Effect of arachidonic acid on the L-type calcium current in frog cardiac myocytes

Jerome Petit-Jacques and H. Criss Hartzell*

The Heart Cell Laboratory, Department of Anatomy and Cell Biology, Emory University School of Medicine, Atlanta, GA 30322, USA

1. External application of the unsaturated fatty acid arachidonic acid (AA) to frog ventricular cells caused a large inhibition (~85%) of the L-type calcium current ($I_{Ca,L}$) previously stimulated by the β-adrenergic agonist isoprenaline (Iso). The concentration producing half-maximal inhibition ($K_{½}$) was 1·52 μM. The inhibitory effect did not affect the peak current–voltage relationship but produced a negative shift in the inactivation curve.

2. The inhibitory effect of AA also occurred in cells internally perfused with cAMP and non-hydrolysable analogues of cAMP. These data suggest that AA is acting by a mechanism located beyond adenylyl cyclase and does not involve changes in intracellular cAMP levels.

3. AA also inhibited the calcium current stimulated by internal perfusion with the catalytic subunit of protein kinase A (PKA), suggesting that AA acts downstream of channel phosphorylation.

4. The inhibitory effect of AA on the isoprenaline- or cAMP-stimulated $I_{Ca,L}$ is largely reduced in cells internally perfused with the thiophosphate donor analogue of ATP, ATPγS, or protein phosphatase 1 and 2A inhibitors like microcystin (MC) or okadaic acid (OA). External application of the phosphatase inhibitor calyculin (Caly) also reduced the AA effect. These data suggested that the AA effect on $I_{Ca,L}$ involves activation of protein phosphatase activity.

5. The effect of AA on $I_{Ca,L}$ was not affected by staurosporine, an inhibitor of protein kinases. It was also unaffected in cells internally perfused with GTPγS. These results suggest that neither a PKC- nor a G-protein-mediated mechanism are likely to be involved in the effect of AA on $I_{Ca,L}$.

6. A saturated fatty acid, myristic acid (MA), had no inhibitory effect on the isoprenaline-stimulated Ca^{2+} current, whereas, in the same cells arachidonic acid produced ~85% inhibition of $I_{Ca,L}$.

7. The inhibitory effect of AA was not affected by exposing the cells to indomethacin (Indo), an inhibitor of the metabolism of AA by cyclo-oxygenase, nor nordihydroguaiaretic acid (NDGA), an inhibitor of the lipoxygenase pathway. However, the non-metabolizable analogue of AA, 5,8,11,14-eicosatetraynoic acid (ETYA), was without effect on the isoprenaline-stimulated $I_{Ca,L}$.

8. These results suggest that AA inhibits $I_{Ca,L}$ via a mechanism which involves, in part, stimulation of protein phosphatase activity. This process could provide a new mechanism in the modulation of calcium channel activity.

Arachidonic acid (AA) and the products of its metabolism (Needleman, Turk, Jakschik, Morrison & Lefkowith, 1986) have a large variety of effects on ion channels in many different tissues (reviewed by Meves, 1994). In *Aplysia* sensory neurons, AA and its lipoxygenase products mimic the effects of FMRFamide (Phe-Met-Arg-Phe-NH_2) and increase the opening of SK channels, causing an inhibition of neurotransmitter secretion (Piomelli *et al.* 1987). AA also inhibits Ca^{2+} channels in different types of nerve cells (Keyser & Alger, 1990; Khurana & Bennet, 1993) and in intestinal smooth muscle cells (Shimada & Somlyo, 1992). In cardiac tissue, AA causes the uncoupling of cells by closing gap junction channels (Massey, Minnich & Burt, 1992). In atrial cells, AA activates outwardly rectifying K^+

* To whom correspondence should be addressed.

channels (Kim & Clapham, 1989) and lipoxygenase metabolites of AA modulate the inwardly rectifying muscarinic-gated K^+ channel (K_{ACh}) (Kim, Lewis, Graziadei, Neer, Bar-Sagi & Clapham, 1989; Kurachi, Ito, Sugimoto, Shimizu, Miki & Ui, 1989). AA can have an inhibitory effect on the T- and L-type Ca^{2+} current in guinea-pig atrial cells (Cohen, Bale & Leibowitz, 1990) or a stimulatory effect on the L-type current in ventricular cells of the same species (Huang, Xian & Bacaner, 1992). Although many different ion channels are affected by AA, the mechanisms by which AA affects the channels and the physiological significance of these effects remain areas of investigation.

In this paper, we have investigated the effects of AA on the voltage-gated L-type Ca^{2+} channel in cardiac myocytes because this channel plays a central role in excitation coupling in heart tissue and has been a model system for studying ion channel regulation. The cardiac L-type Ca^{2+} current ($I_{Ca,L}$) is stimulated by activation of β-adrenergic receptors by a process which involves phosphorylation via the cAMP cascade (Hartzell, 1988; Trautwein & Hescheler, 1990). It remains unclear whether the phosphorylated substrate is the Ca^{2+} channel or a regulatory protein (Hartzell, 1993). Stimulation by β-adrenergic receptors is reversed by several protein phosphatases (Hescheler, Kameyama, Trautwein, Mieskes & Soeling, 1987). We have recently proposed a model for frog cardiac Ca^{2+} channel regulation in which two phosphorylation sites are involved. One site is preferentially dephosphorylated by protein phosphatase 2A and the other site is dephosphorylated by another protein phosphatase. Although one site is clearly phosphorylated by protein kinase A, the other site can also be phosphorylated by a different, as yet unidentified, protein kinase which we call protein kinase X (Frace & Hartzell, 1993; Hartzell, Hirayama & Petit-Jacques, 1995). Here we have investigated the effects of arachidonic acid on $I_{Ca,L}$ in frog ventricular myocytes. The results show that AA inhibits the Ca^{2+} current stimulated by phosphorylation. They also suggest that AA acts, at least in part, via activation of protein phosphatase activity. A preliminary account of these results has appeared (Petit-Jacques & Hartzell, 1995).

METHODS

Frog ventricular cardiomyocytes were isolated from *Rana catesbeiana*. Frogs were killed by double pithing. In some instances an inhalation anaesthetic, methoxyfluorane (2,2-dichloro-1,1-difluoro-ethylmethyl ether), was used prior to pithing and excision of the heart. Animals were cared for and killed according to the guidelines of the Emory University Animal Care and Use Committee in compliance with the US Public Health Service Policy as stated in *The Guide for the Care and Use of Laboratory Animals* (HHS, NIH Pub. No. 85-23, 1985).

Cell isolation and electrophysiological techniques have been described in detail previously (Fischmeister & Hartzell, 1986). Briefly, frog ventricular cells were enzymatically isolated and patch clamped by the whole-cell patch clamp technique. The internal solution for recording $I_{Ca,L}$ was (mM): 118 CsCl, 4 $MgCl_2$, 2·8 Na_2K_2ATP, 10 potassium 1,4-piperazinediethane sulphonic acid (K_2Pipes), 5 sodium creatine phosphate, 5 K_2EGTA, 0·05 GTP (Na^+ salt), pH adjusted to 7·15 with KOH. When lithium adenosine-5′-*O*-3-thiotriphosphate ($Li_4ATP\gamma S$) was included in the internal solution, Na_2K_2ATP was omitted. Various test compounds were applied internally by pipette perfusion. These included: cAMP, 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate (CPT-cAMP), 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP; Sigma), microcystin-LR (MC; GibcoBRL), okadaic acid (OA; Research Biochemicals Inc.), catalytic subunit of the cAMP-dependent protein kinase (Promega, Madison, WI, USA), nordihydroguaiaretic acid (NDGA; Sigma) and GTPγS (Sigma). When GTPγS was present in the internal perfusion, GTP was omitted.

The external solution was (mM): 103 NaCl, 20 CsCl, 10 Hepes, 1·8 $CaCl_2$, 1·8 $MgCl_2$, 5 pyruvic acid, 5 D-glucose, 0·3 μM tetrodotoxin, pH adjusted to 7·4 with NaOH. Additions to external solution included isoprenaline (Iso; Sigma), calyculin A (Caly; LC Laboratories, Woburn, MA, USA), staurosporine, indomethacin (Indo), 5,8,11,14-eicosatetraynoic acid (ETYA), myristic acid (MA) and arachidonic acid (Sigma). Fatty acid (free acid, 99%) stock solutions were prepared biweekly at 0·1 M in dimethylsulphoxide (DMSO) and kept at −80°C in the dark. Inhibitors (NDGA and indomethacin) were made fresh at 0·1 M in DMSO. Final solutions were vortexed and sonicated. Staurosporine stock solution was 1 mM in DMSO. The final concentration of DMSO was $\leqslant$0·1%. All experiments were conducted at room temperature (22–26 °C) and the solutions protected from light.

Cells were patch clamped with soft glass pipettes having resistances of 1–2 MΩ. Total series resistance was usually less than 3 MΩ. $I_{Ca,L}$ was elicited at various frequencies by voltage pulses delivered by a programmable digital stimulator (Challenger DB; W. Goolsby, Kinetic Software, Atlanta, GA, USA and Emory University, Atlanta, GA, USA). Routine pulses were from −80 mV holding potential to 0 mV. The pulse was 200 ms in duration and $I_{Ca,L}$ was measured as the peak inward current minus the current at the end of the pulse. For statistical analysis, results are presented as means ± S.E.M.

