Muscarnic Receptor Activation of Arachidonate-mediated Ca\(^{2+}\) Entry in HEK293 Cells Is Independent of Phospholipase C*

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Receptor-enhanced entry of Ca\(^{2+}\) in non-excitable cells is generally ascribed to a capacitative mechanism in which the activation of the entry pathway is specifically dependent on the emptying of agonist-sensitive intracellular Ca\(^{2+}\) stores. Although such entry can be clearly demonstrated under conditions of maximal or near-maximal stimulation, it is uncertain whether such a mechanism can operate during the oscillatory [Ca\(^{2+}\)]\(_i\) signals that are frequently seen following stimulation with low concentrations of agonists. In this study, we report that the stimulation of human m3 muscarinic receptors stably transfected into HEK293 cells results in the appearance of a novel arachidonate-mediated Ca\(^{2+}\) entry pathway. We show that the generation of arachidonic acid and the activation of this pathway are specifically associated with stimulation at the low agonist concentrations that typically give rise to oscillatory [Ca\(^{2+}\)]\(_i\) signals. At such agonist concentrations, however, the generation of arachidonic acid is independent of the simultaneous activation of the phospholipase C-inositol 1,4,5-trisphosphate pathway. We further show that the arachidonate-mediated Ca\(^{2+}\) entry demonstrates characteristics that distinguish it from the corresponding capacitative pathway in the same cells and therefore is likely to represent an entirely distinct pathway that is specifically responsible for the receptor-enhanced entry of Ca\(^{2+}\) during [Ca\(^{2+}\)]\(_i\) oscillations.

Calcium signaling in non-excitable cells is composed of two components: a release of calcium from intracellular stores and an increased entry of calcium from the extracellular medium. The role of inositol 1,4,5-trisphosphate (InsP\(_3\)),\(^1\) generated as a result of the receptor activation of phospholipase C, in the release of calcium from specific intracellular stores is well established, but the nature of the calcium entry pathway and its regulation is far from clear. To date, discussion of such calcium entry has generally focused on the so-called "capacitative model," in which calcium entry is activated as a direct consequence of the emptying of the intracellular calcium stores and is independent of how this emptying is actually achieved (1, 2). The precise nature of the mechanism for the activation of capacitative entry is, as yet, unclear, but it may involve the release and/or generation of a diffusible signaling molecule within the cell that activates the plasma membrane channels ("store-operated channels") responsible for calcium entry. Alternatively, a more direct molecular coupling between the stores and the plasma membrane channels may occur (3). Although such capacitative entry can be clearly demonstrated in a wide variety of different cells, it is far from certain that such a mechanism is the only one involved in the increase in calcium entry in non-excitable cells following receptor activation (4, 5). For example, many cells show an oscillatory [Ca\(^{2+}\)]\(_i\) signal when stimulated at low agonist concentrations (6, 7), and such signals are associated with an enhanced entry of Ca\(^{2+}\). However, evidence indicates that the activation of capacitative Ca\(^{2+}\) entry generally requires significantly higher levels of agonist-generated InsP\(_3\) than does the release of Ca\(^{2+}\) from the bulk of the agonist-sensitive internal stores (i.e. at low concentrations of InsP\(_3\)) substantial release of Ca\(^{2+}\) from agonist-sensitive stores can occur without any activation of capacitative entry (8–10). Consequently, it is far from clear that the transitory (and/or incomplete) nature of calcium store depletion during [Ca\(^{2+}\)]\(_i\) oscillations would provide an adequate or appropriate signal for the activation of calcium entry via a capacitative mechanism. Such considerations led us previously to investigate the nature of receptor-activated increases in Ca\(^{2+}\) entry during [Ca\(^{2+}\)]\(_i\) oscillations in cells from the exocrine avian nasal gland. In these studies, we showed that such Ca\(^{2+}\) entry was non-capacitative in nature (11) and appeared to involve a novel arachidonate-activated pathway (12).

In the experiments reported here, we extend our earlier findings to another, more widely used and functionally less highly specialized cell type, namely HEK293 cells. The specific cell line chosen had been stably transfected with the human m3 muscarinic receptor (m3-mAChR), thereby avoiding possible complications resulting from the presence of multiple muscarinic receptor subtypes. Using this cell line, we were able to show that the receptor-mediated generation of arachidonic acid is independent of the simultaneous activation of the PLC-InsP\(_3\) pathway, indicating that the m3-mAChR is capable of coupling both to the activation of PLC and the generation of arachidonic acid in a separate but parallel manner. Furthermore, we show that the arachidonate-mediated calcium entry pathway demonstrates characteristics that distinguish it from the more well known capacitative or store-operated entry of calcium.

