British Journal of Pharmacology (1995) 116, 1887-1893

Modulation of calcium channel currents by arachidonic acid in single smooth muscle cells from vas deferens of the guinea-pig

Norihiro Nagano, [1]Yuji Imaizumi & Minoru Watanabe

Department of Chemical Pharmacology, Faculty of Pharmacentical Science, Nagoya City University, 3-1 Tanabe-dohri, Mizuho-Ku, Nagoya, 467, Japan

1 Effects of arachidonic acid (AA) on voltage-dependent Ca channel currents were investigated by whole-cell-clamp methods in single smooth muscle cells freshly isolated from vas deferens of the guinea-pig.

2 Ca channel current was decreased by application of 1-30 μM AA in a concentration-dependent manner. When Ca^{2+} or Ba^{2+} was the charge carrier, Ca channel current (I_{Ca} or I_{Ba}) was reduced by AA to a similar extent (IC_{50} = 10 and 6 μM, respectively). Addition of 15 mM BAPTA to the pipette solution did not affect the reduction of I_{Ba} by 10 μM AA.

3 The effect of AA on I_{Ba} was not prevented by internal application of 1 mM nordihydroguaiaretic acid (NDGA) and 1 mM indomethacin (Indo). When the pipette solution contained 0.1 mM guanosine-5'-triphosphate (GTP), I_{Ba} was decreased slightly but significantly by application of 30 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) but not by PGE_2. This effect of $PGF_{2\alpha}$ was irreversible or not observed when the pipette solution contained 0.3 mM guanosine-5'-(3-thiotriphosphate) (GTPγS) or both GTP or guanosine-5'-O-(2-thiodiphosphate) (GDPβS), respectively.

4 External application of 100 units ml^{-1} superoxide dismutase slightly but significantly attenuated the inhibition of I_{Ba} by 1-30 μM AA. Intracellular application of 1 mM GDPβS or 0.3 mM GTPγS did not significantly change the effect of AA. Intracellular application of 0.1 mM 1-(5-isoquinolinesulphonyl)-2-methylepiperazine (H-7) also did not change the effect of AA.

5 These results indicate that the decrease in Ca channel currents in vas deferens smooth muscle cells is mainly due to AA itself, as opposed to its metabolites. The effect of AA may be due to AA itself, as opposed to its metabolites. The effect of AA may be due to its direct action on Ca channels or membrane phospholipids, but may not be mediated by activation of GTP binding proteins or protein kinase C. The inhibition of Ca channel current by AA may be partly induced by superoxide radicals derived from AA oxidation. $PGF_{2\alpha}$ also reduces Ca channel currents but probably by a separate mechanism via activation of a GTP binding protein.

Keywords: Ca channel; arachidonic acid; smooth muscle; vas deferens; superoxide; prostaglandin.

Introduction

Arachidonic acid (AA) is a major component of cell membrane lipids and is mainly released by activation of phospholipase A_2. A number of AA metabolites, acting through cyclo-oxygenase and lipoxygenase pathways (see review by Needleman *et al.*, 1986) mediate a variety of cell signalling events under both physiological and pathological conditions. It has been reported that AA *per se* and/or its metabolites can modulate activites of several kinds of ionic currents including voltage-dependent Ca channels (Ordway *et al.*, 1991). Ca^{2+} influx through voltage-dependent Ca channels plays a central role in cellular signal transduction in various types of cells. In smooth muscle cells, activation of Ca channels is essential for action potential generation, and triggers Ca^{2+} release from intracellular Ca^{2+} stores, to induce contractions. Modulation of Ca channel activity by transmitters, hormones and autacoids has therefore been studied extensively (see review by McDonald *et al.*, 1994).

The inhibition of Ca channel activity by AA and/or its metabolites has been described in various types of cells, including sympathetic neurones (Ikeda, 1992), cultured ciliary ganglion cells (Khurana & Bennett, 1993), hippocampal CA1 neurones (Keyser & Alger, 1990), intestinal smooth muscle cells (Shimada & Somlyo, 1992) and cultured vascular smooth muscle cells (Serebryakov *et al.*, 1994). In some of these cell types, externally applied AA or prostaglandins reduce Ca channel currents directly or via separate signal transduction pathways. In single smooth muscle cells from the portal vein (Loirand *et al.*, 1990), however, Ca channel currents were not affected by AA. On the other hand, AA increases the Ca current in rat pituitary GH_3/B_6 cells (Vacher *et al.*, 1989; 1992) and ventricular myocytes of the guinea-pig (Huang *et al.*, 1992). Thus the effect of AA on Ca^{2+} channels varies widely.

Regulation of Ca^{2+}-activated K^+ channels in smooth muscle cells by AA, its metabolites or other fatty acids has been extensively studied (Bregestovski *et al.*, 1988; Kirber *et al.*, 1992; Gebremedhin *et al.*, 1992; Hu & Kim, 1993). However, effects of AA on Ca^{2+} channel activity in smooth muscles has received less attention. The present study evaluated the possibility that AA and/or its metabolites are involved in regulation of Ca channel activity in smooth mucsle cells of vas deferens, since prostaglandins play significant physiological roles in this tissue including the regulation of excitatory transmitter release from sympathetic nerve endings (Ambache & Zar, 1970) and contraction induced by sympathomimetics (Clegg, 1966) (see review by Horton, 1969).

Methods

Cell isolation

Single smooth muscle cells were enzymatically isolated from the vas deferens of the guinea-pig. The procedures of cell isolation were the same as described previously (Imaizumi *et al.*, 1991). A few drops of cell suspension were placed in a recording chamber (0.5 ml) mounted on the stage of a phase contrast microscope (Nikon TMD). Cells were continuously perfused with a HEPES buffered solution (see solutions) at 5 ml min^{-1}. Only relaxed cells which had lengths over 100 μM were used for electrical recording.

[1] Author for correspondence.

Solutions

HEPES buffered solution (standard HEPES solution) having the following composition was used for electrical recordings as the external solution (mM): NaCl 137, KCl 5.9, $CaCl_2$ 2.2, $MgCl_2$ 1.2, glucose 14, HEPES 10. The pH was adjusted to 7.2 with NaOH. When the Ba^{2+} current was recorded Ca^{2+} in standard HEPES solution was replaced by equimolar Ba^{2+} (Ba solution).

