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Direct Activation of an Inwardly Rectifying Potassium Channel by Arachidonic Acid

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ABSTRACT

Arachidonic acid (AA) is an important constituent of membrane phospholipids and can be liberated by activation of cellular phospholipases. AA modulates a variety of ion channels via diverse mechanisms, including both direct effects by AA itself and indirect actions through AA metabolites. Here, we report excitatory effects of AA on a cloned human inwardly rectifying K^+ channel, Kir2.3, which is highly expressed in the brain and heart and is critical in regulating cell excitability. AA potently and reversibly increased Kir2.3 current amplitudes in whole-cell and excised macro-patch recordings (maximal whole-cell response to AA was 258 ± 21% of control, with an EC_{50} value of 447 nM at −97 mV). This effect was apparently caused by an action of AA at an extracellular site and was not prevented by inhibitors of protein kinase C, free oxygen radicals, or AA metabolic pathways. Fatty acids that are not substrates for metabolism also potentiated Kir2.3 current. AA had no effect on the currents flowing through Kir2.1, Kir2.2, or Kir2.4 channels. Experiments with Kir2.1/2.3 chimeras suggested that, although AA may bind to both Kir2.1 and Kir2.3, the transmembrane and/or intracellular domains of Kir2.3 were essential for channel potentiation. These results argue for a direct mechanism of AA modulation of Kir2.3.

Inwardly rectifying potassium channels (Kir) are widely expressed in a variety of cells and play a critical role in regulating cell excitability (Hille, 1992). Kir channels are characterized by a subunit topology of two transmembrane domains surrounding a pore region (Kubo et al., 1993) and by the distinctive property of passing current more readily in the inward direction. This inward rectification is largely attributed to voltage- and $[K^+]_o$-dependent channel block by intracellular Mg^{2+} and polyamines (Matsuda et al., 1987; Ficker et al., 1994; Lopatin et al., 1994; Shyng et al., 1996). Seven Kir subfamilies (Kir1.0 to Kir7.0) have been identified by cloning, including four members of the Kir2.0 subfamily, Kir2.1, Kir2.2, Kir2.3 and Kir2.4 (Kubo et al., 1993; Koyama et al., 1994; Makhina et al., 1994; Périer et al., 1994; Töpert et al., 1998). At the molecular level, the Kir2.0 channels share ~50 to 70% amino acid identity. Functionally, all four channels of this subfamily are constitutively active and exhibit strong inward rectification. Of particular interest is Kir2.3, which is highly expressed in the heart and brain (Périer et al., 1994) and is modulated by ATP (Collins et al., 1996), protein kinase C (PKC; Henry et al., 1996), G-protein $\beta\gamma$ subunits (Cohen et al., 1996), Mg^{2+} (Chuang et al., 1997), and H^+ (Coulter et al., 1995; Zhu et al., 1999). In this study, we report the modulation of Kir2.0 channels by another important signaling molecule, arachidonic acid, and examine the mechanisms underlying this modulation.

AA (20:4) is a *cis*-polyunsaturated fatty acid (FA) ubiquitously present in the plasma membrane. It is normally linked covalently to other molecules in the membrane to form phospholipids, but can be liberated by activation of cellular phospholipases (e.g., phospholipase A_2). Unesterified, or free AA, has been shown to modulate the activity of a variety of ion channels, including K^+ channels (for review, see Meves, 1994). AA modulation can occur by both direct and indirect mechanisms. Many indirect effects are apparently mediated by AA metabolites [i.e., metabolic products of cyclooxygenase (COX), lipoxygenase (LOX), or cytochrome P450-dependent epoxygenase (P450) (Piomelli et al., 1987; Scherer and Breitwieser, 1990; Hu and Kim, 1993)]. Other indirect effects of AA have been ascribed to processes ranging from activation of PKC to generation of free oxygen radicals (Keyser and Alger, 1990; Schmitt and Meves, 1993). In other cases, however, AA has also been shown to act directly on the channel

