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Concentration-dependent Modulations of Potassium and Calcium Currents of Rat Osteoblastic Cells by Arachidonic Acid

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Abstract. We show that the voltage-gated K^+ and Ca^{2+} currents of rat osteoblastic cells are strongly modulated by arachidonic acid (AA), and that these modulations are very sensitive to the AA concentration. At 2 or 3 μM, AA reduces the amplitude and accelerates the inactivation of the K^+ current activated by depolarization; at higher concentrations (≥5 μM), AA still blocks this K^+ current, but also induces a very large noninactivating K^+ current. At 2 or 3 μM, AA enhances the T-type Ca^{2+} current, close to its threshold of activation, whereas at 10 μM, it blocks that current. AA (1–10 μM) also blocks the dihydropyridine-sensitive L-type Ca^{2+} current. Thus, the effect of AA on Ca^{2+} entry through voltage-gated Ca^{2+} channels can change qualitatively with the AA concentration: at 2 or 3 μM, AA will favor Ca^{2+} entry through T channels, both by lowering the voltage-gated K^+ conductance and by increasing the T current, whereas at 10 μM, AA will prevent Ca^{2+} entry through voltage-gated Ca^{2+} channels, both by inducing a K^+ conductance and by blocking Ca^{2+} channels.

Key words: K^+ current—Ca^{2+} current—Arachidonic acid—Osteoblast

Introduction

Using the whole-cell configuration of the patch-clamp technique, we found that rat osteoblastic cells show a variety of voltage-gated ionic currents. The main current present in an external ionic solution close to the extracellular physiological solution is a potassium current which is sensitive to tetraethylammonium (TEA) and 4-amino-pyridine (4AP). It is activated by depolarization and inactivates within a few seconds during a prolonged depolarization [7]. A similar current has been described previously in chick osteoblast cultures [43]. We have also detected a TTX-sensitive sodium current and two types of calcium currents: a low-threshold T-type current and a dihydropyridine-sensitive L-type current [6].

The dihydropyridine-sensitive calcium current has since been found in various osteoblast-like cell lines, such as UMR 106 [9, 23], or ROS 17/2.8 [5, 13, 14]. The T-type calcium current has also been detected in some of these studies [1, 23]. In addition to voltage-gated channels, osteoblast-like cells also show various types of mechanosensitive ion channels [8, 9]. Furthermore, spontaneous membrane potential fluctuations have been observed in osteoblast-like cells [11, 34] indicating that voltage-gated currents of osteoblasts can be activated under physiological conditions.

It is known that many hormones, neurotransmitters or local factors are able to modify the intracellular calcium concentration of osteoblastic cells and that some of them can modulate the transmembrane potential of these cells (*see e.g.*, refs included in [5] and [8]). One way to investigate the possible role of the ion channels recently described in these cells (in particular their role in controlling calcium entry through voltage-gated channels) is to determine how they can be modulated by stimulation of "second-messenger" pathways.

In the present study, we investigated the modulations of voltage-gated currents of osteoblasts by arachidonic acid (AA), which is known to affect some ionic channels in other cell types, and is a possible messenger of some hormonally or mechanically activated effects in bone (*see* Discussion). Our experiments revealed that AA has pronounced effects on the main K^+ and Ca^{2+} currents of these cells. Furthermore, these

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modulations appeared to be steeply concentration dependent, indicating that, according to its amplitude, a local change of the AA concentration may have distinct consequences on voltage-dependent processes, in particular on calcium entry.

Materials and Methods

The experiments were performed at room temperature on primary cultures of newborn rat osteoblastic cells, using the whole-cell configuration of the patch-clamp technique.

Cell Preparation

Cells were prepared as described in [6]. Osteoblastic cells were isolated from newborn rat calvaria. The central parts of parietal bones were excised and the periosteal tissues carefully stripped away to eliminate chondrocytes, suture cells and periosteal progenitor cells. Bones were incubated at 37°C during two sequential 10 min periods in a Ca^{2+}-Mg^{2+}-free Earle solution containing 0.5 mg trypsin/ml (Worthington) and 4 mM EDTA. Isolated cells were harvested, washed and seeded at about 10,000 cells/dish (35 mm Falcon) in BGJ medium (Flow Laboratories), supplemented with 10% fetal calf serum (Flow Laboratories), fungizone (2.5 μg/ml), penicillin (100 IU/ml) and streptomycin (50 μg/ml). Experiments were performed on isolated cells from day 4 to day 7 in culture.

Solutions

Unless otherwise indicated, the solutions used to study K^+ currents were the following: *NaCl external solution,* containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid)-NaOH pH 7.4, and *KCl internal solution* containing (in mM): 146 KCl, 0.1 EGTA-Na, 1 $MgCl_2$, 3 ATP-Na_2 and 10 HEPES-NaOH pH 7.3.

In some experiments, we used a *high K^+ external solution* containing (in mM): 148 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES-NaOH pH 7.4, a low-Cl^- *K glutamate internal solution* containing (in mM): 125 K glutamate, 11 KCl, 0.1 EGTA, 1 $MgCl_2$, 3 ATP-Na_2 and 10 HEPES-Na pH 7.3, or a *BAPTA internal solution,* containing (in mM): 117 KCl, 10 BAPTA (1,2-Bis (2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid), 1 $CaCl_2$, 1 $MgCl_2$, 3 ATP-Na_2 and 10 HEPES-KOH pH 7.3.

The solutions used to study Ba^{2+} currents were the following: *NMG-Ba^{2+} external solution,* containing (in mM): 130 NMG (N-methyl-D-glucamine)-glutamate, 20 $BaCl_2$, 10 HEPES-NMG pH 7.4, and *NMG-Cs^+ internal solution,* containing (in mM): 34 NMG-Cl, 56 NMG-glutamate, 28 Cs glutamate, 10 EGTA, 1 $CaCl_2$, 1 Mg Cl_2, 3 ATP-Mg and 10 HEPES-NMG pH 7.3.

Stock solutions of arachidonic acid, arachidonic methyl ester and oleic acid were prepared at 10 mM in hexane, aliquoted and kept at −20°C under N_2. Just before the first fatty acid application on each cell, a new aliquot was thawed, hexane was evaporated under N_2, replaced by ethanol, and the tube was mechanically agitated for 10 sec, leading to a stock solution at 10 (or in a few cases 20) mM in pure ethanol. This stock was immediately diluted a thousandfold in the external solution, leading to a 10 or (20) μM solution that was then diluted to prepare the solutions successively tested on the same cell. Over the time course of recording from one cell, the efficacy of these solutions did not change. The total amount of ethanol in the final solution never exceeded 0.1%. Stock solutions of linoleic acid, myristic acid and ETYA (eicosatetraynoic acid) were prepared at 10 mM in ethanol just before use. Indomethacin and NDGA (nordihydroguiaretic acid) were diluted at 50 mM in DMSO. Staurosporine was diluted at 0.1 mM in DMSO. These stock solutions were freshly prepared on the day of each experiment, and were kept in the dark (at 0°C in the case of staurosporine) for a maximum time of 4 hr before final dilution and immediate use.