The pipette-filling solution (pipette solution) contained (mM): CsCl 140, $MgCl_2$ 4, ATP-Na_2 4, EGTA 5 and HEPES 10. The pH was adjusted to 7.2 with KOH. In some experiments, the pCa of the pipette solution was regulated by adding $CaCl_2$ at a selected concentration. The pCa of the solution was adjusted to a required value with Ca-EGTA buffer (5 mM EGTA and adequate concentrations of $CaCl_2$) according to the methods used by Benham *et al.* (1986). Moreover, in some experiments, 15 mM, 1,2-*bis*(2-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid (BAPTA) was added to the pipette solution. When the pipette solution contained only 5 mM EGTA or 5 mM EGTA plus 15 mM BAPTA, the pCa of the solution is >9.0, respectively, assuming contamination with Ca^{2+} as 10 μM. When effects of a drug were tested, the drug was applied externally by an exchange of perfusion solution or internally by adding to the pipette solution. When internally applied, the effects of the drug reached a steady level in 5–10 min after rupture of the patch membrane, and measurements were taken.

Electrical recording and data analysis

Whole cell voltage clamp was applied by the method originally introduced by Hamill *et al.* (1981). EPC-7 (List, Germany) and CEZ-2200 (Nihon Kohden, Japan) amplifiers were used. The resistance of the pipette ranged from 2 to 5 MΩ when filled with the pipette solution. The seal resistance formed between cell membrane and the tip was approximately 30 GΩ. Series resistance was between 4–8 MΩ and was partly compensated. All electrophysiological measurements were made at room temperature (23±2°C).

Data were stored and analysed by menu-driven software (Robinson & Giles, 1986) as previously described (Imaizumi *et al.*, 1990). Pooled data are presented as mean±s.e.mean. Statistical significance was determined by Student's *t* test.

Drugs

Arachidonic acid (AA), prostaglandin E_2 (PGE_2), and superoxide dismutase (SOD) were obtained from Funakoshi (Tokyo, Japan). Bovine serum albumin (MILES, Fraction V) and 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) were from Seikagaku Kogyo (Tokyo, Japan). Collagenase was from Yakulto (Tokyo, Japan) or Amano (Aichi, Japan). Trypsin inhibitor type II-O, guanosine-5'-O-(2-thiodiphosphate) (GDPβS), guanosine-5'-O-(3-thiotriphosphate) (GTPγS), nordihydroguaiaretic acid (NDGA), indomethacin (Indo) and prostaglandin $F_{2\alpha}$ (PGF_2) were purchased from Sigma Co. Ltd. (St. Louis, U.S.A.). Ethyleneglycol-*bis*-(aminoethyl)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA) and 1,2-*bis*(2-aminophenoxy) ethane-*N*,*N*,*N*',*N*'-tetraacetic acid (BAPTA) were from Dojin Kagaku (Kumamoto, Japan). Collagenase/dispase was from Boehringer Mannheim (Mannheim, Germany). NDGA and Indo were dissolved at a concentration of 1 M in ethanol and used as the stock solutions. When 1 mM NDGA or Indo was added to the pipette solution, the solution contained 0.1% ethanol. The stock solutions of AA and prostaglandins at concentrations of 0.1 or 1 M in dimethyl sulphoxide (DMSO) were stored in ampoules filled with N_2 gas at −20°C or −80°C. The final concentration of DMSO was 0.1% or less. Other drugs and agents were dissolved in water.

Results

Effect of AA on Ca channel currents

Effects of 30 μM AA on Ba current through Ca channels (I_{Ba}) were examined in single vas deferens smooth muscle cells in a solution containing 2.2 mM Ba^{2+} (Figure 1). Inactivation of L-type Ca channels following an elevation of intracellular Ca^{2+} concentration has been demonstrated in various types of cells including vas deferens smooth muscle cells (Nakazawa *et al.*, 1988; Imaizumi *et al.*, 1991). In most of the experiments in the present study, therefore, Ba^{2+} was used as the charge carrier instead of Ca^{2+}. I_{Ba} was elicited by depolarization from a holding potential of −60 mV to a test potential of 0 mV for 150 ms at 0.067 Hz. Application of 30 μM AA reduced the peak I_{Ba} amplitude, although the onset was slow (~20 s). I_{Ba} was almost abolished within 3–4 min from the start of the application (Figure 1a). The slow onset was not due to the solution exchange rate, since application of 0.1 mM Ca^{2+} abolished I_{Ba} within 5 s under the same conditions. When a lower concentation of AA was applied, the onset time was even longer. After washout of AA, I_{Ba} recovered partially. The failure of I_{Ba} to return to the control after washout of AA may be, at least in part, due to 'run-down of I_{Ba}' which was observed consistently. The rates of I_{Ca} and I_{Ba} run-down for 10 min were 37.0±5.9% (n=19) and 33.3±4.7% (n=27, P<0.05 vs. I_{Ca}), respectively. To resolve effects of AA and other agents on Ca channel currents from the run-down, the time-course of changes in peak amplitude of I_{Ba} or I_{Ca} just prior to the addition of an agent was fitted by a straight line and extrapolated to the period after the addition of the agent. The difference between the line and the recorded amplitude at each time was taken as the effect of the agent. Bath application of 0.1% DMSO, the solvent of AA, *per se* did not affect I_{Ba} amplitude significantly (95.5±2.6%, n=5, P>0.05 vs. control).

To examine the voltage-dependence of AA-induced block of I_{Ba}, the current-voltage relationship of I_{Ba} was obtained using a ramp command waveform in the absence and presence of 10 μM AA. The ramp was applied for 100 ms with a depolarizing rate of 1.2 Vs^{-1} from −80 to +40 mV once every 10 s (Figure 2a). The peak amplitude of I_{Ba} developed at approxi-

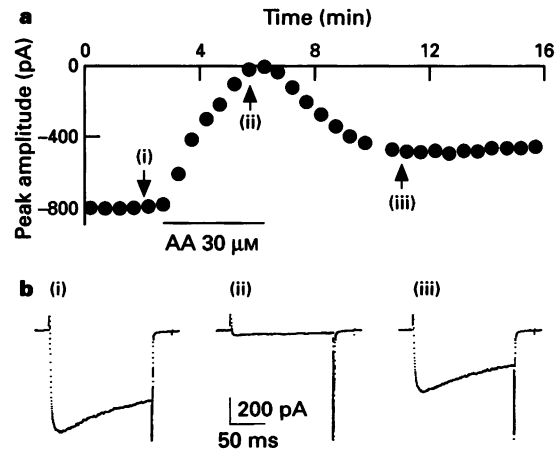

Figure 1 Effects of 30 μM arachidonic acid (AA) on I_{Ba} through Ca channels were examined in single smooth muscle cell isolated from vas deferens of the guinea-pig. I_{Ba} was elicited by 150 ms depolarization from −60 to 0 mV at 0.067 Hz. (a) Time course of change in peak amplitude of I_{Ba}. AA (30 μM) was applied during the period indicated by a horizontal bar. I_{Ba} was almost abolished, and then recovered by about 65% following washout of AA. Time 0 min indicates the start of I_{Ba} recording under the conditions where potassium current was blocked by internal diffusion of Cs from the recording pipette for about 3 min after rupturing the patch membrane. (b) Current traces which were recorded at the times corresponding indicated by (i), (ii) and (iii) in (a).