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ABBREVIATIONS: Kir, inwardly rectifying K^+ channel; PKC, protein kinase C; AA, arachidonic acid; FA, fatty acid; COX, cyclooxygenase; LOX, lipoxygenase; P450, cytochrome P450-dependent epoxygenase; CHO, Chinese hamster ovary; MEBSS, modified Earle's balanced salt solution; SOD, superoxide dismutase; DMSO, dimethyl sulfoxide; PGE_2, prostaglandin E_2; NDGA, nordihydroguaiaretic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; BSA, bovine serum albumin.

protein or a closely associated site, rather than interacting with the channel indirectly (Giaume et al., 1989; Ordway et al., 1989).

We have studied the effects of AA on cloned human Kir2.0 channels. Here, we demonstrate that AA, as well as other FAs, can potently and reversibly potentiate Kir2.3 currents. This effect seems to result from a direct AA action at an extracellular site on or near the channel and is highly selective for the Kir2.3 subtype of the Kir2.0 channels. Given the high levels of Kir2.3 expression in the brain and heart, it is likely that potentiation of the channel by AA and other FAs, which results in membrane hyperpolarization, could play a significant role in increasing electrical stability of these tissues.

Materials and Methods

Construction of Kir2.0 Channel Clones. Kir2.1, Kir2.2, Kir2.3, and Kir2.4 were cloned from human brain tissues using standard molecular biology techniques as described previously (Curran et al., 1992). Chimeric human Kir2.1/2.3 cDNAs were produced using the mega-primer technique as described by Sarkar and Sommer (1990). Double chimeras were joined at the pore-forming sequence "GYGF" facilitated by an amino acid identity region between Kir2.1 and Kir2.3, which spans 14 amino acids. The amino acid composition of these two constructs is: for Kir2.3–2.1, amino acids 1 to 136 from Kir2.3 coupled to amino acids 145-Stop from Kir2.1; for Kir2.1–2.3, amino acids 1 to 144 from Kir2.1 coupled to amino acids 137-Stop from Kir2.3. Triple chimeras were made using two rounds of mega-primer polymerase chain reaction and used regions of identity at the carboxyl end of the first membrane-spanning domain (M1) and the amino end of the second membrane-spanning domain (M2). The amino acid composition of the triple chimeras is: for Kir2.1–2.3–2.1, amino acids 1 to 113 from Kir2.1, 88 to 147 from Kir2.3 and 156-Stop from Kir2.1; for Kir 2.3–2.1–2.3, amino acids 1 to 87 from Kir2.3, 114 to 155 from Kir2.1 and 148-Stop from Kir2.3. All constructs were confirmed by direct DNA sequencing using automated sequencers and Big-Dye termination chemistries as recommended by the manufacturer (ABI310, PE Biosciences, Foster City, CA).

Channel Expression and Cell Culture. The Kir2.0 constructs were subcloned in the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) and transfected into a mutant form of Chinese hamster ovary (CHO) cells (Steglich and Scheffler, 1982). A CHO cell line was selected that stably and robustly expressed Kir2.3 currents. Cells with stable Kir2.3 expression were harvested and plated on glass coverslips 1 to 4 days before recording. Whole-cell recordings of currents through Kir2.3 channels were performed using the stable line. Transiently transfected cells were used for macro-patch recordings of Kir2.3 currents, as well as whole-cell recordings of currents through Kir2.1, Kir2.2, Kir2.4, and Kir2.1/2.3 chimeras. For transient transfection, Lipofectamine 2000 was used according to a procedure provided by the manufacturer (Life Technologies, Rockville, MD). Channel construct DNAs (1 μg) were cotransfected with CD4 cDNA (0.1 μg). Transfected cells were visually identified for electrophysiological recording by binding of CD4 antibody-coated beads (Jurman et al., 1994) and were used 1 to 3 days after transfection.

For all experiments, cells were grown in low glucose Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum, nonessential amino acids, G-418 (400 mg/l), and putrescine (500 μM) in a humidified, 37°C incubator with 10% CO_2.

