We have recently questioned whether the capacitative or store-operated model for receptor-activated Ca\textsuperscript{2+} entry can account for the influx of Ca\textsuperscript{2+} seen at low agonist concentrations, such as those typically producing [Ca\textsuperscript{2+}]\textsubscript{i} oscillations. Instead, we have identified an arachidonic acid-regulated, noncapacitative Ca\textsuperscript{2+} entry mechanism that appears to be specifically responsible for the receptor-activated entry of Ca\textsuperscript{2+} under these conditions. However, it is unclear whether these two systems reflect the activity of distinct entry pathways or simply different mechanisms of regulating a common pathway. We therefore used the known selectivity of the Ca\textsuperscript{2+}-stimulated type VIII adenylyl cyclase for Ca\textsuperscript{2+} entry occurring via the capacitative pathway (Fagan, K. A., Mahey, R., and Cooper, D. M. F. (1996) J. Biol. Chem. 271, 12438–12444) to attempt to discriminate between these two entry mechanisms in HEK293 cells. Consistent with the earlier reports, we found that thapsigargin induced an approximate 3-fold increase in adenylyl cyclase activity that was unrelated to global changes in [Ca\textsuperscript{2+}]\textsubscript{i} or to the release of Ca\textsuperscript{2+} from internal stores but was specifically dependent on the induced capacitative entry of Ca\textsuperscript{2+}. In marked contrast, the arachidonate-induced entry of Ca\textsuperscript{2+} completely failed to affect adenylyl cyclase activity despite producing a substantially greater rate of entry than that induced by thapsigargin. These data demonstrate that the arachidonate-activated entry of Ca\textsuperscript{2+} occurs via an entirely distinct influx pathway.

The agonist-stimulated entry of extracellular Ca\textsuperscript{2+} plays a critical role in the generation and maintenance of intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) signals resulting from activation of receptors coupled to the phospholipase C/inositol trisphosphate signaling pathway. However, in nonexcitable cells, the precise nature and mechanism of activation of such Ca\textsuperscript{2+} entry pathways remains unclear. An activation of a Ca\textsuperscript{2+} entry that is dependent on and subsequent to the emptying of intracellular agonist-sensitive Ca\textsuperscript{2+} stores has been demonstrated in a wide variety of cells. This so-called “capacitative” or store-operated mechanism of Ca\textsuperscript{2+} entry first proposed by Putney (1, 2) appears to be an almost universal feature of cells and can be readily demonstrated to be responsible for the sustained elevations of [Ca\textsuperscript{2+}]\textsubscript{i} following activation of cells with high agonist concentrations, as well as for the refilling of the agonist-sensitive stores on the termination of such signals. Recently however, we have questioned whether the demonstrated properties of such capacitative entry and the characteristics of the channels involved (as far as is currently known) are adequate or appropriate to account for the receptor-activated influx of Ca\textsuperscript{2+} seen at low agonist concentrations, such as those that typically give rise to oscillatory [Ca\textsuperscript{2+}]\textsubscript{i} signals (3, 4). This is an important question for two reasons. First, it is generally considered that such oscillatory [Ca\textsuperscript{2+}]\textsubscript{i} signals are likely to represent the physiologically relevant response for many cells. Second, it is known that the receptor-activated influx of Ca\textsuperscript{2+} during such signals has a marked effect on the oscillation frequency (5–9), which is a key component of the agonist-generated message within the cell. Our studies on the mechanism of Ca\textsuperscript{2+} entry during [Ca\textsuperscript{2+}]\textsubscript{i} oscillations led us to the identification of a novel noncapacitative Ca\textsuperscript{2+} entry pathway that is gated by arachidonic acid and that appears to be specifically responsible for the receptor-activated entry of Ca\textsuperscript{2+} under these conditions (10, 11). Together, our studies have shown that 1) arachidonic acid is generated at the relevant agonist concentrations that are known to produce [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in the same cells; 2) the addition of low concentrations of exogenous arachidonic acid induces an entry of Ca\textsuperscript{2+} that is entirely independent of store depletion; 3) the inhibition of the agonist-induced generation of arachidonic acid specifically and rapidly blocks the Ca\textsuperscript{2+} entry associated with [Ca\textsuperscript{2+}]\textsubscript{i} oscillations, yet it is without effect on capacitative Ca\textsuperscript{2+} entry; 4) inhibition of the metabolism of arachidonic acid converts agonist-induced oscillatory [Ca\textsuperscript{2+}]\textsubscript{i} signals into sustained “plateau” signals, as might be expected if Ca\textsuperscript{2+} entry was further increased by the accumulating arachidonic acid (10, 11). Based on these findings, it is our contention that arachidonic acid fulfills all the generally accepted criteria for being the second messenger responsible for the regulation of the agonist-activated entry of Ca\textsuperscript{2+} during [Ca\textsuperscript{2+}]\textsubscript{i} oscillations.