MATERIALS AND METHODS

 Cultures of the human embryonic kidney cell line HEK293 that were stably transfected with the human m3 muscarinic receptor (m3-HEK cells) were obtained from Dr. Craig Logsdon (University of Michigan, Ann Arbor, MI) (see Ref. 13 for details). These cells were cultured under standard conditions in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and antibiotics. Changes in [Ca\(^{2+}\)]\(_i\) in

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1 The abbreviations used are: InsP\(_3\), inositol 1,4,5-trisphosphate; [Ca\(^{2+}\)]\(_i\), intracellular free calcium ion concentration; mAChR, muscarinic receptor; PLC, phospholipase C; PLD, phospholipase D; PLA\(_2\), phospholipase A\(_2\); PKC, protein kinase C; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; ETYA, 5,8,11,14-eicosatetraenoic acid; PMA, phorbol 12-myristate 13-acetate.
single individual cells were determined following loading with the fluorescent probe indo-1. Loading was achieved by incubation in 4 μM indo-1/AM for 12 min, followed by washing (three times) in saline. Cells were then incubated for a further 30 min at 37 °C to allow for complete hydrolysis of the acetoxyethyl ester. [Ca2+]i was measured as the ratio of the emitted fluorescence, measured as photon counts, at 405 and 485 nm using excitation at 350 nm as described previously (14, 15). The technique utilized for the simultaneous determination of changes in [Ca2+]i and Mn2+ quench was as described previously (15). In the experiments designed to eliminate the effects of increases in [Ca2+]i, cells were loaded with the Ca2+ chelator BAPTA by preincubation in saline containing 5 mM BAPTA/AM for 20 min. Preliminary experiments showed that this was sufficient to completely abolish any detectable [Ca2+]i signals in cells subsequently stimulated with concentrations of carbachol up to 5 μM.

Determinations of arachidonic acid release were essentially as described previously (12). Briefly, m3-HEK cells were cultured in six-well plates until ~50% confluent. 0.5 μM [3H]arachidonic acid was then added to each well, and culture was continued overnight. The cells were then washed three times with saline containing 0.2% fatty acid-free bovine serum albumin prior to addition of agonist and/or drugs as appropriate. Arachidonic acid released during a 5-min incubation was determined by liquid scintillation counting of the supernatant and normalized to the total counts in the cells, determined following solubilization with 1 M NaOH. Total inositol phosphates were determined in a similar manner. Cells cultured in six-well plates were incubated overnight in the presence of 5 μM/ml myo-[3H]inositol, followed by washing three times in saline. To enhance detection of inositol phosphate generation and turnover, experiments were performed in the presence of lithium (10 mM) following a 10-min preincubation in the same concentration of lithium. At the end of the experimental period (5 min), the saline was removed and replaced with 1 ml of 0.5 μM trichloroacetic acid. After incubation for 15 min on ice, the trichloroacetic acid-soluble fraction was extracted with ether and neutralized with sodium bicarbonate, and the extract was applied to Dowex columns. The loaded columns were washed with water, followed by 60 mM sodium formate plus 5 mM borax, before eluting the inositol phosphates with 1.2 M ammonium formate in 100 mM formic acid. Samples of the eluted inositol phosphates were counted by liquid scintillation. Total inositol phosphate generation is expressed as percent total counts in the phosphoinositide pool, determined from counting samples of the trichloroacetic acid-insoluble tissue fractions following their digestion overnight with 1.5 M NaOH.

Arachidonic acid, isotetrandrine, indomethacin, nordihydroguaiaretic acid, 5,8,11,14-eicosatetraynoic acid (ETYTA), and U73122 were from BIOMOL Research Labs Inc., and the phorbol ester phorbol 12-myristate 13-acetate (PMA) was from Calbiochem. [3H]Arachidonic acid and [3H]inositol were from American Radiolabeled Chemicals.

