Signaling Pathways Underlying Muscarinic Receptor-induced \([\text{Ca}^{2+}]_i\) Oscillations in HEK293 Cells

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Summary

We have investigated the signaling pathways underlying muscarinic receptor-induced calcium oscillations in human embryonic kidney (HEK293) cells. Activation of muscarinic receptors with a maximal concentration of carbachol (100 µM) induced a biphasic rise in cytoplasmic calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) comprised of release of Ca\textsuperscript{2+} from intracellular stores and influx of Ca\textsuperscript{2+} from the extracellular space. A lower concentration of carbachol (5 µM) induced repetitive [Ca\textsuperscript{2+}]\textsubscript{i} spikes or oscillations, the continuation of which was dependent on extracellular Ca\textsuperscript{2+}. The entry of Ca\textsuperscript{2+} with 100 µM carbachol and with the sarcoplasmic-endoplasmic reticulum calcium ATPase inhibitor, thapsigargin, was completely blocked by 1 µM Gd\textsuperscript{3+}, as well as 30 – 100 µM of the membrane permeant IP\textsubscript{3} receptor inhibitor, 2-aminoethoxydiphenyl borane (2-APB). Sensitivity to these inhibitors is indicative of capacitative calcium entry. Arachidonic acid, a candidate signal for Ca\textsuperscript{2+} entry associated with [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in HEK293 cells, induced entry that was inhibited only by much higher concentrations of Gd\textsuperscript{3+}, and was unaffected by 100 µM 2-APB. Like arachidonic acid-induced entry, the entry associated with [Ca\textsuperscript{2+}]\textsubscript{i} oscillations was insensitive to inhibition by Gd\textsuperscript{3+}, but was completely blocked by 100 µM 2-APB. These findings indicate that the signaling pathway responsible for the Ca\textsuperscript{2+} entry driving [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in HEK293 cells is more complex than originally thought, and may involve neither capacitative calcium entry nor a role for PLA\textsubscript{2} and arachidonic acid.
Introduction

An increase in the level of intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) plays a central role in signal transduction for a variety of cellular functions, including cellular secretion, muscle contraction, cell growth and differentiation, and apoptosis. Changes in [Ca\(^{2+}\)]\(_i\) in mammalian cells are mediated by mobilization of Ca\(^{2+}\) from internal Ca\(^{2+}\) stores and/or by entry of Ca\(^{2+}\) from the extracellular space. In many nonexcitable cells, Ca\(^{2+}\) signaling by neurotransmitters or hormones is initiated through cell membrane receptors coupled to phospholipase C (PLC) and the production of inositol 1,4,5-trisphosphate (IP\(_3\)) (1). IP\(_3\) as a second messenger produces a biphasic Ca\(^{2+}\) signal, comprised of an initial Ca\(^{2+}\) release from endoplasmic reticulum (ER), followed by a sustained Ca\(^{2+}\) plateau due to Ca\(^{2+}\) entry across the plasma membrane. This Ca\(^{2+}\) entry usually results from the depletion of intracellular Ca\(^{2+}\) stores and in such instances is termed “capacitative Ca\(^{2+}\) entry” (2,3). This mode of entry presumably involves store-operated Ca\(^{2+}\) channels (SOCs) in the plasma membrane. Although capacitative calcium entry has been documented in many different cell types, the signal by which store emptying activates SOCs remains uncertain (4,5).

In addition to the sustained elevation of [Ca\(^{2+}\)]\(_i\) seen with high agonist concentrations, a more complex and subtle repetitive cycling of [Ca\(^{2+}\)]\(_i\), known as [Ca\(^{2+}\)]\(_i\) spiking or [Ca\(^{2+}\)]\(_i\) oscillations, often results from lower concentrations of agonists in some
cell types (1,6,7). The characteristics of \([\text{Ca}^{2+}]_i\) oscillations vary widely among different cell types, and a single mechanism may be insufficient to account for the variety of observed responses (1,7-9). Formation of IP$_3$ and cyclical release of Ca$^{2+}$ from IP$_3$-sensitive stores may underlie the generation of oscillations induced by agonists (1,10). However, a Ca$^{2+}$ induced Ca$^{2+}$ release (CICR) pathway has been suggested in initiating oscillations by caffeine or other agents unrelated to IP$_3$ generation (9,11). Ca$^{2+}$ influx from the external milieu is currently thought to be activated in such situations, and appears to be needed to sustain \([\text{Ca}^{2+}]_i\) oscillations (1,7). However the mechanism whereby Ca$^{2+}$ entry is triggered during \([\text{Ca}^{2+}]_i\) oscillations is not altogether clear. Some models suggest that capacitative calcium entry provides Ca$^{2+}$ entry during oscillations (7,12). More recently a novel, non-capacitative mechanism has been proposed that involves agonist-activated generation of arachidonic acid and arachidonic acid-induced Ca$^{2+}$ entry (13-15).

Arachidonic acid is present in cell membranes esterified in phospholipids and can be released by phospholipase A$_2$ (PLA$_2$) in response to various extracellular stimuli (16,17). Arachidonic acid can also be generated from diacylglycerol, a product of PLC or phospholipase D activation, by action of diglyceride lipase (16). In recent years, an increasing number of reports have suggested that arachidonic acid directly modulates cellular responses, including Ca$^{2+}$ signal transduction. As for IP$_3$, Ca$^{2+}$ release from ER and Ca$^{2+}$ influx from the extracellular space induced by arachidonic acid have been demonstrated.
in a number of cell types (18-23). However, the mechanisms underlying [Ca^{2+}]_{i} changes in response to arachidonic acid are not clear.

