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Muscarinic receptor-operated Ca^{2+} influx in transfected fibroblast cells is independent of inositol phosphates and release of intracellular Ca^{2+}

(calcium channels/receptor chimeras/phorbol ester/arachidonic acid)

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ABSTRACT **Receptor-mediated changes in cytoplasmic calcium concentrations occur either through release from intracellular calcium stores or by the opening of channels in the plasma membrane, allowing influx of calcium from the extracellular fluid. Carbachol, a muscarinic receptor agonist, stimulated both calcium influx and inositol 1,4,5-trisphosphate ($InsP_3$)-mediated intracellular calcium release in A9 fibroblast cells expressing a m3 muscarinic receptor clone. The calcium influx persisted even after pretreatment of cells with phorbol 12-myristate 13-acetate, which completely prevented the rise in inositol phosphates and intracellular calcium levels. The calcium influx was blocked by divalent cations but was not affected by inhibitors of voltage-dependent calcium channels or high potassium depolarization, indicating the presence of a receptor-operated and voltage-insensitive calcium channel in these cells. Calcium influx was not stimulated by the addition of cAMP analogs or arachidonic acid. To examine the possible involvement of G proteins in m3 receptor-activated calcium influx, two chimeric m2 and m3 muscarinic receptors were expressed in A9 cells in which the third cytoplasmic loop (the primary structural determinant in G protein coupling selectivity of muscarinic receptors) had been exchanged between the m2 receptor, which has no effect on calcium influx, and the m3 receptor. Calcium influx was found to be associated with a structural component of the m3 muscarinic receptor other than the third cytoplasmic loop.**

Receptor-stimulated increases in cytosolic free calcium generally occur in two phases, an initial rapid spike followed by a sustained phase. The initial spike is the result of release of calcium from intracellular stores mediated primarily by inositol 1,4,5-trisphosphate ($InsP_3$) generated following receptor-mediated activation of phospholipase C (1). The sustained plateau phase follows influx of calcium from the extracellular medium and requires the continued presence of agonist. Receptor-mediated calcium influx is thought to occur either through the well-studied voltage-sensitive calcium channels or through receptor-operated calcium channels, about which little is currently known. Receptor-operated calcium channels have been identified in a variety of both excitable and nonexcitable cell types (2). Unlike the voltage-sensitive calcium channels, receptor-operated calcium channels have avoided direct measurement when electrophysiological techniques have been used possibly because of their low abundance or extremely low conductance. These channels can be indirectly activated by the second messengers $InsP_3$ (3, 4), inositol 1,3,4,5-tetrakisphosphate (5), or intracellular calcium (6) and in some cells may be independent of second-messenger regulation (7). Plasma membrane calcium channels responsible for the refilling of intracellular calcium stores have been described that open following $InsP_3$-mediated calcium release (8). It is not clear if the refilling channels and receptor-operated calcium channels are identical.

Muscarinic acetylcholine receptor subtypes m1, m3, and m5, when expressed in A9 and Chinese hamster ovary fibroblasts, couple to multiple transmembrane signal transduction pathways, resulting in the generation of intracellular second-messenger molecules arachidonic acid, cAMP, and $InsP_3$ as well as an increase in cytoplasmic calcium originating from intracellular and extracellular sources (9–11). The mechanism by which cytoplasmic free calcium is increased in these cells is uncertain since arachidonic acid, cAMP, and $InsP_3$ have been shown to directly or indirectly mobilize intracellular calcium in a variety of cell types (1, 12, 13). In this study we demonstrate that m3 receptor-mediated activation of calcium influx is independent of $InsP_3$ and intracellular calcium release. Calcium influx was not activated with the exogenous addition of cAMP analogs or arachidonic acid. Alterations in receptor structure was used to determine guanine nucleotide-binding protein (G protein) involvement in calcium influx regulation. Studies using m2 and m3 muscarinic receptor chimeras suggest that m3 receptor-mediated calcium influx is independent of the third cytoplasmic loop (the primary structural determinant of G protein-coupling selectivity of muscarinic receptors).