RESULTS

m3-mAChRs Coupled to Arachidonic Acid Generation to Activate Calcium Entry—We first determined whether activation of the transfected m3 muscarinic receptor in HEK cells was coupled to the generation of arachidonic acid, as described previously for the native muscarinic receptor in avian nasal gland cells (12). Fig. 1 shows that addition of the muscarinic receptor agonist carbachol to cells prelabeled with [3H]arachidonic acid resulted in a marked increase in arachidonic acid generation. This increase was dependent on agonist concentration and was clearly apparent even at agonist concentrations close to the threshold for inducing detectable [Ca2+]i signals in these cells (~1 μM carbachol). Concentrations of carbachol >5 μM were not examined as, based on our earlier studies (12), we believed that the receptor activation of arachidonic acid generation and the associated increase in calcium entry were specifically associated with stimulation with low agonist concentrations, whereas at very high agonist concentrations, [Ca2+]i signals were apparent. To examine the effects of arachidonate as a mitogen, signaling in m3-HEK cells, low concentrations of exogenous arachidonic acid were added to otherwise unstimulated cells. Addition of as little as 3–8 μM arachidonic acid resulted, after a variable lag period of several tens of seconds, in a slow increase in [Ca2+]i (Fig. 2A). Such an increase in [Ca2+]i could result from an increase in calcium entry from the extracellular medium or from release of calcium from intracellular stores. To distinguish between these two alternatives, lanthanum was used as a generic blocker of calcium entry channels. In the presence of 100 μM La3+, addition of exogenous arachidonic acid failed to increase [Ca2+]i, and only when the La3+ was removed was an increase in [Ca2+]i observed (Fig. 2B). The failure to observe any increase in [Ca2+]i in the presence of La3+ confirms that such an increase reflects an increase in Ca2+ entry. These data also rule out the additional possibility that arachidonic acid was inducing an increase in [Ca2+]i, by inhibition of the plasma membrane calcium pump. At high concentrations (~1 mM or higher), La3+ is also known to block the plasma membrane Ca2+-ATPase, but such an effect would be expected to further increase [Ca2+]i, not to block such an increase. To confirm that arachidonic acid was indeed activating calcium entry, simultaneous determinations of changes in [Ca2+]i and Mn2+ quench were performed. In these, extracellular Mn2+ (0.2 mM) was used as a surrogate for Ca2+ as, at low external concentrations, it readily passes through many kinds of Ca2+ channels. On entering the cytosol, the Mn2+ binds to and quenches the fluorescence of such probes as indo-1, and the rate of fluorescence quenching can then be used as an indirect measure of the rate of Mn2+ entry (Fig. 2C). As shown in Fig. 2C, the arachidonate-induced increase in [Ca2+]i was associated with a simultaneous increase in the rate of Mn2+ quench, indicating that Ca2+ entry is increased.

To characterize the effects of the carbachol-induced generation of arachidonic acid and the consequent increase in Ca2+ entry on [Ca2+]i, signals in m3-HEK cells, we examined the effect of the biscoclaurine alkaloid isotetrandrine. This substance acts as an apparently specific and readily reversible inhibitor of agonist-induced arachidonic acid generation (16, 17), as demonstrated in our earlier studies on the avian nasal gland (12). Consistent with these studies, isotetrandrine (10 μM) had no effect on carbachol-induced inositol phosphate generation in m3-HEK cells, but completely blocked the simultaneously carbachol-induced generation of arachidonic acid (Fig. 3). Addition of 10 μM isosafrole to cells demonstrating an oscillatory [Ca2+]i signal in response to low concentrations of carbachol resulted in an immediate cessation of the oscillations, which subsequently rapidly returned with normal amplitude and frequency on removal of the isosafrole (Fig. 4A). This is identical to the effect seen in these cells if Ca2+ entry was inhibited during an oscillatory [Ca2+]i response. For example, addition of La3+ to a cell during an oscillatory [Ca2+]i

**Fig. 1.** Effect of carbachol on the release of arachidonic acid in m3-HEK cells. Cells were incubated overnight in [3H]arachidonic acid and, after washing, exposed to carbachol (CCh) at different concentrations for 5 min in the presence of 0.2% fatty acid-free bovine serum albumin (to act as a sink for released arachidonic acid (AA), as described under “Materials and Methods.” Values are the means ± S.E. (n = 4).
response to carbachol resulted in the immediate cessation of the oscillations, which promptly returned on removal of the
La
2+
(Fig. 4B). To confirm that exposure to isotetrandrine during oscillations was indeed inhibiting Ca
2+
entry, we again used the Mn
2+
quench approach. As previously reported by us (11, 14) and by others (18, 19), oscillations in [Ca
2+]
were associated with a constant enhanced rate of Mn
2+
quench, reflecting a constant Ca
2+
entry. Fig. 4C shows that addition of isotetrandrine induced an immediate inhibition of this rate of Mn
2+
quench, which was rapidly restored on removal of the isotetrandrine. The observed effects on Mn
2+
quench (and hence, presumably Ca
2+
entry) were temporally directly associated with the inhibition of the [Ca
2+]
oscillations. It should be noted that the acute sensitivity to inhibition of Ca
2+
entry during oscillatory [Ca
2+]
signals seen here is not a universal feature in all cell types, some of which will continue to oscillate for some time even after complete removal of extracellular Ca
2+ (e.g. Xenopus oocytes). Such variation most likely reflects differences in surface-to-volume ratios and in the relative activities of the Ca
2+
pumps on the plasma membrane and on the intracellular stores (as well as levels of cytosolic buffers, etc.). However, such differences should not be interpreted as indicating that Ca
2+
entry plays no consistent or critical role in oscillatory [Ca
2+]
signals, as even in Xenopus oocytes, changes in Ca
2+
entry profoundly influence the frequency of agonist-induced [Ca
2+] oscillations (20).