In this study, we have used relatively specific pharmacological probes to analyze and compare capacitative, non-capacitative and arachidonic acid-induced Ca^{2+} entry in HEK293 cells. We confirm earlier reports of a non-capacitative mechanism associated with [Ca^{2+}]_{i} oscillations in these cells. However, our findings indicate a possible role for the IP_{3} receptor in this signaling pathway and call into question the role of arachidonic acid, at least as a direct mediator of Ca^{2+} entry in this cell type.
Materials and Methods

Cell Culture: Human embryonic kidney 293 (HEK293) cells obtained from ATCC were grown at 37° C in Dulbecco’s Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamine in a humidified 95% air, 5% CO2 incubator. For Ca2+ measurements, cells were cultured to about 70% confluence, passaged onto glass coverslips and used 24-48 h after plating.

Fluorescence measurements: Fluorescence measurements were made with Fura2-loaded single or groups of HEK293 cells as described previously (24). In brief, coverslips with attached cells were mounted in a Teflon chamber and incubated in DMEM with 1 µM acetoxymethyl ester of Fura2 (Fura2/AM, Molecular Probes, USA) at 37° C in the dark for 25 min. Before [Ca2+]i measurements, cells were washed 3 times and incubated for 30 min at room temperature (25° C) in HEPES-buffered physiological saline solution (HPSS: NaCl 120; KCl 5.4; Mg2SO4 0.8; HEPES 20; CaCl2 1.8 and glucose 10 mM, with pH 7.4 adjusted by NaOH). Ca2+-free solutions contained no added CaCl2 in the HPSS.

In preliminary experiments, we observed that [Ca2+]i oscillations were not reproducibly observed in cells loaded with 1 µM Fura2/AM, presumably due to excessive cytoplasmic Ca2+ buffering. Thus, for these experiments we used 100 nM Fura2/AM for loading and 1.5 mM extracellular CaCl2 as previously described (25).

Fluorescence was monitored by placing the Teflon chamber with Fura2-loaded cells onto the stage of a Nikon Diaphot microscope (40x Neofluor objective). The cells
were excited alternatively by 340 and 380 nM wavelength light from a Deltascan D101 (Photon Technology International Ltd. USA) light source equipped with a light path chopper and dual excitation monochromators. Emission fluorescence intensity at 510 nM was recorded by a photomultiplier tube (Omega optical, USA). All experiments were conducted at room temperature (25° C) and carried out within 2 h of loading for each coverslip. Changes in \([\text{Ca}^{2+}]_i\) are reported for one single cell in oscillation experiments or a group of cells (6 to 10) in other protocols. The data are expressed as the ratio of Fura2 fluorescence due to excitation at 340 nm to that due to excitation at 380 nm (F340/F380).

**Mn\(^{2+}\) quench measurements:** Mn\(^{2+}\) quench experiments were performed with a group of HEK293 cells in nominally Ca\(^{2+}\)-free medium containing 0.1 or 2 mM MnCl\(_2\). Ftot, which is independent of \([\text{Ca}^{2+}]_i\) responses (26), was obtained by a weighted summing of the fluorescence of 340 and 380, and expressed as % of the initial value in the absence of extracellular Mn\(^{2+}\).

**Materials:** Arachidonic acid and 5,8,11,14-eicosatetraynoic acid were obtained from BioMol (PA, USA). Carbachol, thapsigargin and 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA) were purchased from Calbiochem (CA, USA). 2-aminoethoxydiphenyl borane (2-APB) was synthesized as previously described (27).

**Statistics:** For some experiments, average peak responses (F340/F380) were calculated and expressed as mean ± S.E.M. for the indicated number (n) of experiments. Statistical significance was determined with the Student’s t test (P <0.05).
Results

\([Ca^{2+}]_i\) signaling responses to carbachol in HEK293 cells: In \(Ca^{2+}\)-containing HPSS, 100 µM carbachol induced a large, somewhat transient increase in \([Ca^{2+}]_i\) (F340/F380), followed by a slowly declining but generally sustained elevated level of \([Ca^{2+}]_i\) (Fig. 1A). In nominally \(Ca^{2+}\)-free medium, this same concentration of carbachol induced a transient increase in \([Ca^{2+}]_i\); following readdition of \(Ca^{2+}\) to the medium, a second, sustained entry of \(Ca^{2+}\) was observed (Fig. 1B), indicating release of \(Ca^{2+}\) from internal sites and \(Ca^{2+}\) entry.

\([Ca^{2+}]_i\) oscillations have been reported to be induced by 1 µM carbachol in HEK293 cells transfected with the M₃ muscarinic receptors (28). However, in the current study in which wild type HEK293 cells were used, we failed to consistently produce repetitive transient responses of \([Ca^{2+}]_i\) with 1-3 µM carbachol. When the cells were stimulated with 5 µM carbachol, about 50% of the tested cells (101/205) showed oscillatory \([Ca^{2+}]_i\) responses at a frequency of approximately 0.5-1/min in the presence of 1.5 mM \(Ca^{2+}\) (Fig. 1C). These robust spikes could last up to 1 h, but the frequency progressively slowed with time. Because all cells did not oscillate, and because the frequency varied somewhat among the cells which did oscillate, we adopted the protocol shown in Fig. 1C whereby a cell was stimulated for about 20 min, the carbachol removed by 3 changes of incubation medium, and then, after an additional period of about 25
minutes (data acquisition was halted during this period), the same cell was again stimulated with 5 µM carbachol for an additional 20 min, generally under an altered experimental condition. As shown in Fig. 1C, in normal HPSS, the second stimulation always resulted in an oscillatory response that was similar to, although somewhat slower than the first.

