The m3 muscarinic acetylcholine receptor differentially regulates calcium influx and release through modulation of monovalent cation channels

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Several types of transmembrane receptors regulate cellular responses through the activation of phospholipase C-mediated Ca^{2+} release from intracellular stores. In non-excitabile cells, the initial Ca^{2+} release is typically followed by a prolonged Ca^{2+} influx phase that is important for the regulation of several Ca^{2+} -sensitive responses. Here we describe an agonist concentration-dependent mechanism by which m3 muscarinic acetylcholine receptors (mAChRs) differentially regulate the magnitude of the release and influx components of a Ca^{2+} response. In transfected Chinese hamster ovary cells expressing m3 mAChRs, doses of the muscarinic agonist carbachol ranging from 100 nM to 1 mM evoked Ca^{2+} release responses of increasing magnitude; maximal Ca^{2+} release was elicited by the highest carbachol concentration. In contrast, Ca^{2+} influx was maximal when m3 mAChRs were activated by moderate doses (1–10 μM) of carbachol, but substantially reduced at higher agonist concentrations. Manipulation of the membrane potential revealed that the carbachol-induced Ca^{2+} influx phase was diminished at depolarized potentials. Importantly, carbachol doses above 10 μM were found to couple m3 mAChRs to the activation of an inward, monovalent cation current resulting in depolarization of the cell membrane and a selective decrease in the influx, but not release, component of the Ca^{2+} response. These studies demonstrate, in one experimental system, a mechanism by which a single subtype of G-protein-coupled receptor can utilize the information encoded in the concentration of an agonist to generate distinct intracellular Ca^{2+} signals.

Keywords: calcium influx/cation channels/muscarinic receptors/signal transduction

Introduction

Intracellular calcium ions act as second messenger molecules to regulate a wide range of targets including metabolic enzymes, kinases, phosphatases and ion channels. Alterations in the activity of these proteins can cause broad physiological changes that ultimately affect the excitability, growth and survival of a cell (Tsien and Tsien, 1990;

Cheek, 1991; Clapham, 1995). Agonist stimulation of many different growth factor, antigen and G-protein-coupled receptors leads to an increase in intracellular Ca^{2+} through activation of various isoforms of phospholipase C (PLC) and the subsequent inositol 1,4,5-trisphosphate (IP3)-induced release of Ca^{2+} from intracellular stores (Berridge, 1993). In non-excitabile cells, the transient release of intracellular Ca^{2+} is usually accompanied by the influx of Ca^{2+} across the plasma membrane. This influx, sometimes termed capacitative Ca^{2+} entry, is associated with the emptying of intracellular Ca^{2+} stores and causes elevations of cytosolic Ca^{2+} that are of lesser magnitude than the initial release phase, but of substantially longer duration (Putney and Bird, 1993).

Whereas a variety of different transmembrane receptors regulate cellular responses through the activation of PLC, the intermediary Ca^{2+} signals generated by a given receptor can differ in several characteristics including the source and magnitude of the Ca^{2+} increase, as well as the spatial and temporal pattern of this increase (Lechleiter *et al.*, 1991; Petersen *et al.*, 1991; Fertwell, 1993; Thorn *et al.*, 1993). Moreover, certain Ca^{2+} -dependent effectors exhibit differential sensitivity to specific characteristics of a Ca^{2+} signal. For example, prolonged Ca^{2+} influx, rather than release, is important for the regulation of signaling proteins that mediate antigen-induced T-cell activation (Crabtree and Clipstone, 1994). In contrast, intracellular Ca^{2+} release, not influx, is crucial for the regulation of mitochondrial enzyme activities (Hajnóczky *et al.*, 1995). In the case of neurotransmitter or enzyme release from somatotrophs, salivary glands and pancreatic acinar cells, the frequency of agonist-stimulated Ca^{2+} oscillations has been correlated with regulation of the secretory activity of these cell types (Rapp and Berridge, 1981; Holl *et al.*, 1988; Stauffer *et al.*, 1993). Therefore, the ability of a receptor to generate a particular Ca^{2+} response profile may provide a mechanism for insuring signal specificity by enabling an agonist to selectively regulate only a subset of the Ca^{2+} -dependent effectors expressed within a cell.

Although much is known about the steps in the pathways leading from agonist-activated receptors to IP3-induced elevations of intracellular Ca^{2+} , far less is understood regarding the mechanisms by which receptors modulate particular characteristics of a Ca^{2+} response. In this study, we investigated the regulation of Ca^{2+} signaling by the m3 muscarinic acetylcholine receptor (mAChR) in transfected Chinese hamster ovary (CHO) cells. Previous studies have shown that m3 mAChRs couple to pertussis toxin-insensitive G proteins, such as G_{α_q} , to stimulate PLC β 1 activity in both excitable and non-excitabile cell types (Peralta *et al.*, 1988; Kunkel and Peralta, 1993; Sawaki *et al.*, 1993; Ashkenazi and Peralta, 1994). The increases in intracellular Ca^{2+} evoked by m3 mAChRs can be transient, sustained or oscillatory and are important

for the regulation of several cellular responses including enzyme activation, transcriptional induction and mitogenesis (Ashkenazi *et al.*, 1989; Lechleiter *et al.*, 1991; Felder *et al.*, 1993; Hu and El-Fakahanny, 1993). Here, we describe a signal transduction mechanism that allows m3 mAChRs to differentially regulate the release and influx components of a Ca^{2+} response. At low to moderate doses of the muscarinic agonist carbachol, m3 mAChRs were found to stimulate Ca^{2+} release, as well as maximal levels of Ca^{2+} influx. In contrast, we found that higher carbachol concentrations evoked peak Ca^{2+} release responses, but also triggered an active suppression of the influx phase of the Ca^{2+} signal. Electrophysiological analysis revealed that Ca^{2+} influx, but not release, was voltage sensitive and inhibited by membrane depolarization. Furthermore, we found that m3 mAChRs selectively inhibited Ca^{2+} influx by stimulating an inward, monovalent cation current; this current was only activated by high carbachol concentrations and caused a potent depolarization of the cell membrane. Therefore, these studies demonstrate how a single subtype of G-protein-coupled receptor can transduce agonist signals of varying concentration into distinct forms of Ca^{2+} responses through the selective modulation of ion channel activity.