RESULTS

Effect of arachidonic acid on the calcium current

Figure 1 shows the inhibitory effect of arachidonic acid on $I_{Ca,L}$ previously stimulated by Iso in a ventricular cell. In this experiment the cell was first exposed to 0·5 μM Iso, which increased the amplitude of $I_{Ca,L}$ approximately fifteenfold (net current amplitude increased from −197 to −2918 pA). When the effect of Iso reached steady state, 5 μM AA was added to the Iso-containing solution. This produced a strong inhibition (~85%) of $I_{Ca,L}$. The net $I_{Ca,L}$ decreased from −2918 pA in the presence of Iso to −445 pA in the presence of Iso + AA. The percentage inhibition of $I_{Ca,L}$ by AA was calculated by:

$$P = 100[1 - (I_{Ca,L} \text{ with AA}/I_{Ca,L} \text{ without AA})],$$

where P is percentage inhibition. On average, 5 μM AA reduced $I_{Ca,L}$ by 85·9 ± 1·8% ($n = 16$, see also Fig. 4*B*).

When AA was washed out, the recovery of the current amplitude was ~100%. A second application of 5 μM AA led to a similar decrease in current amplitude. Usually the time required to reach the steady-state effect of AA was faster (10 min in Fig. 1) for the second application than for the first application (13 min in Fig. 1). In sixteen cells, the mean time to reach a steady-state effect of AA was 12·8 ± 1·2 min. The effect of AA was frequently biphasic. This is especially obvious in Fig. 1*A*, where the first application of AA produced a rapid effect with $\tau = 74$ s and a slower effect, which apparently began after a delay with $\tau = 101$ s. We think that these two kinetic processes may reflect different mechanisms.

Figure 2 shows the effects of AA on $I_{Ca,L}$ in more detail. Figure 2*A* quantifies the effect of 5 μM AA on basal $I_{Ca,L}$ and on $I_{Ca,L}$ pre-stimulated by Iso. The calcium current amplitudes are presented as current density. The mean $I_{Ca,L}$ density was 2·9 ± 0·4 pA pF^{-1} under control conditions ($n = 16$), 1·7 ± 0·4 pA pF^{-1} in the presence of 5 μM AA alone ($n = 5$), 37·7 ± 2·4 pA pF^{-1} in the presence of 0·5 μM Iso ($n = 16$) and 5·3 ± 0·7 pA pF^{-1} in the presence of Iso + AA ($n = 16$). The comparison of the $I_{Ca,L}$ density values in the presence of Iso and in the presence of Iso + AA, gives 85·9% inhibition by AA of $I_{Ca,L}$ pre-stimulated by Iso (see also Fig. 4*B*). The effect of AA on basal $I_{Ca,L}$ was quantitatively smaller than the effect on Iso-stimulated $I_{Ca,L}$. In comparison, AA inhibited basal $I_{Ca,L}$ by 31·1 ± 13·2% ($n = 5$) but inhibited Iso-stimulated $I_{Ca,L}$ by 85·9 ± 1·8% ($n = 16$). The effect of AA on basal $I_{Ca,L}$ seemed greater when the basal $I_{Ca,L}$ was large.

Figure 2*B* shows the effect of AA on the current–voltage relationship of Iso-stimulated $I_{Ca,L}$. Iso (0·5 μM) increased $I_{Ca,L}$ density at every potential and shifted the peak of the *I–V* curve from +5 to 0 mV. AA (10 μM) reduced the Iso-stimulated current at every potential without any appreciable change in the shape of the current–voltage relationship. Although AA had no effect on the shape of the *I–V* relationship, AA did alter the inactivation curve by

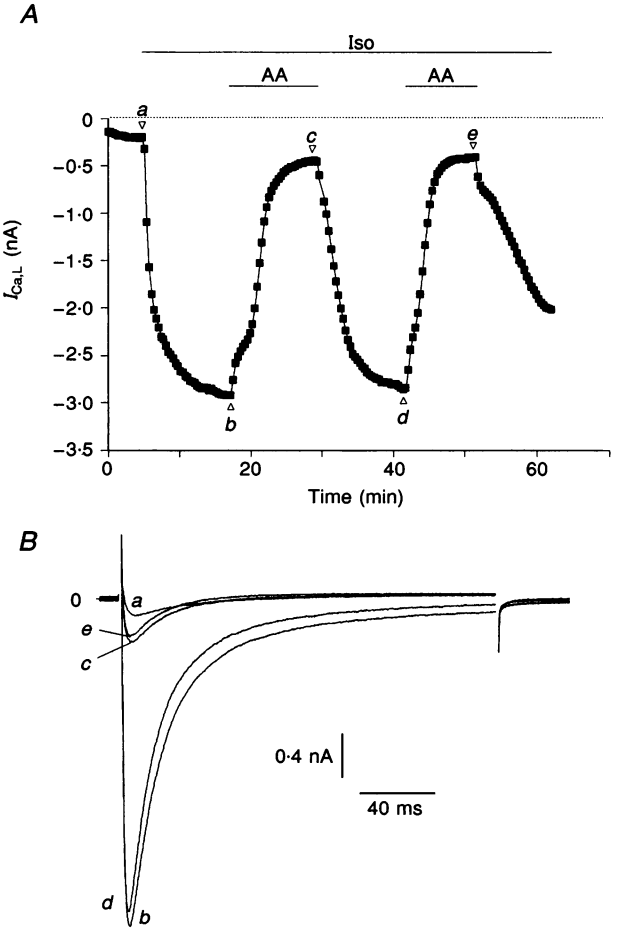

Figure 1. Effect of arachidonic acid on $I_{Ca,L}$ pre-stimulated by isoprenaline

A, a patch clamped ventricular myocyte was stimulated once every 20 s with 200 ms duration pulses to 0 mV from a holding potential of −80 mV. $I_{Ca,L}$ was measured as the difference between peak inward current and current at the end of the pulse. Isoprenaline (Iso, 0·5 μM) and arachidonic acid (AA, 5 μM) were applied to the superfusate as indicated by the horizontal bars. *B*, *a–e* represent Ca^{2+} current traces corresponding to the labels in *A*.

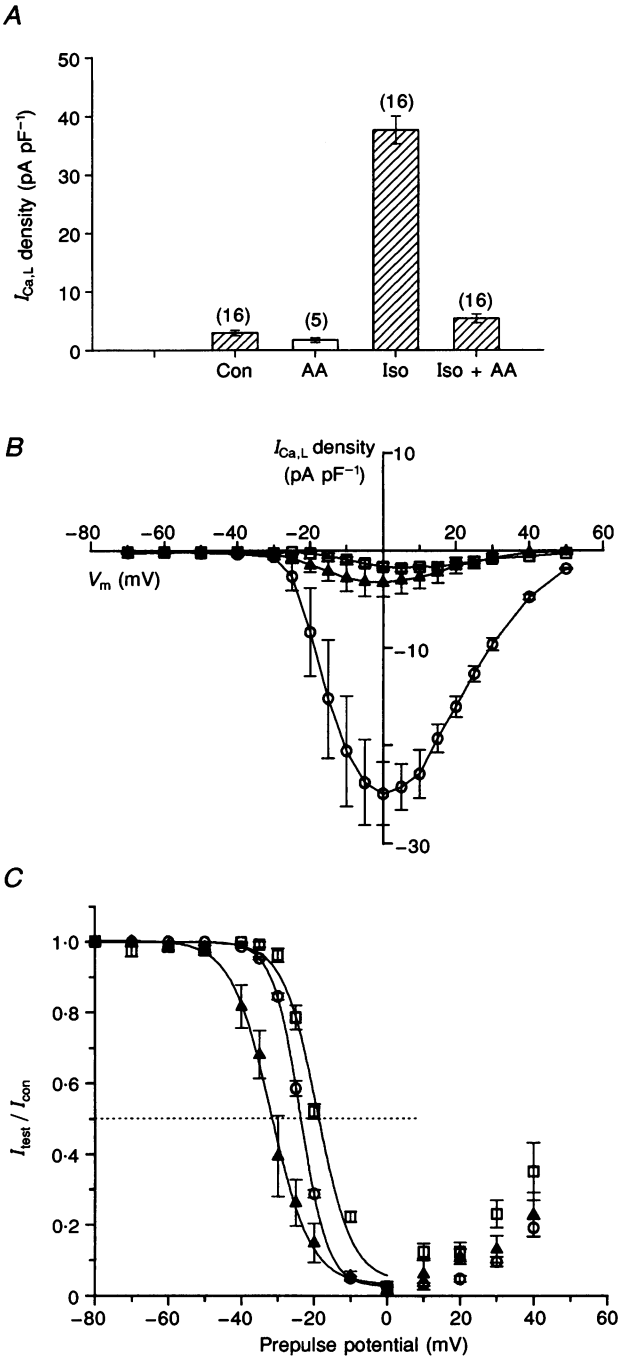

Figure 2. Effect of arachidonic acid on $I_{Ca,L}$ in ventricular cells

A, mean $I_{Ca,L}$ density plotted for control conditions (Con), 5 μM AA, 0·5 μM Iso and 0·5 μM Iso with 5 μM AA (Iso + AA). Number of cells tested in each condition is indicated in parentheses. In this and other figures, error bars are S.E.M. *B*, current–voltage relationships shown for control conditions (□), 0·5 μM Iso (○) and 0·5 μM Iso with 10 μM AA (▲) for 4 cells. Ca^{2+} current amplitudes are reported as current densities. *C*, inactivation curves produced by a double-pulse protocol in control conditions (□), 0·5 μM Iso (○) and 0·5 μM Iso with 10 μM AA (▲) for 3 cells. The pulse protocol consisted of a prepulse to the voltage indicated on the abcissa for 200 ms followed by a 3 ms return to −80 mV and then a 200 ms test pulse to 0 mV. The amplitudes of the responses to the test pulses (I_{test}) were normalized to the control amplitude of a test pulse without a prepulse (I_{con}) and plotted *versus* prepulse potential. Between −80 and 0 mV, the curves were fitted to the Boltzmann equation.