Perfusion System

The culture dish was continuously perfused with an external solution. A four barrel fast perfusion system (made of glass and Teflon tubing, each barrel being connected to a glass syringe via Teflon tap) was used for rapid application of fatty acids or pharmacological agents. One of the barrels was filled with the control external solution, the others with the test solutions. The recorded cell was continuously perfused with one of these barrels (solutions flowing by gravity), and the fast perfusion system was moved laterally to apply the desired solution onto the cell. All barrels of the fast perfusion system always contained the same solvent dilution. A separate fast perfusion system was used for each series of experiments to avoid possible contamination by ions or any agent tested which could have adhered to the perfusion system.

Recording and Analysis

Patch-clamp micropipettes were made from hard-glass (Kimax 51); the shank of each pipette was covered with Sylgard and the tip was fire-polished. The resistance of these electrodes filled with the KCl internal solution was between 3 and 6 MΩ. The cells were voltage-clamped by an EPC7 List amplifier, controlled by a TANDON 38620 computer, via a Cambridge Electronic Design (CED) 1401 interface, using CED patch- and voltage-clamp software. The current monitor output of the amplifier was filtered at 0.1 or 1 kHz before being sampled on-line at 0.2 or 2 kHz for measurements of K^+ or Ca^{2+} currents, respectively. The bath was connected to the ground via an agar bridge, and junction potentials between the pipette solution and the bath solution were measured and taken into account.

The series resistance (*Rs*) was systematically measured several times during each experiment. Particular care was taken to eliminate experiments in which *Rs* changed over time (by continuously controlling the capacitive current before filtering for sampling). *Rs* was between 6 and 12 MΩ when using the KCl internal solution. These values are high enough to introduce a difference of a few mV between the voltage applied to the electrode and that actually applied to the inside of the cell (error of maximum 10 mV for the strongest depolarizing jumps inducing large K^+ currents). However, the current modulations observed upon application of AA occurred without any simultaneous change of *Rs* and thus cannot be considered as resulting from changes in the applied voltage. The capacitance of the cells recorded in the present study was superior to 100 pF, preventing the use of the *Rs* compensation circuit of the EPC7. This capacitance, combined with the large *Rs* values obtained with the NMG-Cs internal solution used for Ca current recordings (25–40 MΩ) explains the apparent slow time course of the Ca currents recorded. However, the amplitude of these currents being small (*see* Figs. 6–8), the voltage error due to *Rs* did not exceed 5 mV in the case of the Ca current experiments, and again, the current modulations induced by AA were observed without any change in *Rs*. The use of larger electrodes (of lower resistance) was excluded, since an extensive exchange of the intracellular medium by the internal solution present in the electrode induced a large outwardly rectifying chloride current and prevented a reliable study of other currents.

When using the standard NaCl external and KCl internal solutions, we usually applied depolarizing jumps from −100 to 0 mV (chloride equilibrium potential), to study K^+ currents. In the present study, under these conditions, the K^+ current recorded at 0 mV was very stable during repetitive jumps from −100 mV. Cells showing a spontaneous progressive K^+ current increase during cell dialysis, I_{K2} [7], were rarely found and were not used for the present study. On the contrary, with these usual NaCl external and KCl internal solutions, the current recorded at −100 mV often changed over time, increasing during the first minutes of cell dialysis and then decreasing towards a stable value. Thus, with these solutions, when we wanted to record K^+ currents over a range of membrane potentials, we waited until the resting currents stabilized over the whole voltage range (Figs. 1 and 3). In experiments where the K^+ currents were measured only at 0 mV, the current traces recorded at the holding potential sometimes showed small changes which were not related to the fatty acid application.

In the experiments performed with the low-Cl^- K glutamate internal solution (Figs. 1*D*, 2 and 4*A*), the current was almost immediately stable over the whole voltage range.

We did not do any leak or capacitive subtraction during the study of K^+ currents (their large amplitude precluded the necessity for such subtraction; furthermore, a linear subtraction procedure would not have been justified, since many cells showed some inward rectification with the solutions used for studying K^+ currents (*not shown*)).

On the contrary, with the solutions used to study Ba^{2+} currents, we had no indication of any inward rectification, so we used the usual linear leak and capacitive subtraction procedures (before each series of depolarizing jumps, we applied three identical hyperpolarizing jumps of 20 mV amplitude). Note that all the effects described could also be observed without leak subtraction, and that the leak current was always very small compared to the Ba^{2+} currents studied.

Results

Modulations of Potassium Currents

Blockade of the Inactivating K^+ Current by Arachidonic Acid

As previously described [7], depolarizing jumps from −100 mV induced a voltage-gated K^+ current which inactivated during the 10 seconds of each depolarization. This current, called I_{K1}, is illustrated by the control traces of Fig. 1*A* and *B* at 0 and +30 mV, respectively, in the usual NaCl external solution. Addition of AA (3 μM) to this solution strongly reduced this current, as shown by the traces labeled AA. This effect was also observed below the Cl^- equilibrium potential (0 mV), at −15 mV (*not shown*), and was slowly reversible (Fig. 1*C*). Figure 1*D* illustrates an experiment performed in a high K^+ external solution (in which the K^+ equilibrium potential was 0 mV), demonstrating that the current affected by AA is selective for K^+ ions. Under these conditions, depolarizing jumps from −100 to −12 mV induced an inward current, whereas depolarizing jumps to +12 mV induced an outward current, and AA reduced both currents.

AA not only reduced the amplitude of I_{K1}, but it also accelerated its kinetics of inactivation.

In the usual 5 mM K^+ external solution, the acceleration of inactivation induced by AA was more pronounced for stronger depolarizations (*compare* records of Fig. 1*A* and *B*). The time $\tau_{1/2}$, at which the K^+ current had decayed by half, was measured in control and in the presence of AA during various depolarizations. In control, $\tau_{1/2}$ was close to 2 sec at 0 mV and was not markedly voltage dependent between −15 and +45 mV. In the experiment illustrated, AA reduced $\tau_{1/2}$ by about 60, 58, 70, 78, and 84% at respectively −15, 0, +15, +30 and +45 mV. The voltage dependence of this kinetic effect was confirmed in two other experiments where 3 μM AA reduced $\tau_{1/2}$ by 49 or 52% at 0 mV, and by 83 or 80% at +45 mV, whereas the control $\tau_{1/2}$ values were very similar at 0 and +45 mV (difference of less than 10%).

The effect of AA on I_{K1} was steeply concentration dependent over the 1–5 μM concentration range. In a series of experiments, performed with the NaCl external solution and KCl internal solution, I_{K1} was measured during depolarizations from −100 to 0 mV (by the difference between the peak outward current and the current remaining after 10 sec at 0 mV) both in control and in the presence of a variable concentration of AA. Whereas 1 μM did not have any effect (four cells), 2 μM induced a 9% reduction in only one out of three cells and had no effect in the two others; 3 μM induced a reduction of 46 ± 13% of the current amplitude and a reduction of 53 ± 15% of $\tau_{1/2}$ (nine cells). At 5 μM, AA induced an almost complete blockade of I_{K1} in eight cells, but it also activated a noninactivating K^+ current (*see below*).