This protocol was utilized for the experiment illustrated in Fig. 1D. In this experiment, 5 min before, and during the second exposure to carbachol, the cell was bathed in a nominally Ca\(^{2+}\)-free medium containing 200 µM BAPTA. With this protocol, carbachol induced one or two spikes but sustained oscillations were not observed. These results confirm that in wild type HEK293 cells, as shown previously for cells trasfected with the M\(_3\) muscarinic receptor, [Ca\(^{2+}\)]\(_i\) oscillations produced by a low concentration of carbachol depend on extracellular Ca\(^{2+}\), presumably indicating a role for Ca\(^{2+}\) influx.

**Effects of Gd\(^{3+}\) on [Ca\(^{2+}\)]\(_i\) responses to thapsigargin and carbachol in HEK293 cells:** Gd\(^{3+}\) is a potent inhibitor of agonist-activated calcium entry (29), and has been shown to discriminate between capacitative and non-capacitative calcium entry (22). In experiments utilizing the same protocol as in Fig. 1B, The effects of Gd\(^{3+}\) on Ca\(^{2+}\) entry due to the SERCA inhibitor, thapsigargin, were determined. Thapsigargin depletes Ca\(^{2+}\) stores passively by virtue of its ability to inhibit the SERCA pumps on the endoplasmic reticulum and the ensuing entry of Ca\(^{2+}\) is therefore assumed to be the very definition of capacitative calcium entry (3,30). As shown in Fig. 2A, Gd\(^{3+}\) inhibited Ca\(^{2+}\) entry induced by the thapsigargin in a concentration-dependent manner, and with no significant effect on the
Ca^{2+} release phase (Fig. 2A and results not shown). Gd^{3+} had no significant effect on basal 
$[\text{Ca}^{2+}]_i$ (data not shown). The sensitivity of thapsigargin-induced capacitative calcium 
entry to inhibition by Gd^{3+} is similar to that reported by Broad et al. (22).

Similar experiments were carried out utilizing a maximal concentration of 
carbachol, and such an experiment is shown in Fig 3A. Ca^{2+} entry due to maximal 
muscarinic receptor activation appeared similarly sensitive to inhibition by Gd^{3+}, leading 
to the conclusion that the entry is largely or entirely capacitative.

However, significantly different results were obtained when cells were stimulated 
to oscillate with the lower, 5 µM concentration of carbachol. As Fig. 3 illustrates, 
concentrations of 1, 10, 30, 100, or 500 µM Gd^{3+} produced little or no effect on the 
$[\text{Ca}^{2+}]_i$ oscillations; only at the highest concentrations tested, 100 and 500 µM Gd^{3+}, was there 
even partial suppression of the oscillatory frequency. The failure of even these very high 
concentrations of Gd^{3+} to block the oscillations was surprising. However, it is known that 
another lanthanide, La^{3+}, can inhibit active membrane extrusion of Ca^{2+} at higher 
concentrations (31). We determined whether Gd^{3+} might have a similar action by examining 
the time course of the $[\text{Ca}^{2+}]_i$ response to thapsigargin in the absence of extracellular 
Ca^{2+} and in the presence of varying concentrations of Gd^{3+}. The decay of the $[\text{Ca}^{2+}]_i$ 
response to thapsigargin under these conditions is due almost entirely to plasma membrane 
extrusion (32). As shown in Fig. 4, Gd^{3+} concentrations of 30 µM or greater caused an
augmentation of the thapsigargin-induced \([\text{Ca}^{2+}]_i\) signal and a slowing of its decay. Thus, at these higher concentrations, oscillations may continue due to "trapping" of intracellular Ca\(^{2+}\), despite an inhibition of Ca\(^{2+}\) entry. However, at 10 µM Gd\(^{3+}\), there was no significant augmentation of the response indicating that the Ca\(^{2+}\) entry channels supporting the oscillations are truly less sensitive to Gd\(^{3+}\) than are capacitative calcium entry channels.

**Arachidonic acid-induced \([\text{Ca}^{2+}]_i\) signaling in HEK293 cells.** Shuttleworth and his co-workers have suggested that the non-capacitative calcium entry occurring in HEK293 cells and other cell types in response to low concentrations of muscarinic agonists is mediated by arachidonic acid, released from membrane lipids by phospholipase A\(_2\) (13,21,28). Thus, we next examined the effects of Gd\(^{3+}\) on Ca\(^{2+}\) mobilization in HEK293 cells in response to arachidonic acid. In preliminary experiments, we found that between 30-300 µM arachidonic acid could reproducibly induce both Ca\(^{2+}\) release and Ca\(^{2+}\) entry in a concentration dependent manner. However arachidonic acid at concentrations > 100 µM occasionally resulted in \([\text{Ca}^{2+}]_i\) levels that saturated the indicator likely due to a non-selective increase in membrane permeability (33). Concentrations in the range of 5 – 10 µM did not induce increases in \([\text{Ca}^{2+}]_i\) in all cells. As shown in Fig. 5A, 30 µM arachidonic acid slowly increased the fluorescence ratio, and the response appeared to occur in two phases. In nominally Ca\(^{2+}\)-free medium, arachidonic acid induced a transient \([\text{Ca}^{2+}]_i\) rise
followed by a sustained elevation of [Ca\(^{2+}\)]\(_i\) after restoration of Ca\(^{2+}\) to the medium (Fig. 5B), indicating that both Ca\(^{2+}\) release and Ca\(^{2+}\) entry are activated by arachidonic acid in HEK293 cells. To examine the possible involvement of metabolites of arachidonic acid in the [Ca\(^{2+}\)]\(_i\) responses, we employed 5,8,11,14-eicosatetraynoic acid (ETYA), an inhibitor of cyclooxygenase, lipoxygenases and cytochrome P450 arachidonic acid metabolizing enzymes (34). 20 µM ETYA had no effect on either Ca\(^{2+}\) release or Ca\(^{2+}\) entry induced by 30 µM arachidonic acid, indicating that the [Ca\(^{2+}\)]\(_i\) changes induced by 30 µM arachidonic acid are unlikely to result from an arachidonic acid metabolite (data not shown). A similar conclusion was reached by Shuttleworth and Thompson based on a somewhat different strategy (28).

