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External blockade of the major cardiac delayed-rectifier K^+ channel (Kv1.5) by polyunsaturated fatty acids

(K^+ conductance/arachidonic acid/fish oil/antiarrhythmics/tedisamil)

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ABSTRACT **The present work shows that arachidonic acid and some other long chain polyunsaturated fatty acids such as docosahexaenoic acid, which is abundant in fish oil, produce a direct open channel block of the major voltage-dependent K^+ channel (Kv1.5) cloned in cardiac cells. The inhibitory action of these selected fatty acids is seen when they are applied extracellularly but not when they are included in the patch pipette. Fatty acids then appear to bind to an external site on the Kv1.5 channel structure. Inhibition of Kv1.5 channel activity by polyunsaturated fatty acids (acceleration of the apparent inactivation and decrease of the peak current) is similar to that produced by the class III antiarrhythmic tedisamil. Docosahexaenoic acid and arachidonic acid also inhibit the delayed-rectifier K^+ channel currents in cultured mouse and rat cardiomyocytes. These results are discussed in the light of the reported fatty acids effects on cardiac function in diseased states. Since Kv1.5 is also present in the brain, the results reported here could also have a significance in terms of processes such as long-term potentiation or depression.**

Fatty acids play an important role in the life and death of cardiac cells because (*i*) they are essential fuels for mechanical, electrical, and synthetic activities of the heart; (*ii*) their level is abnormally high in an ischemia followed by a reperfusion; and (*iii*) dietary fish oil is apparently beneficial for heart function (for a review, see ref. 1).

In heart, voltage-gated K^+ channels determine the resting potential, shape, and length of the action potential, thus controlling cardiac performance. Several different types of voltage-sensitive K^+ channels are expressed in myocytes (for reviews, see refs. 2 and 3), and their intrinsic properties are regulated by guanine nucleotide-binding proteins (4) and by different types of kinases (5). Activation of one class of cardiac K^+ channels by intracellular arachidonic acid (AA) has also been observed (6–8).

Many different types of voltage-sensitive K^+ channels have been recently cloned (for reviews, see refs. 9–11), and some of them have been expressed in cells normally lacking endogenous channels (12–14). We have taken advantage of such an expression system to study the interaction of fatty acids with Kv1.5, a voltage-dependent K^+ channel present at a particularly high level in mammalian heart (including human) (15–17).

MATERIALS AND METHODS

The coding sequence of the Kv1.5 cDNA cloned from mouse heart (18) was inserted into the *Apa* I cloning site of the expression vector pRcCMV (Invitrogen). The recombinant vector was characterized and used to transfect CHO cells by calcium phosphate precipitation. G418-resistant stable cell lines were established, and one of them was selected for its high content of recombinant Kv1.5 RNA and for the expression of a large outward current.

Cardiac cell isolation and culture have been described (19). The patch–clamp technique was used to monitor the expression of Kv1.5 channels. Pipettes were coated with Sylgard resin to reduce pipette capacitance. The patch–clamp amplifier RK300 (Bio-Logic, Grenoble, France), equipped with a 10 Gohm feedback headstage, was used for whole-cell and single-channel recordings. Currents were stored in digitized format on digital audio tapes by using Bio-Logic DTR1201 recorder for further analysis. Currents flowing out of the pipette in the outside–out configuration were represented as positive. The cut-off frequency of the filter was set at 1 kHz, and the sampling frequency was 5 kHz. Voltage stimulation and current analysis were done with the software pClamp (Axon Instruments, Burlingame, CA). In some experiments, the P/4 procedure was used to automatically subtract both capacitive and leakage currents. The external medium was 135 mM NaCl/4 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/10 mM Hepes made pH 7.4 with NaOH. The internal medium was 130 mM KCl/1 mM $MgCl_2$/5 mM Hepes/2 mM EGTA made pH 7.25 with KOH. The K^+-rich external solution contained 140 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes made pH 7.4 with KOH.