Results

To investigate the regulation of Ca^{2+} signaling via a G-protein-coupled receptor, we generated stable transfected CHO cells expressing the m3 mAChR (CHO-m3 cells) and measured agonist-induced responses using a ratiometric Ca^{2+} indicator dye, fura-2. Consistent with previous findings, CHO-m3 cells exhibited a biphasic Ca^{2+} response when challenged with the acetylcholine analog carbachol (Dell'Acqua *et al.*, 1993). Addition of 1 μM carbachol evoked a large, transient rise in intracellular Ca^{2+} , followed by a prolonged phase of elevated Ca^{2+} levels that was due to the influx of extracellular Ca^{2+} (Figure 1A and B). The magnitude of the transient Ca^{2+} release increased with higher doses of carbachol and reached a maximum at concentrations from 10 μM to 1 mM. In contrast, the peak amplitude of the Ca^{2+} influx phase was elicited by low to moderate carbachol concentrations (1–10 μM) and was reduced to near basal levels by higher concentrations of agonist (Figure 1B). The magnitude of the influx phase was not influenced by the size of the initial transient Ca^{2+} release and suggested that these two characteristics of the Ca^{2+} response may be regulated independently by the m3 mAChR in these cells. Consistent with this possibility, the addition of a saturating dose of carbachol (1 mM) during the influx phase generated by the prior application of 10 μM carbachol caused a rapid reduction in cytosolic Ca^{2+} levels, but did not promote any further release of Ca^{2+} from internal stores (Figure 1C). This reduction was not the result of rapid receptor desensitization since other experiments demonstrated that m3 mAChRs are competent to signal following sequential additions of carbachol (see below; Figure 3B). Therefore, these experiments demonstrate that at high levels of agonist, m3 mAChRs selectively reduce the magnitude of the influx component of a Ca^{2+} response.

To examine the mechanism by which m3 mAChRs

Fig. 1. Ca^{2+} release and influx are independently regulated by the m3 mAChR in an agonist concentration-dependent manner. **(A)** Traces depict the average Ca^{2+} response of CHO-m3 cells treated with 1 μM carbachol while bathed in the presence (+ $\text{Ca}^{2+}_{\text{ext}}$) or absence (- $\text{Ca}^{2+}_{\text{ext}}$) of 2 mM extracellular Ca^{2+} . Intracellular Ca^{2+} levels were determined by ratiometric measurement of fura-2 fluorescence. Calibrated Ca^{2+} levels are plotted as a function of time (five experiments, 64 cells/experiment). Carbachol was added at the time indicated by the arrow. **(B)** Dose-response analysis of intracellular Ca^{2+} increases in CHO-m3 cells treated with carbachol. Absolute Ca^{2+} levels are plotted against time. The arrow marks the time of carbachol addition. Carbachol concentrations are shown on the right. **(C)** A representative trace showing the effect of 1 mM carbachol addition during the Ca^{2+} influx phase generated by a previous application of 10 μM carbachol. The trace depicts the change in Ca^{2+} levels plotted against time and is representative of 15 experiments (64 cells/experiment). Carbachol was added at the time indicated by the arrows.

inhibit Ca^{2+} influx, we first investigated the possible involvement of the products of PLC activation, IP₃ and diacylglycerol (DAG). IP₃ increases intracellular Ca^{2+} levels by activating Ca^{2+} release from intracellular stores (Berridge, 1993). Furthermore, the IP₃-derived metabolite

Fig. 2. Carbachol dose-response analysis of IP₃ generation in CHO cells expressing the m3 mAChR. IP₃ levels were measured in CHO-m3 cells 30 s after treatment with doses of carbachol ranging from 0.1–1000 μM. IP₃ levels of unstimulated control cells increased by 17±6 c.p.m. Atropine blocked all carbachol-induced IP₃ generation. Bars represent the SEM for each experiment ($n=2$).

inositol 1,3,4,5- tetrakisphosphate (IP₄) has been implicated in receptor-activated Ca²⁺ influx through membrane channels (Luckoff and Clapham, 1992). Therefore, we tested whether m3 mAChR-induced suppression of Ca²⁺ influx might involve an inhibition of IP₃ formation at high carbachol concentrations. A dose-response analysis of carbachol-mediated IP₃ generation demonstrated that IP₃ levels continued to increase at agonist concentrations up to 1 mM (Figure 2). Thus, the diminished Ca²⁺ influx response does not involve a reduced production of IP₃ and, presumably, IP₄ levels at high carbachol concentrations. It is possible that the other product of PLC activity, DAG, could play a role in modulating the Ca²⁺ influx response through activation of protein kinase C (PKC). For example, PKC-dependent phosphorylation of the IP₃ receptor may increase the flux of Ca²⁺ through this ligand-gated channel (Cameron *et al.*, 1995). To test whether the regulation of PKC activity by the m3 mAChR can affect Ca²⁺ influx, cells were treated with the PKC inhibitors staurosporine and GF109203X, or with the PKC activator phorbol myristate acetate. Neither the inhibition nor activation of PKC by these pharmacological agents had any detectable effect on basal or carbachol (1 μM or 1 mM) stimulated Ca²⁺ release or influx (data not shown; see Materials and methods for additional experimental details). Therefore, these experiments suggest that the mechanism by which m3 mAChRs selectively decrease the Ca²⁺ influx response does not involve an agonist-dependent decrease in PLC activity.

Previous studies have shown that m3 mAChRs stimulate at least two other kinase pathways that could possibly modulate the Ca²⁺ influx response in these cells. For example, high agonist concentrations have been shown to couple m3 mAChRs to the activation of adenylyl cyclase and the subsequent stimulation of cAMP-dependent protein kinase A (Peralta *et al.*, 1988; Conklin *et al.*, 1989; R.C.Carroll, unpublished data). Therefore, to determine whether cAMP signaling may play a role in modulating carbachol-induced Ca²⁺ influx or release, cells were treated with cholera toxin, an activator of the stimulatory G protein of adenylyl cyclase, or dibutyryl cAMP, a membrane

permeable activator of protein kinase A. As with the PKC activator and inhibitors, these agents had no detectable effect on any characteristic of the m3 mAChR-mediated Ca²⁺ response (data not shown). In addition to regulating serine/threonine kinase activity, G_{αq}-coupled mAChRs have also been shown to stimulate non-receptor tyrosine kinases. Carbachol-stimulated tyrosine kinase activity mediates the suppression of a delayed rectifier K⁺ channel termed Kv1.2 and is also required for the influx component of the Ca²⁺ response in certain cells (Huang *et al.*, 1993; Lee *et al.*, 1993; Gronroos *et al.*, 1995; Meucci *et al.*, 1995). A possible role for tyrosine phosphorylation in the regulation of Ca²⁺ influx was tested by treating CHO-m3 cells with the tyrosine kinase-specific inhibitor genistein or with the tyrosine phosphatase inhibitor, pervanadate (see Materials and methods for experimental details). Once again, treatments with pharmacological agents that should either enhance or reduce the effects of tyrosine kinase signaling did not alter the m3 mAChR-induced Ca²⁺ influx or release responses over a wide range of agonist concentrations and incubation times (data not shown). Whereas it is possible that yet unknown kinases or phosphatases that were unaffected by our treatments could be responsible for mediating the inhibition of Ca²⁺ influx observed at high carbachol concentrations, these experiments suggest that the most prevalent serine/threonine and tyrosine phosphorylation pathways associated with m3 mAChR signaling are not involved in this response.