The pattern of [Ca\(^{2+}\)]\(_i\) signaling induced by arachidonic acid is reminiscent of that due to thapsigargin; a release of stored Ca\(^{2+}\) followed by activation of Ca\(^{2+}\) entry across the plasma membrane. Thus, we next examined the effects of Gd\(^{3+}\) on arachidonic acid-induced signaling, since this lanthanide appears to have relatively selective effects on store-operated or capacitative calcium entry. At a concentration of 1 µM, which completely blocked Ca\(^{2+}\) entry due to carbachol and thapsigargin, Gd\(^{3+}\) had no significant effect on Ca\(^{2+}\) entry in response to 30 µM arachidonic acid (Fig. 5B and C). At concentrations of 3 and 10 µM, Gd\(^{3+}\) inhibited Ca\(^{2+}\) influx induced by 30 µM arachidonic
acid with complete blockade at 10 µM. Surprisingly, 10 µM Gd$^{3+}$ also caused a complete
abolishment of arachidonic acid-induced Ca$^{2+}$ release (Fig. 5B, C). After complete
inhibition with 10 µM Gd$^{3+}$ of Ca$^{2+}$ release due to 30 µM arachidonic acid in nominally
Ca$^{2+}$-free medium, a normal release of [Ca$^{2+}$]$_i$ could be evoked on addition of 1 µM
thapsigargin or 100 µM carbachol (not shown). These results indicate that arachidonic acid
induces both Ca$^{2+}$ release and Ca$^{2+}$ entry in HEK293 cells, and both of these responses
are sensitive to inhibition by Gd$^{3+}$; however, this pathway is at least 10-fold less sensitive
to Gd$^{3+}$ than capacitative calcium entry.

Effects of 2-APB on [Ca$^{2+}$]$_i$ responses induced by carbachol, thapsigargin and
arachidonic acid. Recent studies have indicated that capacitative calcium entry involves
interactions between IP$_3$ receptors and the plasma membrane (35). One piece of evidence for
this idea is the sensitivity of capacitative calcium entry to inhibition by 2-APB (36), a
membrane-permeant inhibitor of the IP$_3$ receptor (27). We next examined the actions of this
reagent as a potential inhibitor of Ca$^{2+}$ entry responses to agonists, to thapsigargin, and to
arachidonic acid.

In unstimulated cells, and in the absence of extracellular Ca$^{2+}$, 2-APB at 100 µM
slightly augmented the baseline fluorescence ratio in about 80% of HEK293 cells tested
(n=48). The increment in the baseline was 8.6 ± 3.2 % of that of Ca$^{2+}$ release by 1 µM
thapsigargin in nominally Ca$^{2+}$-free medium (n=9). A weak inhibitory effect on Ca$^{2+}$
ATPase in the ER has been suggested to account for the rise of \([\text{Ca}^{2+}]_i\) by high concentrations of 2-APB (27).

Utilizing a similar protocol as for the Gd\(^{3+}\) experiments, 2-APB produced a concentration-dependent inhibition of \(\text{Ca}^{2+}\) influx induced by 100 \(\mu\text{M}\) carbachol (Fig. 6, Top) or 1 \(\mu\text{M}\) thapsigargin (Fig. 6, Bottom) when \(\text{Ca}^{2+}\) was restored to the bath. Like Gd\(^{3+}\), 2-APB altered the \(\text{Ca}^{2+}\) entry phase with almost the same potency among the two agonists, with 30 \(\mu\text{M}\) 2-APB producing essentially complete block of \(\text{Ca}^{2+}\) entry for both modes of activation. However, 30 \(\mu\text{M}\) 2-APB attenuated the \(\text{Ca}^{2+}\) release peak induced by 100 \(\mu\text{M}\) carbachol only weakly, and this inhibition was still incomplete with 100 \(\mu\text{M}\) 2-APB (Fig. 6). With 100 \(\mu\text{M}\) 2-APB, an approximate 20\% reduction of \(\text{Ca}^{2+}\) release due to 1 \(\mu\text{M}\) thapsigargin could also be seen (Fig. 6), which may be due to the inhibition of \(\text{Ca}^{2+}\) ATPase in the endoplasmic reticulum and a partial reduction of the size of the pool sensitive to thapsigargin.

2-APB at 100 \(\mu\text{M}\), a concentration which caused complete inhibition of capacitative calcium entry, did not alter \(\text{Ca}^{2+}\) entry due to 30 \(\mu\text{M}\) arachidonic acid (Fig. 7). As for thapsigargin, 100 \(\mu\text{M}\) 2-APB caused a slight reduction of \([\text{Ca}^{2+}]_i\) release in response to 30 \(\mu\text{M}\) arachidonic acid (Fig. 7 and results not shown).

These data, including the data obtained with Gd\(^{3+}\), provide evidence that the mechanisms by which arachidonic acid activates \(\text{Ca}^{2+}\) release and \(\text{Ca}^{2+}\) influx are
different from those of the store-depleting agents, thapsigargin and carbachol. As first suggested by Shuttleworth, capacitative calcium entry appears not to be involved in Ca\textsuperscript{2+} entry due to arachidonic acid in HEK293 cells (21).