shifting it to more negative potentials (Fig. 2*C*). Between −80 and 0 mV, the data were fitted to the Boltzmann function:

$$I_{Ca,test}/I_{Ca,control} = 1/\{1 + \exp[(V - V_{½})/S]\}.$$

Under control conditions, $V_{½}$ was −19·1 mV and the slope factor (S) was 4·70. In the presence of Iso, $V_{½}$ was −23·81 mV and S was 3·75. When AA was present with Iso, $V_{½}$ was −31·87 mV and S was 5·46. Thus, application of AA caused a leftward shift (~8 mV compared with Iso, ~13 mV compared with control) of the inactivation curve. The AA effect on the $I_{Ca,L}$ inactivation curve in frog ventricular cells resembled the effect on $I_{Ca,L}$ inactivation curve in rabbit smooth muscle cells (Shimada & Somlyo, 1992).

Figure 3 shows the dose–response relationship for the effect of AA on $I_{Ca,L}$ in ventricular cells. Cells were externally perfused with increasing concentrations of AA. Only one dose was applied to each cell. The percentages of inhibition of the Iso-stimulated $I_{Ca,L}$ were plotted *versus* the AA concentration and the data were fitted with the Hill equation:

$$P = P_{max}/\{1 + (K_{½}/[AA])^{n_H}\},$$

where P is the percentage inhibition, P_{max} is the maximum percentage inhibition and n_H is the Hill coefficient. Under these conditions, the $K_{½}$ for AA inhibition of $I_{Ca,L}$ was 1·52 μM.

The effect of AA is located beyond adenylyl cyclase and does not involve a regulation of intracellular cAMP levels

To investigate further the mechanisms of the effect of AA on $I_{Ca,L}$, we applied AA to cells internally perfused with cAMP or its non-hydrolysable analogues 8-Br-cAMP and CPT-cAMP. In ventricular cells internally perfused with 10 μM cAMP, 10 μM AA led to an 81·6 ± 3·1% ($n = 5$) inhibition of $I_{Ca,L}$ (Fig. 4*B*). This percentage of inhibition was similar to that obtained with 10 μM AA (86·8 ± 2·3%, $n = 11$) and 5 μM AA (85·9 ± 1·8%, $n = 16$) on the Ca^{2+} current stimulated by 0·5 μM Iso (Fig. 4*B*).

Since AA is known to be able to activate guanylate cyclase (Gerzer, Brash & Hardman, 1986), we were interested in whether AA acted via cGMP. In frog ventricular cells, an increase in the cytoplasmic concentration of cGMP leads to an activation of the cGMP-stimulated phosphodiesterase, hydrolysis of cAMP and a decrease in Iso- or cAMP-stimulated $I_{Ca,L}$ (Hartzell & Fischmeister, 1986). However, $I_{Ca,L}$ stimulated by non-hydrolysable analogues of cAMP was not affected by cGMP (Hartzell & Fischmeister, 1986). Thus we tested whether AA affected $I_{Ca,L}$ stimulated by non-hydrolysable analogues of cAMP. In Fig. 4*A*, a ventricular cell was internally dialysed with 10 μM 8-Br-cAMP. $I_{Ca,L}$ amplitude increased eighteenfold (from −124 to −2247 pA). When the effect of 8-Br-cAMP reached steady state, 5 μM AA was added to the superfusion medium. This produced a 72·9% inhibition of $I_{Ca,L}$ amplitude (from −2247 to −608 pA). After washing out AA, $I_{Ca,L}$ returned to the 8-Br-cAMP-stimulated level. A second application of AA led to a 76·9% inhibition of the Ca^{2+} current (from −2231 to −514 pA). In six cells internally perfused with 8-Br-cAMP or CPT-cAMP, the percentage inhibition of $I_{Ca,L}$ by 5 μM AA was 85·3 ± 2·4% (Fig. 4*B*). This inhibition was similar to that obtained with 5 or 10 μM AA in cells exposed to 0·5 μM Iso or internally perfused with 10 μM cAMP (Fig. 4*B*). Thus, AA does not act by reducing cAMP levels, either by a mechanism involving cGMP or another mechanism. The

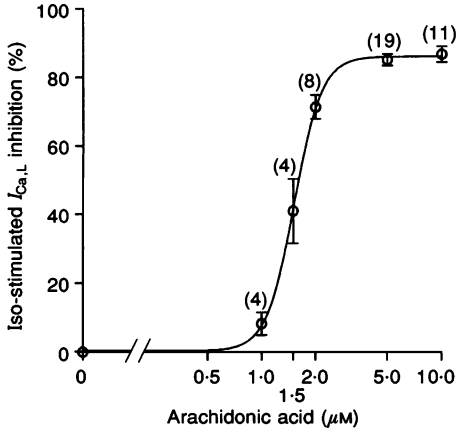

Figure 3. Concentration–response relationship for the effect of AA on $I_{Ca,L}$ in ventricular cells

Data points represent the mean percentage inhibition of $I_{Ca,L}$ by various concentrations of AA. Before application of AA, $I_{Ca,L}$ was stimulated with 0·5 μM Iso. The continuous line represents the Hill equation (see text) with $K_{½} = 1·52$ μM and the Hill coefficient (n_H) = 5·62. Number of cells tested for each concentration is indicated in parentheses.

involvement of cGMP-dependent protein kinase in the effect of AA on $I_{Ca,L}$ also seems unlikely, because activators of this kinase (such as 8-bromo-cGMP) have no effect on cAMP-stimulated $I_{Ca,L}$ in frog ventricular cells (Hartzell, 1988). In summary, these data suggest that AA acts on $I_{Ca,L}$ by a mechanism located beyond adenylyl cyclase which does not involve a regulation of the intracellular cAMP levels.

Effect of AA on $I_{Ca,L}$ stimulated by the catalytic subunit of the cAMP-dependent protein kinase

The above results suggest that the AA effect was exerted on a site different from those which regulate intracellular cAMP. In brush-border membranes of placental tissue, AA is able to inhibit the activity of the cAMP-dependent protein kinase (PKA), but has no effect on the catalytic subunit of the kinase. AA seems to compete with cAMP for binding to the kinase regulatory subunit (Doolan & Keenan, 1994). These authors suggested that the inhibition of PKA by AA could be involved in the inhibitory effect of AA on a chloride current. To test the hypothesis of a possible effect of AA on PKA activity, we studied the effect of AA on $I_{Ca,L}$ elevated by the catalytic subunit of the PKA. In Fig. 5*A*, a ventricular cell was internally perfused with a solution containing 200 μg ml^{-1} of the catalytic subunit of PKA. Due to its high molecular weight (40 kDa), the diffusion of the catalytic subunit into the cell was slow. In 37 min the $I_{Ca,L}$ amplitude increased nearly fifteenfold (from −133 to −2065 pA). External application of 5 μM AA led to about 70% inhibition of the current (from −2065 to −619 pA) in 8 min. After washing out the AA, $I_{Ca,L}$ increased from −619 to −3903 pA. A second application of AA inhibited $I_{Ca,L}$ by 77·7% in 11 min ($I_{Ca,L}$ decreased from −3903 to −870 pA). The second application of AA gave a more accurate percentage of inhibition, because of the achievement of the steady-state effect of the PKA. In