What is not entirely clear, however, is whether arachidonic acid is regulating an entirely distinct Ca\textsuperscript{2+} entry pathway in the plasma membrane or merely modulating the activity of the same pathway activated by store depletion in the capacitative mechanism. We have previously shown that the [Ca\textsuperscript{2+}]\textsubscript{i} signal produced by capacitative Ca\textsuperscript{2+} entry and that produced by the addition of exogenous arachidonic acid demonstrate differences in their sensitivity to a reduction in extracellular pH, suggesting that they may represent distinct entry pathways (11). In the following study, we have used the reported marked selectivity of certain Ca\textsuperscript{2+}-sensitive adenylyl cyclases in cells for Ca\textsuperscript{2+} entering specifically via the capacitative pathway (12). It has been shown in a variety of studies that these adenylyl cyclases, whether endogenously present or following their transient transfection, are largely unresponsive to changes in [Ca\textsuperscript{2+}]\textsubscript{i}, resulting from Ca\textsuperscript{2+} release from internal stores or
from Ca\(^{2+}\) entry via “nonspecific” ionomycin-induced pathways. In contrast, these same adenylyl cyclases are acutely sensitive to the Ca\(^{2+}\) entering via store-operated mechanisms (13), a sensitivity that is believed to reflect a specific co-localization of the adenylyl cyclase with the store-operated Ca\(^{2+}\) channels in the plasma membrane (14). In this study we have utilized the type VIII Ca\(^{2+}\)-stimulated adenylyl cyclase transiently transacted into HEK293 cells. This is the same adenylyl cyclase and cell line used by Fagan et al. (13) who showed that its activity was markedly and specifically increased by Ca\(^{2+}\) entering via the capacitive or store-operated pathway.

**EXPERIMENTAL PROCEDURES**

*Materials—* Thapsigargin, arachidonic acid, isobutylmethylxanthine, and 4-(3-butoxy-4-methoxyphenyl)-2-imidazolidinone were all from Bi- omol Research Laboratories Inc. The cyclic AMP binding assay kits were from Amersham Pharmacia Biotech. Samples of the human embryonic kidney cell line HEK293 were obtained from the ATCC.

*Plasmid Construction and Transient Transfection—* The rat type VIII adenylyl cyclase cDNA clone in the pcDNA3.1 expression vector (Invitrogen) was generously provided by Dr. Dermot Cooper (University of Colorado, Denver, CO). HEK293 cells were cultured under standard conditions in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and antibiotics at 37 °C in a humidified atmosphere of 95% air and 5% CO\(_2\). Prior to transfection, cells were seeded into 75-cm\(^2\) flasks and grown until approximately 50% confluent. Transfection was performed by the calcium phosphate method of Chen and Okayama (15) using 26 μg of plasmid DNA. Eighteen hours after transfection, cells were washed and then harvested using Ca\(^{2+}\)-Mg\(^{2+}\)-free phosphate-buffered saline containing 0.06% EDTA. The harvested cells were centrifuged, resuspended in medium, plated into 6-well culture dishes, and incubated for a further 2 days before experiments were performed.

