cells were pretreated with 50 μM D-tubocurarine to prevent activation of nicotinic receptors. These results are representative of 4 independent experiments.

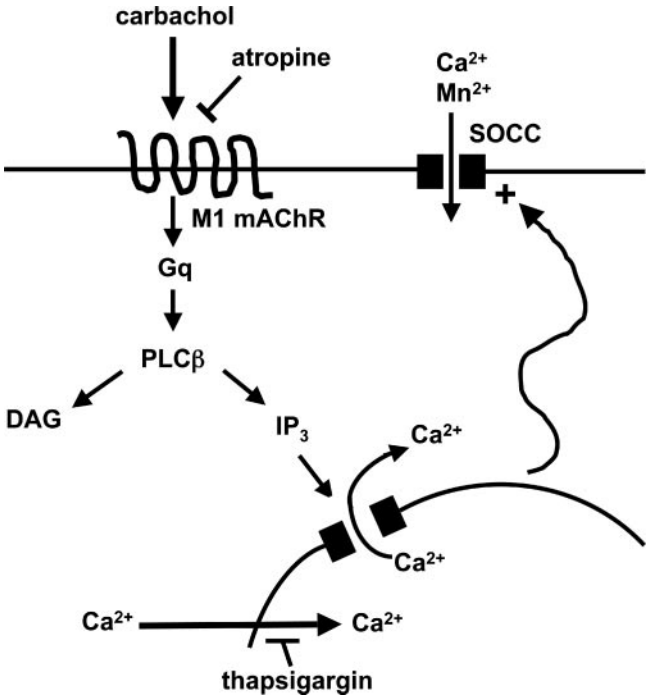

Fig. 8. **Model for store-operated Ca^{2+} influx in PC12D cells.** Stimulation of M1 muscarinic acetylcholine receptors (mAChRs) with carbachol activates Gq and phospholipase C-β (PLX), resulting in the production of the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP3). IP3 binds to the IP3 receptor/channel in the endoplasmic reticulum, causing the channel to open and release Ca^{2+} into the cytoplasm. The emptying of these Ca^{2+} stores causes store-operated Ca^{2+} channels (SOCCs) in the plasma membrane to open, resulting in an increase in Ca^{2+} levels in the cytoplasm. The SOCCs are also permeable to Mn^{2+}. Thapsigargin depletes Ca^{2+} stores by blocking the uptake of Ca^{2+} from the cytoplasm into the endoplasmic reticulum. The mAChR antagonist atropine blocks the activation of M1 mAChRs by carbachol.

Ca^{2+} stores by thapsigargin. In the case of carbachol, the influx of extracellular Ca^{2+} is sustained for as long as the Ca^{2+} stores remain empty, and the stores remain empty as long as the mAChRs remain activated. Inhibition of mAChR by exposure to atropine, however, induces the rapid refilling of intracellular stores and a return to prestimulation levels of intracellular Ca^{2+} (Figs. 2, 3 and 5). By contrast, thapsigargin causes the irreversible depletion of intracellular Ca^{2+} stores. Thus, treatment of PC12D cells with thapsigargin would be expected to block the ability of atropine to decrease Ca^{2+} levels in carbachol-stimulated cells. The data depicted in Fig. 5 shows that this is the case. Another prediction that follows from this model is that the maximal rates of carbachol- and thapsigargin-stimulated entry of Ca^{2+} and Mn^{2+} into the cell should be the same, and these rates should not sum when cells are exposed to both agents simultaneously. The data shown in Figs. 6 and 7 shows that this is the case. Taken together, these observations suggest that most of the mAChR-induced Ca^{2+} influx in PC12D cells can be accounted for by "capacitative" Ca^{2+} influx, without postulating the existence of an independent "receptor operated" Ca^{2+} influx pathway.