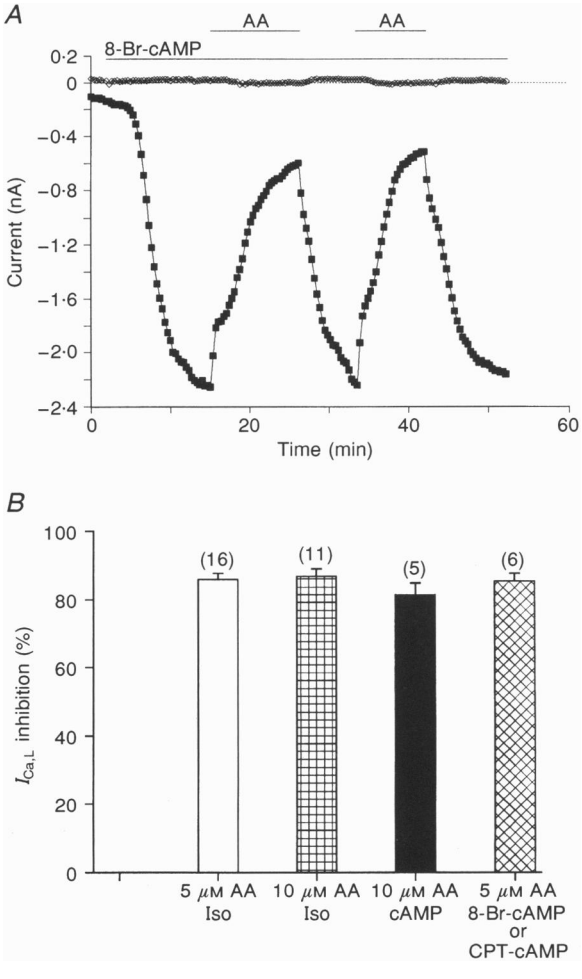

Figure 4. Effect of arachidonic acid on cyclic AMP-stimulated current

A, $I_{Ca,L}$ current values at 0 mV (■) and holding current values at −80 mV (◇) plotted *versus* time. The horizontal bars above indicate when 10 μM 8-Br-cAMP was perfused internally and when the superfusion contained 5 μM AA. *B*, the percentage inhibition by AA (5 or 10 μM) of $I_{Ca,L}$ stimulated by Iso or cAMP for different experimental conditions. Concentrations: Iso, 0·5 μM; cAMP, 8-Br-cAMP or CPT-cAMP, 10 μM.

four cells internally perfused with 200 μg ml^{-1} of the catalytic subunit of PKA, the average inhibitory effect of AA on $I_{Ca,L}$ was 83·1 ± 2·9%. This effect was similar to that of AA on the Iso- (85·2 ± 1·7%, $n = 19$) and the cAMP-analogue-stimulated $I_{Ca,L}$ (85·3 ± 2·4%, $n = 6$) (Fig. 5*B*). These data suggest that the AA effect was at a site beyond phosphorylation by PKA.

The effect of AA on $I_{Ca,L}$ is reduced in presence of protein phosphatase inhibitors

Since AA inhibited $I_{Ca,L}$ stimulated by the catalytic subunit of PKA, we hypothesized that AA was acting on the channel directly or was stimulating dephosphorylation. Thus, we investigated the effect of AA on cells exposed to inhibitors of protein phosphatases. Figure 6*A* shows an experiment utilizing microcystin, an inhibitor of protein phosphatases 1 and 2A (MacKintosh, Beattie, Klumpp, Cohen & Codd, 1990). In previous studies, we showed that 20 μM microcystin produced a maximal effect on $I_{Ca,L}$ (Frace & Hartzell, 1993). By using this high concentration of MC, we supposed we could achieve a 'maximal' inhibition of the activity of type 1 and 2A protein phosphatases. When MC (20 μM) was internally perfused, $I_{Ca,L}$ amplitude increased about tenfold in ~8 min. Iso (0·5 μM) was then applied before the steady-state effect of MC was achieved. Iso further stimulated $I_{Ca,L}$ from −714 to −2252 pA. When the Iso effect had reached a plateau, 5 μM AA was then applied. In ~14 min AA inhibited the

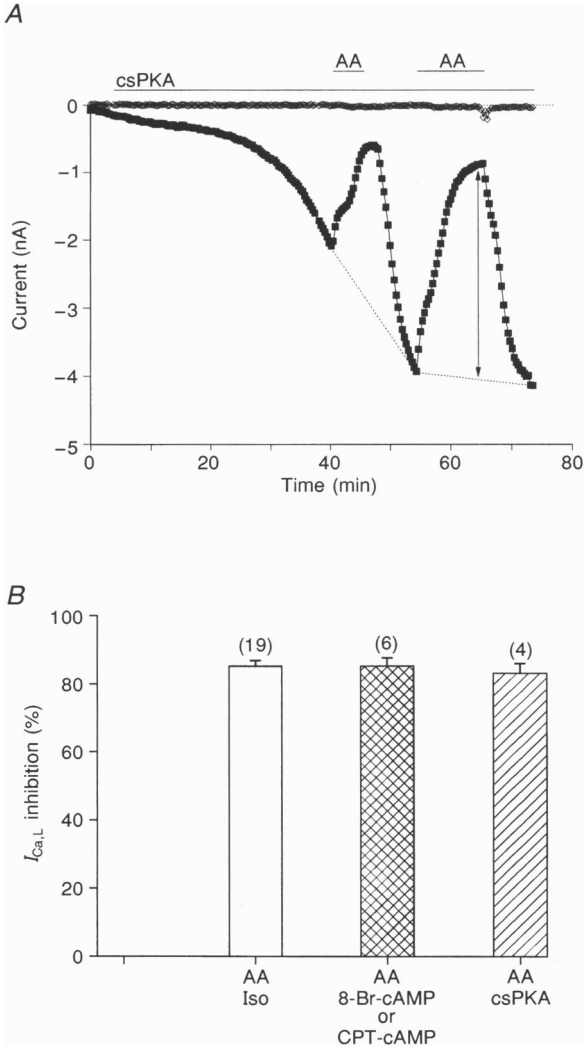

Figure 5. AA inhibits the calcium current stimulated by the catalytic subunit of PKA

A, the amplitudes of net $I_{Ca,L}$ at 0 mV (■) and of holding current at −80 mV (◇) plotted *versus* time. The horizontal bar labelled csPKA indicates internal perfusion with the catalytic subunit of PKA (200 μg ml^{-1}). Due to its high molecular weight, csPKA diffused slowly inside the cells and a second application of AA (5 μM) was necessary to measure the inhibitory effect with more accuracy (indicated by the arrow). *B*, vertical bars represent the mean percentage inhibition by 5 μM AA of $I_{Ca,L}$ stimulated by Iso, cAMP or PKA for different experimental conditions. Concentrations; Iso, 0·5 μM; 8-Br-cAMP or CPT-cAMP, 10 μM; csPKA, 200 μg ml^{-1}.

current by only 43% ($I_{Ca,L}$ diminished from −2252 to −1283 pA). The inhibition exhibited only one component, unlike the inhibition observed in the absence of MC (e.g. Fig. 1). The effect of AA was fully reversible. At the end of the experiment when Iso was washed out, $I_{Ca,L}$ remained at the Iso-stimulated level because of the inhibition of phosphatase activity by MC. This shows that the activity of protein phosphatases was inhibited.

The same result was obtained by utilizing two other phosphatase 1 and 2A inhibitors, okadaic acid and calyculin A. The data are summarized in Fig. 6*C*. In cells internally perfused with 20 μM MC, AA inhibited 45·5 ± 1·7% of the Iso-stimulated $I_{Ca,L}$ ($n = 3$). In cells internally perfused with 25 μM OA and exposed to Iso, the inhibitory effect of AA was 49·7 ± 2·1% ($n = 3$). Calyculin A (a more potent inhibitor of phosphatase 1 than phosphatase 2A (MacKintosh & MacKintosh, 1994)), applied externally to the cell in the presence of Iso, also reduced the effect of AA on $I_{Ca,L}$. In the presence of 200 nM calyculin in the external solution, AA inhibited Iso-stimulated $I_{Ca,L}$ only 37 ± 5·4% ($n = 4$). In comparison,