One of the principal mechanisms by which G-protein-coupled receptors regulate cell physiology is through the modulation of ion channels including delayed and inward rectifier K⁺ channels, Ca²⁺-activated K⁺ channels, voltage-gated Ca²⁺ channels, and non-specific cation channels (Brown and Birnbaumer, 1990; Huang *et al.*, 1993; Kunkel and Peralta, 1995). To determine whether m3 mAChR-induced changes in ion channel activity contribute to the regulation of the Ca²⁺ influx response, we conducted whole-cell voltage clamp and current clamp experiments to test the dose-dependent effects of carbachol on the electrophysiological properties of CHO-m3 cells. Activation of m3 mAChRs with doses of ≥10 μM carbachol evoked a rapid and substantial depolarization of the cell membrane (Figure 3A). For example, addition of 1 mM carbachol caused a rapid depolarization of the membrane from a typical resting potential of -29 ± 8 mV to -8 ± 3 mV. Similar to the dose-dependent reduction of Ca²⁺ influx, carbachol concentrations of <10 μM had little effect on the membrane potential, whereas maximal depolarization was evoked by 1 mM carbachol. The m3 mAChR-mediated depolarization did not undergo desensitization since initial application of low concentrations of carbachol did not prevent the depolarization caused by the subsequent addition of higher carbachol doses (Figure 3B). In addition, cells that were stimulated with a single concentration of carbachol depolarized to the same extent as those that were treated with consecutive doses of agonist.

Since m3 mAChR-induced membrane depolarization and inhibition of Ca²⁺ influx exhibited the same carbachol dose-response characteristics, we investigated whether the two events are functionally related. In some cell types, the Ca²⁺ influx response linked to internal store depletion is sensitive to the hyperpolarized state of the cell membrane (Matthews *et al.*, 1989; Laskey *et al.*, 1990; Girard and

Fig. 3. High carbachol concentrations depolarize the membrane of CHO-m3 cells and block the voltage-sensitive Ca^{2+} influx response. **(A)** Changes in the membrane potential of a CHO-m3 cell held in current clamp following the addition of 1 mM carbachol. The carbachol-induced depolarization was inhibited by the subsequent application of 200 μM atropine, a muscarinic antagonist. Arrows indicate the time of carbachol and atropine addition. The trace is representative of eight experiments. **(B)** The CHO-m3 cell membrane potential is depolarized by carbachol treatment in a dose-dependent manner. The cell was held in whole-cell current clamp to allow measurement of the membrane potential as increasing doses of carbachol (1.0–1000 μM) were added to the bath at the times indicated by the arrows. Each successive carbachol dose was added after the membrane potential had stabilized following the previous agonist addition. Similar results were obtained in four independent experiments. **(C)** Fura-2-loaded CHO-m3 cells bathed in physiological extracellular saline were stimulated with 10 μM carbachol (arrow). During the Ca^{2+} influx phase, the concentration of external K^+ was increased from 5 to 40 mM (by addition of KCl to the bath) at the time indicated by the arrow. Similar results were obtained when K aspartate was used to increase external K^+ . The trace shown is representative of eight experiments (64 cells/experiment). Ca^{2+} measurements were performed as described above. **(D)** The carbachol-stimulated Ca^{2+} influx phase in CHO-m3 cells is reduced when the membrane is held at a depolarized potential using whole-cell voltage clamp. The thin trace represents the average Ca^{2+} response of 20 control (unclamped) cells treated with 10 μM carbachol. The thick line depicts the Ca^{2+} response of a voltage-clamped cell in the same field as the control cells. The clamped cell was depolarized to 0 mV (D) before the addition of 1 μM carbachol. Ca^{2+} influx was restored when the depolarizing voltage clamp was released (R). Similar results were observed in five cells. **(E)** Carbachol-mediated Ca^{2+} influx is greatly potentiated in a CHO-m3 cell which is prevented from depolarizing by voltage clamp. The thin trace shows the average Ca^{2+} response of 20 control cells treated with 1 mM carbachol. The thick trace represents the Ca^{2+} response of a cell held at its normal resting potential (–30 mV) (H) before the addition of 1 mM carbachol. Ca^{2+} influx in the clamped cell was greatly reduced after the cell was released (R) from voltage clamp. This experiment was repeated four times with similar results.

Clapham, 1993). Therefore, we employed complementary methods to test the possible voltage dependence of the Ca^{2+} influx response in CHO-m3 cells. To determine whether depolarization of the cell membrane was sufficient to inhibit carbachol-induced Ca^{2+} influx, we stimulated cells with a dose of carbachol (10 μM) that generated a large Ca^{2+} influx response and then depolarized the cells by increasing the external K^+ concentration from 5 to 40 mM (Figure 3C). The addition of high external K^+ led to an immediate drop in the magnitude of the Ca^{2+} influx phase. Secondly, we tested the voltage sensitivity of the carbachol-induced Ca^{2+} influx by directly manipulating the membrane potential using whole-cell voltage clamp while simultaneously measuring the intracellular Ca^{2+} concentration in carbachol-stimulated cells. CHO-m3 cells were treated first with a dose of carbachol (1 μM) that produced a large Ca^{2+} influx response, but no depolarization of the cell membrane (Figure 3D). Importantly, when these cells were held at a depolarized membrane potential (0 mV) by voltage clamp, we observed a substantially reduced Ca^{2+} influx phase that was restored immediately

upon release of the cells from the depolarized potential (Figure 3D). Similarly, we used voltage clamp and simultaneous Ca^{2+} measurements to determine whether the reduction in the Ca^{2+} influx response caused by high doses of carbachol required membrane depolarization. For this experiment, cells were held by voltage clamp at their normal resting potential (–30 mV) and then treated with a dose of carbachol (1 mM) that normally evokes significant membrane depolarization, but little Ca^{2+} influx (Figures 1B and 3E). Under these conditions, cells exhibited atypically large levels of Ca^{2+} influx as long as the voltage clamp prevented the normal depolarization response caused by 1 mM carbachol (Figure 3E). Together, these results demonstrate that carbachol-induced Ca^{2+} influx in CHO cells is voltage sensitive and inhibited by membrane depolarization.