Electrophysiology. Standard whole-cell voltage-clamp and patch-clamp techniques were used (Hamill et al., 1981). For whole-cell recording, at least 85% of the series resistance (typically 2–5 MΩ) was compensated. Currents were amplified, digitized (2 kHz), and filtered (5 kHz) with an AXOPATCH 200B patch-clamp amplifier and 1200 series DigiData digitizer (Axon Instruments, Foster City, CA). All data were acquired at room temperature (20–22°C).

Solutions and Chemicals. Recording pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) using a Sutter micropipette puller (Sutter Instruments, Novato, CA) and fire polished with a Narishige microforge (Narishige Scientific Instrument Lab, Tokyo, Japan). Solution-filled pipettes typically had a resistance of 1 to 2 MΩ. For whole-cell recordings, cells were perfused with a modified Earle's balanced salt solution (MEBSS) containing 132 mM NaCl, 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES. The pH of the solution was adjusted to 7.4 with NaOH. The intracellular pipette solution contained: 5 mM NaCl, 40 mM KCl, 100 mM KF, 5 mM EGTA, 3 mM EDTA, 10 mM HEPES, and 5 mM glucose. The pH was adjusted to 7.4 with KOH. For whole-cell experiments with superoxide dismutase (SOD), the pipette solution contained 110 mM K-glutamate, 20 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 5 mM K_2-ATP, and 10 mM HEPES, pH 7.4. Outside-out patches were perfused with the same MEBSS as in whole-cell recording except that 70 mM NaCl were replaced with an equal concentration of KCl. The pipette solution was identical to that used in whole-cell recording. For inside-out patches, the pipette solution was identical to the bath solution for outside-out patches, whereas the solution perfusing the patch was the same as the pipette solution for whole-cell recording.

Stock solutions [10 mM in dimethyl sulfoxide (DMSO), unless otherwise noted] of FAs, prostaglandin E_2 (PGE_2), nordihydroguaiaretic acid (NDGA), indomethacin, H7, Ro-31–8220 (1 mM in DMSO), and SOD (30,000 units/ml in deionized H_2O) were stored in small aliquots at −80°C and freshly thawed and diluted into saline buffer each time before use. All the chemicals were purchased from Sigma (St. Louis, MO), except 5,8,11,14-eicosatetraynoic acid (ETYA) and Ro-31–8220, which were acquired from BIOMOL Research Laboratories (Plymouth Meeting, PA).

Data Analysis. Potentiation by FAs was normalized for each cell to its control current amplitude (the value in the absence of an extracellularly applied FA). In experiments where AA was applied along with a second compound (e.g., indomethacin), the control value was taken as the current amplitude in the presence of the second compound alone (either in the bath or pipette solution or in both the bath and pipette solutions). For whole-cell and outside-out patch recordings, 3 mM Ba^{2+} was used to subtract Kir current from leak current. At the end of an inside-out patch experiment, the patch was exposed to a solution containing either MEBSS or 135 mM CsCl to induce complete current rundown or block. Such records were subsequently used for leak current subtraction. Data were expressed as mean ± S.E.M. with three or more independent observations. The AA dose-response data were normalized for each cell to the difference between the response to 10 μM AA and that in control, averaged for each AA concentration and fitted to a logistic function given by $r = 1 - 1/[1 + (c/EC_{50})^{n_H}]$, where r is the normalized response, c is the AA concentration, EC_{50} is the concentration at which 50% of the maximum AA response is reached, and n_H is the Hill coefficient. Where appropriate, a two-tailed t test was performed to determine the statistical significance of compound effects. Statistical significance is denoted by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) (for first comparisons), or †† ($p < 0.01$) (for second comparisons). The junction potential in the whole-cell experiments (estimated to be ~7 mV using the pCLAMP 8 calculator) was corrected. All data were analyzed off-line using pCLAMP6 (Axon Instruments) and Microcal Origin (version 5.0, Microcal Software, Inc., Northampton, MA).