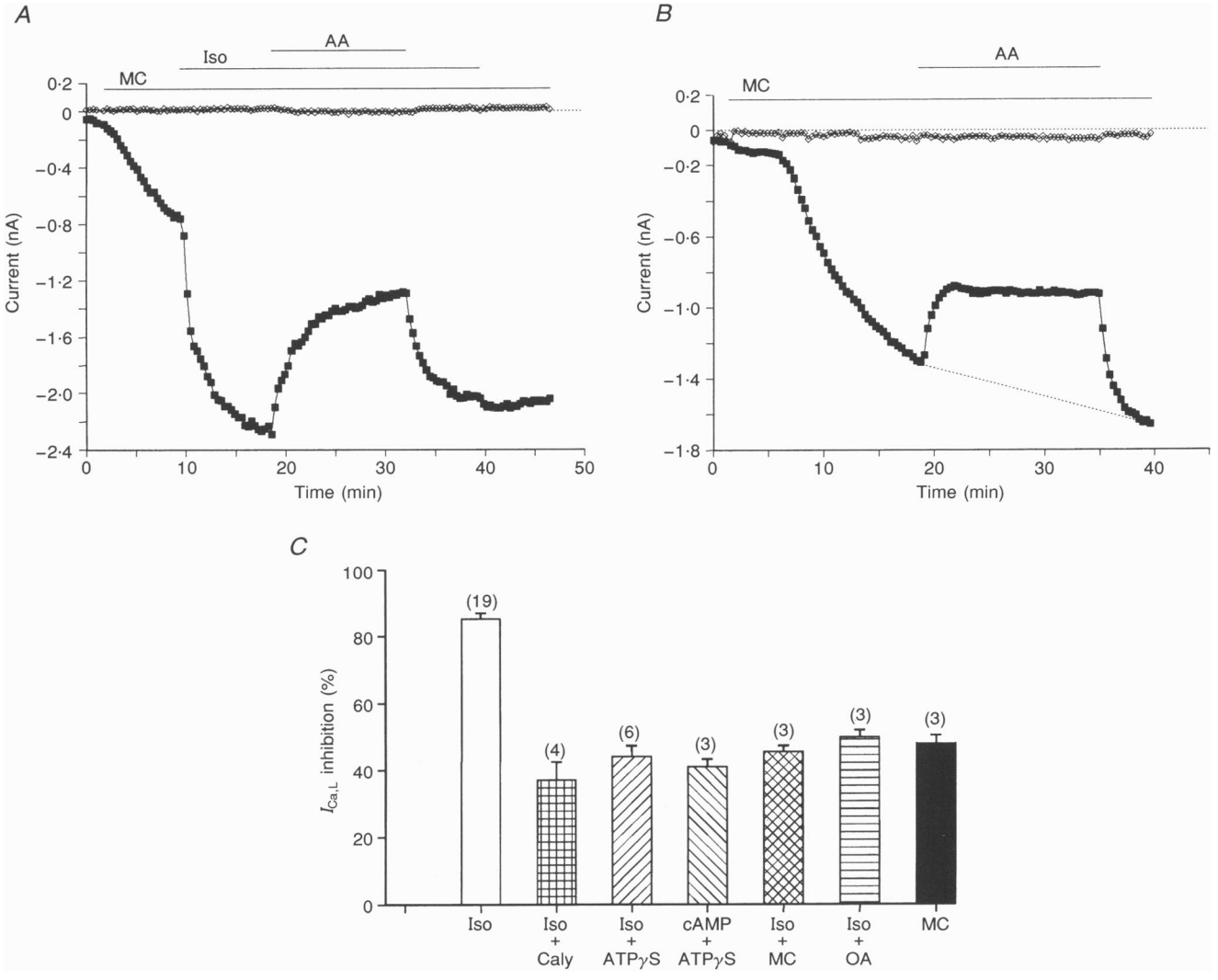

Figure 6. The inhibitory effect of AA is reduced in the presence of inhibitors of protein phosphatases

A, net $I_{Ca,L}$ amplitude at 0 mV (■) and holding current amplitude at −80 mV (◇) plotted *versus* time. Microcystin (MC, 20 μM) was first applied by internal perfusion as indicated. Iso (0·5 μM) and AA (5 μM) were added to the external superfusion as indicated by the labelled horizontal bars. *B*, experiment with MC (20 μM) in the absence of Iso. ■, $I_{Ca,L}$; ◇, holding current. AA (5 μM) was applied as indicated. Due to the slow increase of $I_{Ca,L}$ in MC (dashed line), the percentage of inhibition was calculated with the value of current at the end of the experiment, after wash-out of AA. *C*, summary of different experimental conditions. Vertical bars represent the mean percentage inhibition by AA (5 μM) of $I_{Ca,L}$ stimulated by Iso, cAMP or MC. Number of cells tested is indicated in parentheses. Concentrations: Iso, 0·5 μM; cAMP, 10 μM; calyculin A (Caly), 200 nM; ATPγS, 2·5 mM; MC, 20 μM and okadaic acid (OA), 25 μM.

under control conditions, AA inhibited 85·2 ± 1·7% of the Iso-stimulated $I_{Ca,L}$ ($n = 19$).

To test further the hypothesis that AA reduced $I_{Ca,L}$ by stimulating dephosphorylation, we examined the effect of AA in the presence of ATPγS, which can be used by PKA to thiophosphorylate proteins. Thiophosphorylated proteins are generally more resistant to dephosphorylation than phosphorylated proteins. By using ATPγS in conjunction with Iso or cAMP, we supposed that the thiophosphorylated substrate (the Ca^{2+} channel or a regulatory protein associated with it) would be more resistant to dephosphorylation (Esguerra, Wang, Foster, Adelman, North & Levitan, 1994). When cells were internally perfused with a solution containing 2·5 mM ATPγS, the inhibitory effect of AA on Iso-stimulated $I_{Ca,L}$ was 44 ± 3·1% ($n = 6$). Inhibition was 41 ± 2·3% ($n = 3$) on $I_{Ca,L}$ pre-stimulated by cAMP (Fig. 6*C*). In summary, these data suggest that the AA effect on $I_{Ca,L}$ is, in part, dependent on protein phosphatase activity, probably of the 1 or 2A type. The effects of ATPγS in these studies differs from some of our previously published results. We previously reported that in the presence of ATPγS, $I_{Ca,L}$ was persistently elevated by Iso, but we concluded that this persistence was largely due to persistent activation of adenylyl cyclase as ATPγS was converted to GTPγS by nucleotide diphosphate kinase (Parsons & Hartzell, 1993). The evidence supporting this conclusion was that the stimulatory effect of ATPγS was blocked by *Rp*-cAMPS, an inhibitor of PKA. These experiments differ in that GTP was included in the internal solution in the present experiments, but was absent in the experiments of Parsons & Hartzell (1993). We believe that in the present experiments, $I_{Ca,L}$ was persistently stimulated by thiophosphorylation because the response to forskolin was irreversible in the presence of ATPγS under these conditions.

AA inhibition of $I_{Ca,L}$ is not specific for PKA phosphorylation sites

In frog atrial and ventricular cells, the effect of phosphatase inhibitors on $I_{Ca,L}$ has revealed a new protein kinase activity, which we have termed protein kinase X (PKX). PKX is not PKA or PKC (Frace & Hartzell, 1993; Hartzell *et al.* 1995). Moreover, the Ca^{2+} channels seem to be regulated by two phosphorylation sites that can be affected by PKX and PKA (Hartzell *et al.* 1995). In the presence of MC alone, only one site is phosphorylated, whereas this site and another site are phosphorylated in the presence of Iso. The site phosphorylated by PKX is dephosphorylated by a phosphatase having low sensitivity to MC, whereas the other site seems to be dephosphorylated by phosphatase 2A (Hartzell *et al.* 1995). To determine whether AA affected the dephosphorylation of one of the sites selectively, we applied AA to cells internally perfused with microcystin, in the absence of isoprenaline in the external solution. These conditions result in an $I_{Ca,L}$ increase due to PKX-dependent phosphorylation (Frace & Hartzell, 1993; Hartzell *et al.* 1995). A representative experiment is shown in Fig. 6*B*. In this cell, internal perfusion with 20 μM MC led to an approximately nineteenfold increase in $I_{Ca,L}$ in ~17 min. In three cells, 20 μM MC increased $I_{Ca,L}$ 21·7 ± 5·5-fold. AA (5 μM) was then applied externally and this led to a 44·1% decrease of the MC-stimulated $I_{Ca,L}$ in ~16 min (Fig. 6*B*). In three cells, 5 μM AA led to a 48 ± 2·2% inhibition of the MC-stimulated $I_{Ca,L}$ (Fig. 6*C*, last bar). In comparison, in cells internally perfused with 20 μM MC and exposed to 0·5 μM Iso, AA inhibited $I_{Ca,L}$ by 45·5 ± 1·7% ($n = 3$, Fig. 6*C*). These data suggest that AA affects not only the PKA but also the PKX phosphorylation sites that regulate the Ca^{2+} channels.

Phosphorylation must occur for AA to exert its full effect on $I_{Ca,L}$

The hypothesis that AA stimulates dephosphorylation is reinforced by experiments like the one presented in Fig. 7, which suggest that phosphorylation must occur before AA can exert its inhibitory effect. In this experiment, a ventricular cell was exposed to AA before any other drug. In this particular cell the basal $I_{Ca,L}$ was large (>500 pA) and slowly ran down during the first 4 min of the experiment. AA (10 μM) was applied to the cell at 5 min. However, it was difficult to quantify the effect of AA on basal $I_{Ca,L}$ in this cell because of the presence of the run-down. In the continuous presence of AA, 0·5 μM Iso was then added at time t of ~11 min. In ~2 min, $I_{Ca,L}$ increased and reached a maximum amplitude of −1685 pA. This maximum amplitude however, was not maintained and $I_{Ca,L}$ then decreased to reach a steady-state value of −786 pA at t of ~22 min. AA was then washed out and the full effect of Iso on $I_{Ca,L}$ was observed (the net current increased from −786 to −2658 pA in ~8 min). It should be noted that the maximal effect of Iso in the absence of AA (between $t = 27$ and $t = 31$ min) was stable and the current did not decrease as it did in the presence of AA. At the steady-state effect of Iso, AA was reapplied, leading to a 92·4% inhibition of $I_{Ca,L}$ in ~6 min (from −2658 to −200 pA). Similar data were obtained when 5 μM AA was used and when $I_{Ca,L}$ was stimulated by cAMP. In the example of Fig. 7, the peak $I_{Ca,L}$ amplitude obtained at the beginning of the Iso effect in the presence of AA (−1685 pA) was ~63% of the maximal amplitude reached when the cell was exposed to Iso alone (−2658 pA). However, this peak amplitude was variable from cell to cell (the mean value was 53·4 ± 7·7% ($n = 4$) of the Iso maximal effect). These data suggest that the inhibitory effect of AA is more pronounced after phosphorylation has occurred. This supports the hypothesis that AA is acting via a dephosphorylation process which involves a protein phosphatase activity.