To determine the mechanism by which the m3 mAChR causes membrane depolarization and thereby inhibits Ca^{2+} influx, we characterized the ionic currents that are modulated selectively by high doses of carbachol. In a large proportion of voltage-clamped cells (>50%) an outwardly

Fig. 4. Carbachol activates a monovalent cation channel in CHO-m3 cells. **(A)** Cells were held at -30 mV and stepped from -80 to 60 mV in 20 mV increments for 200 ms. Three recordings were made in physiological saline before and after the addition of 1 mM carbachol; the traces represent the averaged difference current derived from these recordings. **(B)** Current (pA)–voltage (mV) relationship of the carbachol-induced (difference) current shown in (A). See Materials and methods for additional details.

rectified current developed spontaneously (data not shown). Ion substitution experiments revealed that the current was carried by K^+ . We found that the K^+ current was strongly suppressed by carbachol doses ≥ 10 μ M. The current was blocked by Ba^{2+} , Cs^{2+} and Co^{2+} (20 mM), as well as high concentrations of external Ca^{2+} and TEA (100 mM). Complete, carbachol-induced depolarization from normal resting potentials occurred in cells in which this K^+ channel was completely blocked by Co^{2+} or TEA. Furthermore, treatment of cells with TEA had no effect on the agonist-induced Ca^{2+} influx. Therefore, the m3 mAChR-mediated suppression of this K^+ current does not appear to play a direct role in the reduction of Ca^{2+} influx which occurs at high doses of carbachol. However, following the addition of carbachol (≥ 10 μ M), a small ionic current was activated (Figure 4A). This current was inhibited by application of the muscarinic antagonist atropine (data not shown). In normal physiological saline, the m3 mAChR-activated current was not rectified and exhibited a reversal potential of ~ 15 mV (Figure 4B). Shift in the reversal potential of the current was observed when the concentration of either Na^+ or K^+ was altered in the recording solutions, indicating that a monovalent cation channel was responsible for conducting the carbachol-induced current (Materials and methods). Consistent with this possibility, substitution of either Ca^{2+} or Cl^- within the recording solutions did not

affect the reversal potential of this current. The m3 mAChR-activated current was blocked by high concentrations (20 mM) of either Ba^{2+} or Cs^{2+} , but was not affected by the addition of Co^{2+} , Gd^{3+} or tetraethylammonium chloride. Previous studies of receptor-regulated ion channels in non-excitable cells have reported the activation of a Ca^{2+} -permeant cation current; activation of this current has been correlated with the Ca^{2+} influx observed following the release of Ca^{2+} from internal stores (Felder *et al.*, 1991; Vaca *et al.*, 1994). However, the carbachol-activated cation current observed in CHO-m3 cells does not appear to conduct the Ca^{2+} influx response since stimulation of the cation current is associated with reduced influx. Furthermore, the m3 mAChR-activated current was not dependent on extracellular Ca^{2+} .

Whereas the m3 mAChR activated current is small, the near absence of other conductances at negative potentials make it likely that activation of this current is sufficient to cause the observed carbachol-induced changes in membrane potential. Recorded cells had input resistances of 500 M Ω to 1 G Ω , and prior to the addition of carbachol, the only measurable conductance in the CHO cells was that of the outwardly rectified K^+ current which displayed very small currents (<10 pA) around the cells' normal resting potential. The addition of carbachol blocked this K^+ current almost completely and the only conductance activated by carbachol was that of the cationic current. Thus, electrophysiological analysis indicates that high concentrations of carbachol couple m3 mAChRs to activation of an inward monovalent cation current that causes depolarization of the cell membrane.

The role of the monovalent cation channel in regulating Ca^{2+} influx was tested directly by interfering with the inward Na^+ current carried by this channel. For these experiments, carbachol-induced Ca^{2+} responses were recorded from cells that were incubated in an extracellular saline solution in which Na^+ was replaced with *N*-methyl-D-glucamine (NMDG $^+$), a monovalent cation which does not pass through Na^+ and cation channels (Marcus *et al.*, 1992; Inoue *et al.*, 1995). Replacement of Na^+ with NMDG $^+$ eliminated carbachol-induced depolarization of the cell membrane (Figure 5A). Basal Ca^{2+} levels were unaffected by the absence of extracellular Na^+ , however, cells treated with doses of carbachol up to 1 mM exhibited large Ca^{2+} influx responses in the absence of m3 mAChR-induced membrane depolarization (Figure 5B). To establish that the robust Ca^{2+} influx responses evoked by high carbachol concentrations were due to the lack of an m3 mAChR-induced depolarization rather than some unexpected consequence of the lack of extracellular Na^+ , cells in NMDG $^+$ saline were stimulated with 1 mM carbachol and then depolarized by the addition of high external K^+ during the Ca^{2+} influx phase (Figure 5C). As expected for a depolarization-dependent mechanism, addition of 40 mM K^+ resulted in a potent decrease in Ca^{2+} influx in the absence of external Na^+ (Figure 5C). Finally, to confirm that the requirement for external Na^+ was related to the inward current carried by the carbachol-activated monovalent cation current rather than for the activity of a cell membrane Na^+ – Ca^{2+} exchanger, we conducted experiments in which external Na^+ was replaced with Li^+ , a cation which blocks Na^+ – Ca^{2+} exchanger activity, but is permeant through many cation channels (Marcus

et al., 1992; Bouchard *et al.*, 1993; Wacholtz *et al.*, 1993). When Li^+ was substituted for Na^+ , high doses of carbachol evoked a potent depolarization of the cell membrane (data not shown). Furthermore, the Ca^{2+} release and influx responses generated by low and high doses of carbachol exhibited the same profiles as those recorded in the standard Na^+ -containing saline solution (Figure 5D). Together, these results demonstrate that Na^+ influx through the carbachol-activated cation channel causes membrane depolarization and a concomitant reduction in the influx phase of the Ca^{2+} response.