*Effects of 2-APB on [Ca\textsuperscript{2+}]_{i} oscillations and Ca\textsuperscript{2+} entry in response to low concentrations of carbachol.* As shown in Fig. 8, 2-APB inhibited the repetitive transient [Ca\textsuperscript{2+}]_{i} responses in a concentration-dependent manner and 100 µM 2-APB completely blocked the sustained oscillatory response of HEK293 cells to 5 µM carbachol.

The inhibition by 2-APB of the [Ca\textsuperscript{2+}]_{i} response to 5 µM carbachol was unexpected since arachidonic acid-induced Ca\textsuperscript{2+} signaling was unaffected by this drug. However, we considered the possibility that this concentration of carbachol might induce a small influx of Ca\textsuperscript{2+} that is only detectable when amplified through calcium-induced calcium release, and this might depend on functional IP\textsubscript{3} receptors. Therefore, to assess more directly the actions of 2-APB on Ca\textsuperscript{2+} entry during [Ca\textsuperscript{2+}]_{i} oscillations, we utilized Mn\textsuperscript{2+} quench measurements. Mn\textsuperscript{2+} enters cells through divalent cation channels, but quenches Fura2 fluorescence at all wavelengths (37). Thus, the activity of Ca\textsuperscript{2+} influx channels is reported by the rate of Mn\textsuperscript{2+} quench of Fura2. In the presence of 0.1 mM Mn\textsuperscript{2+}, in nominally Ca\textsuperscript{2+}-free medium, a resting rate of Mn\textsuperscript{2+} quench was seen in unstimulated cells, and this was blocked when the cells were pretreated with 100 µM 2-APB (Fig. 9A). 5 µM carbachol increased Mn\textsuperscript{2+} quench, and again the rate of quench in
the presence of carbachol was completely blocked by 100 µM 2-APB (Fig. 9B). Because 2-APB essentially completely blocked even basal Mn$^{2+}$ quench, we repeated the experiments with 2 mM Mn$^{2+}$. Under these conditions, a basal rate of Mn$^{2+}$ quench was seen in the presence of 100 µM 2-APB, but this was not further increased by 5 µM carbachol (Fig. 10); however, addition of 5 µM arachidonic acid induced a substantial increase in quench (Fig. 10A). These results demonstrate that 2-APB attenuated both the resting Mn$^{2+}$ entry and divalent cation influx stimulated by 5 µM carbachol, but not that of exogenous arachidonic acid.

These findings indicate that the signaling mechanism underlying non-capacitative calcium entry and [Ca$^{2+}$]$_i$ oscillations may involve signals other than, or in addition to arachidonic acid. There is previous pharmacological evidence for a role for PLA$_2$; for example, the oscillations are inhibited by the PLA$_2$ inhibitor, isotetrandrine (28). Data shown in Fig. 11A and B essentially replicates the previous findings of Shuttleworth and Thompson (28), showing that isotetrandrine can block [Ca$^{2+}$]$_i$ oscillations, and the addition of a low concentration of arachidonic acid can partially restore the response (in 7 of 14 cells tested). We found that 10 µM isotetrandrine completely blocked the oscillations in 12 of 21 cells tested, while 20 µM blocked completely in 3 of 5. However, like its close cousin, tetrandrine (38), isotetrandrine can also function as a calcium channel blocker. We thus tested the effects of isotetrandrine on the entry of Ca$^{2+}$ directly activated by arachidonic acid. As illustrated in Fig. 12A, 10 µM isotetrandrine consistently inhibited the Ca$^{2+}$ entry in
response to arachidonic acid (3 of 3 with 10 µM isotetrandrine; 3 of 4 with 20 µM isotetrandrine). The inhibition is apparently not due to action as a non-specific channel blocker, or to membrane depolarization, because isotetrandrine caused only slight inhibition of entry in response to thapsigargin (Fig. 12B). These findings call into question the validity of isotetrandrine as a specific tool to demonstrate PLA₂ involvement. In addition, as discussed below, they may indicate that the effects and pharmacological sensitivity of exogenously added arachidonic acid do not faithfully reflect the behavior of arachidonic acid generated endogenously as a component of a physiological signaling cascade. Further, the relative insensitivity of the thapsigargin-induced entry to isotetrandrine further supports the view that the entry driving the \([Ca^{2+}]_i\) oscillations is not capacitative.

Discussion

In the present study, we initially established that Gd³⁺ and 2-APB appear to be relatively specific and potent inhibitors of capacitative calcium entry. Results shown here indicate that Ca²⁺ entry due to two different store depleting agents, thapsigargin, and carbachol, are sensitive to the inhibitory effects of Gd³⁺ and 2-APB in HEK293 cells. Gd³⁺ from 30 nM to 1 µM and 2-APB from 10 to 100 µM in a concentration-dependent manner inhibited this Ca²⁺ entry with similar potency for the two agonists (Fig. 6 and data not shown), indicating a similar mechanism for blocking Ca²⁺ entry, i.e. capacitative calcium entry activated by store depletion in HEK293 cells. At concentrations of 1 µM Gd³⁺ and 100 µM 2-APB, respectively, a complete abolishment of Ca²⁺ influx due to
carbachol and thapsigargin was observed, consistent with previous reports in which a complete blockade of capacitative calcium entry induced by different store depleting drugs could be obtained with 1 µM Gd$^{3+}$ in rat A7r5 cells (22), and with 100 µM 2-APB in DDT1-MF2 cells, A7r5 cells and HEK293 cells (36). These results clearly demonstrate that Gd$^{3+}$ and 2-APB are both potent inhibitors of capacitative calcium entry. 2-APB has also been shown to modulate IP$_{3}$ receptors (27), leading Ma et al. (36) to conclude that the IP$_{3}$ receptor is somehow involved in the activation of capacitative calcium entry.