METHODS

Stable Expression of Muscarinic Receptors in A9 Fibroblasts. A9 cells were obtained from the American Type Culture Collection and transfected with muscarinic m2 and m3 (14, 15) or m2/m3 chimeras (16) as described.

Measurement of Intracellular Calcium Concentration in Single Fura-2-Loaded A9 Cells. Cells grown on glass cover slips coated with Vitrogen (300 μg/ml; Collagen Corp.) were loaded with 5 μM fura-2 acetoxymethyl ester (Molecular Probes) for 30 min at 37°C in growth medium, washed once, and stored in Eagles no. 2 medium containing bovine serum albumin at 1 mg/ml and 20 mM Hepes buffer (pH 7.4) at 25°C for no more than 45 min before the experiment. Fura-2 fluorescence was measured photometrically at an emission wavelength of 510 nm in a single cell mounted on a Nikon Diaphot microscope illuminated alternately with 340-nm and 380-nm light (bandpass, 4 nm) in a SLM Aminco DMX-1000 spectrofluorometer (SLM Aminco, Urbana, IL). Ratios of fluorescence at 340

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Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; G protein, guanine nucleotide-binding protein; PMA, phorbol 12-myristate 13-acetate; BAPTA-AM, bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate acetoxymethyl ester.

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nm/380 nm were converted to calcium concentrations, and calibration was performed as described (17).

Measurement of Inositol Phosphate Release. A9 cells were grown to ≈80% confluence in 24-well cell-culture plates and labeled overnight with 2 μCi (74 kBq) of [^{3}H]inositol per well. Released [^{3}H]inositol phosphates were measured as described (10).

Electrophysiology. Cell-attached single-channel recordings were obtained with an Axon Instruments (Burlingame, CA) Axopatch 1A amplifier. Amplified signals (×100) were filtered at 5 kHz and subsequently stored on videotape with aid of PCM-based A/D converter (Instrutech, Mineolo, NY; VR 100-4; 12-bit resolution; digitization rate, 18 kHz). Single-channel records were visualized by playback of the tape record on a Gould pen recorder filtered at 50 Hz. Responses were obtained with the pipette potential (V_p) at 0 mV. Patch electrodes having a resistance of 5–10 MOhms contained a solution consisting of 140 mM KCl, 5 mM NaCl, 2 mM $CaCl_2$, 10 mM glucose, 10 mM Hepes (pH 7.2–7.3) with osmolarity adjusted to 315–325 mOsm with sucrose. Seals obtained with electrodes varied from 2 to 10 GV. The bathing solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes (pH 7.2–7.3) with osmolarity adjusted to 320–330 mOsm with sucrose. Carbachol was applied to the cell exterior by pressure ejection of Ringer solution with a micropipette placed within 5–10 μm of the cell. The channels activated by carbachol were identified as being potassium channels based on their reversal potential as described (18).

RESULTS

A9 fibroblasts expressing the m3 muscarinic acetylcholine receptor (A9m3 cells), when stimulated with the muscarinic receptor agonist carbachol, generated a peak rapidly, followed by a sustained increase in intracellular free calcium as measured by single-cell microspectrofluorimetry of the calcium-sensitive fluorescent dye fura-2 (Fig. 1). Previous studies suggested that the peak phase is the result of InsP_3-mediated release of intracellular calcium stores and the sustained phase is due to an agonist-dependent influx of extracellular calcium (2). Consistent with this view, removal of extracellular calcium eliminated the sustained phase in carbachol-stimulated A9m3 cells but retained the peak phase (Fig. 1). These results clearly indicate a role for extracellular calcium in the maintenance of the sustained response.

Inhibition of carbachol-stimulated InsP_3 generation would be expected to prevent the intracellular release of calcium and therefore allow the direct visualization of the calcium influx component. Preincubation of A9m3 cells with phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, inhibited carbachol-stimulated inositol phosphate generation to below basal levels (Fig. 2), similar to results demonstrated in several other cell types (19, 20). PMA pretreatment also blocked the carbachol-stimulated peak intracellular calcium release consistent with the suggested role of InsP_3 in mediating this response (Fig. 3*A*). When calcium was added back to the medium of A9m3 cells pretreated with PMA (Fig. 3*B*), carbachol stimulated a slow rise and sustained increase in intracellular calcium levels that remained elevated as long as carbachol was present in the medium. Following PMA pretreatment, carbachol-stimulated calcium influx was eliminated in the absence of extracellular calcium (Fig. 3*B*). These observations indicate that the sustained phase of intracellular free calcium was due to carbachol-stimulated calcium influx and was independent of inositol phosphates and intracellular calcium.

