Arachidonic acid inhibits the store-operated Ca^{2+} current in rat liver cells

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Vasopressin and other phospholipase-C-coupled hormones induce oscillations (waves) of $[Ca^{2+}]_{cyt}$ (cytoplasmic Ca^{2+} concentration) in liver cells. Maintenance of these oscillations requires replenishment of Ca^{2+} in intracellular stores through Ca^{2+} inflow across the plasma membrane. While this may be achieved by SOCs (store-operated Ca^{2+} channels), some studies in other cell types indicate that it is dependent on AA (arachidonic acid)-activated Ca^{2+} channels. We studied the effects of AA on membrane conductance of rat liver cells using whole-cell patch clamping. We found no evidence that concentrations of AA in the physiological range could activate Ca^{2+}-permeable channels in either H4IIE liver cells or rat hepatocytes. However, AA (1–10 μM) did inhibit ($IC_{50} = 2.4 \pm 0.1$ μM) Ca^{2+} inflow through SOCs (I_{SOC}) initiated by intracellular application of Ins(1,4,5)P_3 in H4IIE cells. Pre-incubation with AA did not inhibit I_{SOC} development, but decreased maximal amplitude of the current. Iso-tetrandrine, widely used to inhibit receptor-activation of phospholipase A_2, and therefore AA release, inhibited I_{SOC} directly in H4IIE cells. It is concluded that (i) in rat liver cells, AA does not activate an AA-regulated Ca^{2+}-permeable channel, but does inhibit SOCs, and (ii) iso-tetrandrine and tetrandrine are effective blockers of CRAC (Ca^{2+}-release-activated Ca^{2+}) channel-like SOCs. These results indicate that AA-activated Ca^{2+}-permeable channels do not contribute to hormone-induced increases or oscillations in $[Ca^{2+}]_{cyt}$ in liver cells. However, AA may be a physiological modulator of Ca^{2+} inflow in these cells.

Key words: arachidonic acid, Ca^{2+}-release-activated Ca^{2+} channel (CRAC channel), H4IIE liver cell, iso-tetrandrine, patch-clamp recording, store-operated Ca^{2+} channel.

INTRODUCTION

In non-excitable cells, agonists acting at phospholipase-C-coupled receptors generate repetitive rises in free $[Ca^{2+}]_{cyt}$ (Ca^{2+} concentration in the cytoplasmic space) by releasing Ca^{2+} from intracellular Ca^{2+} stores and activating Ca^{2+} influx through plasma membrane Ca^{2+} channels. It has been generally accepted that the major component of the receptor-activated Ca^{2+} entry into the cell is produced by a store-operated Ca^{2+} entry mechanism, which involves activation of SOCs (store-operated Ca^{2+} channels) in response to emptying intracellular Ca^{2+} stores by Ins(1,4,5)P_3 [1]. At the same time, a significant body of evidence suggests that a non-store-operated Ca^{2+} entry may also be activated by phospholipase-C-coupled receptors [2–4]. In A7r5 vascular smooth muscle cells, avian nasal gland cells and m3HEK293 cells (HEK-293 cells stably transfected with human M_3 muscarinic receptor), non-store-operated Ca^{2+} influx stimulated by AA (arachidonic acid) has been suggested to be a major pathway for Ca^{2+} entry activated by physiological concentrations of specific agonists that induce intracellular Ca^{2+} waves [5–7].

AA, a *cis*-polyunsaturated fatty acid, is a constituent of membrane phospholipids that can be released by cellular phospholipases, particularly by phospholipase A_2 and diacylglycerol lipase [8]. Free AA has been shown to modulate the activity of a number of ion channels, including a range of Ca^{2+}-permeable channels [9–11]. There is no single mechanism of action by which AA modulates ion channels. Thus there is evidence that it binds directly to some channel proteins, but, for other channels, it can also have an indirect effect through its metabolites, free radicals and AA-sensitive protein kinases and phosphatases [12–14]. In some cells in which AA has been shown to inhibit SOCs, it has also been shown to activate a specific Ca^{2+} conductance [15]. Patch-clamping of m3HEK293 cells revealed that, in these cells, AA activates a Ca^{2+} current (I_{ARC}) that is distinctively different from that activated by store depletion (I_{SOC}) [16]. This current was implicated in mediating Ca^{2+} oscillations activated by low concentrations of carbachol in these cells [17].