To isolate K^+ currents in cardiac cells, we used an extracellular medium containing 135 mM Tris chloride, 4 mM KCl, 2.8 mM $MgCl_2$, and 10 mM Hepes made pH 7.4 with HCl. Fatty acids were dissolved weekly in ethanol at the concentration of 0.1 M and stored under nitrogen atmosphere at −20°C.

RESULTS AND DISCUSSION

Fig. 1*A* shows that stably Kv1.5-transfected cells display outward currents that rapidly rise to a peak and display no inactivation during a 300-ms-duration depolarizing pulse from the holding potential of −60 mV to a test potential of +30 mV. No contaminating current was observed under the experimental conditions used in this work. The expressed current was sensitive to tedisamil, a typical class III antiarrhythmic drug (20). Addition of 30 μM tedisamil induced a typical open-channel block that has been described in rat and guinea pig ventricular myocytes (20) with a marked acceleration of the inactivation and a reduction of the peak current (Fig. 1*A*).

Fig. 1*B* shows the effects of increasing concentrations of AA. Addition of 1 μM AA accelerated activation and slightly increased peak current amplitude. AA at 30 μM had a tedisamil-like effect. It reduced the peak current and produced a typical current relaxation with a monoexponential time course. AA at 100 μM produced a 90% block of the

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Abbreviations: AA, arachidonic acid; ETYA, eicosatetraynoic acid; DOHA, docosahexaenoic acid.

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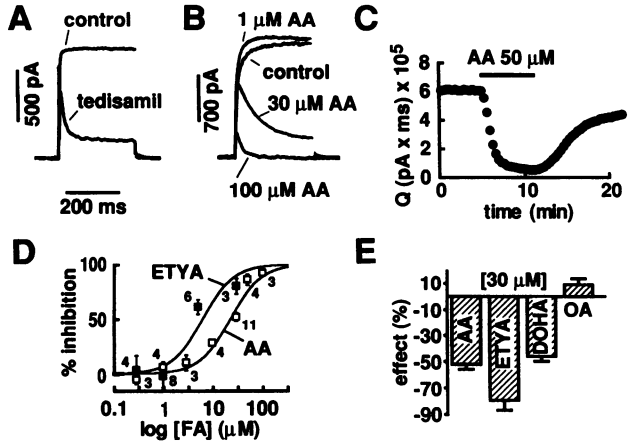

FIG. 1. Long-chain fatty acid inhibition of the Kv1.5 channel. (*A*) Inhibition by 30 μM tedisamil of the Kv1.5 current (expressed in a transfected CHO cell). (*B*) Effect of increasing concentrations of AA (as indicated near each current trace) on the Kv1.5 current. In *A* and *B*, time scales are identical. (*C*) Time-course of channel block by 50 μM AA and reversibility. Charge quantities were measured for each current trace and plotted as a function of time. AA superfusion is indicated by an horizontal bar. (*D*) Dose effect relationships for Kv1.5 channel block with ETYA and AA. The number of cells for each concentration studied is indicated in the graph. (*E*) Effects (expressed as a percentage of control) of various fatty acids at a concentration of 30 μM. Numbers of cells ranged from 5 to 11. Holding potential was −60 mV; test potential was +30 mV. Pulses were delivered with a period of 20 sec.

current. Fig. 1*C* shows the time course and reversibility of the AA effect. Concentration dependences of K^+ channel blockade by AA (ED_{50} = 21 μM) and its nonmetabolizable analog eicosatetraynoic acid (ETYA) (ED_{50} = 6 μM) are shown in Fig. 1*D*. Fig. 1*E* shows that long-chain polyunsaturated fatty acids such as AA, ETYA, and docosahexaenoic acid (DOHA) blocked Kv1.5 (ETYA was the most potent). Linoleic and linolenic acids had no effect on Kv1.5 activity (not shown). Similarly, the long-chain saturated palmitic and stearic acids did not alter Kv1.5 channel activity. The monounsaturated long-chain oleic acid increased the Kv1.5 current by about 10% without modifying its inactivation kinetics. The hydroxylated fatty acid from plant, ricinoleic acid, and the esterified palmitic acid methyl ester were both ineffective. Finally, the AA metabolite prostaglandin E_2 at 30 μM did not alter Kv1.5 activity.