The AA effect on $I_{Ca,L}$ is not mediated via a protein kinase C

In nerve cells, *cis* fatty acids and AA inhibit Na^+ and Ca^{2+} currents via an activation of PKC activity. The effect of

these fatty acids is blocked by inhibitors of PKC like H-7 (1-(5-isoquinolinylsulphonyl)-2-methylpiperazine) or staurosporine (Linden & Routtenberg, 1989; Keyser & Alger, 1990). Thus we examined the effect of AA on cells exposed to staurosporine, an inhibitor of protein kinase C (Tamaoki, Nomoto, Takahashi, Kato, Morimoto & Tomita, 1986). Staurosporine by itself (50 nM) had an inhibitory effect on $I_{Ca,L}$ (Hartzell *et al.* 1995). For example, in two cells, staurosporine inhibited 52% of the Iso-stimulated $I_{Ca,L}$. Nevertheless, in ventricular cells exposed to 50 nM staurosporine, 5 μM AA was able to inhibit the residual Iso-stimulated Ca^{2+} current by 84·3 ± 1·6% ($n = 4$; data not shown). These data suggest that the AA effect is insensitive to staurosporine and is unlikely to be dependent on a protein kinase C activity. Moreover, in our preparation, PKC activators like phorbol esters have no effect on the Ca^{2+} current (Hartzell *et al.* 1995).

The AA effect on $I_{Ca,L}$ is not mediated via a G-protein-dependent mechanism

In atrial batrachian and mammalian cells, arachidonic acid is able to modulate the muscarinic K^+ channel (K_{ACh}). It has been suggested that AA or its metabolites could interfere with the GDP–GTP exchange processes of the G-protein linked to the K_{ACh} channel (Kurachi, Ito, Sugimoto, Shimizu, Miki & Ui, 1989; Kim, Lewis, Graziadei, Neer, Bar-Sagi & Clapham, 1989). To test whether AA acted via a G-protein-mediated mechanism, we internally perfused cells with the non-hydrolysable analogue of GTP, GTPγS. If AA acts via a G-protein, we predicted that the effect of AA would be irreversible under these conditions. In cells internally perfused with 50 μM GTPγS after the effect of 0·5 μM Iso had reached its maximal value, the amplitude of $I_{Ca,L}$ decreased. This was due to the activation of the inhibitory G-protein, G_i, by GTPγS, as previously reported (Parsons & Hartzell, 1993). In four cells, the Ca^{2+} current decreased by 28·1 ± 4·5% in 7·7 ± 0·6 min. However, in these cells, 10 μM AA was still able to inhibit the residual Ca^{2+} current. AA led to a 74·5 ± 1·7% decrease of the Ca^{2+} current in the presence of GTPγS ($n = 4$), compared with a 74·3 ± 1% decrease in three control cells from the same cell dissociations (data not shown). Moreover, in cells dialysed with the GTPγS solution, the AA effect was also fully reversible on wash-out, as in control conditions. These data suggest that the

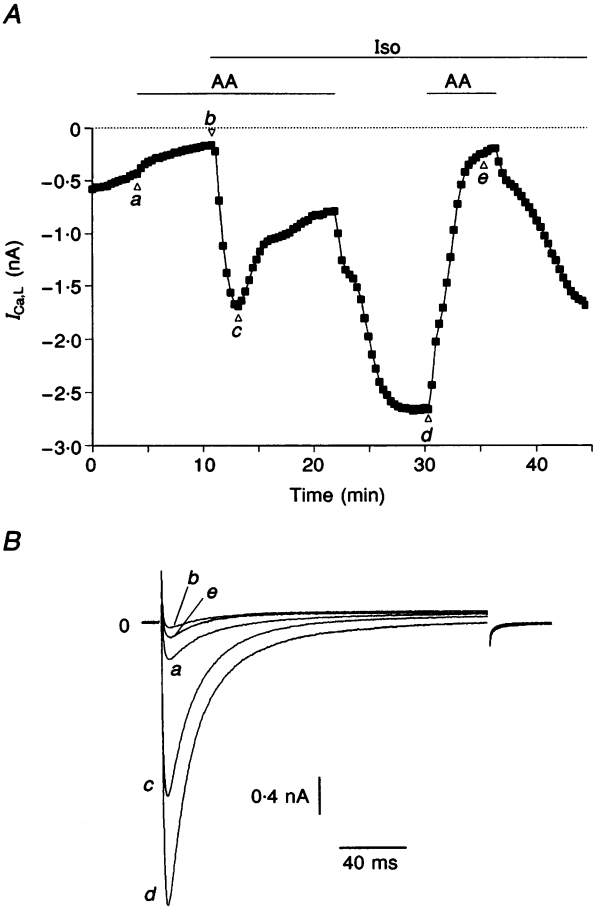

Figure 7. Effect of arachidonic acid on the basal $I_{Ca,L}$ and on $I_{Ca,L}$ stimulated by Iso

A, net $I_{Ca,L}$ at 0 mV is plotted *versus* time. AA (10 μM) and Iso (0·5 μM) were present in the superfusate as indicated by the labelled horizontal bars. *B*, *a–e* represent Ca^{2+} current traces corresponding to the labels in *A*.

AA effect is unlikely to be mediated by a G-protein-dependent mechanism.

A saturated fatty acid, myristic acid, has no inhibitory effect on $I_{Ca,L}$

Myristic acid (MA) is a long-chain saturated fatty acid (C 14:0). It has been shown that MA has a slight stimulatory effect on $I_{Ca,L}$ in rabbit intestinal smooth muscle cells (Shimada & Somlyo, 1992). We thus compared the effects of MA and AA on $I_{Ca,L}$ in frog ventricular cells. In Fig. 8*A*, the cell was first exposed to 10 μM MA, which caused a slight increase in the basal Ca^{2+} current amplitude (+34%). In six cells, 10 μM MA increased the basal $I_{Ca,L}$ by 9·5 ± 7·9%. After MA was washed out and the cell was re-exposed to the control solution for a few minutes, the addition of 0·5 μM Iso led to nearly a thirteenfold increase in $I_{Ca,L}$. At the steady-state effect of Iso, MA was reapplied to the cell. MA had nearly no effect on the Iso-stimulated $I_{Ca,L}$; it caused about a 6% increase in the $I_{Ca,L}$ amplitude in ~11 min. In comparison, in ~10 min 10 μM AA caused a 78·2% inhibition of the Iso-stimulated Ca^{2+} current in the same cell (Fig. 8*A*). In five cells, MA increased the Iso-