The steepness of the concentration dependence of the effect of AA on I_{K1} was confirmed by comparing the effects of two AA concentrations on a same cell. This is illustrated by the experiment of Fig. 2, performed under different ionic conditions (low chloride, glutamate intracellular solution, $E_{Cl} = -62$ mV). Under these conditions, the most remarkable effect of AA is the acceleration of the K^+ current inactivation. Figure 2*A* gives the current value measured at 500 msec after the onset of each repetitive depolarization from −100 to −15 mV, for three successive AA applications on the same cell. It is clear that 2 μM had almost no effect, whereas 3 μM was very active (*see also* traces in Fig. 2*B*).

Induction of a Noninactivating K^+ Current by Arachidonic Acid

In addition to the I_{K1} blocking effect described above, AA concentrations equal to or greater than 5 μM were shown to *activate* a K^+ current different from I_{K1}. This is illustrated by Fig. 3. In this experiment, a series of

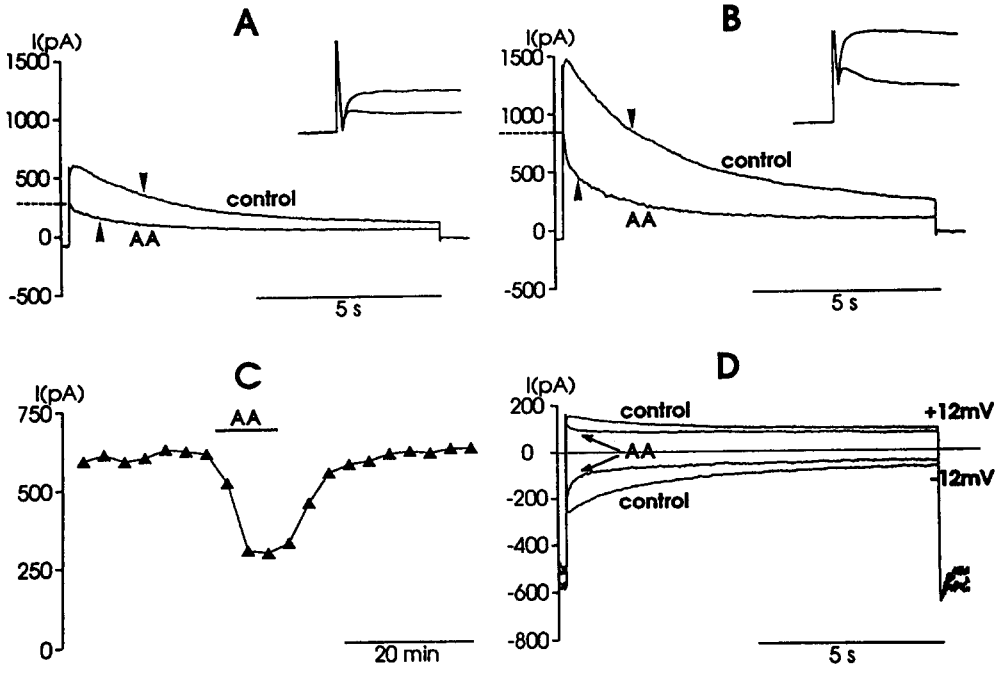

Fig. 1. The slowly inactivating K^+ current activated by depolarizing jumps (I_{K1}) is reversibly affected by AA. (*A*, *B* and *C*) Experiment performed with the usual NaCl external solution and KCl internal solution (E_{Cl} = 0 mV, E_K = −84 mV). Current traces recorded during depolarizing jumps from −100 to 0 mV (*A*), or to +30 mV (*B*) in control, and in presence of 3 μM AA (*AA*). The dotted lines indicate the peak current of the AA traces. On each trace, the arrow indicates the time $\tau_{1/2}$ for half decay between the peak current and the current remaining at the end of the depolarizing jump. $\tau_{1/2}$ (measured from the onset of the depolarization) was not markedly voltage dependent in control: 1.7, 2, 2, 1.9 and 1.85 sec at −15, 0, +15, +30 and +45 mV, respectively. AA clearly reduced $\tau_{1/2}$, and this effect was more pronounced for stronger depolarizations (*see text*). In *A* and *B*, the insets show part of the current traces on a 40 times expanded time scale (and twice smaller current scale). The peak outward current measured during successive jumps from −100 to 0 mV is plotted in *C*, showing that the blocking effect induced by AA was slowly reversible. (*D*) Experiment performed on another cell with the high K^+ external solution and the low-Cl^- K glutamate internal solution (E_{Cl} = −62 mV, E_K = 1 mV). Current traces recorded during depolarizing jumps from −100 mV to +12 or −12 mV, in control and in presence of 2.5 μM AA. Note that, in the conditions of this experiment, the current affected by AA reverses around 0 mV, as expected for a K^+ current.

depolarizing jumps (from −100 mV to −80, −60, −40, −20 and 0 mV) was applied before, during and after an application of 5 μM AA, under ionic conditions where E_K was −84 mV and E_{Cl} was 0 mV. AA induced a small inward current at −100 mV and a large outward current at 0 mV which no longer inactivate during the 10 sec jump to 0 mV (*see* current traces recorded during depolarizing jumps from −100 to 0 mV in Fig. 3*A*). We interpret this observation as the combination of two effects: the blockade of the inactivating I_{K1} current and the induction of a noninactivating current, which must be carried by K^+ ions since it reversed around E_K (*see* Fig. 3*C*). Figure 3*B* shows the current values measured either at −100 mV (○) or after 10 sec at 0 mV (●), for successive jumps from −100 to 0 mV. The AA-induced current disappeared after washing. Subtraction of the traces recorded in control from the traces recorded in the presence of AA during identical voltage jumps gives the *I-V* curve of the AA-induced current, by measuring the current at the end of each voltage jump after inactivation of I_{K1} (Fig. 3*C*). This *I-V* curve shows that the AA-induced current reverses close to E_K and displays some outward rectification in the presence of physiological asymmetrical K^+ concentrations.

The experiment illustrated by Fig. 4*A*, performed with the high K^+ external solution and the low-Cl^- internal solution (E_{Cl} = −62 mV, E_K = 1 mV), confirms that the current activated by high concentrations of AA is selective for K^+ ions. Indeed, under these ionic conditions, the AA-induced current reversed around 0 mV, as expected for a K^+ current, and its *I-V* curve was almost linear between −100 and +24 mV (*not shown*).