In intact cells, arachidonic acid frequently undergoes rapid oxidation, resulting in the production of a variety of eicosanoids that are, themselves, known to possess signaling activities. To determine whether the observed effects were due to arachidonic acid itself or to one of the many products of arachidonic acid metabolism, we examined the effects of inhibition of the cyclooxygenase pathway, using indomethacin. Addition of arachidonic acid itself or to one of the many products of arachidonic acid metabolism, we examined the effects of inhibition of the cyclooxygenase pathway, using indomethacin. Addition of arachidonic acid was further confirmed in experiments showing that addition of the non-metabolizable arachidonic acid...
analog ETYA to unstimulated cells caused an increase in 
$[\text{Ca}^{2+}]_i$ similar to that seen with arachidonic acid (Fig. 5C). In these experiments, it was noted that the effects of ETYA were frequently more rapid in onset than those seen with arachidonic acid, but required somewhat higher concentrations ($20 \mu M$ versus $5 \mu M$). Such data are consistent with the response to exogenously added arachidonic acid being muted by metabolism in the intact cell, yet showing some selectivity for arachidonic acid over its analog ETYA. Together, the above data indicate that it is arachidonic acid itself that is the active moiety responsible for the observed effects on $[\text{Ca}^{2+}]_i$. They also suggest that both lipoxygenase and cyclooxygenase pathways are active in HEK293 cells and, in vivo, act to limit increases in receptor-stimulated levels of arachidonic acid.

mAChR Activation Stimulates Arachidonic Acid Generation Independently of PLC Activation—The m3-mAChR is known to couple, via a member of the $G_q$ family of G proteins, to the activation of phospholipase C and the generation of diacylglycerol and inositol 1,4,5-trisphosphate. The former activates protein kinase C, whereas the latter is integral to the initiation of $[\text{Ca}^{2+}]_i$ signals. To determine whether the observed increased generation of arachidonic acid was a downstream effect of the simultaneous activation of this PLC pathway, various approaches were employed. The possible involvement of InsP3-mediated increases in $[\text{Ca}^{2+}]_i$ was examined by determining arachidonic acid generation in cells loaded with the calcium chelator BAPTA (as described under “Materials and Methods”) prior to stimulation with carbachol. As shown in Fig. 6, the carbachol-induced generation of arachidonic acid was completely unimpaired in such BAPTA-loaded cells, indicating that this effect was not dependent on the receptor-activated $[\text{Ca}^{2+}]_i$ signals.

We next examined the possible involvement of the PLC-
mediated generation of diacylglycerol and the consequent activation of PKC in the observed mAChR-induced arachidonic acid release. Direct stimulation of PKC activity by addition of the phorbol ester PMA (preincubation for 5 min in the presence of 0.1 μM PMA) resulted in a modest, but significant, inhibition of arachidonic acid release in unstimulated cells (0.14 ± 0.01% in PMA-treated cells versus 0.18 ± 0.1% in control cells). More significantly, pretreatment with PMA completely abolished the normal mAChR-induced arachidonic acid release seen following addition of carbachol (Fig. 7). These data indicate that the principal effect of PKC activation appears to be to uncouple the stimulation of arachidonic acid release by mAChRs. Although the precise basis for this effect is unknown, these data clearly indicate that the activation of PKC does not result in the stimulation of arachidonic acid generation. Based on the above data from BAPTA-loaded cells and PMA-treated cells, it seems that neither the PLC-mediated increase in [Ca^{2+}], nor the activation of PKC is involved in the observed activation of arachidonic acid release. Nevertheless, we were concerned that the above experiments may not entirely eliminate a potential involvement of the PLC pathway. For example, it has been reported in several cell types that the stimulation of PKC can exert a negative feedback on receptor-activated PLC activity. It was therefore possible that the failure of PMA to stimulate arachidonic acid release may have simply reflected the concomitant inhibition of PLC activity. Experiments on m3-HEK cells confirmed that PMA (0.1 μM with preincubation for 5 min) did indeed significantly inhibit the carbachol-induced increase in inositol phosphate generation, an effect consistent with a reduction of agonist-activated PLC activity (data not shown). Alternatively, it is possible that the mAChR activation of arachidonic acid generation requires an increase in both [Ca^{2+}], and PKC activity. We therefore carried out an additional series of experiments examining the effect of the drug U73122 (21) on mAChR activation of arachidonic acid generation. Although not always entirely specific, U73122 has been extensively used as an inhibitor of PLC activity, and consistent with this, we found that in the presence of 10 μM U73122, carbachol-induced inositol phosphate generation was completely abolished (Fig. 8A). However, under identical conditions, carbachol-induced arachidonic acid generation was unaffected (Fig. 8B). These data confirm that the observed receptor-activated arachidonic acid generation is not a downstream effect of any PLC activity.