In contrast to the well-characterized voltage-sensitive calcium channels, attempts to examine receptor-operated calcium channels by using whole-cell patch-clamp techniques have been unsuccessful (2). Our own electrophysiological

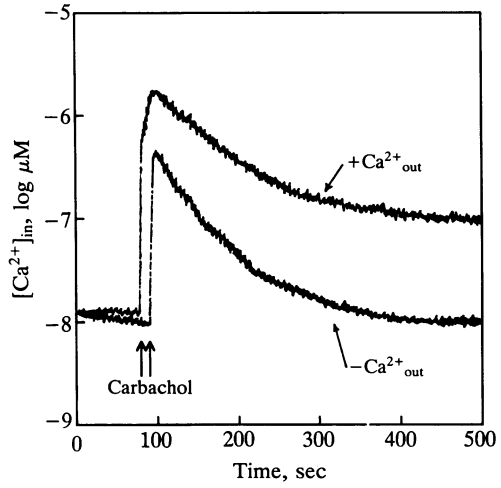

FIG. 1. Intracellular free calcium concentration ($[Ca^{2+}]_{in}$) in single fura-2-loaded A9 cells expressing the m3 muscarinic acetylcholine receptor stimulated with muscarinic receptor agonist carbachol (10 μM). Curves: upper curve, generated in the presence of 1 mM calcium-containing medium, demonstrates typical peak and plateau phases of intracellular free calcium; lower curve, generated in the absence of external calcium (Ca^{2+}_{out}) shows only peak phase with loss of plateau. Data are representative of at least 10 separate experiments.

studies on A9 cells and those of others (18) were able to detect m3 receptor-activated calcium-dependent potassium channels (Fig. 4), but were unsuccessful in identifying m3 receptor-sensitive calcium channels possibly because of their extremely low abundance or low conductance. PMA pretreatment of A9m3 cells did not prevent potassium channel activation by carbachol (Fig. 4), suggesting that a source of calcium other than through InsP_3 activation may be involved in potassium channel regulation. Potassium channel activation was prevented by prior incubation of the cells in Ringer's solution containing BAPTA-AM, which quickly buffered the rise in internal calcium ion concentration preventing activation of potassium channels. Similar results were obtained by removing extracellular calcium from the bathing medium, implying that the potassium channels were dependent on the presence of calcium in the external medium. The robust potassium-channel response may have obscured direct detection of any receptor-operated calcium channels. Whole-

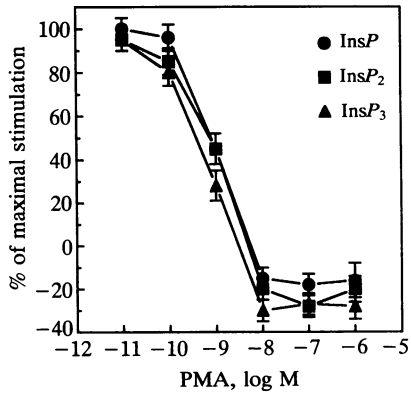

FIG. 2. Carbachol (100 μM)-stimulated inositol phosphate generation in A9 cells expressing the m3 muscarinic receptor was inhibited by preincubation with the phorbol ester PMA for 30 min. Carbachol was added to cells, and the assay was terminated 15 min later. Actual mean cpm of 100% and 0% values are: 12,823 and 569 for inositol monophosphates (Ins*P*), 2026 and 293 for inositol bisphosphates (InsP_2), and 709 and 86 for InsP_3. Data are the means ± SEM of three independent experiments performed in triplicate.