Hepatocytes are polarized epithelial cells in which Ca^{2+} oscillations can be activated by a variety of Ca^{2+}-mobilizing hormones. They exhibit a Ca^{2+}-selective current mediated by SOCs (I_{SOC}) that has been characterized by patch-clamp recording [18,19]. The hepatocyte I_{SOC} exhibits many of the characteristics, including high Ca^{2+} selectivity, of I_{CRAC} studied in lymphocytes and mast cells [18,19]. Indirect evidence indicates that hormone-induced Ca^{2+} oscillations in hepatocytes are maintained by a store-operated Ca^{2+} entry mechanism, as they are inhibited by known blockers of SOCs [19]. On the other hand, there is evidence that Ca^{2+}-mobilizing hormones induce AA release [20].

The present study was designed to elucidate the effects of AA on SOCs in liver cells, and to establish whether AA activates a specific Ca^{2+} conductance in these cells. The results indicate that AA, at concentrations within the estimated physiological range, inhibits I_{SOC} in rat liver H4IIE cells, but does not itself activate any type of membrane conductance in either H4IIE cells or rat hepatocytes. We also show that iso-tetrandrine, commonly used to inhibit activation of phospholipase A_2 by receptors and therefore AA release [21], is a potent blocker of the I_{SOC} in H4IIE cells.

EXPERIMENTAL

Cell culture

H4IIE cells (A.T.C.C. CRL 1548) were cultured at 37 °C in 5 % (v/v) CO_2 in air in DMEM (Dulbecco's modified Eagle's medium;

Abbreviations used: AA, arachidonic acid; $[Ca^{2+}]_{cyt}$, Ca^{2+} concentration in cytoplasmic space; CRAC, Ca^{2+}-release-activated Ca^{2+}; DMEM, Dulbecco's modified Eagle's medium; m3HEK293 cells, HEK-293 cells stably transfected with human M_3 muscarinic receptor; I_{ARC}, current mediated by AA-regulated channels; I_{CRAC}, current mediated by CRAC channels; I_{SOC}, current mediated by store-operated Ca^{2+} channels; SOC, store-operated Ca^{2+} channel.

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Gibco) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 10 mM Hepes (pH 7.4) and 10 % (v/v) foetal bovine serum (complete DMEM) [22]. The cells were subcultured for a maximum of 15 passages. Hepatocytes were isolated from male Hooded Wistar rats by liver perfusion with collagenase, plated on glass coverslips in complete DMEM (as above) and used for patch clamping on the following day [23].

Electrophysiology

Whole-cell patch clamping was performed at room temperature (24 °C) using a computer-based patch-clamp amplifier (EPC-9, HEKA Electronics, Lambrecht/Pfalz, Germany) and PULSE software (HEKA Electronics). The usual bath solution contained 140 mM NaCl, 4 mM CsCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose and 10 mM Hepes, adjusted to pH 7.4 with NaOH. The internal solution 1 contained 120 mM caesium glutamate, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM MgATP, 10 mM EGTA and 10 mM Hepes, adjusted to pH 7.2 with NaOH. The internal solution 2 contained 130 mM caesium glutamate, 10 mM CsCl, 0.5 mM $CaCl_2$, 6 mM MgATP, 1 mM EGTA and 10 mM Hepes, adjusted to pH 7.2 with NaOH. The calculated internal free Ca^{2+} concentration for each internal solution was approx. 100 nM (EQCAL; Biosoft, Cambridge, U.K.). Depletion of the intracellular Ca^{2+} stores was achieved by addition of 20 μM Ins(1,4,5)P_3 (D-*myo*-inositol 1,4,5-trisphosphate hexapotassium salt; Sigma) to internal solution 1 or 1 μM thapsigargin (Sigma) to the bath solution. Patch pipettes were pulled from borosilicate glass, coated with Sylgard and fire-polished; pipette resistance ranged between 2 and 4 MΩ. In order to monitor the development of I_{SOC}, voltage ramps between -138 and $+102$ mV were applied every 2 s, starting immediately after achieving the whole-cell configuration. Acquired currents were filtered at 2.7 kHz and sampled at 10 kHz. Traces presented in the Figures were further digitally filtered at 1.5 kHz. All voltages shown have been corrected for the liquid junction potential of -18 mV between the bath and electrode solutions (estimated by JPCalc [24]). The holding potential was -18 mV throughout. Cell capacitance was compensated automatically by the EPC9 amplifier. AA and tetrandrine were purchased from Sigma; iso-tetrandrine was purchased from Calbiochem.