Results

AA Potently and Selectively Potentiates Kir2.3 Currents. In Fig. 1A, a series of voltage steps (−127 to +23 mV) elicited whole-cell Kir2.3 currents that were strongly inwardly rectifying. Bath application of AA (10 μM) significantly and reversibly enhanced the current amplitude throughout this voltage range. This effect was neither ob-

served in nontransfected cells or cells transfected with the expression vector alone (in which Kir currents were never seen) nor induced by 0.1% DMSO (vehicle control, data not shown). To further address the possibility that the potentiation might be due to a nonspecific detergent effect or potential up-regulation of other currents in the stable cell line, we coapplied AA (10 μM) and Ba^{2+} (3 mM) during a voltage ramp protocol (Fig. 1B). The control ramp current had a reversal potential of −84 mV, close to the equilibrium potential for K^+ of −83 mV predicted by the Nernst equation under these ionic conditions. The AA-induced current did not change this reversal potential, remained inwardly rectifying, and was completely blocked by Ba^{2+}. In addition, AA potentiation was also observed in CHO cells transiently transfected with Kir2.3 cDNA (Fig. 6; whole-cell data not shown). These results are consistent with an AA effect on Kir2.3. The AA potentiation was concentration dependent with an EC_{50} value of 447 nM (Fig. 2A) and maximum potentiation of 2.5-fold at −97 mV (Fig. 2B).

To better understand the nature of this potentiation, we also examined the effect of AA on whole-cell currents through other Kir2.0 channels. The results are shown in Fig. 3. Despite sharing ~60% primary sequence identity with Kir2.3, the currents of Kir2.1, Kir2.2, and Kir2.4 were not affected by AA (10 μM). This suggests that the selective potentiation of Kir2.3 by AA reflects a specific interaction between AA and the Kir2.3 channel protein that is not present for the other Kir2.0 channels.

Potentiation of Kir2.3 Is Independent of AA Metabolism. Metabolic products of AA are known to modulate ion channel activity. To determine whether AA metabolism was involved in the potentiation of Kir2.3, we first examined the