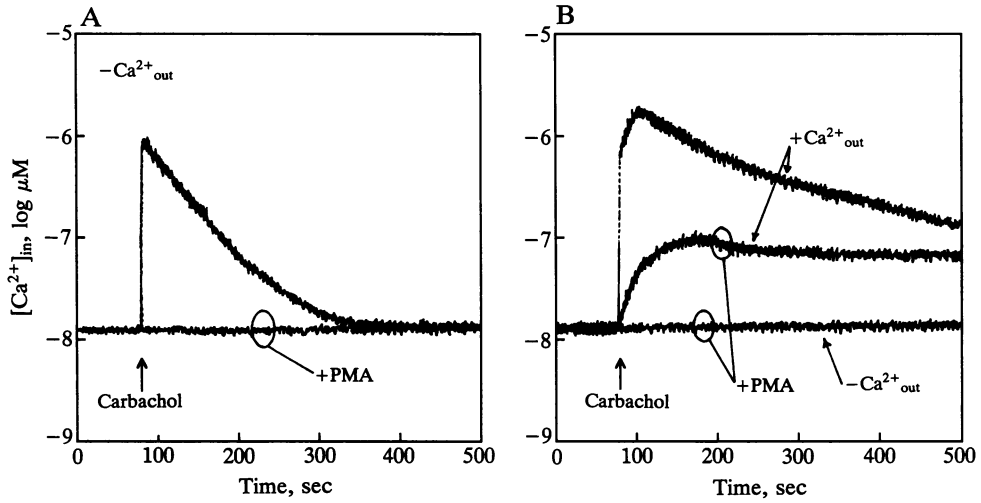

FIG. 3. (*A*) Intracellular free calcium concentration ($[Ca^{2+}]_{in}$) in single fura-2-loaded A9 cell expressing the m3 muscarinic acetylcholine receptor stimulated with the muscarinic receptor agonist carbachol (10 μM). Both curves were recorded in the absence of extracellular calcium (Ca^{2+}_{out}) and with added 0.5 mM EGTA. Curves: upper curve shows typical InsP_3-mediated calcium peak; lower curve shows complete loss of intracellular calcium release after preincubating the cells with PMA (100 nM) for 15–45 min to inhibit inositol phosphate formation. Data are representative of at least six separate experiments. (*B*) The top curve is shown without PMA addition for reference. In the other two curves A9m3 cells were preincubated with PMA (100 nM) for 15–45 min. The middle curve was generated in the presence of 1 mM extracellular calcium where carbachol (10 μM) stimulated a slow influx of intracellular free calcium that remained above basal levels for the duration of the experiment. The bottom curve was generated after removal of extracellular calcium with added 0.5 mM EGTA, which eliminated the influx of calcium to the cell interior. Data are representative of at least six experiments.

cell recordings in which the potassium channels were blocked by internal perfusion of the cell with cesium chloride also did not reveal any calcium current. Therefore, a pharmacological approach was used to further characterize receptor-mediated calcium influx. Several voltage-dependent calcium-channel blockers were examined for their effect on m3 receptor-operated calcium influx. Nifedipine, verapamil, and diltiazem, which are dihydropyridine, phenylalkylamine, and benzothiazepine L-type calcium channel blockers respectively (21) had no effect (up to 100 μM) on m3 receptor-stimulated calcium influx. ω-Conotoxin, an N-type calcium channel blocker (22) also had no effect (up to 100 μM). Membrane depolarization with 70 mM KCl as well as addition of Bay K 8644, a L-channel agonist (23), did not induce calcium influx. Carbachol-stimulated calcium influx (1 mM) was blocked with 5 mM $CoCl_2$, $NiCl_2$ or $LaCl_3$ (data not shown). These results suggest the presence of voltage-insensitive receptor-operated calcium channels in A9 cells.