Arachidonic acid, an unsaturated fatty acid produced by the action of PLA$_{2}$ or diacylglycerol lipase on membrane lipids, is mobilized in a variety of cell types by the actions of neurotransmitters and hormones. Arachidonic acid also induces Ca$^{2+}$ fluxes (39-41) as well as a variety of Ca$^{2+}$-dependent effects in cells, and thus has been suggested as a second messenger modulating Ca$^{2+}$ signal transduction (13,16,17,33). However, the mechanisms underlying Ca$^{2+}$ signaling modulation due to arachidonic acid remain unclear. In order to better define the mechanisms of calcium signaling in response to arachidonic acid, we examined the effects of the two inhibitors of capacitative calcium entry, Gd$^{3+}$ and 2-APB, on arachidonic acid mediated Ca$^{2+}$ signaling. Arachidonic acid at concentrations at or above 30 µM released Ca$^{2+}$ from intracellular Ca$^{2+}$ stores and induced Ca$^{2+}$ entry (Fig. 5), consistent with findings in other cell lines (18,19,41). Interestingly, we found that 10 µM Gd$^{3+}$ completely blocked Ca$^{2+}$ release in response to arachidonic acid (Fig 5C, D), but did not affect release due to thapsigargin or carbachol (Fig. 6). The mechanism for this effect is not
known. Direct interaction of Gd$^{3+}$ with arachidonic acid seems unlikely since the concentration of Gd$^{3+}$ (10 µM) is less than that of arachidonic acid (30 µM).

Ca$^{2+}$ entry induced by arachidonic acid is also attenuated by Gd$^{3+}$, but only in concentrations in excess of 1 µM. As for the inhibition of Ca$^{2+}$ release due to arachidonic acid, 10 µM Gd$^{3+}$ was required to produce complete blockade of Ca$^{2+}$ entry (Fig. 5C, D). A similar result was reported for A7r5 cells (22). The mechanism by which Gd$^{3+}$ inhibits both Ca$^{2+}$ release and influx is not know, nor is it clear as to whether these two effects are even related. The significant point for the current study, however, is that 1 µM Gd$^{3+}$, which is more than sufficient for complete inhibition of capacitative calcium entry, is without effect when arachidonic is used as an activator of Ca$^{2+}$ mobilization.

In addition to Gd$^{3+}$, 2-APB is emerging as a relatively specific inhibitor of capacitative calcium entry. Ma et al. (36) demonstrated that 2-APB blocked capacitative calcium entry in HEK293 cells, as well as the entry ascribed to the transfected Trp3 channel. In the latter case, previous work had shown that transfected Trp3 can be activated either through an interaction with subplasmalemmal IP$_3$ receptors (35), or more directly by diacylglycerol (42). 2-APB blocked Trp3 channels when activated by phospholipase C-linked agonists, but not when activated by diacylglycerol. This finding led Ma et al. (36) to conclude that 2-APB was not acting as a channel blocking drug, and that its mechanism of action in the case of Trp3 channels, and probably also in the case of capacitative calcium entry, involved inhibition of IP$_3$ receptors. These results have been considered strong evidence
for the conformational coupling model (4,43) for activation of capacitative calcium entry because this model invokes an obligatory role for the IP$_3$ receptor interacting with plasma membrane capacitative calcium entry channels (44). Clearly, 2-APB is one of the more specific inhibitors of capacitative calcium entry. It completely blocks capacitative calcium entry in concentrations that are without effect on voltage-operated calcium channels ((27) and Luo, Broad, Bird and Putney, unpublished observations) and in the current study, we have found that it does not block channels activated by arachidonic acid (Fig. 7 and 10). To our knowledge no other organic antagonist of capacitative calcium entry channels shows this degree of specificity (see also (45)). The drug has only one other known site of action, the IP$_3$ receptor, and it is reasonable for the present to accept the interpretation of Ma et al. (36) that this action underlies its actions on calcium entry. However, we note, as was somewhat evident in the work of Ma et al., that Ca$^{2+}$ entry appears to be more sensitive to inhibition by 2-APB than the intracellular, IP$_3$-mediated release of Ca$^{2+}$ (Fig. 6). Thus, it is possible that 2-APB may also have direct, albeit highly specific, actions on capacitative calcium entry channels, although this distinction is not critical to arguments based on its effects in this study.

Finally, with this background of clear and relatively specific actions of Gd$^{3+}$ and 2-APB, we have utilized these reagents to evaluate the role of the capacitative calcium entry pathway in the complex Ca$^{2+}$ signaling response giving rise to [Ca$^{2+}$]$_i$ oscillations in HEK293 cells (28). In our study of wild type HEK293 cells, repetitive [Ca$^{2+}$]$_i$ spikes could
be induced by a relatively low concentration of carbachol and the generation of this
signaling pattern is dependent on extracellular Ca\(^{2+}\) (Fig. 1D). Although previous models
have implicated a role for capacitative calcium entry in the maintenance of [Ca\(^{2+}\)]\(_i\)
oscillations (7,12), this view has been recently questioned by Shuttleworth (13). In the current study,
the insensitivity of [Ca\(^{2+}\)]\(_i\) oscillations to Gd\(^{3+}\) provides clear pharmacological evidence
that the entry pathway activated by low concentrations of carbachol is distinct from the
capacitative pathway seen with higher concentrations of carbachol or with store depletion
by the SERCA inhibitor, thapsigargin. There is considerable evidence that arachidonic acid
can serve as an activator of a non-capacitative pathway in HEK293 cells (46). Consistent with
this idea, arachidonic acid-induced entry of Ca\(^{2+}\) was relatively insensitive to inhibition
by Gd\(^{3+}\), curiously, despite the ability of arachidonic acid to deplete intracellular Ca\(^{2+}\)
stores\(^1\). However, the association between arachidonic acid-induced entry and the entry
associated with oscillations is lost when the effects of 2-APB are examined. 2-APB was
capable of completely inhibiting both the [Ca\(^{2+}\)]\(_i\) oscillations and increased Mn\(^{2+}\) quench
due to 5 µM carbachol, but was without effect on arachidonic acid-induced entry of either
Ca\(^{2+}\) or Mn\(^{2+}\).