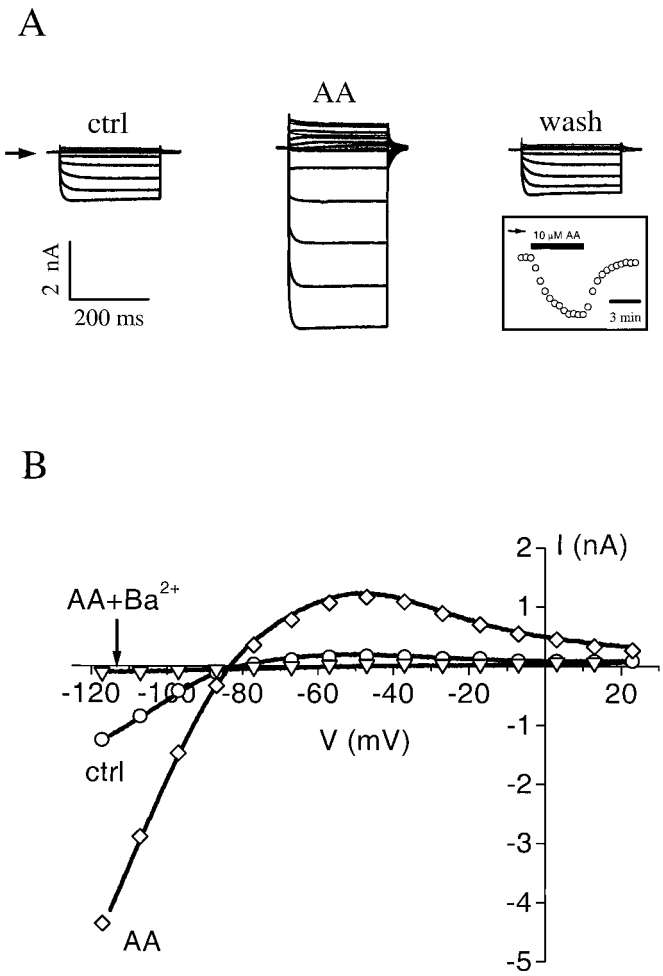

Fig. 1. A, whole-cell Kir2.3 currents in response to a series of 250 ms voltage steps from −127 mV to +23 mV (10 mV increments) before (ctrl), during (AA), and after (wash) application of 10 μM AA. Interpulse intervals were 3 s. Arrow indicates zero current level. The holding potential was −77 mV. Inset: time course of AA activation and wash (−97 mV; arrow is at zero current level). B, Kir2.3 current-voltage relationships. Same cell as in A. Lines represent whole-cell currents elicited by a voltage-ramp protocol (−115 mV to +23 mV; sweep rate: 0.3 V/s). Reversal potential was −84 mV both in the absence and presence of 10 μM AA. AA-induced current was completely blocked by 3 mM Ba^{2+}. Superimposed (open symbols) are steady-state I-V curves under the same conditions (control, 10 μM AA and 10 μM AA + 3 mM Ba^{2+}) as in the ramp protocol.

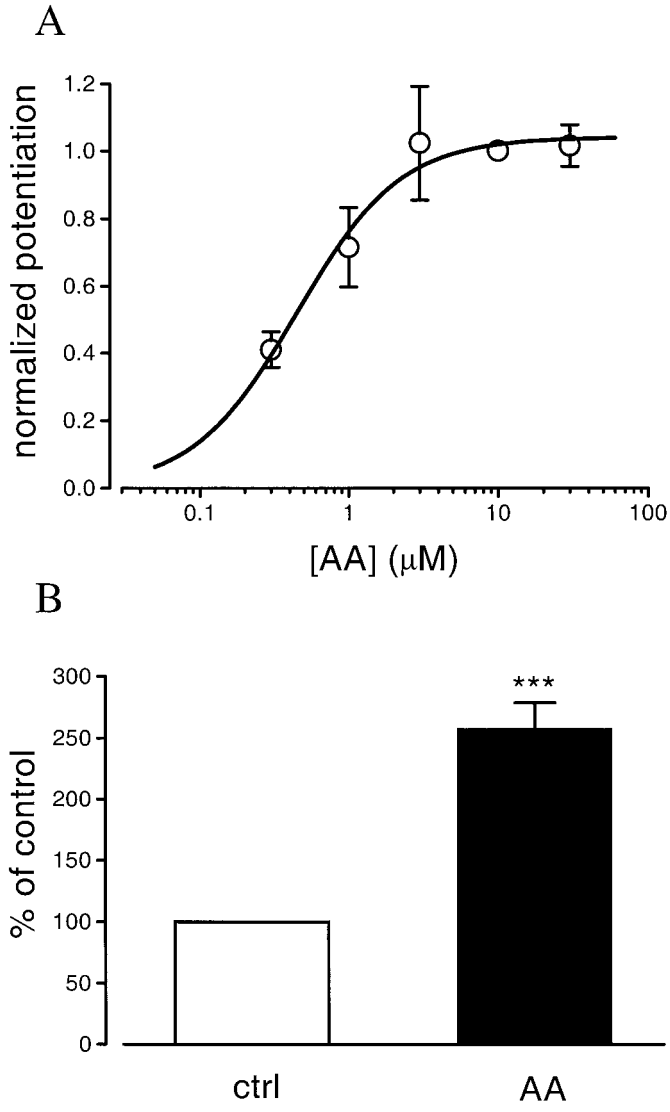

Fig. 2. A, dose dependence of AA potentiation of Kir2.3 whole-cell current. Data from seven cells were individually normalized to the response to 10 μM AA for each cell and fitted to a logistic function as described in the text. The EC_{50} and Hill coefficient values from the fit were 447 nM and 1.3, respectively. B, potentiation of Kir2.3 whole-cell current by 10 μM AA (257.9 ± 20.6% of control, n = 21). For both A and B, cells were hyperpolarized to −97 mV for 250 ms once every 30 s from a holding potential of −77 mV. The average current amplitudes during the last 50 ms of the responses at −97 mV were used in the calculations.