Our simple model differs from a model proposed by Clementi and coworkers for another spontaneously arising PC12 subline, PC12-64 (*21*). In that study, the authors carried out experiments similar to the experiments reported here, but obtained very different results. The most important differences can be summarized as follow: (i) These authors reported that there was no inhibition of carbachol-stimulated Mn^{2+} influx by atropine in PC12-64 cells when atropine was added 2–120 s prior to the addition of $MnCl_2$. By contrast, we observe a complete inhibition of Mn^{2+} influx within 90 s of addition of atropine (Fig. 3). (ii) They found that intracellular Ca^{2+} stores in PC12-64 cells refilled within 3 to 4 min following exposure to carbachol and that this rate of refilling was slowed when mAChR were blocked with atropine. By contrast, we find that Ca^{2+} stores remain empty in the continuous presence of carbachol for at least 10 min (Fig. 2) and that refilling

of the stores is accelerated by atropine (Figs. 2 and 4). (iii) In Ca^{2+}-reintroduction experiments, Clementi and coworkers observed that Ca^{2+} influx was largely blocked when the cells were treated with atropine just prior to adding $CaCl_2$ to the medium. By contrast, we observe no immediate inhibition of Ca^{2+} influx by atropine, but rather can observe an inhibition only after a lag of about 30–60 s (data not shown). This lag-time is about the same time that it takes for intracellular Ca^{2+} to begin to refill (Fig. 2).

Based upon their experiments, Clementi and coworkers concluded that the failure of atropine to rapidly inhibit the Mn^{2+} influx suggested that carbachol must activate two types of Ca^{2+} channels in PC12-64 cells: (i) channels that are impermeable to Mn^{2+} and directly regulated by mAChR (since exposure to atropine does cause intracellular Ca^{2+} levels to decline) and (ii) channels that are permeable to Mn^{2+} and Ca^{2+} and indirectly activated by mAChR *via* depletion of intracellular Ca^{2+} influx. The fact that atropine is immediately effective in inhibiting Ca^{2+} influx in the Ca^{2+} reintroduction experiments was taken as evidence for the existence of a distinct class of Ca^{2+} channels that are directly regulated by mAChR, without the intermediary of signals generated by the emptying of intracellular Ca^{2+} stores. Thus, even among lines of a single cell type, there appears to be significant variations in the mechanism by which mAChRs regulate the influx of extracellular Ca^{2+}. Activation of Ca^{2+} influx in the absence of IP_3-stimulated release of Ca^{2+} from internal stores has also been reported in A9 fibroblasts exogenously expressing the m3 subtype of mAChR (*10*).

Stimulation of mAChRs or exposure to thapsigargin does not induce a sustained Ca^{2+} influx in all cell lines. For example, in neuroblastoma-glioma hybrid cell line NG-108 expressing exogenous M1 mAChRs, exposure to acetylcholine induces only the release of Ca^{2+} from internal stores (*29*). Likewise, treatment of neuronal NG115-40L cells with thapsigargin causes the release of Ca^{2+} from internal stores without a subsequent increase in Ca^{2+} influx (*30*). Also, in primary cultures of cerebellar granule cells, exposure to acetylcholine or thapsigargin (following pretreatment with 40 mM KCl to charge intracellular stores) results in only the release of Ca^{2+} from intracellular calcium stores and does not stimulate Ca^{2+} influx (*31*, *32*). Thus, not all types of cells exhibit the phenomenon of capacitative influx, and in these cells, stimulation of mAChRs causes only a transient increase in intracellular Ca^{2+}.

In summary, activation of mAChRs in PC12D cells stimulates the influx of extracellular Ca^{2+} primarily via SOCCs, which are also activated following depletion of intracellular Ca^{2+} stores with thapsigargin. In the accompanying paper (Zhang, Guo, Kim and Saffen, 2006) we present data showing that there is, in fact, an additional carbachol-activated pathway for Ca^{2+} influx in PC12D cells that is regulated independently of the intracellular Ca^{2+} stores.

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