At this stage, it was of course important to know whether fatty acid blockade of the Kv1.5 channel occurred from the intracellular or the extracellular side of the membrane. Fig. 2*A* shows that intracellular DOHA included in the pipette medium did not alter the K^+ current, although, on the same cell, addition of DOHA to the external medium inhibited the current within 20 sec (n = 9). Similar results were obtained with AA (n = 5) and ETYA (n = 3). Conversely, internal tedisamil inhibited the Kv1.5 current (n = 4), and inhibition was further enhanced when DOHA was added to the superfusion medium (Fig. 2*B*). These results strongly suggest that inhibition of the Kv1.5 channel activity by DOHA (and other fatty acids) is due to binding of fatty acids to an extracellular site, whereas inhibition by tedisamil is due to binding to an intracellular site. Cell-attached, inside-out, and outside-out patch–clamp recordings were performed to confirm this interpretation. In the cell-attached configuration, channel activity occurred in bursts of fast openings and closings with frequent openings to subconductance states (Fig. 2*C*). The unitary conductance determined from the slope of the *I–V* curve was 8 pS (Fig. 2*C*). Superfusion of DOHA or AA in the cell-attached configuration for 10 min—i.e., when fatty acids had no direct external access to the recorded channels (n = 6), did only slightly affect current relaxation (Fig. 2*D*). When patches were excised in the inside-out configuration in an internal medium containing DOHA, current inhibition occurred within <20 sec (n = 3). When DOHA was included in the pipette medium (n = 5), currents recorded during cell-attached experiments showed the typical current relaxation (Fig. 2*E*). Excision of the patches in the inside-out configuration in an internal medium lacking fatty acid did not modify current relaxation (n = 3). When outside-out patch membranes were exposed to extracellular AA, single-channel currents were inhibited within a few seconds (Fig. 2*F*). A direct action of AA and probably other fatty acids on an extracellular domain of the channel protein itself is therefore a probable mechanism for fatty acid-induced block of the Kv1.5 channel. Experiments with inside-out patches show that fatty acids are able to traverse the patch membrane from the intracellular side and possibly block channels with binding to an extracellular site. Therefore, both extracellular and intracellular AA would block the Kv1.5 channel. Other potential and more indirect mechanisms are very unlikely. For example, a nonspecific cell membrane permeabilization by fatty acids was ruled out by the fact that the detergent saponin (0.005%) could not mimic the effects of fatty acids (although it increased the leakage current) (not shown). Nonspecific effects due to fatty acids partitioning into the membrane are also unlikely because AA has a sidedness specificity and also because K^+ channel inhibition was selectively observed with only three of the long-chain fatty acids. Since ETYA, the nonmetabolizable AA analog, is even more potent than AA itself, the possible involvement of fatty acid metabolites (21) through lipoxygenase or cyclooxygenase pathways is also ruled out. Moreover, a 2-hr treatment with both nordihydroguaiaretic acid and indomethacin, two blockers of the lipoxygenase and cycloxygenase pathways, respectively, failed to alter the AA-induced block. A last possibility would be an indirect action of nonesterified fatty acids via fatty acid-sensitive subtypes of protein kinase C (22), as recently observed for an ATP-sensitive K^+ channel (23). Kv1.5 has one consensus site for phosphorylation by protein kinase C, which is located on the intracellular loop between S4 and S5 and also four different consensus sites for protein kinase A located in both amino- and carboxyl-terminal domains (18). Possible involvement of both protein kinases A and C in nonesterified fatty acid effects is unlikely because treatment of the transfected CHO cells for 3 hr with the kinase C inhibitor staurosporine (0.1 μM) and the nonspecific kinase inhibitor H7 (10 μM) did not modify the fatty acid-induced block of Kv1.5. Therefore, all of these data taken together strongly reinforce the conclusion that inhibitory fatty acids most probably directly interact with the Kv1.5 channel at an external site. Such an interpretation is not without precedent. Interestingly, the ion-channel activity associated with the *N*-methyl-D-aspartate (NMDA) receptor in cerebellar granule cells was recently shown to be modulated by AA (24). In that particular case, a potential fatty acid-binding site has been tentatively identified in the NMDA receptor structure (25).