RESULTS

First, the effect of AA on the membrane conductance of H4IIE rat liver cells was investigated to establish if AA activates a specific Ca^{2+} current. As it has been suggested that pre-activation of capacitative Ca^{2+} entry may inhibit AA-induced Ca^{2+} entry [15], in these experiments, we used an intracellular solution containing 6 mM Mg ATP and Ca^{2+} buffered to 100 nM with 1 mM EGTA. This would prevent the spontaneous development of I_{SOC}, and would also minimize the contribution of Mg^{2+}-regulated non-selective cation current attributed to TRPM7 (transient receptor potential melastatin-7 cation channel) [25]. Addition of 10 μM AA to the bath solution under these conditions failed to activate any current within 10 min of recording (Figure 1A).

H4IIE cells are likely to have lost receptors that are normally present in rat hepatocytes, as they do not respond to ATP (<100 μM) or vasopressin (G. Rychkov and G. Barritt, unpublished work). It might be argued that AA-activated Ca^{2+} entry requires the presence of functional G-protein-coupled receptors on the plasma membrane, or that AA-activated Ca^{2+} channels themselves have been lost in this particular cell line. Therefore similar experiments were performed on rat hepatocytes in primary culture that are known to generate cytoplasmic Ca^{2+} waves in response to a variety of hormones [19,26,27]. AA (<20 μM) in the bath solution failed to activate any current in rat hepatocytes within 10 min of recording (Figure 1B), indicating that the failure to observe any AA-activated current in H4IIE cells is unlikely to be due to the loss of expression of G-protein-coupled receptors, and that the absence of AA-activated channels is not unique to H4IIE cells.

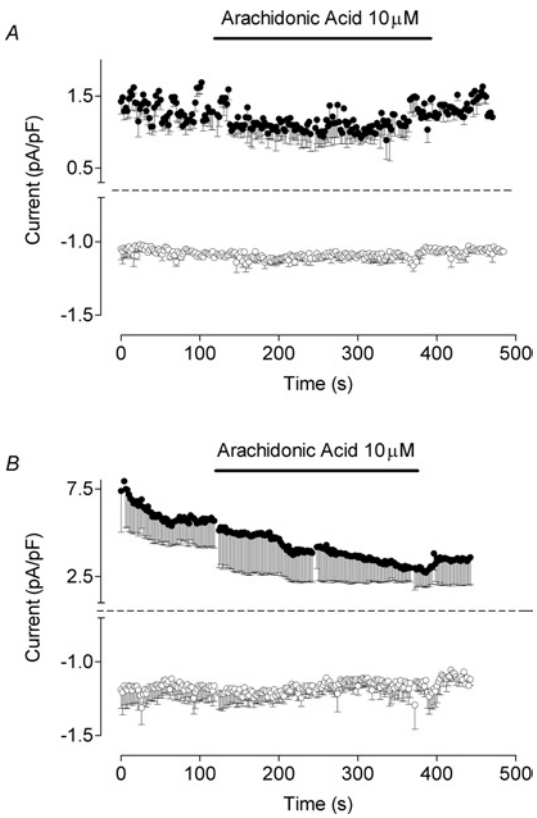

Figure 1 AA fails to activate any current in either H4IIE cells or in rat hepatocytes

Time courses of the inward and outward membrane currents measured at -118 mV (bottom trace) and 82 mV (top trace) in H4IIE cells ($n=5$) (**A**) and in rat hepatocytes ($n=5$) (**B**). Application of 10 μM AA in the bath is indicated by a horizontal bar. Internal solution 2 (see the Experimental section) was used in the pipette in order to prevent development of I_{SOC}.