It is possible that m3 muscarinic receptor-operated calcium influx may be mediated through a G protein, as are other second-messenger pathways coupled to this receptor. To examine this possibility, two chimeric m2/m3 muscarinic receptors were constructed in which the third cytoplasmic loop was exchanged between the m2 and m3 receptors (Fig. 5*A*). This region has been shown to represent the primary structural determinant of G protein-coupling selectivity for receptors belonging to the family of G protein-coupled receptors (16, 24, 25). The wild-type m2 receptor couples primarily to adenylate cyclase inhibition, and the m3 receptor couples primarily to stimulation of arachidonic acid and inositol phosphate generation. Exchange of the third cytoplasmic loop between the two receptors has been shown to reverse the functional specificity of the resultant chimeric receptors (16). In contrast to the wild-type m2 receptor, which had no effect on intracellular calcium levels (data not shown), cells expressing the chimeric m2 receptor/m3 loop construct now responded to carbachol by generating a calcium curve typical of an InsP_3-mediated response, a rapidly attained peak that fell to baseline (Fig. 5*B*). This response was also completely inhibited with phorbol ester preincubation

FIG. 4. Carbachol activates a calcium-dependent potassium channel in PMA-treated A9 cells expressing the m3 receptor. (*A*) Carbachol applied to the outside of the patch activates potassium channels recorded by on-cell patch clamp technique. (*B*) Activation of these channels can be attenuated by incubation of the cells with bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate acetoxymethyl ester (BAPTA-AM; 10 μM for 20 min), a membrane permeant chelator of intracellular calcium. Similar results were obtained by removing extracellular calcium (data not shown). The bar in *A* shows the length of puffer application of carbachol for both records. Data are representative of at least five separate experiments.

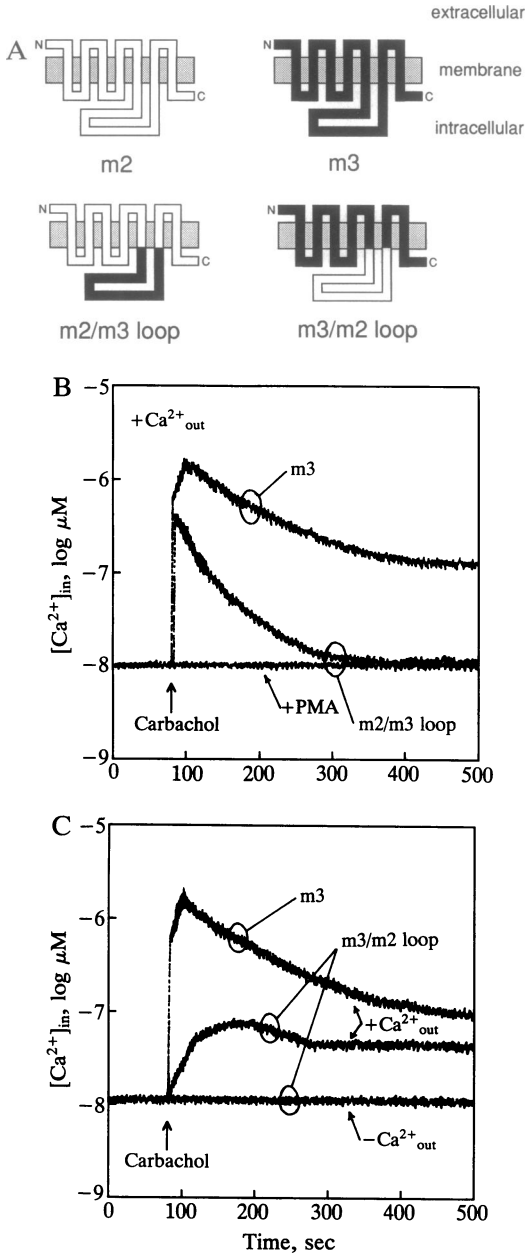

FIG. 5. Chimeric m2/m3 muscarinic receptors were expressed in A9 cells to test the role of the third cytoplasmic loop (the primary structural determinant of G protein-coupling selectivity) in regulation of both intracellular release and influx-mediated increases in intracellular free calcium ($[Ca^{2+}]_{in}$). (*A*) Schematic representations of wild-type m2 and m3 receptors and chimeric m2/m3 loop and m3/m2 loop receptors. (*B*) Carbachol (10 μM) stimulation of the m2 muscarinic receptor containing the m3 third cytoplasmic loop (middle curve) is compared to the typical response seen for the wild-type m3 receptor (top curve). The middle calcium curve resembles the InsP_3-mediated intracellular release with a loss of the plateau phase (compare to Fig. 3*A*). The bottom curve verifies the requirement for inositol phosphate generation on this curve by its disappearance after PMA (100 nM, 15–45 min) pretreatment. Carbachol (100 μM) had no effect on intracellular calcium levels in A9 cells expressing the wild-type m2 receptor or in sham-transfected cells (data not shown). (*C*) Carbachol (10 μM) stimulation of the m3 receptor containing the m2 third cytoplasmic loop (middle curve) is compared to the typical response seen for the wild-type m3 receptor (top curve). The middle calcium curve resembles the influx curve shown in Fig. 3*B*. The extracellular origin of the calcium was verified when removal of extracellular calcium completely eliminated the increase in intracellular free calcium (bottom curve). Data for each curve are representative of at least six experiments.