A more detailed biophysical analysis of the effects of fatty acids on the activity of the Kv1.5 channel is shown in Fig. 3. Its purpose is to establish the mechanism of AA-induced blockade of the Kv1.5 channel. The effects of AA on inward (Fig. 3 *Left*) and outward (Fig. 3 *Right*) K^+ currents recorded in a cell bathed in a K^+-rich solution are presented in Fig. 3*A*. At a membrane potential of −20 mV, AA accelerated current activation, increased peak current amplitude, and induced current inactivation to proceed with a monoexponential time course (n = 5). The large inward deactivation tail current, which decayed with a monoexponential time-course, was much slower in the presence of AA. With a voltage pulse to +40 mV, outward current activation was fast, and no inac-

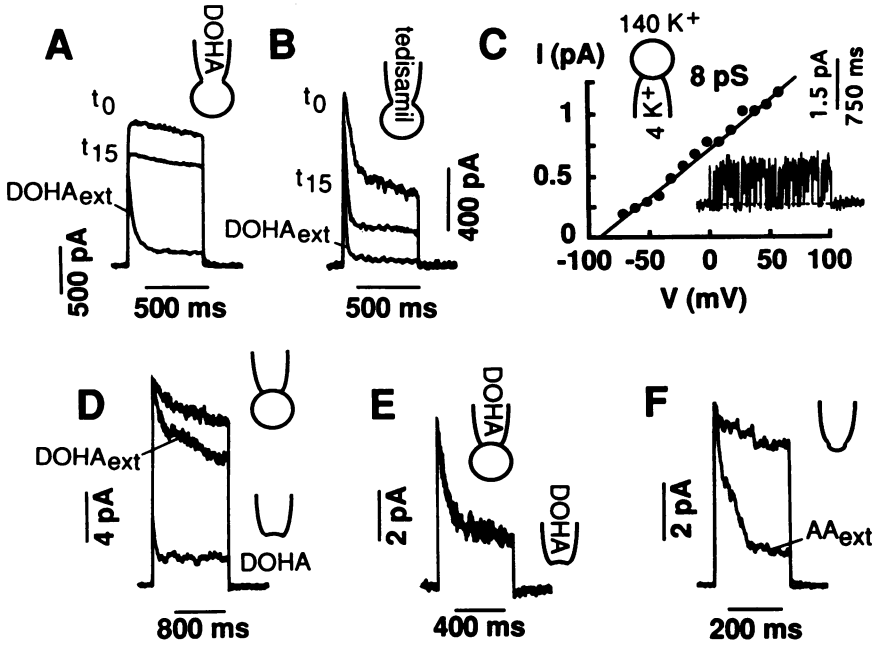

FIG. 2. Inhibition of the Kv1.5 channel by fatty acids at the external face of the membrane. (*A*) DOHA (50 μM) was included in the patch pipette, and the cell in the whole-cell configuration was allowed to stabilize for 15 min (t_{15}). The control trace is the first current elicited after breaking the patch (t_0). Then, 50 μM DOHA was added to the external medium, and the current was recorded after 20 sec of superfusion. (*B*) Effects of 30 μM internal tedisamil. As in *A*, the cell was first allowed to stabilize for 15 min (t_{15}) after breaking the patch (t_0). Then, 50 μM DOHA was added to the external medium, and the current was recorded after 20 sec of superfusion. In *A* and *B*, the cells were stimulated with a period of 20 sec from −60 mV to +30 mV. (*C*) *I–V* relationship of the single-channel Kv1.5 currents recorded in the cell-attached mode. The cell was bathed with the K^+-rich solution (140 mM K^+), and the pipette contained the external medium (4 mM K^+). The linear slope conductance was 8 pS. Single-channel currents illustrated here were recorded at +60 mV. (*D*) Effects of DOHA (50 μM) on single channels in the cell-attached mode. Representations show average (15 pulses) single-channel current traces from an experiment in control conditions and then in the presence of extracellular DOHA. After 10 min of superfusion with DOHA, the patch was excised in the inside-out configuration in the presence of internal DOHA. (*E*) Effects of DOHA (50 μM) on the averaged (15 pulses) single-channel activities recorded in the cell-attached configuration. DOHA was included in the pipette. After 7 min of recording, the patch was excised in the inside-out configuration in a DOHA-free internal medium. (*F*) Effects of 30 μM AA on average single-channel activities (15 pulses averaged) in an outside-out patch. In *D*, *E*, and *F*, the holding potential was −60 mV, and the patch membrane was depolarized to +30 mV every 20 sec.