The fact that AA does not activate Ca^{2+} conductance in liver cells does not imply that it has no role in Ca^{2+} signalling in these cells. AA has been shown to inhibit store-operated Ca^{2+} entry in various cell types [15,28–30]. However, its effects on SOCs in liver cells and on similar CRAC (Ca^{2+}-release-activated Ca^{2+})-type SOCs in other cell lines are not known. Therefore, in the next set of experiments, we investigated the effect of AA on I_{SOC} in H4IIE cells. We have characterized I_{SOC} in H4IIE cells previously and have shown that it has many of the properties of I_{CRAC} in lymphocytes and mast cells [18]. In the absence of AA, depletion of intracellular Ca^{2+} stores in H4IIE cells activated an inward current (I_{SOC}) with a magnitude of approx. -2.5 pA/pF, measured at -118 mV, as described previously [18] (Figure 2A). The current reached its maximum within 60 s after achieving the whole-cell configuration with the pipette containing 20 μM Ins(1,4,5)P_3, and then slowly decayed to a level of 60–70 % of the maximal amplitude. Addition of 10 μM AA to the bath solution after full development of the current produced an almost complete block of I_{SOC} within 150 s. At lower concentrations, the block was slower in onset and less complete. The

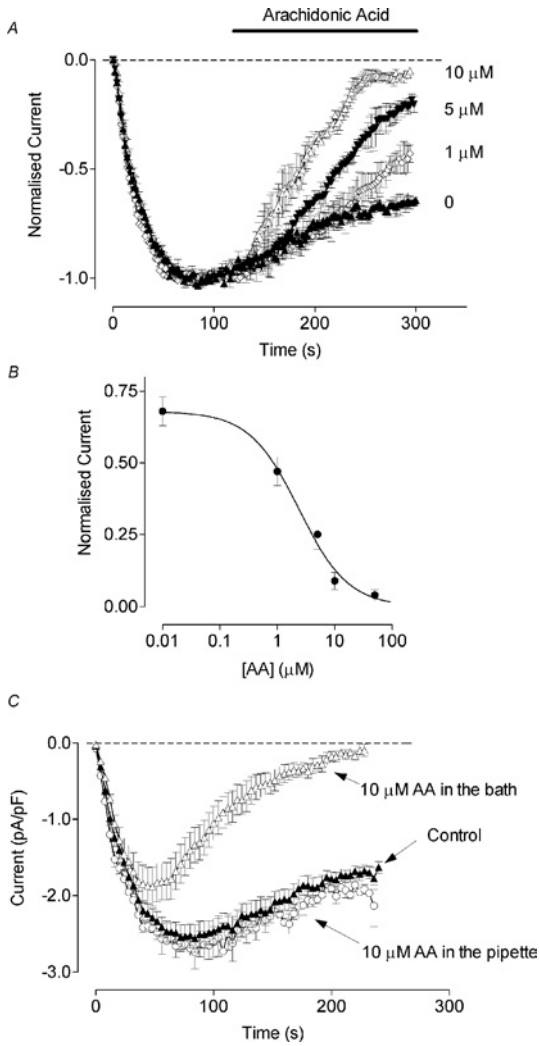

Figure 2 Effects of AA on I_{SOC} in H4IIE cells

(**A**) Time course of I_{SOC} inhibition by AA. Amplitude of I_{SOC} measured at −118 mV is plotted against time. Application of AA is indicated by a horizontal bar ($n = 4$–9). (**B**) Dose-dependent inhibition of I_{SOC} by AA. Each point on the graph is the normalized average amplitude of I_{SOC} at −118 mV measured after 200 s of application of the corresponding concentration of AA in the bath ($n = 4$–9). (**C**) Effect of AA on I_{SOC} development. Cells were either pre-incubated with AA in the bath for at least 2 min before achieving the whole-cell configuration or AA was added to the pipette solution ($n = 6$).

lowest concentration of AA that produced a significant effect within 200 s of application was 1 μM (Figure 2A). Higher concentrations of AA (20–50 μM) blocked I_{SOC} faster; however, they frequently caused development of a non-specific leakage and electrical breakdown of the membrane during steps to negative potentials (results not shown). The apparent IC_{50} for AA measured 200 s after AA application was 2.4 ± 0.1 μM ($n = 4$) (Figure 2B).

Pre-incubation of H4IIE cells with 10 μM AA in the bath for at least 2 min before achieving whole-cell configuration did not prevent development of I_{SOC} (Figure 2C). However, the maximal amplitude of the current was significantly smaller and it was completely inactivated within 200 s of recording. The time constants of I_{SOC} development were 24 ± 2 s ($n = 6$) for the control cells and 20 ± 3 s ($n = 6$) for the cells pre-incubated with AA. These results indicate that it is unlikely that AA interferes with the mechanism of I_{SOC} activation, or that it inhibits I_{SOC} by binding to the closed channel. It is more likely that AA either inhibits the open channel directly or modulates the mechanism by which I_{SOC} is slowly inactivated [31]. Addition of 10 μM AA to the internal solution containing 20 μM Ins(1,4,5)P_3 had no effect on the development or amplitude of I_{SOC} (Figure 2C).