*Cyclic AMP Accumulation—* Adenylyl cyclase activity in vivo was assayed indirectly by determining the accumulation of cAMP in cells preincubated for 10 min in the presence of the phosphodiesterase inhibitors (500 μM isobutylmethylxanthine, 100 μM 4-(3-butoxy-4-methoxyphenyl)-2-imidazolidinone). By eliminating the metabolism of generated cAMP, these inhibitors allowed an estimate of overall adenylyl cyclase activity in the intact cells. Furthermore, the use of phosphodiesterase inhibitors eliminated any possible influence of changes in [Ca\(^{2+}\)], affecting cAMP levels as a result of influencing phosphodiesterase activities (16, 17). Except where indicated, cAMP accumulation was determined over a 3-min period following the addition of 10 μM forskolin (21.7 pmol/mg protein (3)); the use of thapsigargin (250 nM) presumably reflecting the activity of endogenous adenylyl cyclases. Following transient transfection with the type VIII adenylyl cyclase, resting cAMP accumulation was 157.2 ± 23.5 pmol/mg protein and was stimulated approximately 15-fold to 2250.3 ± 345.8 pmol/mg (n = 7) in the presence of forskolin. This amounts to a 4.6-fold increase in forskolin-stimulated cAMP accumulation following transfection. These data represent the results of separate transfections and demonstrate that reasonably consistent levels of expression were obtained in each case.

The overall aim of the study was to compare the ability of arachidonate-activated and store-operated (capacitative) Ca\(^{2+}\) entry pathways to induce an activation of the transfected adenylyl cyclase. We chose the sarcoplasmic-endoplasmic reticulum calcium pump inhibitor thapsigargin as the agent to activate the capacitative entry of Ca\(^{2+}\). The use of thapsigargin and exogenous arachidonic acid to activate capacitative and noncapacitative Ca\(^{2+}\) entry, respectively, avoids the potential activation or generation of additional signaling moieties such as Ge, Gq, protein kinase C, and G protein βγ subunits, which are known to have type-specific effects on adenylyl cyclase activity (20). Examination of the overall [Ca\(^{2+}\)]\(_i\) responses induced in each cell revealed that the addition of 250 nM thapsigargin to the AC8-HEK cells produced a [Ca\(^{2+}\)]\(_i\) signal that was closely paralleled that produced by 8 μM exogenous arachidonic acid (Fig. 1). This [Ca\(^{2+}\)]\(_i\) signal comprised an initial increase to a peak value over the first approximately 150 s followed by a slow decline (equivalent to approximately 30% of the initial increase) over the succeeding 200 s. Comparison of the thapsigargin and arachidonic acid responses indicated a close similarity in the rate of the initial increase, peak values attained, and rate of subsequent slow decline. The only consistent difference was a somewhat more rapid onset of the rise in [Ca\(^{2+}\)]\(_i\) in the case of thapsigargin addition. These concentrations were subsequently used in all remaining experiments.

Changes in adenylyl cyclase activity during the period when [Ca\(^{2+}\)]\(_i\) rose to its maximum values were assessed by determining cAMP accumulation in the presence of phosphodiesterase inhibitors and forskolin during the first 3 min after the addition of either 8 μM arachidonic acid or 250 nM thapsigargin (Fig. 2). It can be seen that thapsigargin produced a greater than 2.5-fold stimulation of adenylyl cyclase during this period. In