(Fig. 5*B*). The loss of the sustained phase observed with this chimeric receptor provides additional evidence that InsP_3 generation and intracellular calcium release are not sufficient for calcium-influx stimulation. In contrast to the wild-type m3 receptor, the m3 receptor/m2 loop construct failed to stimulate a rapid rise in calcium levels but instead stimulated a slow rise and sustained phase of intracellular free calcium (Fig. 5*C*). This calcium elevation was verified to be from an extracellular source when it was eliminated following removal of extracellular calcium (Fig. 5*C*). The m3 receptor/m2 loop construct had no appreciable effect on inositol phosphate release (16), arachidonic acid, or cAMP generation (data not shown), further dissociating activation of these second-messenger pathways from calcium-influx regulation. Therefore, the sustained phase of calcium is associated with a structural component of the m3 receptor other than the third cytoplasmic loop and is independent of second messengers known to be stimulated through m3 receptor activation.

DISCUSSION

These studies demonstrate the dissociation of receptor-operated calcium influx from calcium release from intracellular calcium stores. Although both influx and intracellular release appear to be initiated simultaneously, the influx component reaches its maximum more slowly, is of lower magnitude, and persists as long as agonist is present. In contrast, cytoplasmic release exhibits a rapid peak and is transient even in the presence of agonist. The m3 receptor-operated calcium influx in A9 cells appears to be similar to the ATP-stimulated calcium influx previously described in smooth muscle (7) in that influx did not require soluble second messengers. Unlike our electrophysiological studies in A9 cells, the channel present in smooth muscle was detectable using patch clamp techniques, suggesting possible differences in ion conductance. Calcium influx also has been described following the depletion of intracellular calcium stores, which is involved in the refilling of InsP_3-sensitive pools (8). However, in this study, calcium influx was observed in A9 cells, although intracellular stores had not been depleted previously. Thapsigargin, a non-phorbol-ester tumor promoter that stimulates the emptying of calcium stores and subsequently activates calcium influx, can stimulate the opening of a putative refilling channel in several cell types (26). Thapsigargin was ineffective in the A9 cell up to a concentration of 100 μM (unpublished observations), providing further evidence that a refilling channel is not involved in m3 receptor-activated calcium influx. It is not clear if a refilling channel similar to that seen in other cells is present in the A9 cell.

The activation by cholinergic agonists of the calcium-dependent potassium channels in this study is similar to that previously seen in A9 cells and has been attributed to the release of InsP_3 (27). While a component of the activation is due to InsP_3, our results demonstrate that activation of calcium-dependent potassium channels may also arise from an influx of extracellular calcium. Although our electrophysiological studies provided further evidence of m3 receptor-operated calcium influx, they failed to directly demonstrate the presence of a receptor-operated calcium channel similar to other studies (2). Receptor-operated calcium channels may be of very low conductance, low abundance, or both, making their direct measurement difficult.

We have shown that the third cytoplasmic loop of the m3 receptor is involved in triggering the cytoplasmic release of intracellular calcium, presumably due to InsP_3 generation (16). Calcium influx appears to be associated with a domain(s) of the m3 receptor other than the third cytoplasmic loop, since replacement of this domain from the m3 receptor with the analogous region of the m2 receptor (which had no

effect on intracellular calcium levels) failed to eliminate this response. Previous studies have demonstrated a role for second-messenger InsP_3 (3, 4), inositol 1,3,4,5-tetrakisphosphate (5), and intracellular calcium (6) in receptor-operated calcium influx. Our studies provide an additional and possibly more direct mechanism for receptor-operated calcium entry, one obviating the requirement for arachidonic acid, cAMP, inositol phosphates, or mobilization or depletion of intracellular calcium.