effect of inhibitors of COX, LOX, and P450 pathways on AA potentiation of the channel. Indomethacin (10 μM, COX inhibitor), NDGA (50 μM, LOX inhibitor), or ETYA (30 μM, inhibitor of all three pathways) applied through the whole-cell recording pipette did not significantly affect Kir2.3 current (to allow for diffusion of the compound in the pipette into the cell, we generally waited for 10–30 min after whole-cell formation before starting an experiment). Moreover, in the presence of intracellular ETYA (30 μM) or indomethacin (10 μM), additional extracellular application of the same compound (at the same concentration) had little or no effect on the current (90.7 $\pm$ 2.3% of control, $n = 4$ for ETYA and 100.4 $\pm$ 5.6% of control, $n = 4$ for indomethacin). However, externally applied NDGA (50 μM) completely blocked the whole-cell Kir2.3 current (data not shown). Therefore, experiments with this inhibitor were performed only with intracellular application (50 μM). As shown in Fig. 4A, AA potentiation of Kir2.3 was not affected by any of these metabolic inhibitors.

Further evidence that the AA effect is not the result of metabolism is indicated by the observation that FAs that are not substrates for COX or LOX hydrolysis also mimicked the AA effect. These included *cis-* (oleic acid, 18:1 and palmitoleic acid, 16:1), *trans-* (linolelaidic acid, 18:2), as well as saturated (myristic acid, 14:0) fatty acids (Fig. 4B). Moreover, PGE_2 (10 μM, applied extracellularly), one of the major AA metabolites via the COX pathway, also had no effect on Kir2.3 current (105.6 $\pm$ 2.0% of control, $n = 3$). Finally, AA also increased Kir2.3 current in excised membrane patches (Fig. 6).

PKC and Oxygen Radicals Are Unlikely to Be Involved in AA Potentiation. AA has been reported to modulate ion channels through activation of PKC or generation of oxygen free radicals. Two of the PKC inhibitors we examined, H-7 (50 μM) and Ro-31–8220 (1 μM), had no effect on AA potentiation (Fig. 4C). A third PKC inhibitor, chelerythrine (20 μM), blocked the majority of Kir2.3 current when applied alone either externally in the bath or internally via pipette (data not shown) and was not studied further.[2] Inclusion in the pipette of SOD (100 units/ml), a superoxide free radical scavenger, was also ineffective in preventing AA potentiation (Fig. 4C). In addition, as was reported by others (Bannister et al., 1999), bath application of hydrogen peroxide (0.3%) blocked, rather than enhanced, Kir2.3 current (data not shown).

AA Acts at an Extracellular Site. Because AA can readily cross the membrane (Glatz et al., 1997), it was possible that externally applied AA increased Kir2.3 current by acting at an intracellular site. To address this possibility, we performed a series of whole-cell and excised macro-patch experiments. We first examined if intracellular AA was necessary for potentiation of Kir2.3 current. To this end, 10 μM bovine serum albumin (BSA) was added to the whole-cell recording pipette as an intracellular AA scavenger.[3] Under

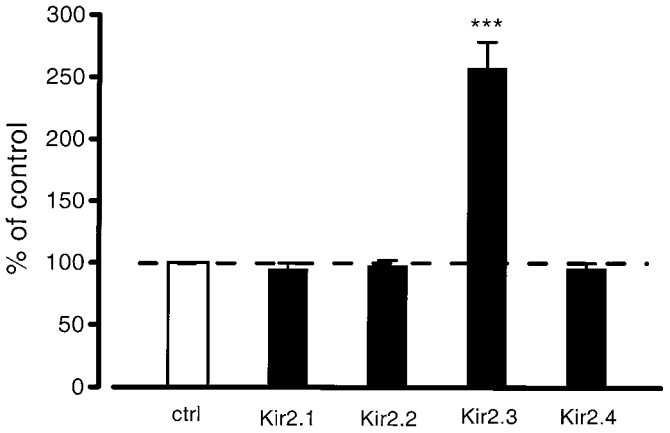

Fig. 3. Selective modulation of Kir2.3 by AA. Whole-cell Kir2.3 current responses to 10 μM extracellular AA (from Fig. 2B) were plotted along with those of Kir2.1, Kir2.2, and Kir2.4 (three cells each). Same voltage protocol as in Fig. 2. Dashed line represents the control (100%) level.