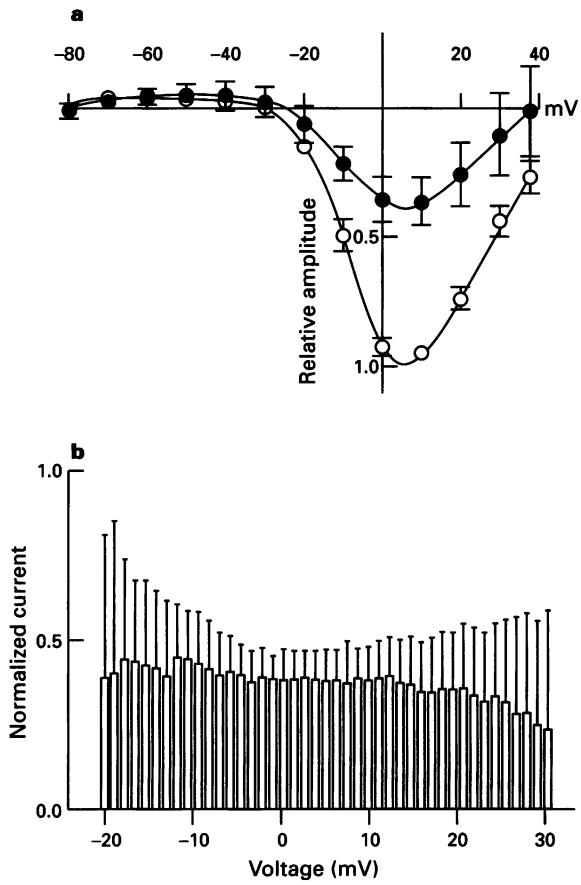

Figure 2 Current voltage relationship of I_{Ba} through Ca channels obtained using ramp command waveformes. The ramp depolarized the cell from −80 mV to +40 mV at the rate of 1.2 V s^{-1}, once every 10 s. The solid lines and circles indicate the average amplitude of I_{Ba} from 4 cells. Values are mean ± s.e.mean: I_{Ba} in the absence (○) and presence (●) of 10 μM arachidonic acid (AA). (b) The voltage-dependence of the inhibition of I_{Ba} by 10 μM AA was examined by replotting the data shown in (a) after normalizing the I_{Ba} amplitude in the presence of AA to that in the control. Note that normalized currents at any voltage are not significantly different from one another ($P<0.05$, $n=4$).

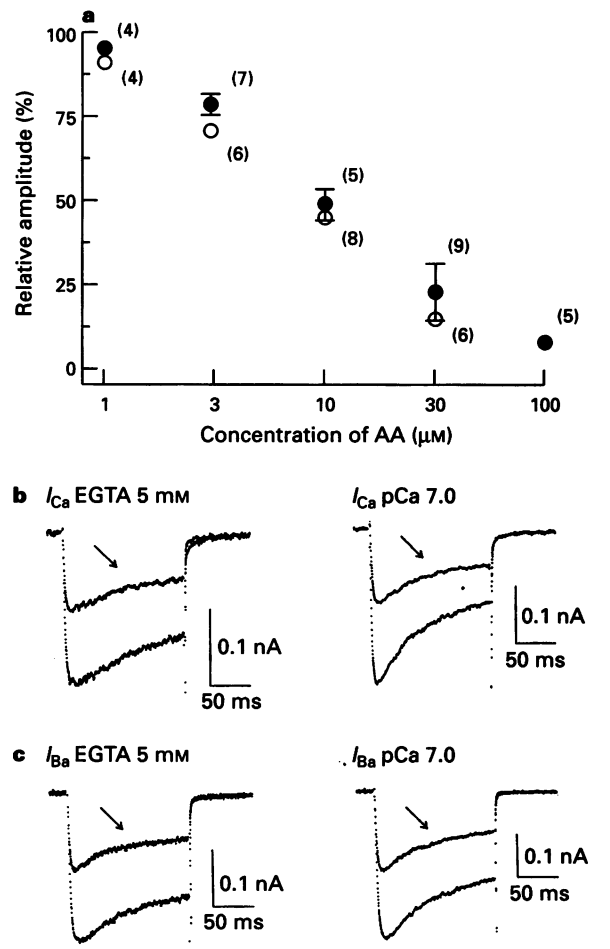

Figure 3 Concentration-dependence of arachidonic acid (AA)-induced block of I_{Ba} or I_{Ca} (a) and influence of internal Ca^{2+} (b). Experiments were performed using a similar protocol to that shown in Figure 1. (a) The relative amplitude of I_{Ba} after application of AA was corrected for 'run-down' (see Methods). The pipette solution contained 5 mM EGTA and no additional Ca^{2+}. (●) Peak amplitude of I_{Ca} recorded at 0 mV in a standard solution containing 2.2 mM $CaCl_2$; (○) I_{Ba} recorded in Ba solution containing 2.2 mM $BaCl_2$. Concentrations of AA required for half inhibition of I_{Ca} or I_{Ba} (IC_{50}s) are about 10 and 6 μM, respectively. The numbers in parentheses denote the number of cells studied. There was no statistically significant difference between I_{Ca} and I_{Ba} at any concentrations of AA examined ($P>0.05$). (b and c) Original traces of I_{Ca} (b) and I_{Ba} (c) recorded using pipette solutions containing 5 mM EGTA and no additional Ca^{2+} (left) and at pCa 7.0 (right), respectively. Arrows indicate I_{Ca} (b) or I_{Ba} (c) in the presence of 10 μM AA. Note that the decay of I_{Ca} was faster when the pipette solution containing pCa 7.0 solution (b, right) than when 5 mM EGTA but no additional Ca^{2+} was included (b, left, pCa = ~9.5). The decay was not significantly affected by application of AA (see text).

mately +5 mV in both the absence (+4.3 ± 0.6 mV; 370 ± 55 pA, $n=4$) or the presence of 10 μM AA (+4.9 ± 1.3 mV, 166 ± 21 pA, $n=4$). The amplitude of I_{Ba} in the presence of 10 μM AA relative to that before AA application at each potential is shown in Figure 2b. These results indicate that the AA-induced block of I_{Ba} is not dependent on test potentials in the range of −20 to +30 mV.