I_{K1} is sensitive to external TEA (reduced by about 40% by 1 mM TEA [7]). For comparison, we looked for possible effects of 1 mM TEA on the noninactivating K^+ current induced by AA. In two experiments performed on different cells, in which 10 sec depolarizing jumps were regularly applied from −100 to 0 mV, 20 μM AA was first applied without TEA and induced a large noninactivating K^+ current (of 0.8 and 2.5 nA, respectively); then, without intermediate wash, AA was applied together with 1 mM TEA. In the presence of AA, addi-

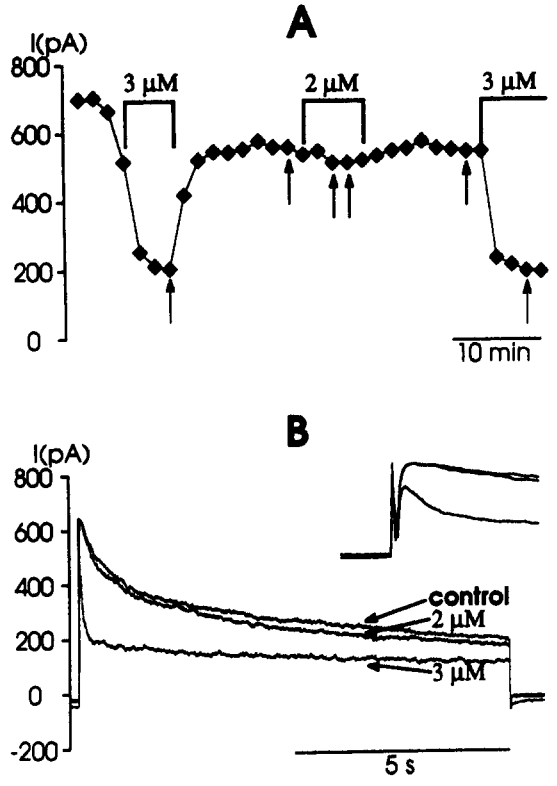

Fig. 2. The dose-response curve of the effect of AA on I_{K1} is very steep. Effects of 2 or 3 μM AA on the current induced by depolarization from −100 to −15 mV, in a cell dialyzed with the low-Cl^- K glutamate intracellular solution. (*A*). Plot of the current recorded 500 msec after the onset of each depolarizing jump, for the three successive AA applications, at 3, 2 and 3 μM. (*B*). Current traces recorded in control, during the application at 2 μM and during the applications at 3 μM (each trace is the mean of two traces, indicated by arrows on Fig. 2*A*). The inset shows part of the same current traces on a 40 times expanded time scale and twice smaller current scale.

tion of TEA did not have any effect. This result confirms that the control inactivating TEA-sensitive I_{K1} current is completely blocked by high concentrations of AA. It also shows that the AA-induced K^+ current is distinct from I_{K1}, pharmacologically, as well as with respect to its kinetic properties and voltage dependence.

The Blockade of I_{K1} Is the Dominant K^+ Current Modulation at Low Concentrations of Arachidonic Acid

Only 4 among 16 cells showed a slight K^+ current increase (of 18, 20, 85 or 180 pA at 0 mV) in response to AA concentrations of 2.5 or 3 μM (which clearly reduced I_{K1}). On the contrary, a K^+ current increase was induced in 8 of 9 cells by 5 μM AA and in 11 of 12 cells by 10 or 20 μM AA; this response was usually of large amplitude (between 100 and 2,500 pA at 0 mV, mean value 820 pA).

Thus, the blockade of I_{K1} was the only (or the major) effect observed for small AA concentrations (3 μM or below) whereas, usually, a large K^+ current increase was also activated for higher concentrations (≥5 μM).

The experiment described by Figs. 1*D* and 4*A* (same cell) illustrates this difference in dose dependence between the two K^+ current modulations which can be induced by AA. In this experiment, a first application of 2.5 μM AA clearly reduced the amplitude of I_{K1} and accelerated its inactivation, but did not activate any other K^+ current (Figs. 1*D* and 4*A*), whereas a second application of AA, at 10 μM, both blocked I_{K1} and induced a large noninactivating K^+ current (Fig. 4*A*). Similar results were obtained in three other experiments where different AA concentrations were successively tested on the same cell, using the usual NaCl external and KCl internal solutions.

In all cases where AA induced a K^+ current, it also induced a blockade of I_{K1}, and after wash of AA, the K^+ current increase (whatever its amplitude) was always more rapidly reversible than the effect on I_{K1}. This difference in the kinetics of reversibility of the two effects is compatible with the observation that the blockade of I_{K1} is the main effect induced by low concentrations of AA.

This difference is illustrated in Fig. 4*B* in the case of an experiment where 10 sec depolarizing jumps from −100 to 0 mV were applied every 20 sec and where 10 μM AA was applied after a first application of the same concentration of arachidonic methyl ester (MAA). Whereas 10 μM MAA did not have any effect (which was confirmed in three other cells), AA both reduced I_{K1} and induced a noninactivating K^+ current. The AA-induced K^+ current increase was immediately reversible (*see* current measurements at the end of the depolarizing jumps (●)), whereas the effect on I_{K1} required more than 4 min to be completely reversed (*see* peak current measurements (▲)).

Effects of Arachidonic Acid on K^+ Currents Do Not Require its Metabolism

To study the possible involvement of AA metabolism in the effects described above, we performed two different types of experiments. First, we tried to block the effects by preincubation with either indomethacin (10 μM), to inhibit cycloxygenase, or nordihydroguaiaretic acid, NDGA (10 μM), to inhibit lipoxygenases. In a second series of experiments, we tried to reproduce the effects of AA with an analogue, eicosatetraynoic acid, ETYA, known to inhibit cycloxygenase, lipoxygenases and cytochrome P450 enzymes, or with other fatty acids, in particular oleic acid, which is not metabolized (whether by cycloxygenase, lipoxygenase or by epoxygenase).