Discussion

Receptor-activated Ca^{2+} signals can differ in several characteristics including the magnitude of intracellular Ca^{2+} increase, the relative contribution of Ca^{2+} release and influx, as well as the sustained or repetitive nature of the Ca^{2+} signal (Rooney *et al.*, 1990; Tsien and Tsien, 1990; Lechleiter *et al.*, 1991; Petersen *et al.*, 1991). Depending upon the cell type, these distinct qualities of a Ca^{2+} signal can be interpreted by Ca^{2+} -sensitive effector proteins to regulate multiple physiological responses including transcription induction, neurotransmitter or hormone secretion, enzyme activation and cell differentiation (Stauffer *et al.*, 1993; Carroll *et al.*, 1995; Hajnoczky *et al.*, 1995; Rusanescu *et al.*, 1995; Dolmetsch *et al.*, 1997). Therefore, delineation of the mechanisms by which neurotransmitters and other agonists modulate specific aspects of intracellular Ca^{2+} signals is fundamentally important to the understanding of many different signal transduction pathways. In this work, we have characterized a mechanism in CHO cells by which a single subtype of G-protein-coupled receptor can utilize the information encoded in the concentration of an agonist to differentially regulate the influx and release components of a Ca^{2+} response. We found that the magnitude of carbachol-induced intracellular Ca^{2+} release was maximal when high concentrations of agonist were applied to CHO-m3 cells. In contrast, m3 mAChRs activated maximal Ca^{2+} influx at low to moderate doses of agonist, whereas higher carbachol concentrations caused a selective inhibition of this response. One possibility for the observed reduction of the Ca^{2+} influx phase at high doses of carbachol may be the activation of membrane Ca^{2+} ATPases. It has been reported that $\text{G}_{\beta\gamma}$ subunits released from $\text{G}_{\alpha s}$ -coupled receptors may activate a plasma membrane ATPase (Lotersztan *et al.*, 1992). However, our electrophysiological assays demonstrated that the Ca^{2+} influx signal is voltage sensitive and that m3 mAChRs inhibited this component of the Ca^{2+} response by activating a monovalent cation channel which caused depolarization of the cell membrane. Interestingly, the voltage sensitivity of the

Fig. 5. Removal of extracellular Na^+ blocks carbachol-dependent depolarization of CHO-m3 cells and prevents the reduction of Ca^{2+} influx normally observed at high doses of carbachol. (A) Recording of the membrane potential of a CHO-m3 cell held in current clamp and bathed in extracellular saline containing Na^+ (thin trace) or extracellular saline in which Na^+ was replaced by NMDG^+ (thick trace). In normal (Na^+ -containing) extracellular saline the cell depolarizes following the addition of 1 mM carbachol, however, in the absence of extracellular Na^+ there is no carbachol-mediated change in the membrane potential. Similar results were obtained in five cells. (B) Ca^{2+} levels of cells stimulated with 1 μM (thick traces) or 1000 μM (thin traces) carbachol maintained in either normal saline (dashed lines) or Na^+ -depleted (NMDG^+ containing) saline (solid lines). The arrow indicates the time of carbachol addition. Traces represent the average of seven experiments (64 cells/experiment). (C) A representative trace of the intracellular Ca^{2+} concentration in a cell maintained in NMDG^+ saline (without Na^+). Arrow indicates the time of 1 mM carbachol application. Arrow indicates the time at which 40 mM KCl was added to the bath to depolarize the membrane. Similar results were obtained in six experiments. (D) Traces represent the Ca^{2+} response of cells stimulated with 1 μM (thick traces) or 1000 μM (thin traces) carbachol bathed in either normal extracellular saline (Na^+ , dotted lines) or saline in which Na^+ was replaced by Li^+ (Li^+ , solid lines). The arrow indicates the time of carbachol addition. Each trace represents the average of six experiments.

Ca^{2+} influx signal and the dose-dependent activation of the monovalent cation channel allow the m3 mAChR to translate even small differences in agonist concentration into subtle alterations in the overall shape of the Ca^{2+} signal (Figure 1B). Moreover, this mechanism extends the range of agonist concentrations over which m3 mAChRs can generate distinct Ca^{2+} signals beyond the moderate doses that cause maximal activation of both Ca^{2+} release and influx. Although the signaling pathway characterized here is in CHO cells, the components involved are widely expressed indicating potential significance for this mechanism in other systems.

Depolarization of the cell membrane may cause a reduction of Ca^{2+} influx through at least two mechanisms. It is possible that the channels which conduct Ca^{2+} across the cell membrane following the initial release phase are themselves inactivated at depolarized potentials. We were unable to examine this directly since we have not isolated the ionic current responsible for Ca^{2+} influx. However, previous studies in other cell types have described currents that may be related to the Ca^{2+} release-activated Ca^{2+} influx observed in CHO-m3 cells. These include a highly Ca^{2+} -selective current first recorded in mast cells, as well as a less selective cation current carried by the ion channel protein encoded by the *Drosophila trp* gene (Hoth and Penner, 1993; Vaca *et al.*, 1994). Neither the Ca^{2+} -selective mast cell channel nor the *trp* cation channel display voltage-dependent inactivation (Hoth and Penner, 1993; Vaca *et al.*, 1994). Therefore, if similar proteins conduct the influx response in CHO cells, it is unlikely that the carbachol-induced reduction of Ca^{2+} influx involves a voltage-dependent change in the gating properties of these channels. Alternatively, rather than directly regulating the gating of a Ca^{2+} channel, the mechanism by which receptor-mediated depolarization inhibits the influx response may involve a reduction in the electrochemical driving force for the inward flow of Ca^{2+} . The depolarizing Na^+ current conducted by the carbachol-activated monovalent cation channel may simply reduce the driving force for inward Ca^{2+} flow carried by a separate population of influx channels; under these conditions, Ca^{2+} efflux mechanisms may predominate and thus result in a dramatic reduction in the concentration of cytosolic Ca^{2+} . Significantly, both the Ca^{2+} -selective mast cell current and *trp*-mediated current exhibit a sharp increase in inward Ca^{2+} movement at hyperpolarized potentials. Two characteristics of the m3 mAChR-induced inhibition of Ca^{2+} influx support a driving force-dependent mode of action. First, the magnitude of the carbachol-induced depolarization correlated directly with the extent of Ca^{2+} influx suppression as predicted for a mechanism involving a reduction in driving force (Laskey *et al.*, 1990). Secondly, we have recently found that by increasing the driving force for Ca^{2+} influx through addition of a high extracellular Ca^{2+} recording solution, m3 mAChRs evoke large influx signals even at high carbachol concentrations that normally inhibit this response (R.C.Carroll, unpublished observation).