**Calcium Entry Is Distinct from Capacitative Entry**—As discussed above, the activation of the capacitative mechanism of Ca^{2+} entry is solely dependent on the emptying of intracellular stores of calcium no matter how this is achieved. However, under normal circumstances of receptor stimulation, the discharge of the intracellular Ca^{2+} stores occurs as a result of the PLC-mediated generation of inositol 1,4,5-trisphosphate, which activates Ca^{2+}-permeable channels on the stores. The demonstration that mAChR activation of arachidonic acid generation is not a downstream effect of the simultaneous activation of PLC (see above) sug-

![Fig. 6. Effect of carbachol on the release of arachidonic acid in BAPTA-loaded cells.](image)

![Fig. 7. Effect of PMA on carbachol-stimulated arachidonic acid release.](image)

![Fig. 8. Effect of U73122 on carbachol-induced inositol phosphate generation (A) and arachidonic acid release (B).](image)
gests that the arachidonate-activated Ca\(^{2+}\) entry is unlikely to be part of a capacitative mechanism. This is further supported by the fact that isetetrandrine, which, as shown, is an effective inhibitor of receptor-activated arachidonic acid generation, has no effect on the capacitative entry of Ca\(^{2+}\) induced by thapsigargin (data not shown). Similar data were obtained in our earlier studies on avian nasal gland cells (12) and have been reported for the related compound tetrandrine in adrenal glo-merulosar cells (22). However, this does not entirely preclude the possibility that arachidonic acid is involved in some way in the capacitative mechanism. For example, the complete depletion of the stores induced by thapsigargin may result in such a profound stimulation of the capacitative mechanism that isetetrandrine is not able to reverse it significantly. Alterna-

merulosa cells (22). However, this does not entirely preclude gargin (data not shown). Similar data were obtained in our hanced Ca\(^{2+}\) donic acid results in the immediate inhibition of receptor-en-

We have also shown that inhibition of the generation of arachi-
donic acid (Fig. 9A, reducing the pH of the superfusing saline to 6.7 induced a marked decrease in the sustained [Ca\(^{2+}\)]\(i\), seen in thapsigargin-treated cells, consistent with an inhibition of ca-

pacitative entry. This effect was readily reversed on restoration of normal extracellular pH. An identical reduction in extracellu-

lar pH was, however, completely without effect on the similar sustained [Ca\(^{2+}\)]\(i\), seen in cells exposed to exogenous arachi-
donic acid (Fig. 9B). This marked difference in the effect of extracellular pH indicates that the Ca\(^{2+}\) entry channel acti-
vated by arachidonic acid is unlikely to be the same as that acti-
vated by thapsigargin-induced depletion of intracellular stores.

**DISCUSSION**

In the studies reported here, we have shown that activation of the m3-mAChR stably transfected in HEK293 cells results in the generation of arachidonic acid and that this specifically activates a Ca\(^{2+}\) entry pathway that is critical to the regulation of the oscillatory [Ca\(^{2+}\)]\(i\), signals generated by low concentra-
tions of appropriate agonists. Although detailed concentration-
response curves for the generation of arachidonic acid were not determined, it is clear that this response shows a sensitivity to muscarinic agonists that is very similar to that demonstrated by the activation of PLC and is therefore consistent with a potential role in the regulation or generation of [Ca\(^{2+}\)]\(i\), signals. We have also shown that inhibition of the generation of arachidonic acid results in the immediate inhibition of receptor-en-
hanced Ca\(^{2+}\) entry and the cessation of the oscillatory [Ca\(^{2+}\)]\(i\), response. The data further indicate that this is an effect of arachidonic acid itself and not of a product of the commonly recognized metabolic pathways for arachidonic acid. Regarding our data on the activation of Ca\(^{2+}\) entry by exogenous application of arachidonic acid, such application has been reported to produce a variety of effects on [Ca\(^{2+}\)]\(i\), signals in a many differ-
ent cells, including effects on Ca\(^{2+}\) release from intracellular stores (28) as well as on Ca\(^{2+}\) entry (29). However, most of these studies involved the use of very high concentrations of arachidonate (>50 \(\mu\)M), raising the possibility of a variety of nonspecific effects. In contrast, the effects on Ca\(^{2+}\) entry we have observed are seen at very low concentrations of exog-