Figure 3a shows the concentration-dependent block of I_{Ba} by AA in the concentration-range of 1 and 100 μM. I_{Ba} was elicited by depolarization from −60 to 0 mV for 150 ms every 15 s. A 50% decrease in peak I_{Ba} was obtained with 6 μM AA (IC_{50}). Block of Ca current (I_{Ca}) recorded under the same conditions was also observed when a similar concentration-range of AA (1 − 100 μM) was applied ($IC_{50}=10$ μM, Figure 3a).

To examine the contribution of Ca^{2+}-dependent inactivation of I_{Ca} or I_{Ba} to AA-induced inhibition, the concentration of Ca^{2+} in the pipette solution was changed. When selected concentrations of Ca^{2+} were added to the pipette solution containing 5 mM EGTA to keep pCa at 7.0 or 7.5, I_{Ca} (elicited by depolarization from −60 to 0 mV, see Figure 3b) was reduced to 51.9 ± 5.9% of the control by 10 μM AA ($n=4$). Without the addition of Ca^{2+} to the pipette solution (pCa = ~9.5, assuming contaminated Ca^{2+} of 20 μM), the I_{Ca} was reduced to 48.3 ± 4.4% by 10 μM AA ($n=6$, $P>0.05$ vs. pCa 7.0 or 7.5). There was a tendency for the decay of I_{Ca} during the depolarization to be larger when the Ca^{2+} concentration in the pipette solution was higher, and was also larger than that of I_{Ba} when the pipette solution was the same (Figure 3b and c). The ratio of I_{Ca} amplitude at the end of pulse versus the amplitude at the peak (end/peak ratio of I_{Ca}) was 0.62 ± 0.05 ($n=4$) and that of I_{Ba} was 0.75 ± 0.04 ($n=4$, $P<0.05$ vs. I_{Ca}). Application of 10 μM AA did not affect either the end/peak ratio of I_{Ca} (0.60 ± 0.03, $n=4$, $P>0.05$ vs. 0.62 ± 0.05) or that of I_{Ba} (0.72 ± 0.03, $n=4$, $P>0.05$ vs. 0.75 ± 0.04) under these conditions. When the pCa of the pipette solution was 7.0 or 7.5, the end/peak ratio of I_{Ca} was 0.59 ± 0.06 ($n=4$, $P>0.05$ vs. 0.62 ± 0.05) and 0.53 ± 0.02 ($n=4$, $P>0.05$ vs. 0.59 ± 0.06) in the absence or presence of 10 μM AA, respectively. Therefore, the decay of I_{Ca} during a depolarization was slightly but not significantly increased by

10 μM AA. Even when 15 mM BAPTA was added to the pipette solution, I_{Ba} was reduced to 47.3 ± 9.2% by 10 μM AA ($n=4$, $P>0.05$ vs. 44.5 ± 2.4% without addition of Ca^{2+} to the pipette solution, $n=8$). Taken together, the results strongly suggest that an intracellular Ca^{2+}-dependent mechanism is not substantially involved in AA-induced block of Ca channels.

AA metabolites and AA-induced block of Ca channels

The possibility that AA affects Ca channels after being metabolized into prostaglandins, leukotrienes, etc. through cyclo-oxygenase and lipoxygenase pathways (AA cascade) was examined. The effects of indomethacin (Indo), a cyclo-oxygenase inhibitor, and NDGA, a lipoxygenase inhibitor, on the AA-induced inhibition of I_{Ba} were tested. When 10 μM Indo or 10 μM NDGA was added to the bathing solution, I_{Ba} at 0 mV was reduced by more than 30% ($n=3$ for each). Direct block of Ca current by these agents has been suggested in other preparations (Burch *et al.*, 1983; Korn & Horn, 1990). When the pipette solution contained 1 mM Indo or NDGA, the I_{Ba} amplitude at 0 mV was 370 ± 55 pA ($n=4$) and 345 ± 49 pA ($n=7$), respectively ($P>0.05$ vs. control 343 ± 70 pA, $n=5$, 0.1% ethanol). Intracellular application of 0.1% ethanol, the solvent of Indo and NDGA, *per se* did not change significantly either the rate of run-down (28.2 ± 6.7% during 10 min, $n=5$) or the inhibition of I_{Ba} by 10 μM AA (a decrease to 46.4 ± 4.2% of the control, $n=5$, $P>0.05$ vs. 44.5 ± 2.4%). The run-down of I_{Ba} in the presence of Indo or NDGA in the pipette solution was 34.8 ± 9.7 and 33.8 ± 10% for 10 min, respectively ($n=4$ and 7, $P>0.05$ vs. 28.2%). Therefore, we preferred to apply 1 mM Indo or 1 mM NDGA internally by addition to the pipette solution. After more than 5 min from the start of a recording, AA was applied. In our apparatus, it took less than 5 min for an applied low molecular chemical to diffuse to a substantial extent from the pipette solution into the cytosol, since internal application of 1 mM EGTA from a KCl-rich pipette solution markedly reduced Ca-dependent K current within 3 min after rupturing the patch membrane to establish the whole cell clamp configuration (not shown). The relative amplitude of I_{Ba} after application of 10 μM AA in the presence of Indo or NDGA in a pipete solution averaged 46.9 ± 5.8% ($n=4$) and 49.1 ± 2.5% ($n=7$) of the control, respectively, and was not significantly different from that in their absence (44.5 ± 2.4%; $P>0.05$ vs. Indo or NDGA).

Effects of prostaglandins on Ca channel currents

Figure 4 shows effects of $PGF_{2\alpha}$ and PGE_2 on I_{Ba}. GTP-binding proteins are involved in signal transduction induced by various kinds of neurotransmitters, hormones or autacoids (see review by Gilmam, 1987). In the absence of GTP in the pipette solution, external application of 30 μM $PGF_{2\alpha}$ did not significantly affect I_{Ba}, whereas 30 μM AA reduced I_{Ba} by about 70% in the same cell (Figure 4a). Application of 10 μM PGE_2 affected I_{Ba} slightly in the absence of GTP (Figure 4b). As summarized in Figure 4d, averaged values of relative I_{Ba} amplitude in the presence of PGE_2 or $PGF_{2\alpha}$ at a concentration of 30 μM were not significantly different from those in the control. When the pipette solution contained 0.1 mM GTP, application of 30 μM $PGF_{2\alpha}$ reduced I_{Ba} by approximately 30%,