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**FIG. 1.** Comparison of the effect of thapsigargin and arachidonic acid on [Ca\(^{2+}\)]\(_i\), levels in AC8-HEK293. Cells loaded with indo-1 were exposed to either thapsigargin (250 nM) or arachidonic acid (8 μM) at the point indicated by an arrow. Changes in [Ca\(^{2+}\)]\(_i\), recorded as the ε485/ε508 emission ratio were determined in a group of 30–50 cells as described under “Experimental Procedures.” Typical responses are illustrated.
marked contrast and despite the very similar rate and magnitude of the overall increase in [Ca\(^{2+}\)], arachidonic acid failed to produce any significant increase in adenylyl cyclase activity. This inability of the arachidonic acid-induced changes in [Ca\(^{2+}\)], to effectively increase cAMP accumulation was not due to any independent inhibitory action of the fatty acid on the adenylyl cyclase activity as, in a separate series of experiments, simultaneous addition of 8 \(\mu\)M arachidonic acid to cells exposed to 250 \(\mu\)M thapsigargin had no significant effect on the ability of the latter to increase cAMP accumulation (7.75 ± 0.28 \(\mu\)mol/mg protein for thapsigargin alone compared with 7.08 ± 0.28 \(\mu\)mol/mg protein for thapsigargin in the presence of arachidonic acid, \(n = 5, p = 0.07\)).

Despite the overall similarity in the [Ca\(^{2+}\)], changes, it is important to appreciate that there are marked differences in the origin of these changes in the two situations. In the case of thapsigargin, Ca\(^{2+}\) is initially released from intracellular stores as a result of inhibition of the sarcoplasmic-endoplasmic reticulum calcium pump on the store membranes (21). The resulting depletion of the intracellular stores subsequently activates Ca\(^{2+}\) entry via a capacitative mechanism of unknown nature. In contrast, we have previously shown that the increase in [Ca\(^{2+}\)], induced by exogenous arachidonic acid results entirely from an increased Ca\(^{2+}\) entry (10, 11). As stimulation of the type VIII adenylyl cyclase has been reported to be specifically dependent on Ca\(^{2+}\) entry via capacitative pathways (13) it was important to compare the effects of thapsigargin and arachidonic acid at a time when [Ca\(^{2+}\)], changes were specifically dependent on Ca\(^{2+}\) entry alone. Furthermore it is known that, both in the case of thapsigargin and exogenous arachidonic acid, activation of Ca\(^{2+}\) entry is rather slow to develop (11, 19). To examine the time course of the activation of Ca\(^{2+}\) entry in the experiments performed here, a Mn\(^{2+}\) quench protocol was employed on the AC8-HEK cells. Fig. 3 illustrates the response to the addition of 8 \(\mu\)M arachidonic acid. As can be seen, although an increase in the rate of Mn\(^{2+}\) quench could be detected approximately 1 min after the addition of arachidonic acid, maximal rates were not achieved until some 2 min later. A similar time course for the increase in the rate of Mn\(^{2+}\) quench was obtained with the addition of 250 \(\mu\)M thapsigargin (data not shown).

Based on these data we sought to examine the effects on adenylyl cyclase activity over the period 3–6 min after addition, at which time Ca\(^{2+}\) entry was at its maximum. Consistent with the reported specific dependence of the Ca\(^{2+}\)-sensitive adenylyl cyclase on capacitative Ca\(^{2+}\) entry and with the above data on the rates of Mn\(^{2+}\) quench, examination of the thapsigargin-stimulated rate of cAMP accumulation during this period was somewhat higher (approximately 38%) than that seen during the first 3 min despite the observed decline in overall values of [Ca\(^{2+}\)], (Fig. 4). This indicates that activation of the adenylyl cyclase is independent of overall values of [Ca\(^{2+}\)], as reported previously. Furthermore, nominal removal of extracellular Ca\(^{2+}\) during this period completely obliterated the observed thapsigargin-induced stimulation in adenylyl cyclase activity (Fig. 4). This did not reflect an inhibitory effect of extracellular Ca\(^{2+}\) on the adenylyl cyclase as activities in the absence of thapsigargin were not significantly affected. This demonstrates that any possible contribution from Ca\(^{2+}\) release from intracellular stores can be ignored at this stage of the response.