\(^2\) R. Penner, personal communication.
lations induced by cholecystokinin B receptors transfected into Chinese hamster ovary cells (30). An arachidonate-activated Ca\textsuperscript{2+} entry that is believed to be involved in basic fibroblast growth factor-induced responses in Balb/c 3T3 fibroblasts has also been recently described (31), and low concentrations of exogenous arachidonic acid have been shown to activate Ca\textsuperscript{2+}-permeable, voltage-insensitive cation channels in chromaffin cells (32). Direct regulation of ion channels by fatty acids, including arachidonic acid, has been shown for a variety of different channel types (33, 34) and includes both stimulatory and inhibitory effects. More important, although the full characterization of the arachidonate-mediated pathway we have described must await further studies, we have shown that it possesses characteristics that distinguish it from the more well-known capacitative or store-operated pathway. For example, in our previous study of this pathway in avian nasal gland cells (12), we showed that its activation was apparently independent of any InsP\textsubscript{3}-induced release of Ca\textsuperscript{2+} from intracellular stores. In fact, the data indicated that, at the low agonist concentrations that typically give rise to oscillatory [Ca\textsuperscript{2+}], signals, generated levels of InsP\textsubscript{3} alone were not adequate to induce any detectable release of Ca\textsuperscript{2+} from the stores, and such release was absolutely dependent on the additional effect of the arachidonate-mediated entry of Ca\textsuperscript{2+}. However, the possibility remained that the entry we observed was, in fact, dependent on a capacitative mechanism that was activated by the emptying of a small specific subset of the overall agonist-sensitive store whose discharge was undetectable in our experiments. Published evidence suggests this is unlikely because, as noted earlier, data from a variety of different cells indicate that if such a subset exists, its discharge would require higher concentrations of InsP\textsubscript{3} than those required to empty the bulk of the agonist-sensitive stores in the cell (8–10). Nevertheless, in the study reported here, we have made an additional critical observation that supports our hypothesis that the arachidonate-mediated Ca\textsuperscript{2+} entry is entirely distinct from the capacitative pathway activated as a result of the depletion of intracellular stores. We showed that, consistent with reports from various different cell types, such capacitative entry in HEK293 cells is acutely sensitive to reductions in extracellular pH. In marked contrast, the Ca\textsuperscript{2+} entry activated by arachidonic acid in the same cells is entirely insensitive to such changes in extracellular pH. Given that the observed effects on the capacitative entry are reported to reflect a direct effect on the store-operated Ca\textsuperscript{2+} channel (at least in the case of I\textsubscript{CRAC}), such a clear difference indicates that the arachidonate-activated pathway must be distinct from that activated by store depletion. This conclusion is further supported by our demonstration that the receptor-mediated increase in arachidonic acid generation that we have observed is independent of the simultaneous activation of the PLC-InsP\textsubscript{3} pathway (see below). Clearly, such a finding is inconsistent with the idea that the muscarinic receptor stimulation of the arachidonate-mediated pathway is downstream of any InsP\textsubscript{3}-mediated release of stored Ca\textsuperscript{2+}.

Data from a wide range of different cell types suggest that there are generally two principal sources of receptor-mediated increases in arachidonic acid generation. The first involves the action of diacylglycerol/monoaoylglycerol lipases on diacylglycerol. This, in turn, is mainly derived from the hydrolysis of phosphatidylcholine as a result of receptor-induced increases in either PLD activity (via phosphatidic acid) (35, 36) or PLC (37, 38). In this context, it has previously been reported that the m3-mACHR stably transfected into HEK293 cells is capable of coupling to a PLD (39, 40). However, several characteristics of this response in the m3-mACHR-transfected HEK293 cells indicate that this is unlikely to be the basis for the observed carbachol-induced increase in arachidonic acid generation. First, the activation of PLD by the m3-mACHR stably transfected into HEK293 cells has been shown to completely desensitize within 2 min of stimulation, even at the low concentrations of agonist used in this study (40). This desensitization is apparently not a result of the loss of cell-surface receptors, but reflects a rapid and sustained uncoupling of the receptors from the activation of PLD. Second, it has been shown that addition of the phorbol ester PMA induces a pronounced stimulation of PLD activity in m3-HEK cells (40, 41) as well as in many other mammalian cells (42, 43). Examination of the published data indicate that 0.1 μM PMA increased PLD activity to levels ~4-fold higher than those seen with a maximal concentration of carbachol (40). This is clearly in marked contrast to our data showing that, at the same concentration, PMA had a marked inhibitory effect on the carbachol-induced arachidonic acid generation. Based on these findings, we conclude that the reported characteristics of the PLD response in HEK293 cells are not consistent with our findings on the mACHR-activated arachidonic acid generation and that the PLD-mediated generation of diacylglycerol is unlikely to account for the observed mACHR-induced arachidonic acid response.