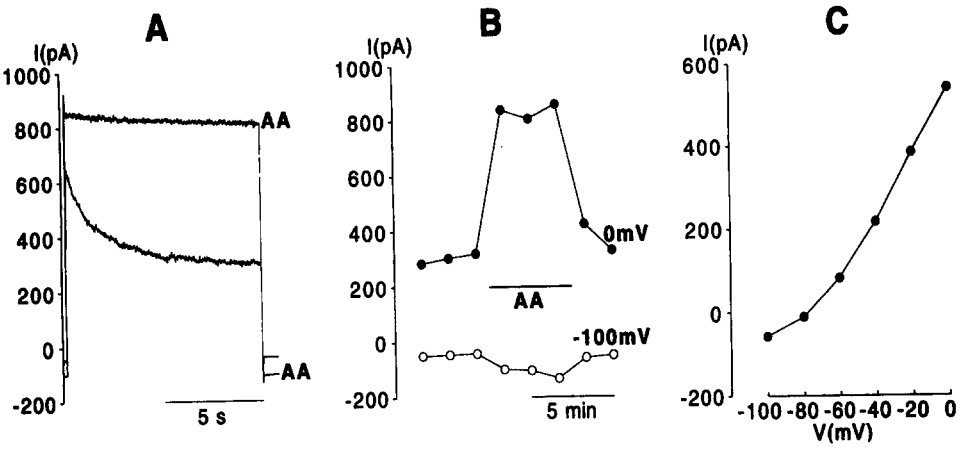

Fig. 3. Induction of a noninactivating K^+ current by 5 μM AA. A series of 10 sec depolarizing jumps from −100 to −80, −60, −40, −20 or 0 mV was regularly applied, and two effects of 5 μM AA were observed: the blockade of the inactivating I_{K1} current and the induction of a noninactivating K^+ current. (*A*) Current traces recorded during depolarizations from −100 to 0 mV in control and in the presence of 5 μM AA. (*B*) Plot of the current measured at the end of each successive jump to 0 mV (●) or at −100 mV (○), before, during and after the application of AA (indicated by the bar). (*C*) *I-V* curve of the AA-induced current, obtained by subtracting control traces from traces recorded during the application, and by measuring the current at the end of each depolarizing jump, or at −100 mV. Note that the induced current reverses around E_K (−84 mV). To obtain a reliable measure of the reversal potential of the AA-induced current, we had to apply AA when the basal current recorded at negative membrane potentials was very stable. The situation was usually not satisfactory during the first few minutes of cell dialysis, where we observed the progressive development of an inward current below 0 mV. This current then gradually disappeared, creating a stable baseline for experimentation.

Indomethacin (10 μM) did not affect the K^+ current and preincubation with 10 μM indomethacin during 15–30 min did not prevent the effects of 10 μM AA (four cells; *not shown*).

Surprisingly, 10 μM NDGA alone reduced I_{K1} and accelerated its inactivation. In three cells, I_{K1} (measured by the difference between the peak current recorded at 0 mV and the current measured after 10 sec at 0 mV) was reduced by 61 ± 18%, whereas the time for half decay, $\tau_{1/2}$, was reduced by 31 ± 13%. These effects of NDGA are illustrated by Fig. 5*A*. After wash of NDGA, a slow and partial reversibility was observed (*not shown*).

Preincubation with 10 μM NDGA during 10 min did not prevent the effects of AA. This is illustrated by Fig. 5*B* where 10 μM AA (applied in the presence of NDGA, after preincubation with NDGA) both blocked I_{K1} and activated a noninactivating K^+ current. This result was confirmed in another similar experiment.

As shown by Fig. 5*C*, 30 μM ETYA strikingly affected I_{K1}; it reduced its amplitude, and considerably accelerated its inactivation. These effects were immediately reversible after wash of ETYA (*not shown*). Similar results were obtained in two other experiments using 30 μM ETYA. The effect of only 10 μM ETYA was tested in three experiments: the current amplitude (difference between peak current and current measured after 10 sec at 0 mV) was reduced by 32 ± 9% and the time for half decay at 0 mV, $\tau_{1/2}$, was reduced by 72 ± 2%. In a total of 11 cells, ETYA (10 μM (3), 20 μM (1), 30 μM (3) or 50 μM (4)), did not induce any K^+ current increase.

Oleic acid, an unsaturated fatty-acid (C_{18}: 1 cis-9), was able to induce both effects of AA, as shown by Fig. 5*D:* 10 μM oleic acid both reduced I_{K1} and induced a K^+ current increase, detectable at the end of the depolarizing jump. Both effects of oleic acid were simultaneously observed in three cells (at 5 μM (1) or 10 μM (2)), whereas only the reduction in amplitude of I_{K1} and the acceleration of its inactivation were observed in three additional cells (5 to 10 μM). In two experiments where oleic acid and AA were successively tested on the same cell at the same concentration (10 μM in one case, 20 μM in the other), oleic acid was less efficient than AA both in reducing I_{K1} and in inducing the noninactivating K^+ current.

Linoleic acid 5 μM, an unsaturated fatty-acid (C_{18}: 2, cis-9, 12), induced the same responses as AA at the same concentration.

In the case of oleic acid and linoleic acid, as in the case of AA, the blockade of I_{K1} developed more rapidly during the application and reversed more slowly during wash than did the fatty acid-induced K^+ current (*not shown*), suggesting that the dominant effect, at low concentrations, is the I_{K1} blockade, whereas at high concentrations, it is the K^+ current increase.

No effect of *myristic acid,* a saturated fatty acid

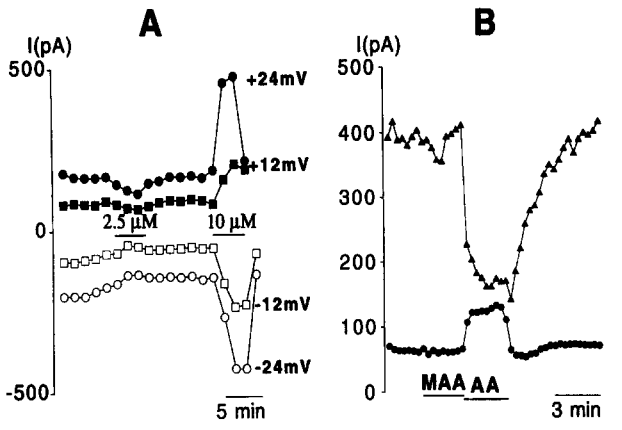

Fig. 4. (*A*) K^+ selectivity of the current activated by 10 μM AA. Same cell as Fig. 1*D*: experiment performed with the high K^+ external solution and the low-Cl^- K glutamate internal solution (E_K close to 0 mV). In this experiment, 10 sec depolarizing jumps from −100 to −24, −12, 0, +12 and +24 mV were regularly applied and two applications of AA were successively performed. As illustrated by Fig. 1*D*, the first AA application, at 2.5 μM, only induced a decrease of I_{K1}. The second AA application, at 10 μM, induced both a complete blockade of I_{K1} (*not shown*) and the induction of a noninactivating K^+ current, measured at the end of each successive depolarization to −24, −12, +12 or +24 mV. The current activated by 10 μM AA reversed around E_K (0 mV). It was also observed at the holding potential, −100 mV, as an inward current of 950 pA, reversible during wash of AA (*not shown*). (*B*) Difference between the kinetics of reversibility of the two effects of AA on K^+ currents and absence of effect of methyl arachidonic acid (MAA). Experiment performed with the usual NaCl external solution and KCl internal solution. Two current values are plotted for each successive 10 sec depolarizing jump from −100 to 0 mV: the peak outward current measured at 0 mV) (▲), and the current measured at the end of the depolarization, after inactivation of I_{K1} (●). MAA (10 μM) was ineffective, whereas 10 μM AA induced both a blockade of I_{K1} (*see* difference between the two current measurements (▲ − ●)) and an increase in a noninactivating K^+ current, visible at the end of the depolarizations. The induced K^+ current decrease was only slowly reversible, whereas the second effect was immediately reversible. This difference can be explained by the fact that the low concentrations of AA remaining in the membrane during the first minutes of wash can still reduce I_{K1}, but are below threshold for induction of the noninactivating K^+ current.