tivation could be observed during the depolarizing pulse (note the different current scales used for inward and outward currents). AA slightly decreased the peak current (about 15%) and induced a typical monoexponential decay of the current (Fig. 3*A Right*). To represent as accurately as possible the effects of fatty acids *I–V*, conductance curves were constructed by measuring charge quantities during voltage pulses of increasing amplitudes (Fig. 3*B*). Addition of AA increased current amplitude at potentials below −10 mV (Fig. 3*A Left*). AA produced a voltage shift of the conductance curve by 16 mV in the negative direction (Fig. 3*B Inset*). AA inhibited both inward and outward currents with similar potencies for voltages higher than −10 mV. The time constant of the monoexponential current decay (60 ms) was constant for these voltages in the presence of AA. All of these data suggest that AA binds primarily to the open state of the channel and that fatty acid-induced inhibition is not voltage-dependent. The open-state block by AA is also indicated by the experiment illustrated in Fig. 3*C*, where a conditioning prepulse potential was imposed to the cell before a test pulse was delivered (see the protocol in Fig. 3*C*). In control conditions, half-inactivation of Kv1.5 occurred with a prepulse potential to 10 mV. In the presence of AA, this value was shifted in the negative direction by 40 mV. This apparent inactivation observed in the presence of AA increased steeply between −50 mV and −10 mV, and this voltage dependence closely paralleled the voltage dependence of the activation indicated by the conductance curve shown in Fig. 3*B Inset*. This parallelism again suggested that AA associates with its binding site to produce blockade only when the channel is in the open state. In agreement with that proposal, it was shown that when the cell was maintained long enough at a negative holding potential (−60 mV), channels became unblocked. A two-pulse protocol was used to measure the rate at which AA leaves channels at rest. In experiments presented in Fig. 3*D*, two pulses were successively delivered, and the time interval between them was gradually increased. In control conditions, the amplitude of the two successively measured K^+ currents remained constant even for very short intervals (not shown). In the presence of AA, kinetic recovery from block occurred in a single phase with a time constant of 568 ms, corresponding to the rate at which AA dissociates from its binding site at −60 mV.