The alternative mechanism for receptor-induced increases in arachidonic acid generation involves the action of a cytosolic PLA\textsubscript{2} releasing arachidonic acid from appropriate phospholipids. Two main types of cytosolic PLA\textsubscript{2} with distinct properties and structures are currently identified: the “classic” type IV Ca\textsuperscript{2+}-dependent cPLA\textsubscript{2} and the more recently characterized type VI Ca\textsuperscript{2+}-independent cPLA\textsubscript{2} (PPLA\textsubscript{2}) (44–46). This latter group is thought to be principally involved in general membrane phospholipid remodeling (47), whereas it is the type IV Ca\textsuperscript{2+}-dependent cPLA\textsubscript{2} that has usually been shown to be associated with the receptor activation of arachidonic acid release (48). In this respect, a particularly significant finding of the studies reported here is that the increase in arachidonic acid generation we have observed was shown to be independent of the simultaneous activation of PLC. Receptor activation of cPLA\textsubscript{2} has been generally reported to involve two distinct processes: a Ca\textsuperscript{2+}-dependent translocation of the cPLA\textsubscript{2} to the membrane to allow interaction with its phospholipid substrate and a phosphorylation that is usually mediated via a mitogen-activated protein kinase, whose activity may, in turn, be modulated by PKC (48). Alternatively, PKC may itself directly phosphorylate the cPLA\textsubscript{2} to increase its activity. The relative importance of the Ca\textsuperscript{2+}-dependent translocation and the phosphorylation steps in the activation of cPLA\textsubscript{2} appears to vary in different cell types and under different circumstances (48). In marked contrast, the studies we have presented here clearly show that neither an increase in [Ca\textsuperscript{2+}], nor the activation of PKC (either individually or together) was required for the observed carbachol-induced generation of arachidonic acid. In this regard, it is perhaps important to point out that most studies investigating the receptor-activation of cPLA\textsubscript{2} have generally utilized maximal (or near-maximal) concentrations of the relevant agonist. Consequently, although the data presented do not exclude the presence of these activation pathways in HEK293 cells, it is clear that neither a Ca\textsuperscript{2+}-dependent translocation nor a PKC-dependent phosphorylation step can play any significant role in the activation of arachidonic acid generation under the specific conditions employed, i.e. at low concentrations of muscarinic agonists. This was further supported by the data obtained using the drug U73122, which confirmed that the mACHR stimulation of arachidonic acid generation we observed is not downstream of PLC activation. Moreover, because the HEK293 cell line utilized here possesses only a single muscarinic receptor subtype (m3), the data show
that this receptor is capable of coupling both to the activation of PLC and the generation of arachidonic acid in a separate but parallel manner. We therefore conclude that the two signaling pathways are independently activated by the m3-mAChR. Given the critical role that such arachidonic acid generation plays in the activation of the Ca\(^{2+}\) response required to drive receptor-activated oscillatory [Ca\(^{2+}\)]\(_i\) signals in these and other cells, the identification of the mechanism responsible for coupling of muscarinic receptors to arachidonic acid generation at these physiologically relevant levels of stimulation is clearly of considerable importance.