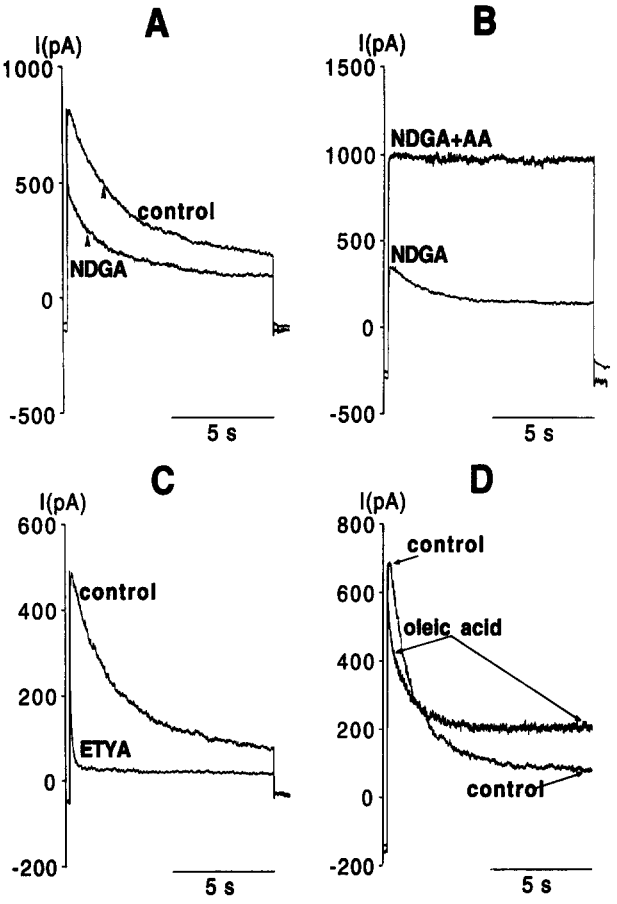

Fig. 5. Effects of NDGA, ETYA and oleic acid. NDGA (10 μM) itself affects the voltage-gated K^+ current (*A*), but does not prevent the two K^+ current modulations induced by AA (*B*). (*A*) Current traces recorded during depolarizing jumps from −100 to 0 mV in control and after 1 min 20 sec in the presence of 10 μM NDGA. Arrows indicate the time for half decay $\tau_{1/2}$ between the peak current and the current remaining after 10 sec at 0 mV. (*B*) Current traces recorded during depolarizing jumps from −100 to 0 mV in another cell, in the presence of NDGA alone (10 μM), and in the presence of both NDGA and AA (10 μM, applied after a 10 min preincubation with NDGA). (*C*) ETYA (30 μM) strongly affects I_{K1}, reducing its amplitude and accelerating its inactivation. These effects were maximum within the 20 sec separating two successive depolarizing jumps (from −100 to 0 mV) and were immediately and entirely reversible during wash. (*D*) Oleic acid mimics AA in inducing both a blockade of I_{K1} and an increase of a noninactivating K^+ current visible at the end of the depolarization. Current traces recorded during depolarizing jumps from −40 to 0 mV are illustrated in control and after 3 min 20 sec in the presence of oleic acid 10 μM. This picture is very similar to that usually observed transiently at the beginning of an application of 5 or 10 μM AA, even though the final effects of AA are usually more pronounced. The effects of oleic acid were reversible during wash (*not shown*).

(C_{14}: 0), could be observed in response to 10–20 min applications at 5 μM on three different cells, or to a 10 min application at 20 μM on another cell.

Tests of Some Possible Indirect Mechanisms

AA is well known to activate protein kinase C [26]. Pretreatment of the cells for 10 to 20 min with staurosporine 0.1 μM, an inhibitor of protein kinase C, did not prevent the effects of 5 μM AA (three cells), whereas a similar treatment of mouse neuroblastoma cells completely suppressed the effects of some fatty acids on the Na^+ and Ca^{2+} currents of those cells [21].

We could observe both AA effects in cells which had been loaded with 10 mM BAPTA for more than 12 min before the AA application (two cells, *see* Materials and Methods).

Finally, since we had observed that I_{K1} can be re-

duced by permeable cyclic GMP analogues [7], and since it was reported that AA can stimulate the guanylate cyclase of other cell types [4, 40], we wondered whether I_{K1} blockade induced by AA could result from the accumulation of cyclic GMP. This does not seem to be the case since AA was still able to reduce I_{K1} when the intracellular solution was supplemented with 0.1 mM cyclic GMP (three cells).

Modulations of Calcium Currents

Effects of Arachidonic Acid on the Low-Threshold Transient (T-Type) Calcium Current

In most cells, using internal and external solutions designed to block K^+ currents and to facilitate the detection of Ba^{2+} currents through calcium channels (*see* Materials and Methods: K^+-free NMG-Cs^+ internal solution, Na^+-free and K^+-free NMG-Ba^{2+} external solution), by applying depolarizing jumps from −80 mV to −45, −30, −15, 0 or +15 mV, we could record a T-type current which was maximum close to −30 mV and about half-activated at −45 mV. In some cells, this current was the only one, whereas in many cells, strong depolarizations (to −15, 0, or +15 mV) also activated a sustained L-type current which was maximum close to 0 mV and half-activated close to −15 mV.

We could study the modulation of the T-type current without any possible contamination by the L-type current, either by choosing cells which had a very small L-type current, or by current measurements below the threshold of activation of the L-type current (for example at −45 mV, since the *I-V* curves of the two currents were clearly separated, as already described [6]).

Figure 6, derived from a cell which had almost no L current, shows that a low concentration of AA (3 μM) clearly increased the T current induced by depolarizing jumps from −80 to −45 mV. This stimulatory effect was reversible (Fig. 6*A*). It was voltage dependent, becoming less pronounced for stronger depolarizations, and was no longer detectable for depolarizing jumps from −80 to +15 mV (Fig. 6*C*). Similar results were observed in four other experiments, using 2–3 μM AA.

The T current inactivation properties were compared in control and in the presence of AA (2 or 2.5 μM) in three experiments: a depolarization to a constant test potential V (below the threshold of activation of the L-type current) was preceded by a 1 sec prepulse to a variable potential V_H, and the amplitude of the T current measured for each jump from V_H to V was normalized with respect to the amplitude of the T current activated by depolarization from −80 mV to V. In control, half-inactivation was observed at −67 ± 1 mV, and the current was completely inactivated by a V_H of −50 mV. AA induced a small shift (of about 4 mV) of the T current inactivation curve towards more negative membrane potentials (*not shown*).

Whereas, as shown above, 2 to 3 μM AA could increase the T current close to its threshold of activation, 10 μM AA strongly *reduced* this current over the whole voltage range (inhibition ≥85% in four cells). This effect was not markedly different for the various depolarizing jumps alternatively applied from −80 mV to −45, −30, −15 and +15 mV. Figure 7 shows that, on a single cell, AA could induce both a reversible increase of the T current recorded at −45 mV during a first application at 3 μM, and a reversible decrease of this same current, during a second application at 10 μM.