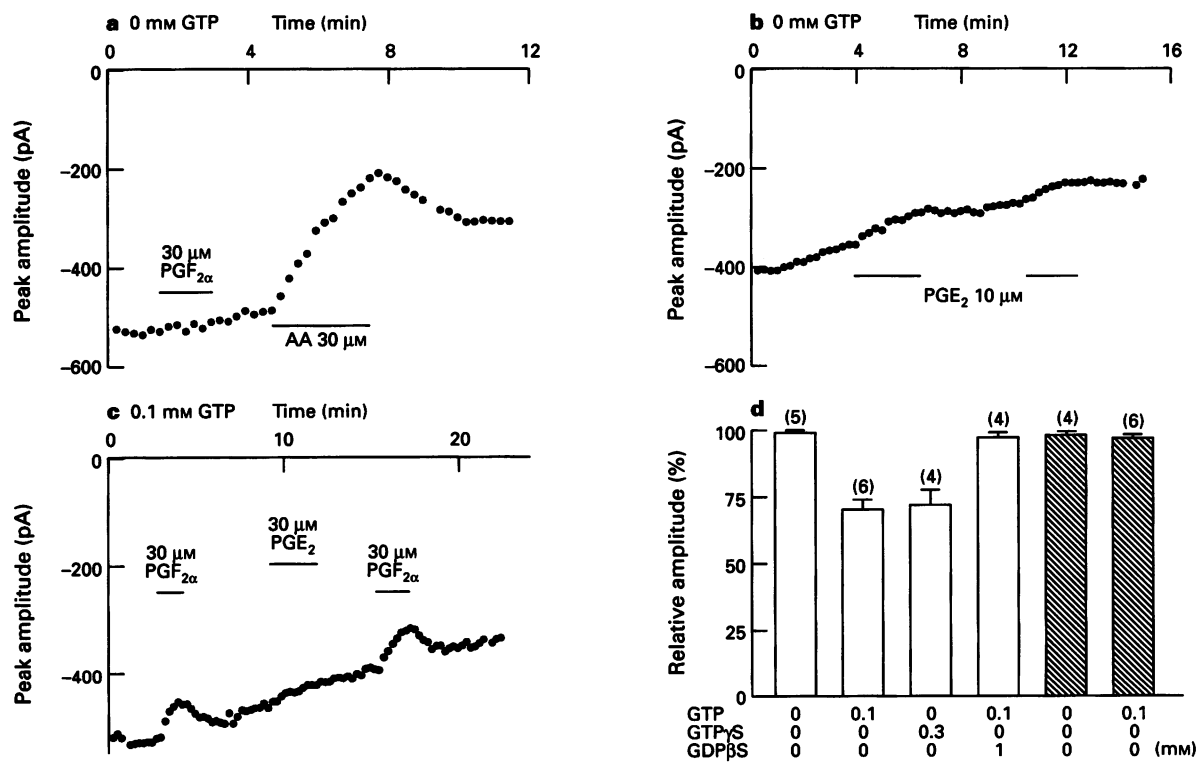

Figure 4 Effects of prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ on I_{Ba} with or without intracellular application of GTP. When 30 μM $PGF_{2\alpha}$ (a) or 10 μM PGE_2 (b) was applied externally without GTP in the pipette solution, I_{Ba} was not markedly reduced. In the same cell (a), 30 μM AA reduced I_{Ba} by over 60%. On the other hand, in the presence of 0.1 mM GTP in the pipette solution, 30 μM $PGF_{2\alpha}$ significantly reduced I_{Ba}, but not 30 μM PGE_2 (c). I_{Ba} was evoked by depolarization from −60 to 0 mV at 0.067 Hz. Summarized data describing the effects of 30 μM prostaglandins on I_{Ba} with or without guanosine nucleotides are shown in (d): Open and hatched columns indicate the mean values of relative I_{Ba} amplitude after application of 30 μM $PGF_{2\alpha}$ or 30 μM PGE_2, respectively. The effects of 30 μM $PGF_{2\alpha}$ with GTP or GTPγS in the pipette are statistically significant ($P<0.05$) in comparison with that without GTP. $PGF_{2\alpha}$ was not effective when the pipette solution contained both 0.1 mM GTP and 1 mM GDPβS. Numbers above column indicate number of cells examined. The relative amplitude of I_{Ba} after application of prostaglandin was corrected for 'run-down' as described in the text.

whereas 30 μM PGE_2 had no significant effect in the same cell (Figure 4c). This effect of 30 μM $PGF_{2\alpha}$ was almost completely removed by washout and was reproducible. Effects of GDPβS and GTPγS, nonhydrolysable analogues of GDP and GTP, respectively, on the decrease in I_{Ba} by $PGF_{2\alpha}$ were also examined. The peak amplitude of I_{Ba} was not changed by internal application of 1 mM GDPβS or 0.3 mM GTPγS. When the pipette solution contained both 1 mM GDPβS and 0.1 mM GTP, I_{Ba} was not significantly affected by 30 μM $PGF_{2\alpha}$ ($n=4$, 97.5±1.5%). The decrease in I_{Ba} by 30 μM $PGF_{2\alpha}$ was irreversible in the presence of 0.3 mM GTPγS in the pipette solution; the relative amplitude was 71.6±6.0% of the control ($n=4$). Pooled data are illustrated in Figure 4d.

Peroxides of AA in Ca channel block

Free radicals generated by AA oxidation may contribute to AA-induced decrease in I_{Ba} in vas deferens smooth muscle cells, as has been reported in hippocampal CA1 neurones (Keyser & Alger, 1990). To prevent generation of free radicals, 100 units ml^{-1} superoxide dismutase (SOD) was added to the bathing solution. Application of this concentration of SOD did not affect I_{Ba} itself, but significantly attenuated the decrease in I_{Ba} by AA at all concentrations examined (1–30 μM, $P<0.05$, Figure 5), whereas the attenuation was the largest when 30 μM AA was applied.

GTP-binding proteins and protein kinase C in AA-induced block of Ca^{2+} channels

To determine whether the activation of GTP-binding protein is involved in AA-induced inhibition of I_{Ba} in vas deferens smooth muscle cells, GDPβS or GTPγS was added to the pipette solution. The decrease in I_{Ba} by 10 μM AA was not affected significantly by the presence of 1 mM GDPβS or 0.3 mM GTPγS. Figure 6 shows summarized data of effects of GTP analogues on AA-induced decrease in I_{Ba} and I_{Ca}.