The mechanism by which m3 mAChRs activate the monovalent cation channel remain unknown. Interestingly, maximal stimulation of this channel required saturating concentrations of agonist and therefore resulted from activation of the majority of the m3 mAChRs expressed on a cell. In contrast, substantial PLC stimulation and

Ca^{2+} release were evoked when only a fraction of the m3 mAChR population was activated by lower concentrations of agonist. Previous studies have identified other examples of G-protein-coupled receptors that regulate multiple signaling pathways with different agonist efficacies. Most notably, m2 mAChRs, α_2 adrenergic and luteinizing hormone receptors have been shown to regulate adenylyl cyclase at low agonist concentrations, but also activate PLC signaling when treated with higher levels of agonist (Ashkenazi *et al.*, 1987; Raymond *et al.*, 1991; Guderman *et al.*, 1992). The selective regulation of effector enzymes by distinct concentrations of agonist may reflect the differential coupling of a receptor to two or more types of heterotrimeric G proteins. Therefore, it is possible that stimulation of the monovalent cation channel involves activation of a low abundance G protein or one that is inefficiently coupled to the m3 mAChR. Alternatively, it has been proposed that a single heterotrimeric G protein may regulate multiple effectors as a result of the differential sensitivity of these effectors to either G_α or $G_{\beta\gamma}$ signals derived from the same population of heterotrimers (Ashkenazi *et al.*, 1989; Birnbaumer, 1992). For example, effectors such as adenylyl cyclase are regulated by relatively low concentrations of G_α subunits, whereas activation of other targets, including certain β isoforms of PLC, requires higher amounts of $G_{\beta\gamma}$ (Birnbaumer, 1992). Thus, it is possible that m3 mAChR-mediated activation of the monovalent cation channel may require the substantially higher levels of $G_{\beta\gamma}$ subunits that are only achieved when cells are treated with large concentrations of agonist. Future studies will examine the relative contributions of G protein subunits to activation of the monovalent cation current by m3 mAChRs, as well as investigate the role of this current in modulating Ca^{2+} influx and other downstream cellular responses.

Materials and methods

The PKC inhibitor GF109203X was obtained from LC Laboratories. Fura-2AM and Pluronic F127 were purchased from Molecular Probes. All cell culture materials were obtained from Gibco-BRL. All other chemicals were purchased from Sigma.

Cell culture

CHO cells were transfected with a plasmid encoding the human m3 mAChR under transcriptional control of the SV40 early promoter as described previously (Peralta *et al.*, 1987, 1988). Stable transfections were selected and amplified in 25 nM methotrexate; similar results were obtained with several independent cell lines expressing the m3 mAChR. The CHO-m3 line expressed $\sim 4 \times 10^5$ receptors per cell, as determined by Scatchard analysis (Peralta *et al.*, 1988). Cells were grown in DMEM/F12 media containing 10% dialyzed FBS, penicillin/streptomycin, and L-glutamine and maintained at 37°C in 5% CO_2 .

Calcium imaging and cell treatments

CHO cells were trypsinized and plated on glass coverslips 48 h prior to each experiment. Cells were loaded with fura-2 AM in the presence of 0.03% pluronic F127 for 1 h at room temperature in Dulbecco's modified Eagle's medium without phenol red. Ca^{2+} responses were averaged from 64 cells per experiment. Coverslips with fura-2-loaded cells were placed in a glass Petri dish containing physiological saline (120 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 23 mM glucose, 52.4 mM sucrose pH 7.3). The dish was placed in a heating chamber on a Nikon Diaphot microscope and maintained at 37°C for the duration of the experiment. Intracellular Ca^{2+} concentrations were determined by ratiometric analysis as described previously (Grynkiewicz *et al.*, 1985; Dell'Acqua *et al.*, 1993).

In experiments using pervanadate (100 μ M), vanadate (100 μ M), staurosporine (1 mM), GF109203X (15 μ M) and dibutyryl cAMP (20 μ M), the drugs were added 10 min before treatment with either 1 μ M or 1 mM carbachol while the cells were maintained at 37°C. In experiments using cholera toxin (6 μ g/ml), genistein (30 μ g/ml) and PMA (10 μ M), cells were treated with the drug for incubation times ranging from 5–45 min prior to the addition of carbachol.

Electrophysiology

Whole-cell voltage clamp studies were carried out in extracellular saline containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 23 mM glucose, 52.4 mM sucrose (pH 7.3). In ion replacement studies, Na^+ was replaced with equimolar NMDG $^+$. For experiments involving an increase in extracellular K^+ , the increased K^+ was derived by the addition of KAsp. The recording pipette saline used, contained 100 mM KAsp, 5 mM NaCl, 270 nM MgCl_2 , 5 mM HEPES, 2 mM ATP and 100 μ M GTP (pH 7.2).

Electrodes were pulled with an L/U-3P (List-Medical) puller in two stages from soda-lime glass (VWR). The pipettes had an average resistance of 1 M Ω . Cells were cultured as described for calcium imaging experiments. Cells were viewed on a Nikon microscope and electrodes were positioned using Narashige and Burleigh manipulators. Grounding was achieved using silver chloride wires inserted into an agar bridge of 2% agar in extracellular saline.

An Axopatch 200 was used for voltage and current clamp recordings. Data acquisition and analysis were carried out on a Gateway 2000 computer using pClamp6 software (Axon). Data were low-pass filtered at 1 kHz. Series resistance was compensated for electronically.

Experiments recording the activity of the small, linear, carbachol-activated current were carried out in the presence of 20 mM Co^{2+} to eliminate any interference from the K^+ current described above. Cells were held at -30 mV and stepped from -80 to 60 mV in 20 mV increments for 200 ms. The current was detected by subtracting the average of three consecutive recordings taken after carbachol stimulation from the average of three consecutive recordings made before the addition of agonist. The shift of the reversal potential of the carbachol-activated current to more hyperpolarized potentials in the presence of reduced external Na^+ or to more depolarized potentials in the presence of increased external K^+ defined this as a cation current. Specifically, a reversal potential of 15 ± 2 mV was observed in normal saline (120 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 23 mM glucose, 52.4 mM sucrose pH 7.3), -5 ± 3 mV in 55 mM external Na^+ (NMDG $^+$ substituted for Na^+), and -25 ± 2 mV in 0 mM external Na^+ (NMDG $^+$ substituted for Na^+). The reversal potential of the carbachol-activated current was shifted to 30 ± 1 mV when external K^+ was elevated from 5 to 45 mM. In contrast, the reversal potential was not significantly altered (17 ± 6 mV) when Ca^{2+} was removed from the external solution. All reversal potential measurements are given $\pm$ SEM from recordings of 4–7 cells performed under each saline condition.