At this point it was of course important to check whether the fatty acid-induced open-channel block demonstrated in cells transfected with the Kv1.5 channel could also be observed in original cardiomyocytes. Effects of fatty acids were investigated on cultures of rat and mouse embryonic ventricular cardiac cells. In mouse neonatal cardiomyocytes, AA and ETYA induced the same characteristic block of the outward delayed-rectifier K^+ current as illustrated for DOHA in Fig. 4 *A* and *B* (while in the same cells, the inward rectifier K^+ current was not affected by DOHA); 30 μM DOHA depressed the outward current measured at +30 mV in mouse neonatal cardiomyocytes by 52 ± 8% (n = 4). AA also depressed the outward K^+ current recorded from rat embryonic ventricular cells and accelerated its kinetics of inactivation (Fig. 4 *C* and *D*); 30 μM AA decreased the outward current amplitude measured at +30 mV in rat neonatal cardiomyocytes by 63 ± 6% (n = 7). Then, clearly the same effects of fatty acids on outward K^+ currents were found in both Kv1.5-transfected CHO cells and in cultured

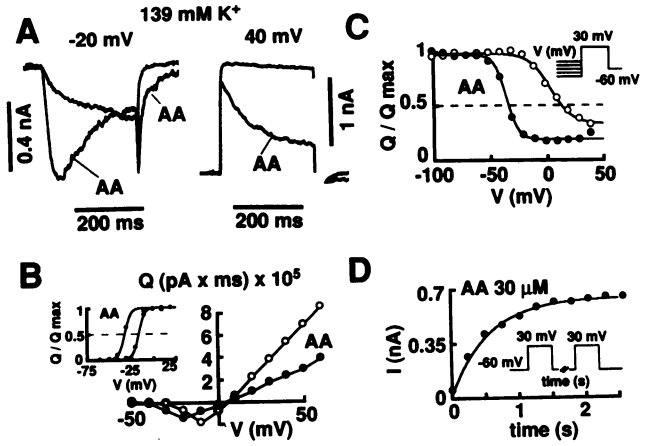

FIG. 3. Biophysical properties of the inhibiting effect of AA. (*A*) Effect of AA (30 μM) on K^+ currents recorded in a cell bathed in a K^+-rich medium (139 mM K^+). The holding potential was −60 mV, and the test potentials were −20 mV or +40 mV. Tail currents were truncated at +40 mV. Pulses were delivered with a period of 20 sec. Note the different current scales in *A Left* and *A Right*. (*B*) The *I–V* curve was constructed by using charge quantities determined for each current trace in controls (○) and in the presence of 30 μM AA (●). (*Inset*) Normalized conductance curves were constructed by measuring charge quantities during each tail current. Normalized curves were fitted with $y = c/[1 + \exp(a - x)/b]$ with $a = -16.2$ mV, $b = 3.3$ mV, and $c = 1$ in controls and with $a = -32.1$ mV, $b = 3.4$ mV, and $c = 1$ for AA. (*C*) Inactivation curves were obtained with a double-pulse protocol. A conditioning prepulse of 7-sec duration and increasing voltage preceded a test pulse of 300-msec duration to +30 mV. Charge quantities were determined during the test pulse and normalized. Curves were fitted with $y = c/[1 + \exp(a - x)/b]$, with $a = 3.8$ mV, $b = -9.3$ mV, and $c = 0.65$ in controls and with $a = -35$ mV, $b = -5.1$ mV, and $c = 0.78$ in the presence of AA. (*D*) A double-pulse protocol was used to determine how fast AA leaves its binding site. Two pulses of 250-ms duration from −60 mV to +30 mV were delivered with an increasing coupling interval every 20 sec. In control conditions, no inactivation occurred (not shown). In the presence of 30 μM AA, recovery from block occurred with single exponential kinetics. The curve was fitted with $y = a[1-\exp(-bx)]$ with $a = 642$ pA and $b = 1.76$ s^{-1}. Amplitudes of the currents for a stimulation from −60 mV to +30 mV with a period of 20 sec was 2348 pA in the control and 1180 pA in the presence of 30 μM AA.