Under conditions used in the experiments with H4IIE cells described above (Figure 1), AA did not seem to activate any specific conductance. However, in other cell types, AA has been shown to activate highly selective Ca^{2+} channels with properties distinct from that of the SOCs in those cells [16]. One of the main differences between I_{SOC} and the current activated by AA (I_{ARC}) in HEK-293 cells is the lack of the fast inactivation in the latter [16]. I_{SOC} in H4IIE cells also shows a significant fast Ca^{2+}-dependent inactivation at negative potentials [32], and therefore can be distinguished easily from a current that shows no fast inactivation. While there was no evidence that AA activated any current in H4IIE cells, the possibility of a transient activation of such a current when I_{SOC} is blocked could not be excluded. Therefore we compared current traces obtained in response to −138 mV voltage steps before, and 60 s after, application of AA. The kinetics of the I_{SOC} inactivation and the relative amplitude of the non-inactivating component at negative potentials remained unaffected in the presence of AA (Figures 3A and 3B). This argues against the presence of any non-inactivating Ca^{2+} current additional to I_{SOC}. Moreover, the I–V plot remained inwardly rectifying in the presence of AA, with no evidence for the development of any outward current or a shift in the reversal potential (which would be expected if any other conductance had developed) (Figure 3C).

One of the methods of distinguishing between Ca^{2+} influxes carried by I_{SOC} and I_{ARC} in m3HEK293 cells, where I_{ARC} was first described, was to use iso-tetrandrine. Iso-tetrandrine is known to inhibit the activation of phospholipase A_2 by receptors [21], and has therefore been used to inhibit agonist-induced AA release [6,15]. Iso-tetrandrine is also one of the stereoisomers of tetrandrine that blocks L-type and T-type Ca^{2+} channels [33]. The effects of either compound on store-operated Ca^{2+} entry have not been investigated previously in most cell types. However, in avian nasal gland cells, iso-tetrandrine had no effect on thapsigargin-activated Ca^{2+} inflow [6]. In H4IIE cells, both tetrandrine and iso-tetrandrine caused a dose-dependent inhibition of I_{SOC} (Figure 4, only tetrandrine is shown). The apparent IC_{50} for tetrandrine was 8.6 ± 0.3 μM ($n = 4$–12) (Figure 4B). At concentrations of 10 and 100 μM, iso-tetrandrine showed potency similar to that of tetrandrine in inhibiting I_{SOC} (results not shown). The onset of the block induced by tetrandrine and iso-tetrandrine was as rapid as that observed with La^{3+} (T. Litjens and G. Rychkov, unpublished work) and was easily reversible upon washout (results not shown). Both tetrandrine and iso-tetrandrine had the same effect when I_{SOC} was activated by either thapsigargin or Ins(1,4,5)P_3.

DISCUSSION

The existence of AA-activated Ca^{2+} entry has been shown in a variety of cell types [5,6,34,35]. In some of these cell types, AA-activated Ca^{2+} channels provide a major pathway for Ca^{2+} entry during Ca^{2+} oscillations triggered by receptor stimulation [5,17,29]. In contrast, in astrocytes, AA inhibits Ca^{2+} oscillation activated by ATP and activates sustained Ca^{2+} influx [30]. The results of the present study demonstrate that AA, in the predicted physiological range of concentrations [36], does not activate Ca^{2+}-permeable channels in H4IIE liver cells, but strongly inhibits Ca^{2+} entry through SOCs (cf. [15,28–30]). The observation that AA also did not activate Ca^{2+}-permeable channels in primary rat hepatocytes indicates that the failure to observe an AA-activated