Measurement of phosphoinositide hydrolysis

Agonist-stimulated PI hydrolysis was assayed in 6-well plates with adherent CHO cells labeled for 12 h with [^3H]myo-inositol by measuring the accumulation of IP_3 using anion exchange chromatography (Berridge *et al.*, 1983; Peralta *et al.*, 1988; Ashkenazi *et al.*, 1989). During the assays 10 mM LiCl was included to inhibit inositol monophosphatase activity. Reactions were stopped with ice cold 15% TCA 30 s after the addition of agonist. In the absence of agonist, basal IP_3 levels in CHO-m3 cells increased by 17 ± 6 c.p.m./ 10^6 cells during the 30 s incubation. The results were derived from two experiments, with each point determined in triplicate.

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References

Ashkenazi, A. and Peralta, E.G. (1994) Muscarinic acetylcholine receptors. In Peroutka, S.J. (ed.), *Handbook of Receptors and Channels*. CRC Press, Ann Arbor, MI, pp. 1–27.

Ashkenazi, A., Winslow, J.W., Peralta, E.G., Peterson, G.L., Schimerlik,

M.I., Capon, D.J. and Ramachandran, J. (1987) An M2 muscarinic receptor subtype coupled to both adenylyl cyclase and phosphoinositide turnover. *Science*, **238**, 672–675.

Ashkenazi, A., Ramachandran, J. and Capon, D. (1989) Acetylcholine analogue stimulates DNA synthesis in brain-derived cells via specific muscarinic receptor subtypes. *Nature*, **340**, 146–150.

Berridge, M.J. (1993) Inositol trisphosphate and calcium signalling. *Nature*, **361**, 315–325.

Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P. and Irvine, R.F. (1983) Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J.*, **212**, 473–482.

Birnbaumer, L. (1992) Receptor-to-effector signaling through G proteins: roles for $\beta\gamma$ dimers as well as α subunits. *Cell*, **71**, 1069–1072.

Bouchard, R.A., Clark, R.B. and Giles, W.R. (1993) Role of sodium–calcium exchange in activation of contraction in rat ventricle. *J. Physiol.*, **472**, 391–413.

Brown, A. and Birnbaumer, L. (1990) Ionic channels and their regulation by G protein subunits. *Annu. Rev. Physiol.*, **52**, 197–213.

Cameron, A.M., Steiner, J.P., Roskams, A.J., Ali, S.M., Ronnett, G.V. and Snyder, S.H. (1995) Calcineurin associated with the inositol 1,4,5-trisphosphate receptor–FKBP12 complex modulates Ca^{2+} flux. *Cell*, **83**, 463–472.

Carroll, R.C., Morielli, A.D. and Peralta, E.G. (1995) Coincidence detection at the level of phospholipase C activation mediated by the m4 muscarinic acetylcholine receptor. *Curr. Biol.*, **5**, 536–544.

Cheek, T.R. (1991) Calcium regulation and homeostasis. *Curr. Opin. Cell Biol.*, **3**, 199–205.

Clapham, D.E. (1995) Calcium signaling. *Cell*, **80**, 259–268.

Conklin, B.R., Brann, M.R., Buckley, N.J., Ma, A.L., Bonner, T.I. and Axelrod, J. (1988) Stimulation of arachidonic acid release and inhibition of mitogenesis by cloned genes for muscarinic receptor subtypes stably expressed in A9L cells. *Proc. Natl Acad. Sci. USA*, **85**, 8698–8702.

Crabtree, G.R. and Clipstone, N.A. (1994) Signal transmission between the plasma membrane and nucleus of T lymphocytes. *Annu. Rev. Biochem.*, **63**, 1045–1083.

Dell'Acqua, M.L., Carroll, R.C. and Peralta, E.G. (1993) Transfected 2 muscarinic acetylcholine receptors couple to $\text{G}_{\alpha 2}$ and $\text{G}_{\alpha 3}$ in Chinese hamster ovary cells. *J. Biol. Chem.*, **268**, 5676–5685.

Dolmetsch, R.E., Lewis, R.S., Goodnow, C.C. and Healy, J.L. (1997) Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature*, **386**, 855–858.

Felder, C.C., Poulter, M. and Wess, J. (1991) Muscarinic receptor-operated Ca^{2+} influx in transfected fibroblast cells is independent of inositol phosphates and release of intracellular Ca^{2+} . *Proc. Natl Acad. Sci. USA*, **89**, 509–513.

Felder, C.C., MacArthur, L., Ma, A.L., Gusovsky, F. and Kohn, E.C. (1993) Tumor suppressor function of muscarinic acetylcholine receptors is associated with activation of receptor-operated calcium influx. *Proc. Natl Acad. Sci. USA*, **90**, 1706–1710.

Fertwell, C. (1993) Ca^{2+} oscillations in non-excitable cells. *Annu. Rev. Physiol.*, **55**, 427–454.

Girard, S. and Clapham, D. (1993) Acceleration of intracellular calcium waves in *Xenopus* oocytes by calcium influx. *Science*, **260**, 229–232.

Gronroos, E., Schippert, A., Engstrom, M. and Sjölander, A. (1995) The regulation of leukotriene D-4-induced calcium influx in human epithelial cells involves protein tyrosine phosphorylation. *Cell Calcium*, **17**, 177–186.

Gryniewicz, G.M., Poenic, M. and Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

Gudermann, T., Birnbaumer, M. and Birnbaumer, L. (1992) Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and calcium mobilization: Studies with the cloned murine luteinizing hormone receptor expressed in L cells. *J. Biol. Chem.*, **267**, 4479–4488.

Hajnóczky, G., Robb-Gaspers, L.D., Seitz, M.B. and Thomas, A.P. (1995) Decoding of cytosolic calcium oscillations in the mitochondria. *Cell*, **82**, 415–424.

Holl, R.W., Thorner, M.O., Mandell, G.L., Sullivan, J.A., Sinha, Y.N. and Leong, D.A. (1988) Spontaneous oscillations of intracellular calcium and growth hormone secretion. *J. Biol. Chem.*, **263**, 9682–9685.

Hoth, M. and Penner, R. (1993) Calcium release-activated calcium current in rat mast cells. *J. Physiol.*, **465**, 359–386.