With the evidence presently available, we cannot definitively determine the
mechanisms of Ca\(^{2+}\) signaling that underlie [Ca\(^{2+}\)]\(_i\) oscillations in HEK293 cells. We
suggest three possible alternatives that may be testable in the future by continued
experimental work:

(i) The simplest explanation for our data is that the entry associated with \([Ca^{2+}]_i\) oscillations is non-capacitative in nature, but involves some mode of activation other than arachidonic acid. At least some of the previous evidence for PLA_2 involvement (28), based on inhibitory effects of the PLA_2 inhibitor, isotetrandrine, is open to question (Fig. 12). Furthermore, regardless of the precise mechanism of action of 2-APB, its ability to distinguish clearly between arachidonic acid-induced entry and the Ca^{2+} entry associated with \([Ca^{2+}]_i\) oscillations suggests that the arachidonic acid pathway is not involved.

(ii) These arguments notwithstanding, it is still possible, given other points of evidence that suggest an involvement of PLA_2 and arachidonic acid, that PLA_2 is involved in the oscillations. However, exogenously added arachidonic acid may act through a different mechanism than arachidonic acid generated by PLA_2 in the cell, perhaps in the vicinity of Ca^{2+} channels.

(iii) Finally, we consider that it still possible that the Ca^{2+} entry underlying oscillations is a store-operated entry, albeit involving different store-operated channels than those seen with maximal store depletion. While only Gd^{3+}-sensitive entry was observed in HEK293 cells following maximal depletion of Ca^{2+} stores, Gd^{3+}-insensitive store-operated channels have been observed in other experimental systems.
(29). In the absence of extracellular Ca\(^{2+}\), at least one \([\text{Ca}^{2+}]_i\) spike is always observed with 5 \(\mu\text{M}\) carbachol, suggesting that intracellular release is triggered independently of, and likely prior to entry. Also, the \([\text{Ca}^{2+}]_i\) oscillations and Mn\(^{2+}\) entry due to 5 \(\mu\text{M}\) carbachol are both blocked by 2-APB, a drug with documented specificity for capacitative calcium channels. Thus, it is possible that with minimal and short-lived Ca\(^{2+}\) discharge, a different population of capacitative calcium entry channels are activated with pharmacological distinctions and as well as pharmacological similarities to the channels activated upon maximal store depletion.

The conclusion from this study is that the interplay among various Ca\(^{2+}\) signaling pathways that result in \([\text{Ca}^{2+}]_i\) spikes and oscillations may be much more complex than originally envisioned. However, some cell types may utilize a simpler mechanism involving strictly phospholipase C, intracellular Ca\(^{2+}\) release, and capacitative calcium entry mechanisms. For example, in mouse lacrimal cells, which produce a characteristic sinusoidal pattern of \([\text{Ca}^{2+}]_i\) oscillations (47), there are no Gd\(^{3+}\)-insensitive responses seen at low agonist concentrations, and the cells appear completely insensitive to the addition of exogenous arachidonic acid (Luo, Broad, Bird and Putney, unpublished observations). Clearly additional work will be needed to understand the varied patterns and mechanisms that control the biologically important phenomenon known as \([\text{Ca}^{2+}]_i\) oscillations.
Footnotes

1 The failure of store depletion by arachidonic acid to activate capacitative calcium entry may be due to the previously documented ability of arachidonic acid to inhibit capacitative calcium entry (48).
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References


**Figure Legends**

**Figure 1.** *Calcium release, calcium entry and [Ca$^{2+}$]$_i$ oscillations due to muscarinic receptor activation.* Fura-2-loaded HEK293 cells were activated as indicated by either 100 µM (A and B) or 5 µM (C and D) carbachol. In A, extracellular Ca$^{2+}$ was present throughout the experiment. In B, Ca$^{2+}$ was initially absent from the medium and was restored to 1.8 mM as indicated. In C and D, a single HEK293 cell was activated with 5 µM carbachol, and then the medium removed with three consecutive washes, and the cell allowed to recover for 25 min. Data collection was interrupted during this interval of washes and recovery, and is indicated by the arrow labeled W. Subsequently, the same cell was activated a second time with 5 µM carbachol. In D, prior to the second stimulation, the extracellular medium was changed to one containing 0.2 mM BAPTA and no added Ca$^{2+}$. Similar results to the ones depicted were obtained in a total of 5-8 experiments.

**Figure 2.** *Effect of Gd$^{3+}$ on thapsigargin-activated Ca$^{2+}$ entry.* In A, the protocol was identical to that in Fig. 1B, except that 1µM thapsigargin was utilized as agonist. In one of the traces (as indicated) 1 µM Gd$^{3+}$ was added to the medium when Ca$^{2+}$ was removed and was present throughout the readdition of Ca$^{2+}$. In B, the agonist was 100 µM carbachol. Results shown are representative of 6 (A) and 5 (B) independent determinations.

**Figure 3.** *Effect of increasing concentrations of Gd$^{3+}$ on [Ca$^{2+}$]$_i$ oscillations induced by 5 µM carbachol.* The top left panel depicts a control experiment carried out according to the
protocol described for Fig. 1C. In subsequent panels, Gd$^{3+}$, at the concentrations shown, was added during the interval indicated. Similar findings were obtained in a total of 4-6 experiments.