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1. Berridge, M. J. & Irvine, R. F. (1989) *Nature (London)* **341,** 197–205.
2. Meldolesi, J., Clementi, E., Fasolato, C., Zacchetti, D. & Pozzan, T. (1991) *Trends Pharmacol. Sci.* **12,** 289–292.
3. Bird, G. St. J., Rossier, M. F., Hughes, A. R., Shears, S. B., Armstrong, D. L. & Putney, J. W., Jr. (1991) *Nature (London)* **352,** 162–165.
4. Kuno, M. & Gardner, P. (1987) *Nature (London)* **326,** 301–304.
5. Irvine, F. & Moore, R. M. (1986) *Biochem. J.* **240,** 917–920.
6. von Tscharner, V., Prod'hom, B., Baggiolini, M. & Reuter, H. (1986) *Nature (London)* **324,** 369–372.
7. Benham, C. D. & Tsien, R. W. (1987) *Nature (London)* **328,** 275–278.
8. Putney, J. W. (1986) *Cell Calcium* **7,** 1–12.
9. Felder, C. C., Dieter, P., Kinsella, J., Tamura, K., Kanterman, R. Y. & Axelrod, J. (1991) *J. Pharmacol. Exp. Ther.* **255,** 1140–1147.
10. Felder, C. C., Kanterman, R. Y., Ma, A. L. & Axelrod, J. (1989) *J. Biol. Chem.* **264,** 20356–20362.
11. Conklin, B. R., Brann, M. R., Buckley, N. J., Ma, A. L., Bonner, T. I. & Axelrod, J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8698–8702.
12. Wolf, B. A., Turk, J., Sherman, W. R. & McDaniel, M. L. (1986) *J. Biol. Chem.* **261,** 3501–3511.
13. Horn, V. J., Baum, B. J. & Ambudkar, I. S. (1988) *J. Biol. Chem.* **263,** 12454–12460.
14. Bonner, T. I., Buckley, N. J., Young, A. C. & Brann, M. R. (1987) *Science* **237,** 527–532.
15. Wess, J., Brann, M. R. & Bonner, T. I. (1989) *FEBS Lett.* **258,** 133–136.
16. Wess, J., Bonner, T. I., Dorje, F. & Brann, M. R. (1990) *Mol. Pharmacol.* **38,** 517–523.
17. Grynkiewics, G., Poenie, M. & Tsien, R. Y. (1986) *J. Biol. Chem.* **260,** 3440–3450.
18. Jones, S. V. P., Barker, J. L., Bonner, T. I., Buckley, N. J. & Brann, M. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 4056–4060.
19. Orellana, S., Solski, P. A. & Brown, J. H. (1987) *J. Biol. Chem.* **262,** 1638–1643.
20. Smith, C. D., Uhing, R. J. & Snyderman, R. (1987) *J. Biol. Chem.* **262,** 6121–6127.
21. Janis, R. A., Silver, P. & Triggle, D. J. (1987) *Adv. Drug Res.* **16,** 309–591.
22. Kasai, H., Aosaki, T. & Fuduka, J. (1987) *Neurosci. Res.* **4,** 228–235.
23. Fox, A. P., Nowycky, M. C. & Tsien, R. W. (1987) *J. Physiol. (London)* **394,** 173–200.
24. Kubo, T., Bujo, H., Akiba, I., Nakai, J., Mishina, M. & Numa, S. (1988) *FEBS Lett.* **241,** 119–125.
25. Kobilka, B. K., Kobilka, T. S., Daniel, K., Regan, J. W., Caron, M. G. & Lefkowitz, R. J. (1988) *Science* **240,** 1310–1316.
26. Koshiyama, H. & Tashjian, A. H. (1991) *Biochem. Biophys. Res. Commun.* **177,** 551–558.
27. Jones, S. V. P., Barker, J. L., Goodman, M. B. & Brann, M. R. (1990) *J. Physiol. (London)* **421,** 499–519.