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REFERENCES

1. Putney, J. W., Jr. (1986) Cell Calcium 7, 1–12
2. Putney, J. W., Jr. (1990) Cell Calcium 11, 611–624
3. Berridge, M. J. (1993) Biochem. J. 312, 1–11
4. Penner, R., Fasolato, C., and Hoth, M. (1993) Curr. Opin. Neurobiol. 3, 368–374
5. Fasolato, C., Innocenti, B., and Pozzan T. (1994) Trends Pharmacol. Sci. 15, 77–83
6. Berridge, M. J. (1993) Nature 361, 315–325
7. Berridge, M. J. (1994) Biochem. J. 302, 545–550
8. Parekh, A., Fleig, A., and Penner, R. (1997) Cell 89, 973–980
9. Hartzell, C. (1996) J. Gen. Physiol. 108, 157–175
10. Liu, K.-Q., Bunnell, S. C., Gurniak, C. B., and Berg, L. J. (1996) J. Exp. Med. 187, 1721–1727
11. Shuttleworth, T. J., and Thompson, J. L. (1996) Biochem J. 316, 819–824
12. Shuttleworth, T. J. (1996) J. Biol. Chem. 271, 21720–21725
13. Yang, J., Williams, J. A., Yule, D. I., and Legsdon, C. D. (1995) Mol. Pharmacol. 48, 477–485
14. Martin, S. C., and Shuttleworth, T. J. (1994) FEBS Lett. 352, 32–36
15. Shuttleworth, T. J. (1994) Cell Calcium 15, 457–466
16. Akiba, S., Kato, R., Sato, T., and Fujii, T. (1992) Biochem. Pharmacol. 44, 45–50
17. Hashizume, T., Yamaguchi, H., Sato, T., and Fujii, T. (1991) Biochem. Pharmacol. 41, 419–423
18. Jacob, R. (1990) J. Physiol. (Lond.) 421, 55–77
19. Thorn, P. (1995) J. Physiol. (Lond.) 482, 275–281
20. Girard, S., and Clapham, D. E. (1993) Science 260, 229–232
21. Smith, R. J., Sam, L. M., Justen, J. M., Bundy, G. L., Bala, G. A., and Bleasdale, J. E. (1990) J. Pharmacol. Exp. Ther. 253, 688–697
22. Rossier, M. F., Python, C. P., Capponi, A. M., Schlegel, W., Kwan, C. Y., and Valloton, M. B. (1988) Endocrinology 123, 1035–1043
23. Muallem, S., Pandol, S. J., and Becker, T. G. (1989) Am. J. Physiol. 257, G917–G924
24. Negulescu, P. A., and Machen, T. E. (1995) Am. J. Physiol. 269, G770–G778
25. Wakabayashi, I., and Grouchko, K. (1996) Biochem. Biophys. Res. Commun. 221, 762–767
26. Hug, M. J., Pahl, C., and Novak, I. (1996) Pflügers Arch. Eur. J. Physiol. 432, 278–285
27. Malaney, A., and Nelson, D. J. (1995) J. Membr. Biol. 146, 101–111
28. Packham, D. E., Jiang, L., and Congreve, A. D. (1995) Cell Calcium 17, 399–408
29. Van der Zee, L., Nelemans, A., and den Hertog, A. (1995) Biochem. J. 305, 859–864
30. Akagi, K., Nagao, T., and Urasihidani, T. (1997) Jpn. J. Pharmacol. 75, 33–42
31. Munaron, L., Antoitiotti, S., Distasi, C., and Lorisio, D. (1997) Cell Calcium 22, 179–188
32. Michizuki-Oda, N., Negishi, M., Mori, K., and Ito, S. (1993) J. Neurochem. 61, 1882–1890
33. Ordway, R. W., Singer, J. T., and Walsh, J. V., Jr. (1991) Trends Neurosci. 14, 96–100
34. Meves, H. (1994) Prog. Neurobiol. (Oxf.) 43, 175–186
35. Lin, P., Wiggan, G. A., and Gillillan, A. M. (1991) J. Immunol. 146, 1609–1616
36. Sato, T., Ishimoto, T., Akiba, S., and Fujii, T. (1995) FEBS Lett. 323, 23–26
37. Dixon, J. F., and Hokin, L. E. (1984) J. Biol. Chem. 259, 14418–14425
38. Hou, W., Artia, Y., and Muriset, J. (1996) Am. J. Physiol. 271, C1735–C1742
39. Sandmann, J., Peralta, E. G., and Wurtman, R. J. (1991) J. Biol. Chem. 266, 6031–6034
40. Schmidt, M., Fassett, B., Rümenapp, U., Bieneck, C., Wieland, T., van Koppen, C. J., and Jacobs, K. H. (1996) J. Biol. Chem. 271, 2422–2426
41. Ekman, K. H. (1997) Physiol. Rev. 77, 303–320
42. Morris, A. J., Hammond, S. M., and Sciorr, V. A., Rudge, S. A., and Frohman, M. A. (1997) Biochem. Soc. Trans. 25, 1151–1157
43. Dekker, E. A. (1994) J. Biol. Chem. 269, 13057–13060
44. Ackermann, E. J., Kempner, E. S., and Dennis, E. A. (1994) J. Biol. Chem. 269, 9227–9233
45. Dennis, E. A. (1997) Trends Biochem. Sci. 22, 1–2
46. Balázs, J., and Dennis, E. A. (1997) J. Biol. Chem. 272, 16669–16672
47. Leslie, C. C. (1997) J. Biol. Chem. 272, 16709–16712
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