Blockade of the L-Type Calcium Current by Arachidonic Acid (2–10 μM)

The L-type current was measured, under the same ionic conditions as the T-type current, either during depolarizing jumps from −50 mV (holding potential which completely inactivates the T-type current), or at the end of depolarizing jumps from −80 mV (after time-dependent inactivation of the T-type current).

As shown by Fig. 8, AA induced a concentration-dependent blockade of the L-type current. In the experiment of Fig. 8*A*, a 3 min application of 3 μM AA reduced by about 81% the L-type current activated by −50 to 0 mV; this effect was slowly reversible. In all the experiments, the L-type current was alternatively measured at −15, 0 and +15 mV, and the reduction induced by AA was not markedly voltage dependent (*not shown*).

In the experiment of Fig. 8*B*, lower concentrations of AA (1 and 2 μM) were applied to determine whether, as for the T-type current, the L-type current could be enhanced, rather than reduced, at the threshold of the dose-response curve. The results illustrated, which were confirmed in another cell maintained at a holding potential of −80 mV, show that whereas 2 μM AA still reduced the L-type current (by about 22% in this experiment and by about 32% in the other cell, at 0 mV), 1 μM AA did not affect this current. Two additional experiments performed in cells maintained at −100 mV confirmed that 0.5 or 1 μM AA does not increase the L current.

Thus, AA reduces the L-type current in a dose-dependent way between 1 and 10 μM. The percent reductions of the L-current at 0 mV, induced by a 3 min exposure to AA were: at *1 μM*, 0% (four cells), at *2 μM*, 22%, 32%, at *3 μM* 46%, 54% (in two cells maintained at −80 mV) and 81%, 87% (in two cells maintained at −50 mV), at *10 μM*, 83%, 100% (in two cells maintained at −80 mV) and 86%, 100% (in two cells maintained at −50 mV).

The main qualitative results of the present paper concerning the sign of the modulations of the K^+ and

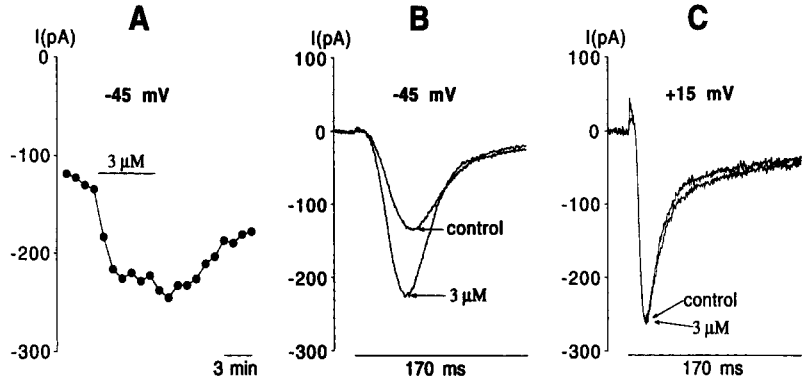

Fig. 6. Voltage-dependent stimulatory effect of 3 μM AA on the T-type Ca^{2+} current. NMG-Ba^{2+} external solution, NMG-Cs^{+} internal solution. (*A*) Plot of the peak inward current activated by successive depolarizations from −80 to −45 mV, before, during and after an application of 3 μM AA (indicated by the bar). (*B*) Current traces obtained in the same cell during depolarizations from −80 to either −45 mV (*B*) or +15 mV (*C*), in control and after 2 min 40 sec in the presence of 3 μM AA. Note that this cell showed almost no L current.

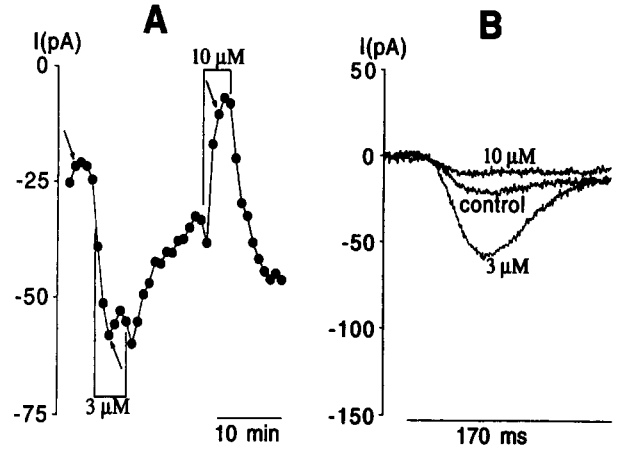

Fig. 7. Dose dependence of the effect of AA on the T-type current. NMG-Ba^{2+} external solution, NMG-Cs^{+} internal solution. Two successive AA applications were performed (at 3 and 10 μM) on the same cell. (*A*) Plot of the peak inward current activated by successive depolarizations from −80 to −45 mV. The T current was increased by 3 μM AA, whereas it was blocked by 10 μM AA. (*B*) Current traces showing the T current at −45 mV in control, after 2 min 20 sec in the presence of 3 μM AA, and after the same time in the presence of 10 μM AA (at the points indicated by arrows in *A*).

Ca^{2+} currents studied are summarized in the Table for two AA concentrations.

Discussion

K^+ Current Modulations by AA

The main effect of AA at concentrations higher than 5 μM is usually the induction of the noninactivating K^+ current which dominates the simultaneous blockade of the inactivating K^+ current I_{K1}. In contrast, at concentrations of 2 or 3 μM, AA usually does not induce any K^+ current increase, but strongly reduces I_{K1} and accelerates its inactivation. Thus, the global effect of AA on K^+ currents changes qualitatively with the AA concentration. Except in a recent case concerning modulations of the neuronal M current [2], such a behavior had not been described previously. Activation of K^+ currents by concentrations of AA ≥ 10 μM is well documented in other cell types (*Aplysia* neurons [29, 30], cardiac cells [16, 17, 20], smooth muscle cells [18, 28], hippocampal neurons [31, 35], insulinoma cells [25]). The observation that AA can reduce some voltage-gated K^+ currents has also been reported, briefly, in neurons (*see* Fig. 1*E* in [15]) and in smooth muscle cells (*see* Fig. 8 in [36]). Furthermore, in neuroblastoma cells, some fatty acids, in particular oleic acid and linoleic acid, were shown to accelerate the inactivation of a voltage-gated K^+ current ([33], possible effects of AA not described).

K^+ Current Activation by AA

Several results show that in osteoblasts, the K^+ current activated by high concentrations of AA (≥5 μM) is distinct from the control voltage-gated I_{K1} current. Whereas I_{K1} appears only above −30 mV [7], the K^+ current observed in the presence of AA can be detected even at −100 mV (*see* Fig. 3), and shows a linear *I-V* curve between −100 and +24 mV in symmetrical K^+ conditions. Furthermore, whereas 1 mM TEA reduces I_{K1} by 40%, it has no effect on the AA-induced K^+ current. Finally, whereas I_{K1} inactivates during a 10 sec depolarization, the AA-induced current does not show any inactivation.

In some other cell types where AA also induces a K^+ conductance increase, it has been shown that either lipoxygenase metabolism [29, 30, 35] or protein kinase C activation [25] is required for the K^+ current activation.