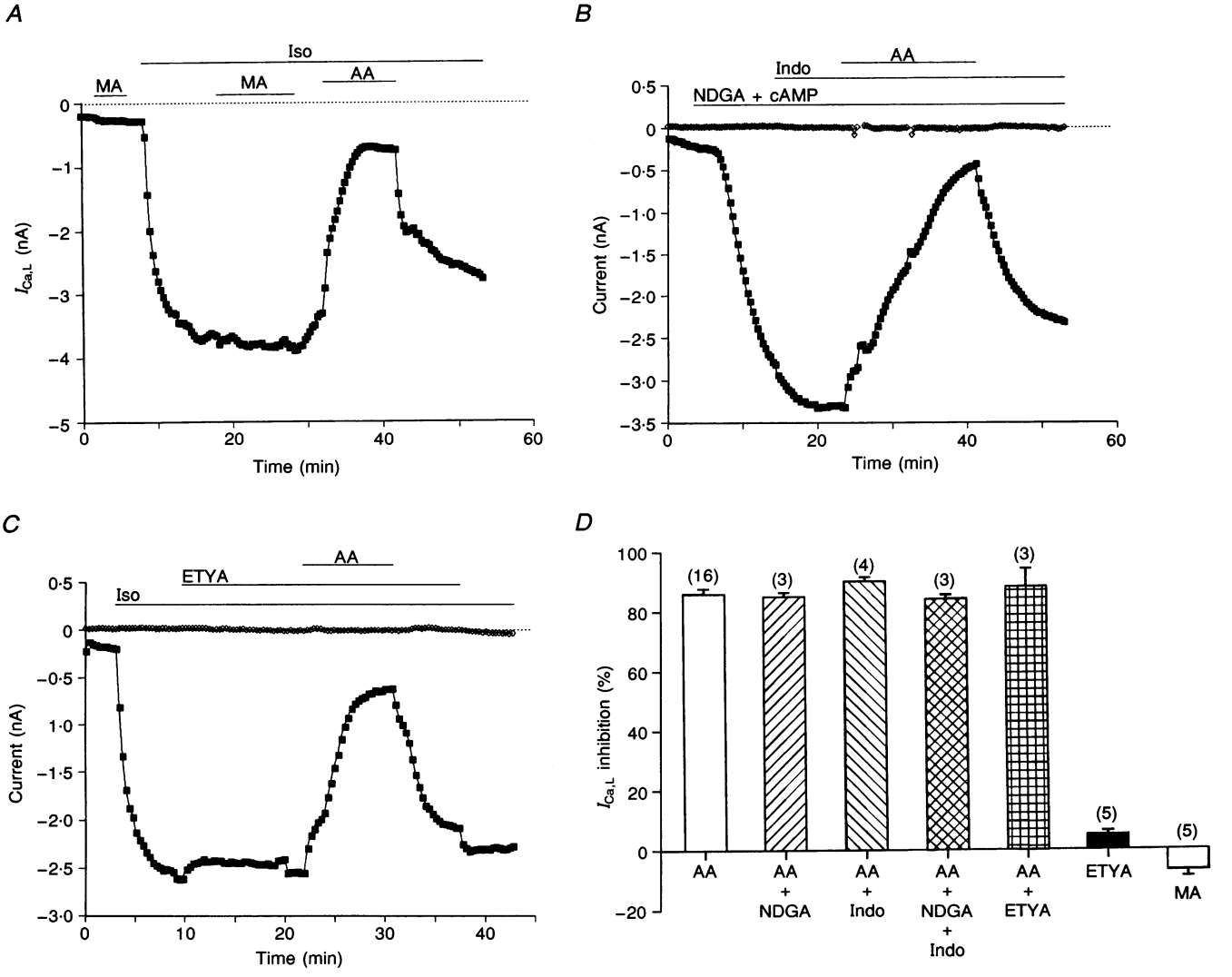

Figure 8. Effect of myristic acid on $I_{Ca,L}$, and effect of AA on $I_{Ca,L}$ in presence of inhibitors of cyclo-oxygenase and lipoxygenase

A, net $I_{Ca,L}$ current at 0 mV plotted *versus* time. Myristic acid (MA, 10 μM), 0·5 μM Iso and 10 μM AA were applied as indicated by the labelled horizontal bars. *B*, net $I_{Ca,L}$ at 0 mV (■) and holding current at −80 mV (◇) plotted *versus* time. As indicated by the bar, nordihydroguaiaretic acid (NDGA, 10 μM) and cAMP were applied at the same time via the internal perfusion system. Then, Indo (10 μM) and AA (5 μM) were applied externally via the superfusion system. *C*, effect of ETYA on $I_{Ca,L}$ (■). Holding current, ◇. Iso (0·5 μM), 5,8,11,14-eicosatetraynoic acid (ETYA, 5 μM) and AA (5 μM) were applied externally as indicated by the labelled horizontal bars. *D*, summary of the effects of drugs. The vertical bars represent the mean percentage inhibition of the $I_{Ca,L}$ pre-stimulated by Iso or cAMP. Concentrations: Iso, 0·5 μM; cAMP, 10 μM; AA, 5 μM; ETYA, 5μM; Indo, 10 μM; NDGA, 10 μM; MA, 10 μM.

stimulated $I_{Ca,L}$ by 7 ± 2·1% (Fig. 8*D*), and AA decreased it by 84·4 ± 2·6%.

The AA effect on $I_{Ca,L}$ is not blocked by inhibitors of cyclo-oxygenase or lipoxygenase pathways

In mammalian atrial cells, the stimulatory effect of AA on K_{ACh} is mediated by 5-lipoxygenase metabolites. The effect of AA is blocked by nordihydroguaiaretic acid, an inhibitor of lipoxygenases, but is insensitive to indomethacin, an inhibitor of cyclo-oxygenase (Kurachi *et al.* 1989; Kim *et al.* 1989). In smooth muscle cells, neither indomethacin nor NDGA affect the inhibitory effect of AA on $I_{Ca,L}$ (Shimada & Somlyo, 1992). We thus examined the effect of AA on $I_{Ca,L}$ in the presence of these metabolic inhibitors. Figure 8*B* shows an experiment on a ventricular cell where AA was applied in the presence of both inhibitors. In experiments using NDGA, we were obliged to apply this inhibitor via the internal perfusion system because when present in the external solution it caused a rapid and near total suppression of $I_{Ca,L}$ (data not shown). Such an inhibitory effect of NDGA has been observed in pituitary cells (Korn & Horn, 1990). We usually applied NDGA in conjunction with cAMP, so that we could verify that a good dialysis of the cells by the NDGA-containing solution was performed. In the cell of Fig. 8*B*, the internal application of 10 μM NDGA and 10 μM cAMP led to a large increase (>14-fold) of $I_{Ca,L}$. Thus NDGA seemed not to affect the action of cAMP on $I_{Ca,L}$. Then, 10 μM indomethacin was externally applied to the cell. Because we wanted to treat the cell with the inhibitor for a relatively long time (~10 min) before the exposure to AA, indomethacin was introduced before the cAMP effect had reached its maximum. When the Ca^{2+} current had reached its maximal steady-state amplitude, 5 μM AA was applied to the cell in the presence of indomethacin. Application of AA led to an 87% inhibition of the current (from −3992 to −429 pA in ~18 min).

We also examined the effect of a non-metabolizable analogue of AA, eicosatetraynoic acid (ETYA). In the cell shown in Fig. 8*C*, exposure to 0·5 μM Iso led to a fifteenfold increase of $I_{Ca,L}$. ETYA (5 μM) was subsequently added to the superfusion. After more than 10 min of application of the analogue, $I_{Ca,L}$ remained at more than 95% of its value in Iso, showing that ETYA had nearly no effect. Then, 5 μM AA was added in the presence of Iso and ETYA, resulting in a 76·2% inhibition of $I_{Ca,L}$.

The data obtained with the metabolic inhibitors are summarized in Fig. 8*D* where the effect of AA is expressed as the percentage inhibition of the Iso- (or cAMP-) stimulated current. In cells internally perfused with 10 μM NDGA, 5 μM AA inhibited $I_{Ca,L}$ by 85 ± 1·4% ($n = 3$). In the presence of 10 μM indomethacin, AA (5 μM) inhibited $I_{Ca,L}$ by 90·1 ± 1·4% ($n = 4$). In cells internally perfused with 10 μM NDGA and superfused with 10 μM indomethacin, $I_{Ca,L}$ was decreased 84·1 ± 1·4% by 5 μM AA ($n = 3$). When the cells were exposed to 5 μM ETYA, AA (5 μM) inhibited 88·1 ± 5·9% of the Ca^{2+} current ($n = 3$). In comparison, in control conditions, 5 μM AA depressed the Iso-stimulated $I_{Ca,L}$ by 85·9 ± 1·8% ($n = 16$). However, ETYA (5 μM) itself was ineffective, inhibiting only 5·1 ± 1·1% of the Iso-stimulated current ($n = 5$). A higher dose of ETYA (15 μM) also had no inhibitory effect on $I_{Ca,L}$. These data suggest that the AA effect seems not to be mediated by the cyclo-oxygenase or lipoxygenase pathways. Since NDGA is also an inhibitor of non-specific lipid peroxidation (Huang *et al.* 1992), it is unlikely that this mechanism is involved. However, the absence of effect of ETYA suggests that perhaps another metabolism of AA is participating in the inhibitory effect on $I_{Ca,L}$.

DISCUSSION

The data presented in this study shows that AA inhibits the L-type Ca^{2+} current in frog cardiac myocytes. We hypothesize that AA inhibits $I_{Ca,L}$ by two mechanisms. We further hypothesize that one mechanism involves stimulation of protein phosphatase activity because the effect of AA was reduced by protein phosphatase inhibitors (MC, OA and calyculin A) and by ATPγS, and was larger after phosphorylation had occurred in response to Iso or cAMP. The other mechanism remains unknown, but its existence is demonstrated by the observation that the inhibition of protein phosphatase activity with very high concentrations of MC or OA only partially inhibited the effect of AA. Furthermore, the inhibitory effect of AA on Iso- or cAMP-stimulated $I_{Ca,L}$ invariably exhibited a biphasic time course (Figs 1, 4, 5 and 8). In contrast, in the presence of protein phosphatase inhibitors only one component was evident (Fig. 6). Neither process, however, involved decreases in cAMP levels or changes in protein kinase activity, because the effect of AA on $I_{Ca,L}$ stimulated by Iso, non-hydrolysable analogues of cAMP and the catalytic subunit of PKA was the same.

Specificity of the effect of AA on $I_{Ca,L}$

We are confident in the specificity of the effects of AA described in this study. The doses of AA we used (1–10 μM) were below the critical micellar concentration (CMC). It is known that above their CMC, fatty acids in solution can form micelles (Meves, 1994) and can cause various non-specific effects, such as detergent effects, on biological membranes and ion channels (Yamada, Terzig & Kurachi, 1994). The CMC for AA has been estimated to be >10 μM (Richieri, Ogota & Kleinfeld, 1992). Due to non-specific binding of AA to the external perfusion tubing system (Massey, Minnich & Burt, 1992; Richieri *et al.* 1992) we suppose that the concentration of AA delivered to the cells during our experiments was less than the nominal concentration. The experiments with MA and ETYA, which showed nearly no effect of these compounds on $I_{Ca,L}$, provide additional evidence that the effect of AA is not due to a non-specific detergent effect (Meves, 1994). Moreover, the AA concentrations we utilized were in the same range

used in many previous patch clamp studies, in cardiac (Kurachi *et al.* 1989; Kim *et al.* 1989; Huang *et al.* 1992) and smooth muscle (Shimada & Somlyo, 1992) tissues, which demonstrated specific effects of AA.