The possibility that activation of protein kinase C (PKC) mediates the AA-induced decrease in I_{Ba} was examined by use of H-7, which inhibits PKC as well as cyclic AMP- or cyclic GMP-dependent protein kinases (PKA and PKG) (Hidaka *et al.*, 1984). When H-7 at a concentration of 100 μM was applied

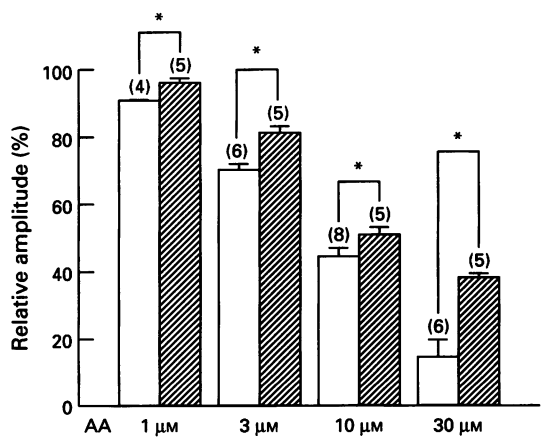

Figure 5 To examine the involvement of free radical generation in arachidonic acid (AA)-induced inhibition of I_{Ba}, 100 units ml^{-1} superoxide dismutase (SOD) was added to the external solution. I_{Ba} was evoked by depolarization from −60 to 0 mV at 0.067 Hz. AA was added about 5 min after the start of SOD application. Open columns, control; hatched columns, SOD 100 units ml^{-1}. The numbers in parentheses denote the number of cells used. Concentrations of AA are shown below columns. The effect of AA on I_{Ba} was reduced in the presence of SOD. Although the attenuation of AA-induced decrease by SOD was statistically significant ($P<0.05$) at all concentrations of AA examined, the difference was most marked when 30 μM AA was applied.

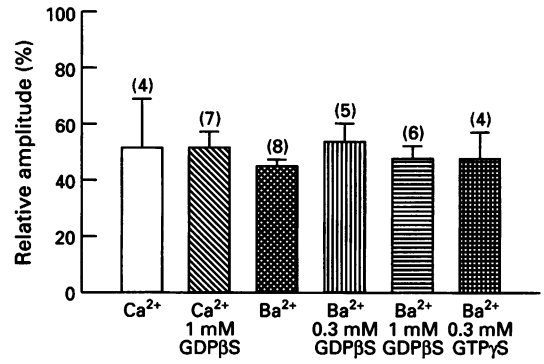

Figure 6 Summarized data describing the effects of GTP or GDP analogues on arachidonic acid (AA)-induced inhibition of Ca channel current. I_{Ba} or I_{Ca} was evoked by depolarization from −60 to 0 mV at 0.067 Hz. Ca^{2+} or Ba^{2+} below columns indicates current carrier. GDPβS or GTPγS at the concentrations shown was added to the pipette solution. AA (10 μM) was added approximately 10 min after rupturing the patch membrane. The numbers in parentheses denote the number of cells studied.

internally I_{Ba} was reduced by 10 μM AA to a similar extent to that observed in the absence of H-7 (control: 44.5±2.4%, $n=8$; H-7: 38.6±4.9%, $n=5$; $P>0.05$).

Discussion

Our results demonstrate that AA reduces voltage-dependent Ca channel current in smooth muscle cells isolated from the guinea-pig vas deferens. A similar inhibition of Ca current by AA has been observed in intestinal smooth muscle cells (Shimada & Somlyo, 1992). In portal vein smooth muscle cells, however, it has been shown that an addition of 1 mM AA to the pipette solution does not change the Ca current (Loirand *et al.*, 1990). Since the L-type Ca channel current is predominant in vas deferens smooth muscle cells under the conditions used in the present study (Imaizumi *et al.*, 1991), this inhibitory effect of AA may be mainly on L-type channels. The fact that the effect of AA did not depend on the potentials used for Ca channel activation may also support this. However, the possibility can be ruled out completely that T-type channel block was also partly involved in AA-induced decrease in Ca channel current in the present study.

When the charge carrier was either calcium or barium ions, comparable effects of AA on Ca channel current were observed. Moreover, the effect of AA was not changed when the Ca^{2+} concentration in the pipette solution was changed in the pCa range of 7.0 and 10.0 by use of Ca-EGTA buffer, 5 mM EGTA or 15 mM BAPTA. The AA-induced decrease in I_{Ba} was, therefore, not modulated by the intracellular calcium concentration, although it is known that AA and its metabolites change Ca^{2+} release from storage sites (Force *et al.*, 1990; Ling *et al.*, 1992; Maruyama, 1993). This suggests that the inactivation of Ca channels by intracellular Ca^{2+} which is increased by Ca^{2+}-influx and Ca^{2+}-release from intracellular Ca storage sites is not involved in the mechanism of AA-induced decrease in Ca channel current. It may also suggest that the mechanism does not depend upon the activation of enzymes or proteins by an increase in intracellular Ca^{2+} concentration.

In ciliary ganglion cells, leukotrienes synthesized from AA by lipoxygenase, are the second messengers responsible for AA-induced block of Ca channels (Khurana & Bennet, 1993). K channels coupled to muscarinic receptors in cardiac myocytes (Kurachi *et al.*, 1989; Kim *et al.*, 1989; Scherer *et al.*, 1993) and M channels in hippocampal neurones (Schweitzer *et al.*, 1990) are activated by AA via leukotriene production. It has been reported that PGE_2 can reduce Ca channel currents in rat sympathetic neuronal cells (Ikeda, 1992). A decrease of I_{Ba} induced by PGE_1 and PGE_2 has also been observed in cultured

smooth muscle cells of the rat thoracic aorta (Serebryakov *et al.*, 1994). In the present study, internal application of 1 mM Indo or NDGA from the pipette solution did not significantly affect the AA-induced decrease in I_{Ba}, implying that AA metabolites from the cyclo-oxygenase pathways may not be involved in this AA effect in vas deferens smooth muscle cells. Application of $PGF_{2\alpha}$ in the presence of GTP in the pipette solution slightly but significantly reduced I_{Ba}, whereas that of PGE_2 did not. The effect of $PGF_{2\alpha}$ was not observed when the pipette solution did not include GTP or included both GTP and GDPβS. The $PGF_{2\alpha}$-induced inhibition of I_{Ba} may be mediated by activation of GTP-binding protein and, therefore, by a different mechanism from that for AA. It is worthy of note that the inhibition of I_{Ba} by 30 μM $PGF_{2\alpha}$ (about 30% decrease) was far smaller than that of 30 μM AA (about 75% decrease).