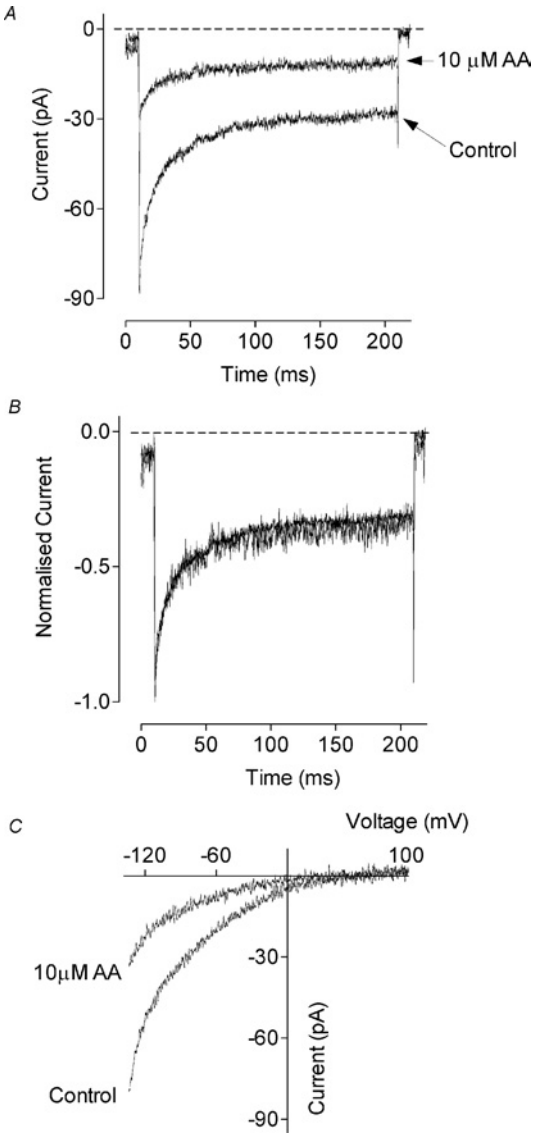

Figure 3 AA does not alter the kinetics of fast inactivation or the inward rectification of I_{SOC} in H4IIE cells

(**A**) Current traces were obtained in response to voltage steps to −138 mV before and after application of 10 μM AA in the bath for 60 s. (**B**) Current traces shown on (**A**) were normalized to the peak value and superimposed. (**C**) Current–voltage plots in the absence and presence of AA in the bath.

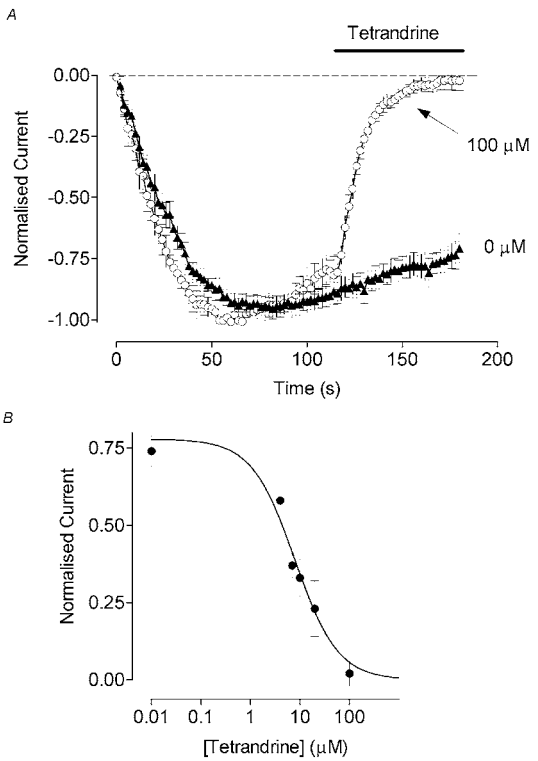

Figure 4 Inhibition of I_{SOC} by tetrandrine in H4IIE cells

(**A**) Time course of I_{SOC} inhibition by 100 μM tetrandrine (application is indicated by the horizontal bar). (**B**) Dose-dependent inhibition of I_{SOC} by tetrandrine ($n = 4$–12).

current in H4IIE liver cells is unlikely to be due to the absence of receptors for agonists in this immortalized cell line.

These results imply that, unlike some other cell types, including vascular smooth muscle cells and m3HEK293 cells, liver cells do not rely on AA-activated Ca^{2+} entry to maintain Ca^{2+} oscillations generated in response to phospholipase-C-coupled hormones. Moreover, the idea that SOCs are responsible for Ca^{2+} inflow that maintains Ca^{2+} oscillations in liver cells [19] is consistent with the present observations of the absence of AA-activated Ca^{2+}-permeable channels in liver cells.