Comparison of the effects of thapsigargin and of arachidonic acid on the adenylyl cyclase activity during this period of maximal increase in Ca\(^{2+}\) entry (3–6 min after addition) reveals that, once again, whereas thapsigargin induces a marked stimulation in activity (approximately 2.5-fold), arachidonic acid completely fails to have any influence on the adenylyl cyclase (Fig. 5A). Importantly, this observed inability of arachidonate-induced changes in [Ca\(^{2+}\)], to induce any stimulation of adenylyl cyclase activity was not because of a lower rate of Ca\(^{2+}\) entry during this period as comparison of the rates of Mn\(^{2+}\) quench induced by thapsigargin (250 \(\mu\)M) and exogenous arachidonic acid (8 \(\mu\)M) during the period 3–6 min after the addition clearly show that, in fact, the arachidonate-induced rates of Mn\(^{2+}\) quench were more than twice that seen during the same period after the addition of thapsigargin (Fig. 5B).

**DISCUSSION**

The data obtained allow us to assess the contributions of the various components of the induced changes in [Ca\(^{2+}\)], to the observed stimulation of the transfected type VIII adenylyl cyclase. The reported absence of endogenous Ca\(^{2+}\)-stimulated
higher in the second 3-min period after the addition despite the obvious decline in [Ca\(^{2+}\)], during the same period. Furthermore, during the second 3-min period after the addition of thapsigargin, we have shown that the continued high adenylyl cyclase activity is entirely dependent on the presence of extracellular Ca\(^{2+}\). This clearly excludes any possible contribution from Ca\(^{2+}\) released from stores, at least during this period of the response. Together, these data clearly demonstrate that, at least in the thapsigargin-treated cells during the second 3-min period after addition, the adenylyl cyclase is being stimulated specifically by the entry of Ca\(^{2+}\). As such, these data are consistent with previous reports that the Ca\(^{2+}\)-stimulated type VIII adenylyl cyclase (13), as with other Ca\(^{2+}\)-sensitive adenylyl cyclases, responds particularly to the Ca\(^{2+}\) entry component of the overall Ca\(^{2+}\) signal (12, 13, 23). However, in marked contrast, the adenylyl cyclase is entirely unresponsive to the Ca\(^{2+}\) entry activated by arachidonic acid. This is despite the fact that, at the respective concentrations used, the rate of Ca\(^{2+}\) entry activated by arachidonic acid would appear to be greater than twice that induced by thapsigargin.

In the previous reports examining the Ca\(^{2+}\)-sensitive adenylyl cyclases in vivo, it has been clearly demonstrated that the endogenous type VI Ca\(^{2+}\)-inhibited adenylyl cyclase of C6-B2 glioma cells, as well as the type I and VIII Ca\(^{2+}\)-stimulated adenylyl cyclases transiently transfected into HEK293 cells, respond specifically to entry via a capacitative pathway; an even more substantial but nonspecific entry induced by ionomycin fails to affect adenylyl cyclase activity (13, 14). A similar tight coupling between capacitative Ca\(^{2+}\) entry and the type III adenylyl cyclase has been reported as underlying the potentiation of adrenocorticotrophin-induced CAMP formation by angiotensin II (24) and in the sustained potentiation of isoproterenol-stimulated CAMP generation by carbachol in mouse parotid (25). In all cases, it has been suggested that this high degree of specificity reflects an intimate association and colocalization of the enzyme with the capacitative channel sites in the plasma membrane. Obviously, this tight association applies both to endogenous as well as to heterologously expressed adenylyl cyclases. Additional experiments by Fagan et al. (14) have shown that this association between the adenylyl cyclase and sites of capacitative Ca\(^{2+}\) entry does not involve the cytoskeleton, implying either some form of co-compartmentalization within the plasma membrane or a direct protein-protein interaction. The same authors also demonstrated that the activation of the adenylyl cyclase was a direct consequence of the Ca\(^{2+}\) influx via the capacitative channel and not to any conformational change associated with the channel opening. Given this demonstrated intimate spatial relationship between capacitative Ca\(^{2+}\) entry sites and the adenylyl cyclase, the complete failure of the Ca\(^{2+}\) entry induced by arachidonic acid to produce any change in adenylyl cyclase activity clearly demonstrates that the arachidonate-induced Ca\(^{2+}\) entry channel must be an entirely distinct entity from that responsible for capacitative entry.

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