- Hu,J. and El-Fakahanny,E.E. (1993) Role of intercellular and intracellular communication by nitric oxide in coupling of muscarinic receptors to activation of guanylate cyclase in neuronal cells. *J. Neurochem.*, **61**, 578–585.
- Huang,X.Y., Morielli,A.D. and Peralta,E.G. (1993) Tyrosine kinase-dependent suppression of a potassium channel by the G protein-coupled m1 muscarinic acetylcholine receptor. *Cell*, **75**, 1145–1156.
- Inoue,K., Nakazawa,K., Inoue,K. and Fujimori,K. (1995) Nonselective cation channels coupled with tachykinin receptors in rat sensory neurons. *J. Neurophys.*, **73**, 736–742.
- Kunkel,M.T. and Peralta,E.G. (1993) Charged amino acids required for signal transduction by the m3 muscarinic acetylcholine receptor. *EMBO J.*, **12**, 3809–3815.
- Kunkel,M.T. and Peralta,E.G. (1995) Identification of domains conferring G protein regulation on inward rectifier potassium channels. *Cell*, **83**, 443–449.
- Laskey,R.E., Adams,D.J., Johns,A., Rubanyi,G.M. and Van-Breemen,C. (1990) Membrane potential and Na⁺–K⁺ pump activity modulate resting and bradykinin-stimulated changes in cytosolic free calcium in cultured endothelial cells from bovine atria. *J. Biol. Chem.*, **265**, 2613–2618.
- Lechleiter,J., Girard,S., Clapham,D. and Peralta,E. (1991) Subcellular patterns of calcium release by G protein-specific residues of muscarinic receptors. *Nature*, **350**, 505–508.
- Lee,K.M., Toscas,K. and Villereal,M.L. (1993) Inhibition of bradykinin- and thapsigargin-induced Ca²⁺ entry by tyrosine kinase inhibitors. *J. Biol. Chem.*, **268**, 9945–9948.
- Lotersztan,S. *et al.* (1992) Role of G protein $\beta\gamma$ subunits in the regulation of the plasma membrane Ca²⁺ pump. *J. Biol. Chem.*, **267**, 2375–2379.
- Luckoff,A. and Clapham,D.E. (1992) Inositol 1,3,4,5-tetrakisphosphate activates an endothelial Ca²⁺-permeable channel. *Nature*, **335**, 356–358.
- Marcus,D.C., Takeuchi,S. and Wangmann,P. (1992) Calcium activated nonselective cation channel in apical membrane of vestibular dark cells. *Am. J. Physiol.*, **262**, C1423–C1429.
- Matthews,G., Neher,E. and Penner,R. (1989) Second messenger-activated calcium influx in rat peritoneal mast cells. *J. Physiol.*, **418**, 105–130.
- Meucci,O., Scorziello,A., Avallone,A., Florio,T. and Schetti,G. (1995) α -1B, but not α -1A, adrenoreceptor activates calcium influx through the stimulation of a tyrosine kinase/phosphotyrosine phosphatase pathway, following noradrenaline-induced emptying of IP3 sensitive calcium stores, in PC C13 rat thyroid cell line. *Biochem. Biophys. Res. Comm.*, **209**, 630–638.
- Peralta,E.G., Ashkenazi,A., Winslow,J.W., Ramachandran,J. and Capon,D.J. (1988) Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. *Nature*, **334**, 434–437.
- Petersen,C.C.H., Toescu,E.C. and Petersen,O.H. (1991) Different patterns of receptor-activated cytoplasmic Ca²⁺ oscillations in single pancreatic acinar cells: dependence on receptor type, agonist concentration and intracellular Ca²⁺ buffering. *EMBO J.*, **10**, 527–533.
- Putney,J.W.Jr and Bird,G.St J. (1993) The signal for capacitive calcium entry. *Cell*, **75**, 199–201.
- Rapp,P.E. and Berridge,M.J. (1981) The control of transepithelial potential oscillations in the salivary gland of *Caliphora erithrocephala*. *J. Exp. Biol.*, **93**, 119–132.
- Raymond,J.R., Albers,F.J., Middleton,J.P., Lefkowitz,R.J., Caron,M.G., Obeid,L.M. and Dennis,V.W. (1991) 5-HT-1-A and histamine H-1 receptors in HeLa cells stimulate phosphoinositide hydrolysis and phosphate uptake via distinct G protein pools. *J. Biol. Chem.*, **266**, 372–379.
- Rooney,R.A., Sass,E.J. and Thomas,A.P. (1990) Agonist-induced cytosolic calcium oscillations originate from a specific locus in single hepatocytes. *J. Biol. Chem.*, **265**, 10792–10796.
- Rusanescu,G., Haiqing,Q., Thomas,S.M., Brugge,J.S. and Halegoua,S. (1995) Calcium influx induces neurite growth through a SRC-Ras signaling cassette. *Neuron*, **15**, 1415–1425.
- Sawaki,K., Hiramatsu,Y., Baum,B.J. and Ambudkar,I.S. (1993) Involvement of G $\alpha_q/11$ in m3 muscarinic receptor stimulation of phosphatidylinositol-4-5-bisphosphate-specific phospholipase C in rat parotid gland membranes. *Arch. Biochem. Biophys.*, **305**, 546–550.
- Stauffer,P.L., Zhao,H., Luby-Phelps,K., Moss,R.L., Star,R.A. and Maullem,S. (1993) Gap junction communication modulates calcium oscillations and enzyme secretion in pancreatic acini. *J. Biol. Chem.*, **268**, 19769–19775.
- Thorn,P., Lawrie,A.M., Smith,P.M., Gallacher,D.V. and Petersen,O.H. (1993) Local and global cytosolic calcium oscillations in exocrine cells evoked by agonists and inositol trisphosphate. *Cell*, **74**, 661–668.
- Tsien,R.W. and Tsien,R.Y. (1990) Calcium channels, stores, and oscillations. *Annu. Rev. Cell Biol.*, **6**, 715–760.
- Vaca,L., Sinkins,W.G., Hu,Y., Kunze,D.L. and Shilling,W.P. (1994) Activation of recombinant *trp* by thapsigargin in Sf9 cells. *Am. J. Physiol.*, **267**, C15101–C15105.
- Wacholtz,M.C., Cragoe,J.Jr and Lipsky,P.E. (1993) Delineation of the role of a sodium, calcium exchanger in regulating intracellular calcium in T cells. *Cell. Immunol.*, **147**, 95–109.

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