**Figure 4.** Effects of Gd$^{3+}$ on Ca$^{2+}$ extrusion in HEK293 cells. To assess inhibitory actions of Gd$^{3+}$ on Ca$^{2+}$ extrusion, cells were activated by 1 µM thapsigargin (TG) in the absence of external Ca$^{2+}$, and in the presence of the indicated concentrations of Gd$^{3+}$. Concentrations of Gd$^{3+}$ of 30 µM or greater delayed the decay of the thapsigargin transient, indicating a degree of inhibition of plasma membrane Ca$^{2+}$ extrusion.

**Figure 5.** Calcium signaling in HEK293 cells due to arachidonic acid, and the effects of Gd$^{3+}$. In A, a fura-2 loaded HEK293 cell was exposed to 30 µM arachidonic acid (AA) as indicated, resulting in a biphasic rise in [Ca$^{2+}$]$_i$. The protocol for B and C was as for Figs. 2 and 3. In C, summary data are included for both the entry (filled circles) and release (open circles) phases of the response to arachidonic acid. The data in C are means ± SEM from 6-7 experiments.

**Figure 6.** Effect of 2-APB on Ca$^{2+}$ release and Ca$^{2+}$ entry due to 100 µM carbachol. The protocol for the two panels is identical to that for Fig. 3B and C except that different concentrations of the membrane permeant IP$_3$ receptor inhibitor, 2-APB, were used. In the top panel, the agonist was 100 µM carbachol, and in the bottom panel the agonist was 1 µM thapsigargin. The results illustrate findings from 6 (A) and 5 (B) independent
determinations.

**Figure 7.** Effect of 2-APB on Ca$^{2+}$ signaling due to 30 µM arachidonic acid (AA). 30 µM arachidonic acid was applied to a HEK293 cell when indicated, in the absence of external Ca$^{2+}$, and Ca$^{2+}$ restored to 1.8 mM during the indicated interval. The cells were pretreated with either 100 µM 2-APB (dashed line), or the solvent control, DMSO (solid line). 100 µM 2-APB failed to inhibit arachidonic acid-induced Ca$^{2+}$ entry in a total of 6 experiments.

**Figure 8.** Effect of 2-APB on [Ca$^{2+}$]$_i$ oscillations due to 5 µM carbachol. The protocol was as for Fig. 4. Sequential stimulations with 5 µM carbachol are shown in A, and the effects of increasing concentrations of 2-APB on the second stimulation depicted in B – D. Similar findings were obtained in a total of 5-6 experiments.

**Figure 9.** Effects of 5 µM carbachol and 100 µM 2-APB on Mn$^{2+}$ entry into HEK293 cells. The Ca$^{2+}$-insensitive fluorescence (Ftot, see Methods) of fura-2-loaded HEK293 cells was monitored. In control cells (A), addition of 0.1 mM Mn$^{2+}$ to the medium causes an accelerated quench of fura-2 (solid line) and this effect is blocked by 2-APB (dotted line). Treatment of the cells with 5 µM carbachol (B) results in an enhanced rate of fura-2 quench (solid line) that is again completely blocked by 2-APB (dotted line). A total of 7 experiments produced similar findings.

**Figure 10.** Effects of 5 µM carbachol, 100 µM 2-APB and 5 µM arachidonic acid (AA) on
Mn$^{2+}$ entry into HEK293 cells. As in Fig. 11, the Ca$^{2+}$-insensitive fluorescence (Ftot, see Methods) of fura-2-loaded HEK293 cells was monitored. In A, in cells treated with 100 µM 2-APB, addition of 2.0 mM Mn$^{2+}$ to the medium causes an accelerated quench of fura-2, but subsequent addition of carbachol does not further increase the rate of quench (solid line). However, with this same protocol, arachidonic acid stimulates the rate of quench (dotted line). In B, addition of 2 mM Mn$^{2+}$ to control, 2-APB-treated cells (solid line), or 2-APB-treated cells stimulated with 5 µM carbachol (dotted line) results in a similar increase in the rate of quench of fura-2. A total of 4-5 experiments produced similar findings.

Figure 11. Effects of isotetrandrine on carbachol-induced \([Ca^{2+}]_i\) oscillations. The protocol for A was as in Fig. 1C. Addition of 10 µM isotetrandrine to the medium blocked the sustained oscillations due to carbachol (7 of 21 cells). In B, 10 µM isotetrandrine was added during the oscillations, and the oscillations ceased. Addition of 5 µM arachidonic acid restored a partial response in 7 of 14 cells tested.

Figure 12. Effects of isotetrandrine on arachidonic acid- and thapsigargin-induced Ca$^{2+}$ entry. In A, Ca$^{2+}$ entry was activated by 70 µM arachidonic acid. Where indicated, 10 µM isotetrandrine was added. Similar results were obtained in a total of 3 experiments with 10 µM isotetrandrine, and in 3 of 4 experiments with 20 µM isotetrandrine. In B, 1 µM thapsigargin was used to activate capacitative calcium entry. The addition of 10 µM isotetrandrine induced only a slight diminution of Ca$^{2+}$ entry, and further addition of 20
μM isotetrandonine inhibited to a slightly greater extent. Similar results were obtained in a total of 5 experiments.
Figure A: Graph showing the effect of 30 µM AA over time.

Figure B: Graph showing the effect of Gd$^{3+}$ concentrations on the ratio (F340/F380) with 30 µM AA and 1.8 mM Ca$^{2+}$.

Figure C: Graph showing the relationship between [Gd$^{3+}$] (µM) and the ratio (F340/F380).
A

10 μM Iso

Arachidonic Acid

B

10 μM Iso

20 μM Iso

Thapsigargin

Ratio

Seconds
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