The observation that the intracellular application of AA had no effect on I_{SOC} development and amplitude suggests an extracellular or membrane-delimited site of action for AA. However, an intracellular site cannot be unequivocally ruled out, since application of a membrane-permeable substance, such as AA, through a patch pipette may not be as effective in maintaining the exogenous AA concentration, since the AA will diffuse into the infinitely large (compared with the cell volume) bath faster than it diffuses through a patch pipette. AA may also be metabolized faster than it is incorporated into the plasma membrane, if the site of action is membrane-delimited. This appears to be the case in studies of the regulation of I_{CRAC} by sphingosine, another membrane-permeable compound. Sphingosine that accumulates in the membrane, as assessed by the changes in the membrane capacitance, and inhibits I_{CRAC} in RBL (rat basophil leukaemia) cells when applied in the bath has no effect on either capacitance or I_{CRAC} when applied through the pipette [37]. Regardless of the site of action in liver cells, AA may be an important modulator of Ca^{2+} entry through SOCs when these are activated by physiological concentrations of hormones that induce the formation of AA. Under physiological conditions, AA is produced in the membrane [8], and, *in vivo*, when cells are packed close to each other in an intact organ, the concentration of AA is likely to rise both inside and outside the cells.

In m3HEK293 cells, Ca^{2+} currents activated by AA and depletion of Ca^{2+} stores were additive [16]. Thus addition of AA after I_{SOC} development in m3HEK293 cells increased membrane current further. In contrast, in the present study with H4IIE cells, I_{SOC} was inhibited completely by similar concentrations of AA. This suggests that I_{SOC} in HEK-293 cells and I_{SOC} in H4IIE cells are likely to be mediated by different types of SOC. Moreover, these results emphasize that conclusions about the nature of Ca^{2+}-permeable pathways in the plasma membrane for one cell type cannot necessarily be applied to another cell type.

Another important observation of the present study is that isotetrandrine and tetrandrine are potent inhibitors of I_{SOC} in liver cells. The mechanism of inhibition is unlikely to involve an effect

of these agents on phospholipase A_2, as the block is very rapid and readily reversible. A direct effect of tetrandrine and iso-tetrandrine on the SOC is consistent with these observations. In addition, if iso-tetrandrine and tetrandrine were acting via phospholipase A_2, they would be expected to enhance I_{SOC}, not to inhibit it, since the present results show that AA, a product of phospholipase A_2 activity, inhibits SOCs in liver cells. In avian nasal gland cells, iso-tetrandrine has been shown to inhibit Ca^{2+} oscillations activated by a low concentration (0.5 μM) of carbachol, but was found to be ineffective in inhibiting Ca^{2+} entry activated by a high concentration (10 μM) of carbachol or by thapsigargin [6]. It was concluded from these experiments that, in avian nasal gland cells, iso-tetrandrine does not affect SOCs, that SOCs are not activated by low concentrations of carbachol, and therefore that the major physiological pathway for Ca^{2+} entry in these cells is through Ca^{2+} channels activated by AA. In view of the present study, caution should be exercised in interpretation of the results obtained using these compounds as inhibitors of receptor-activation of phospholipase A_2 in studies involving the measurement of SOCs, especially when employing Ca^{2+} fluorescent dyes to measure Ca^{2+} inflow, and when the nature of the channels underlying that inflow is not entirely certain.

The observations made by several research groups with different cell types on the effects of AA on plasma membrane Ca^{2+}-permeable channels can be summarized as follows: in different cell types, AA has been shown either to induce Ca^{2+} oscillations [6], or to inhibit them [30]; to inhibit SOC-mediated Ca^{2+} entry [15,28–30], or to have no effect on SOCs [16]; to activate Ca^{2+} channels with a high selectivity for Ca^{2+} [16], or to activate Ca^{2+}-permeable non-selective cation channels [38]; and to activate Ca^{2+}-permeable channels which are very sensitive to inhibition by Gd^{3+} [34], or channels which are insensitive to Gd^{3+} [5,39,40]. On the basis of all these results, it can be suggested that AA plays different physiological roles in regulating Ca^{2+} entry, and may activate or inhibit different types of Ca^{2+}-permeable channels in different cell types. The results also indicate that SOCs are likely to be different in different cell types, and no single model of the mechanism of Ca^{2+} oscillations can be applied to all cell types.

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