In CA1 neurones, the effect of AA on the Ca channel is mediated by activation of PKC and also be oxidation of the membrane surface by peroxide radicals derived from AA (Keyser & Alger, 1990). Since the decrease in I_{Ba} by AA was not changed by the internal application of 100 μM H-7, it is unlikely that the activation of PKC is one of major signal transductions. Moreover, activation of PKA or PKG is unlikely to be involved in the mechanism based upon the non-specific inhibition of these kinases by H7 (Hidaka *et al.*, 1984). Application of 100 units ml^{-1} SOD slightly but significantly attenuated the decrease in I_{Ba} by 1–30 μM AA. These findings suggest that generation of superoxides is one of the mechanisms for the inhibition of Ca channels by AA in vas deferens smooth muscle cells.

It has been suggested that AA directly inhibits Ca channels in intestinal smooth muscle cells (Shimada & Somlyo, 1992). This type of direct action has also been suggested for the mechanism of the AA-induced increase in large conductance Ca-dependent K channel (BK channel) activity in pulmonary smooth muscle cells (Kirber *et al.*, 1992). Since internal application of 0.3 mM GTPγS or 1 mM GDPβS did not affect significantly the AA-induced decrease in I_{Ba} (Figure 6), activation of a GTP binding protein is unlikely to be involved in this AA effect, unlike the AA-induced regulation of the cardiac K^+ channel (Kim *et al.*, 1989; Scherer & Breitwieser, 1990). One mechanism by which AA acts directly on Ca channel proteins or phospholipids, is by being incorporated into the membrane bilayer. In this case, the reversibility of I_{Ba} reduction by AA after washout should be minimal. It has been reported in intestinal smooth muscle cells that *cis*-unsaturated fatty acids, such as palmitoleic acid (16:1), oleic acid (18:1) and linoleic acid (18:2), decrease I_{Ba} in a very similar manner to AA whereas myristic acid (14:0) and lauric acid (12:0) increase I_{Ba} and lenolelaidic acid (18:2, *trans*), myristoleic acid (14:1, *cis*) and palmitic acid (16:0) do not affect I_{Ba} (Shimada & Somlyo, 1992). This pattern of results suggests that the effect of AA is not due to simple physicochemical changes such as an increase in membrane lipid fluidity. Specific experiments of this type were not performed in the present study.

In our previous study (Imaizumi *et al.*, 1991), it was reported that α-adrenoceptor stimulation by noradrenaline (NA) reduces the Ca channel current in vas deferens smooth muscle cells via two mechanisms; (1) Ca-dependent inactivation of Ca channels by Ca release from intracellular Ca^{2+} store following production of inositol trisphosphate and (2) Ca-independent and GTP binding protein-mediated inhibition of Ca channel activity. For some types of receptor, stimulation by transmitters, hormones or autacoids is transduced to at least two intracellular signals by activating both phospholipase A_2 and C (Brinbaumer *et al.*, 1990). In some smooth muscles, α-adrenoceptor stimulation results in activation of both phospholipases and thereby increases inositol turnover and AA release (Exton, 1988). Moreover, it has been reported that production of PGE- and PGF-like materials is decreased and increased, respectively, by α-adrenoceptor stimulation in vas deferens (Borda *et al.*, 1983). Based upon the results in the present study, it is possible that a release of AA or $PGF_{2\alpha}$ is involved in the NA-induced decrease in Ca channel current in vas deferens smooth muscle cells. If this mechanism can be proven, it would give a new insight into the functional significance of AA and prostaglandins as second messengers in neurotransmitter-induced regulation of Ca channels activity in smooth muscle cells. Further studies using a specific PLA_2 inhibitor are planned.

In conclusion, L-type Ca channel activity is reversibly reduced by external application of 1–30 μM AA in smooth muscle cells of the guinea-pig vas deferens. This decrease may not be mediated by activation of GTP binding proteins or PKC or by biosynthesis of lipoxygenase or cyclo-oxygenase products. However, in addition to the direct action of AA, a contribution of superoxide radicals derived from high concentrations of AA (>10 μM) must still be considered.

Y.I. is supported by Grant in Aid from the Japanese Ministry of Education, Science and Culture. We thank Dr Wayne Giles (University of Calgary) for providing the data acquisition and analysis programmes for the IBM-AT and also for his critical reading of the manuscript.

References

AMBACHE, N. & ZAR, M.A. (1970). An inhibitory effect of prostaglandin E_2 on neuromuscular transmission in the guinea-pig vas deferens. *J. Physiol.*, **208**, 30–32.

BENHAM, C.D., BOLTON, T.B., LAND, R.J. & TAKEWAKI, T. (1986). Calcium-activated potassium channels in single smooth muscle cells of rabbit jejunum and guinea-pig mesenteric artery. *J. Physiol.*, **371**, 45–67.

BORDA, E.S., PEREDO, H. & GIMENS, M.F. (1983). Alpha adrenergic stimulation modified prostaglandins release in vas deferens. *Prostaglandins*, **26**, 701–709.

BREGESTOVSKI, P.D., BOLTINA, V.M. & SEREBRYAKOV, V.N. (1989). Fatty acid modifies Ca^{2+}-dependent potassium channel activity in smooth muscle cells from human aorta. *Proc. R. Soc. B.*, **237**, 259–266.

BRINBAUMER, L., ABRAMOWITZ, J. & BROWN, A.M. (1990). Receptor-effector coupling by G proteins. *Biochem. Biophys. Acta*, **1031**, 163–224.

BURCH, R.M., WISE, W.C. & HALUSHKA, P.V. (1983). Prostaglandin-independent inhibition of calcium transient by nonsteroidal anti-inflammatory drugs: Differential effects of carboxylic acid and piroxicam. *J. Pharmacol. Exp. Ther.*, **227**, 84–91.

CLEGG, P.C. (1966). Antagonism by prostaglandins of the responses of various smooth muscle preparations to sympathomimetics. *Nature*, **209**, 1137–1139.

EXTON, J.H. (1988). The role of calcium and phosphoinositides in the mechanisms of α_1-adrenergic and other agonists. *Rev. Physiol. Biochem. Pharmacol.*, **111**, 118–224.

FORCE, T., HYMAN, G., HAJJAR, R., SELLMAYER, A. & BONVENTRE, J.V. (1990). Noncyclooxygenase metabolites of arachidonic acid amplify the vasopressin-induced Ca^{2+} signal in glomerular mesangial by releasing Ca^{2+} from intracellular stores. *J. Biol. Chem.*, **266**, 4295–4302.

GEBREMEDHIN, D., MA, Y.-H., FALCK, J.R., ROMAN, R.J., VAN ROLLINS, M. & HARDER, D.R. (1992). Mechanism of action of cerebral epoxyeicosatrienoic acid on cerebral arterial smooth muscle. *Am. J. Physiol.*, **263**, H519–H525.