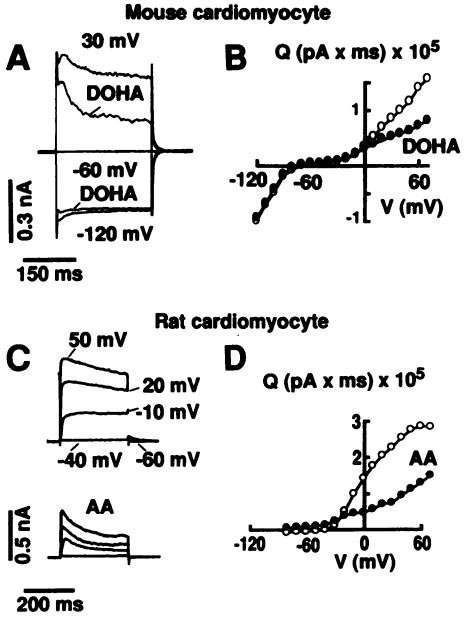

FIG. 4. Fatty acid inhibition of the delayed-rectifier K^+ current in mouse and rat ventricular cardiomyocytes. (*A*) DOHA (30 μM) superfusion blocked the delayed-rectifier K^+ current in a mouse ventricular cultured cardiomyocyte and did not affect the inward-going rectifier. The holding potential was −60 mV, and the test potentials were +30 mV and −120 mV. (*B*) *I–V* curves were constructed by measuring charge quantities. (*C*) Effects of 30 μM AA on K^+ currents in cultured rat ventricular cardiac cells. The holding potential was −60 mV, and the cell was depolarized to −40, −10, +20, and +50 mV. (*D*) *I–V* relationships were constructed by measuring charge quantities.

neonatal mouse and rat ventricular cardiomyocytes. The mRNA encoding the Kv1.5 channel studied in this work is one of the most abundant K^+ channel transcripts in rat and mouse heart and is present at a high level throughout heart development (ref. 16 and our unpublished results). It is thus very tempting to postulate that this effect of fatty acids on intact cardiac cells could possibly be due to their action on the Kv1.5 channel. It remains to be seen whether observations made in this work apply to all animal species (including man), to all cardiac cell types and to all stages of development.

Ischemia and reperfusion are known to increase both intracellular and extracellular levels of AA (reviewed in ref. 1). Previous work (6) has shown that intracellular AA activates, particularly at low intracellular pH (i.e., in conditions corresponding to ischemia), an AA-sensitive K^+ channel. This effect is expected to be beneficial because opening of the AA-activated K^+ channel during ischemia [probably in parallel with ATP-sensitive K^+ channel (3, 26, 27) and Na^+-activated K^+ channels (reviewed in ref. 3)] will tend to hyperpolarize the cardiac cell, thus lowering the activation of Ca^{2+} channels and the exaggerated Ca^{2+} entry via these channels.

This work has identified an external effect of AA and some other fatty acids on the major voltage-dependent cardiac K^+ channel. This effect closely resembles that of the class III antiarrhythmic tedisamil (20) and therefore is also expected to be rather beneficial in ischemia situations.

The American Heart Association has recently reported (28) that dietary fish oil prevents ventricular fibrillation associated with ischemic insult to the heart, one of the major causes of death from heart attacks. In addition, fish oil feeding to rats was shown to prevent ventricular fibrillation from occurring in association with ischemia and reflow produced by permanent or temporary experimental occlusion of a coronary artery (29, 30). The highly unsaturated fatty acids from fish oil, DOHA and eicosapentaenoic acid, prevent the toxicity of high concentrations of ouabain in isolated neonatal rat cardiac myocytes (31, 32). This protective effect might take place via a modulation of L-type Ca^{2+} channels (32). DOHA has also been shown in this work to be particularly efficient in blocking the Kv1.5 channel. It might then be assumed that part of the beneficial effect of fish oil fatty acids may be due also to the potent tedisamil-like effects of DOHA on the Kv1.5 channel.

The Kv1.5 channel is known to be present in many different tissues and particularly in the brain (15). Therefore, it is to be expected that AA also regulates the activity of neuronal Kv1.5 channels. This could have important implications, since AA has recently been given an important intercellular role as a retrograde messenger in long-term potentiation (24, 33).

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