Involvement of a protein phosphatase activity in the AA effect on $I_{Ca,L}$

Our results are consistent with an involvement of a protein phosphatase activity in the inhibition of $I_{Ca,L}$ by AA. About half of the effect of AA could be explained by protein phosphatase stimulation because treatments which inhibit phosphatase activity block > 40% of the AA effect (Fig. 6). The phosphatase activity involved could be of the 1 or 2A type because it is sensitive to calyculin, MC and OA, but the concentrations of these inhibitors were supramaximal for inhibition of phosphatases 1 and 2A. Thus, it is not possible to determine from these studies the species of protein phosphatase involved. The involvement of protein phosphatase 2B (calcium-dependent phosphatase) seems unlikely because in our experiments internal $[Ca^{2+}]$ was buffered to $<10^{-9}$ M. Participation of phosphatase 2C (magnesium-dependent phosphatase) in the effects of AA is unknown. An intervention of this phosphatase in the modulation of $I_{Ca,L}$ in our preparation has been previously suggested (White & Hartzell, 1988). However, no specific inhibitor of this phosphatase is available.

Even with high doses of phosphatase inhibitors, AA was still able to inhibit about half of the $I_{Ca,L}$. There are several possible explanations of this result. (1) The effect of AA is dependent on a type of phosphatase which shares characteristics with type 1 and 2A but is less sensitive to inhibition by MC, OA and calyculin. Thus, these inhibitors may have blocked the phosphatase only partially. (2) Two protein phosphatase activities are stimulated by AA. One activity is due to the types 1 or 2A, and is blocked by MC, OA or calyculin. The other activity is due to another protein phosphatase insensitive to type 1 and 2A inhibitors. The existence of two phosphorylation sites, which are dephosphorylated by different phosphatases, regulating the Ca^{2+} channel has been shown in rabbit (Ono & Fozzard, 1993) and guinea-pig ventricular cells (Wiechen, Yue & Herzig, 1995). Moreover, also in our preparation, there is evidence supporting the existence of two different phosphorylation sites whose sensitivities to dephosphorylation by phosphatases are different (Frace & Hartzell, 1993; Hartzell *et al.* 1995). The data in this present study shows that the action of AA affects both phosphorylation sites because: (a) AA inhibits the Ca^{2+} current stimulated by Iso, cAMP and the catalytic subunit of PKA (sites affected by PKA); (b) AA decreases the Ca^{2+} current stimulated by MC alone (site affected by PKX); (c) AA inhibits the basal Ca^{2+} current which may be regulated by PKX (Frace & Hartzell, 1993; Hartzell *et al.* 1995). (3) The third possible explanation is that two different mechanisms are involved in the inhibitory effect of AA on $I_{Ca,L}$. One is due to the activity of phosphatase 1 or 2A and is blocked by the phosphatase inhibitors. Another mechanism is independent of a protein phosphatase activity but leads also to an inhibitory modulation of the Ca^{2+} current. The idea that there are two separate mechanisms of action of AA is supported by two main observations. (a) The effect of AA is inhibited to the same extent with inhibitors of phosphatase (MC, OA, calyculin) and ATPγS. The observation that ATPγS, which would be expected to reduce dephosphorylation non-selectively, has the same effect as the more selective phosphatase inhibitors, suggests that the second mechanism does not involve a protein phosphatase activity. (b) In addition, we invariably see that the inhibition of Iso- or cAMP-stimulated $I_{Ca,L}$ occurs with two different components. Although the two components have similar exponential time constants, one component seems to begin after a lag period of ~1 min relative to the other component. In the presence of protein phosphatase inhibitors, only one component of inhibition is observed. In preliminary experiments, we have been able to inhibit further the effect of AA by internally perfusing cells with a medium containing 20 μM MC and 2 mM BAPTA. The Ca^{2+} current in these conditions was only inhibited ~28% by AA ($n = 4$). Thus the second component of the AA effect may be mediated by increases in intracellular free Ca^{2+} near the membrane.

To our knowledge the only report of a modulation of a protein phosphatase activity by AA has been made in smooth muscle cells by Gong *et al.* (1992) showing a inhibition of the myosin light-chain phosphatase at high AA concentrations (> 20 μM) and modest stimulatory effect at low AA concentrations (< 20 μM). Moreover these authors report that the catalytic subunit of myosin light-chain phosphatase is known to be identical to phosphatase 1 and they suggest a possible direct effect of AA on the enzyme.

Is the effect of AA on $I_{Ca,L}$ a direct effect?

AA and other fatty acids have direct effects on ion channel activity (Ordway, Walsh & Singer, 1989; Anderson & Welsh, 1990; and see Meves, 1994 for review). These direct effects are generally mimicked by ETYA and sometimes by saturated fatty acids (Ordway *et al.* 1989) and are generally insensitive to metabolic inhibitors (Meves, 1994). These direct effects are supposed to occur at the cytoplasmic side of the channels, because they can be seen in inside-out patches. In our preparation, even though the effect of AA on $I_{Ca,L}$ is still present with NDGA and indomethacin, the participation of a direct effect on the channel remains unlikely for several reasons. (1) More than 40% of the AA effect is blocked by MC, OA, calyculin and ATPγS. (2) The AA effect develops more slowly (the steady-state effect is reached in ~13 min, see Results) than one would expect for a supposed direct effect on the channel (in comparison with inhibitory dihydropyridines, which act in a few seconds). (3) Several experiments were made by applying AA via the internal perfusion system, and under these conditions, AA

had no effect on the Iso-stimulated Ca^{2+} current. (4) The saturated fatty acid myristic acid had no inhibitory effect on $I_{Ca,L}$ (Fig. 8). (5) ETYA had nearly no effect on $I_{Ca,L}$ (Fig. 8). Another hypothesis is that AA interacts directly with the protein phosphatases (Gong *et al.* 1992) which regulate the Ca^{2+} channels. Direct effects of AA on different enzyme activities have been described (Meves, 1994).

Comparison with other studies

Different studies report effects of AA on the Ca^{2+} current in heart tissue. In guinea-pig atrial cells, AA reversibly blocks the L- and T-type Ca^{2+} currents, an effect which is insensitive to inhibitors of 5-lipoxygenase and cyclo-oxygenase (Cohen, *et al.* 1990). In contrast, in guinea-pig ventricular cells, AA and other fatty acids stimulate the L-type Ca^{2+} current (Huang *et al.* 1992). This enhancement of $I_{Ca,L}$ is also present in cells exposed to leukotrienes and prostaglandins, suggesting that 5-lipoxygenase and cyclo-oxygenase metabolism of AA is probably involved (Huang, Xian & Bacaner, 1990). PKA-, PKC- or G-protein-mediated mechanisms seemed not to be involved (Huang *et al.* 1992). The difference between the results of Huang *et al.* (1992) and ours is perhaps related to the difference in animal species, mammalian *versus* batrachian. However, our results in frog ventricular cells seems to confirm those of Cohen *et al.* (1990) in atrial guinea-pig cells. We also found many similarities between our study and the effects of AA on the L-type $I_{Ca,L}$ in mammalian smooth muscle cells (Shimada & Somlyo, 1992).

Physiological significance

The levels of AA and other fatty acids are elevated in the circulation during physiopathological states like ischaemia (Meves, 1994). An inhibition of $I_{Ca,L}$ by AA could participate in a protective action against arrhythmias and cell calcium overload. By reducing the stimulatory action of β-adrenergic agonists, the inhibitory effect of AA on the Ca^{2+} current could provide a control of excitability and a protection against myocardial cell damage.

Our results show that AA inhibits the L-type Ca^{2+} current in frog ventricular myocytes by a mechanism involving, in part, activation of a protein phosphatase activity. Such a stimulation of phosphatase activity could provide a new pathway in the modulation of the Ca^{2+} channels by external stimuli. The regulation of $I_{Ca,L}$ by phosphatase 1 has already been shown in mammalian cardiac cells (Hescheler *et al.* 1987) and we have evidence of a role of protein phosphatases in the regulation of $I_{Ca,L}$ in our preparation (Frace & Hartzell, 1993; Hartzell *et al.* 1995). Also a protein phosphatase-dependent mechanism has been demonstrated for the effect of somatostatin on Ca^{2+}-activated K^+ channels in pituitary cells (White, Schonbrunn & Armstrong, 1991). However, further experiments are required to understand better the mechanism of action of AA on the L-type calcium current.

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Acknowledgements

We thank Dr Louis DeFelice for helpful comments on the manuscript and Ms Alyson Ellingson, Mr Bill Goolsby and Mr Alex Daniel for technical assistance. Supported by NIH grant HL 21195.

Received 3 August 1995; accepted 27 December 1995.