GILMAM, A.G. (1987). G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.*, **56**, 615–649.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMAMM, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 58–100.

HIDAKA, H., INAGAKI, M., KAWAMOTO, S. & SASAKI, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry*, **23**, 5036–5041.

HORTON, E.W. (1969). Hypotheses on physiological roles of prostaglandins. *Physiol. Rev.*, **49**, 122–161.

HU, S. & KIM, H.S. (1993). Activation of K^+ channel in vascular smooth muscles by cytochrome P450 metabolites of arachidonic acid. *Eur. J. Pharmacol.*, **230**, 215–221.

HUANG, J., XIAN, H. & BACANER, M. (1992). Long-chain fatty acids activate calcium channels in ventricular myocytes. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 6452–6456.

IKEDA, S.R. (1992). Prostaglandin modulation of Ca^{2+} channels in rat sympathetic neurons is mediated by guanine nucleotide binding proteins. *J. Physiol.*, **458**, 339–359.

IMAIZUMI, Y., MURAKI, K. & WATANABE, M. (1990). Characteristics of transient outward current in single smooth muscle cells from the ureter of the guinea-pig. *J. Physiol.*, **427**, 301–324.

IMAIZUMI, Y., TAKEDA, M., MURAKI, K. & WATANABE, M. (1991). Mechanisms of NE-induced reduction of Ca current in single smooth muscle cells from guinea pig vas deferens. *Am. J. Physiol.*, **260**, C17–C25.

KEYSER, D.O. & ALGER, B.E. (1990). Arachidonic acid modulates hippocampal calcium current via protein kinase C and oxygen radicals. *Neuron*, **5**, 545–553.

KHURANA, G. & BENNETT, M.R. (1993). Nitric oxide and arachidonic acid modulation of calcium currents in postganglionic neurons of avian cultured ciliary ganglia. *Br. J. Pharmacol.*, **109**, 480–485.

KIM, D., LEWIS, D.L., GRAZIADEI, L., NEER, E.J., BAR-SAGI, D. & CLAPHAM, D.E. (1989). G-protein $\beta\gamma$-subunits activate the cardiac muscarinic K^+-channel via phospholipase A_2. *Nature*, **337**, 557–560.

KIRBER, M.T., ORDWAY, R.W., CLAPP, L.H., WALSH, J.V.J. & SINGER, J.J. (1992). Both membrane stretch and fatty acid directly activate large conductance Ca^{2+}-activated K^+ channels in vascular smooth muscle cells. *FEBS Lett.*, **297**, 24–28.

KORN, S.J. & HORN, R. (1990). Nordihydroguaiaretic acid inhibits voltage-activated Ca^{2+} currents independently of lipoxygenase inhibition. *Mol. Pharmacol.*, **38**, 524–530.

KURACHI, Y., ITO, H., SUGIMOTO, T., SHIMIZU, T., MIKI, I. & UI, M. (1989). Arachidonic acid metabolites as intracellular modulators of the G protein-gated cardiac K^+ channel. *Nature*, **337**, 555–557.

LING, B.N., WEBSTER, C.L. & EATON, D.C. (1992). Eicosanoides modulate apical Ca^{2+}-dependent K^+ channels in cultured rabbit principal cells. *Am. J. Physiol.*, **263**, F116–F126.

LOIRAND, G.P., PACAUD, P., MIRONNEAU, C. & MIRONNEAU, J. (1990). GTP-binding proteins mediate noradrenaline effects on calcium and chloride currents in rat portal vein myocytes. *J. Physiol.*, **428**, 517–529.

MARUYAMA, Y. (1993). Control of inositol polyphosphate-mediated calcium mobilization by arachidonic acid in pancreatic aciner cells of rats. *J. Physiol.*, **463**, 729–746.

MCDONALD, T.F., PELZER, S., TRAUTWEIN, W. & PELZER, D.J. (1994). Regulation and modulation of calcium channels in cardiac, skeletal and smooth muscle cells. *Physiol. Rev.*, **74**, 365–507.

NANAZAWA, K., SAITO, H. & MATSUKI, N. (1988). Fast and slowly inactivating components of Ca-channel currents and their sensitivities to nicardipine in isolated smooth muscle cells from rat vas deferens. *Pflügers Arch.*, **411**, 289–295.

NEEDLEMAN, P., TURK, J., JAKSCHIK, B.A., MORRISON, A.R. & LEFKOWITCH, J.B. (1986). Arachidonic acid metabolism. *Annu. Rev. Biochem.*, **55**, 69–102.

ORDWAY, R.W., SINGER, J.J. & WALSH, J.V. (1991). Direct regulation of ion channels by fatty acids. *Trends Neurosci.*, **14**, 96–100.

ROBINSON, K. & GILES, W. (1986). A data acquisition, display and plotting program for IBM PC. *Comput. Programs Biomed.*, **23**, 319–327.

SCHERER, R.W. & BREITWIESER, G.E. (1990). Arachidonic acid metabolites alter G protein-mediated signal transduction in heart: effects on muscarinic K^+ channels. *J. Gen. Physiol.*, **96**, 735–755.

SCHERER, R.W., LO, C.F. & BREITWIESER, G.E. (1993). Leukotriene C_4 modulation of muscarinic K^+ current activation in bullfrog atrial myocytes. *J. Gen. Physiol.*, **102**, 125–141.

SCHWELTZER, P., MADAMBA, S. & SIGGINS, G.R. (1990). Arachidonic acid metabolites as mediators of somatostatin-induced increase of neuronal M-current. *Nature*, **346**, 464–467.

SEREBRYAKOV, V., ZAKHARENKO, S., SNETKOV, V. & TAKEDA, K. (1994). Effects of prostaglandins E_1 and E_2 on cultured smooth muscle cells and strips of rat aorta. *Prostaglandins*, **47**, 353–365.

SHIMADA, T. & SOMLYO, A.P. (1992). Modulation of voltage-dependent Ca channel current by arachidonic acid and other long-chain fatty acids in rabbit intestinal smooth muscle. *J. Gen. Physiol.*, **100**, 27–44.

VACHER, P., MCKENZIE, J. & DUFY, B. (1989). Arachidonic acid affects membrane ionic conductance of GH_3 pituitary cells. *Am. J. Physiol.*, **257**, E203–E211.

VACHER, P., MCKENZIE, J. & DUFY, B. (1992). Complex effect of arachidonic acid and its lipoxygenase products on cytosolic calcium in GH_3 cells. *Am. J. Physiol.*, **263**, E903–E912.

(Received September 28, 1994
Revised April 19, 1995
Accepted May 19, 1995)