In osteoblasts, the K^+ current activation induced by AA does not seem to require cycloxygenase, lipoxygenase or epoxygenase metabolism, since it is blocked by neither indomethacin nor NDGA (Fig. 5*B*) and can be

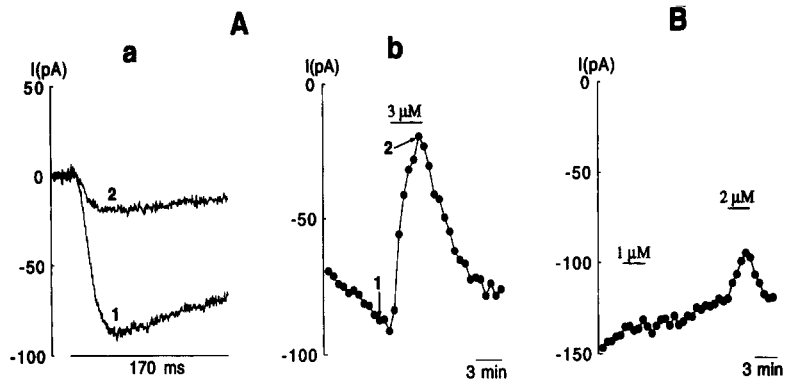

Fig. 8. AA reduces the L-type Ca^{2+} current in a dose-dependent way. NMG-Ba^{2+} external solution and NMG-Cs^{+} internal solution. (*A*) (*a*) L current traces during depolarizing jumps from −50 to 0 mV, in control (*1*), and after 3 min 10 sec in the presence of 3 μM AA (*2*). (*b*) Peak L current values measured during successive depolarizing jumps from −50 to 0 mV before, during and after the AA application. (*B*) Experiment performed in another cell, on which AA was applied first at 1 μM and then at 2 μM. Plot of the peak L current for successive depolarizing jumps from −50 to 0 mV.

Table 1. Qualitative summary of the modulations induced by AA according to its concentration

[AA] (μM) / Current type	3	10
I_{K1}	↓	↓↓
I_K(AA)	0	↑
I_{CaT}	↑	↓
I_{CaL}	↓	↓↓

I_K(AA) is the noninactivating K^+ current activated (↑) by 10 μM AA (in >90% of the cells), but not by 3 μM AA (in 75% of the cells: 0). I_{CaT} is the T-type calcium current measured close to its threshold, at −45 mV. (↑), (↓) and (↓↓) indicate, respectively, potentiation, inhibition and stronger inhibition of the currents.

induced by oleic acid (Fig. 5*D*). Furthermore, it does not seem to require protein kinase C activation since it is not blocked by staurosporine pretreatment and is very rapidly reversible. Note, however, that in the absence of a positive control of the efficacy of staurosporine in our experiments, we cannot completely eliminate a possible role of PKC. Finally, we do not think that this effect results from a Ca^{2+} concentration increase since it persists in BAPTA-loaded cells.

In some previous studies [16, 18, 28], it has been proposed that AA directly activates some K^+ channels. Our results are compatible with such a mechanism. However, it remains to explain why, in osteoblasts, ETYA did not activate any K^+ current.

K^+ Current Blockade by AA

Likewise, we did not get any indication that AA metabolism is required for modulation of the inactivating K^+ current of osteoblasts, since ETYA (Fig. 5*C*) and oleic acid (Fig. 5*D*) can reproduce the effects of AA, and since these effects are not prevented by indomethacin or NDGA (Fig. 5*B*). Thus, the modulation of I_{K1} by AA could also be a direct effect of AA. Since the induced acceleration of inactivation was more pronounced for stronger depolarizations and since MAA was ineffective, it appears that the negative charge of the fatty acid may have an important role.

That NDGA itself has a K^+ current blocking effect (Fig. 5*A*) could result either from a direct pharmacological effect of NDGA, or from an increase in the endogenous AA concentration due to the NDGA-induced inhibition of the AA metabolism. Note that the blocking effect of 10 μM NDGA on I_{K1} was not complete and did not prevent further blockade by the addition of 10 μM exogenous AA (Fig. 5*B*).

The concentration dependence of the AA blocking effect on I_{K1} was very steep. A similar property was described recently in the case of secretory K^+ channels from apical renal membranes, blocked by low concentrations of AA applied to the intracellular face of the membrane [42]. However these channels, voltage independent and insensitive to 5 mM TEA[41], are different from the osteoblastic channels responsible for I_{K1}.

Effects of AA on Voltage-Gated Calcium Currents

Whereas modulations of high-threshold calcium currents by AA had already been described in other cell types (current blockade in hippocampal neurons [15], L current increase in GH_3 cells [39] and cardiac myocytes [24]), modulations of the low-threshold T-type current have not been described. Even though further studies are obviously necessary to investigate their mechanism, the Ca^{2+} current modulations that we observed seem particularly interesting when considered together with the associated K^+ current modulations. At low concentrations, AA reduces I_{K1} and enhances the T-type Ca^{2+} current close to its threshold of activation, whereas at higher concentrations, AA activates a large K^+ current and blocks the T-type Ca^{2+} current (*see* the Table). For each concentration range, both the net effect on K^+ currents and the effect on the T-type Ca^{2+} current

will have the same physiological consequence: low concentrations of AA will favor Ca^{2+} entry during small depolarizations (below the threshold of the L-type Ca^{2+} current), and high concentrations will, on the contrary, oppose Ca^{2+} entry through voltage-gated Ca^{2+} channels (both by increasing the K^+ conductance, even at negative membrane potentials, and by blocking the voltage-gated Ca^{2+} currents).

Physiological Stimuli Involving AA

AA can be released either by activation of phospholipase A_2 (from membrane phospholipids), or by diacylglycerol lipase, following production of diacylglycerol by phospholipase C, or by diglyceride lipase, following activation of phospholipase D. Some of these pathways can be activated by hormones. Parathyroid hormone has been shown to stimulate an increase of free AA in glucocorticoid-pretreated osteoblasts [37]. Recently, in the case of an osteoblast-like cell line, MC3T3-E1, prostaglandin E_2, which has a major role in bone physiology (*see* [27], for a review), has been shown to release AA very potently [19]. AA can stimulate cell growth of this cell line [12]. AA concentration changes might be involved in some inflammatory processes. For example, interleukin-1 α stimulates the production of prostaglandin E_2 [10,38]. Osteoblasts also show receptors for bradykinin, coupled to prostaglandin formation [22]. Finally, activation of PLA_2 has been proposed as an important step in the transduction of mechanical force in bone cells [3, 32].

From the present study, it appears that AA concentration changes can be responsible for modulations of voltage-dependent calcium entry in osteoblasts, in particular through the T-type channels which are likely to play a major role over the voltage range of the membrane potential oscillations of these cells [11, 34]. It will be of interest to further investigate the effects of hormonal (or mechanical) stimulations inducing AA concentration changes, both on the AA-sensitive ionic currents and on the intracellular calcium concentration.

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