these conditions, external application of BSA alone (10 μM) had no effect on Kir2.3 currents; nor did coapplication of external BSA and AA (10 μM each), as would be expected from the high-affinity binding of AA to BSA. However, subsequent application of external AA alone (10 μM) in the presence of intracellular BSA drastically increased the current amplitude (Fig. 5, A and C), just as observed in the

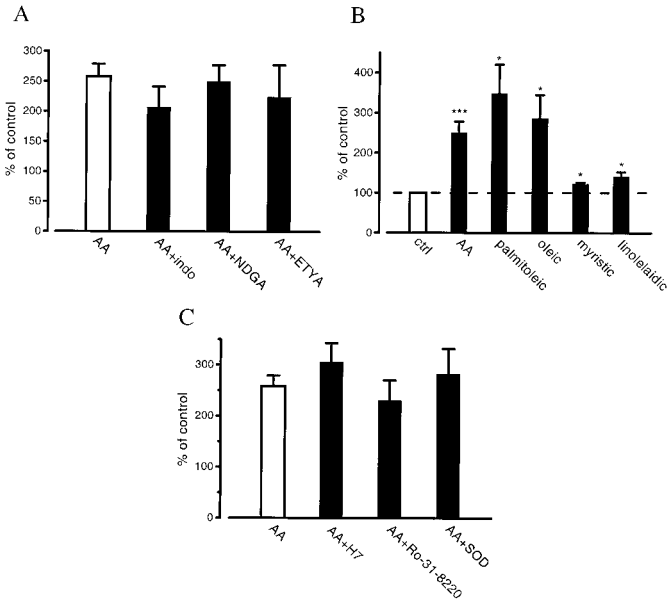

Fig. 4. A, AA potentiation of Kir2.3 whole-cell current in the presence of metabolic inhibitors: indomethacin (indo; 10 μM, $n = 5$), NDGA (50 μM, $n = 8$), and ETYA (30 μM, $n = 5$). NDGA was added only to the recording pipette. ETYA and indomethacin were applied both via pipette and bath perfusion. Responses to AA (10 μM) were normalized to the appropriate control in the presence of an inhibitor (except for AA alone). B, potentiation of Kir2.3 whole-cell current by nonhydrolyzable FAs: palmitoleic (10 μM, $n = 4$), oleic (10 μM, $n = 4$), myristic (3 μM, $n = 3$), and linolelaidic (3 μM, $n = 5$) acids. All responses were readily reversible except for 10 μM oleic acid, which was not reversed after 5 min of wash. Dashed line represents the control (ctrl) level. C, effects of PKC inhibitors, H7 (50 μM in pipette, $n = 6$) and Ro-31–8220 (1 μM in bath, $n = 4$), as well as a superoxide scavenger, SOD (100 units/ml in pipette, $n = 4$), on AA potentiation of Kir2.3 whole-cell current. None of the inhibitors alone seemed to affect the current. Responses to AA (10 μM) were normalized to the appropriate control in the presence of an inhibitor (except for AA alone). All experiments in A, B, and C were performed using the voltage protocol described in Fig. 2.

[2] This block was probably unrelated to PKC inhibition which would be expected to increase (if any), rather than decrease Kir2.3 current (Henry et al., 1996). One possibility is that chelerythrine blocked the Kir2.3 channel directly.

[3] This 66-kDa protein (BSA) has a large capacity and high affinity for binding free FAs, including AA (Bojesen and Bojesen, 1994). It is therefore an ideal AA scavenger here. To ensure proper diffusion of BSA to the cytoplasmic side, we typically selected small-diameter cells (whole-cell capacitance: 10–20 pF), used relatively large pipette tips (1–2 MΩ resistance), and waited for long periods of time (10–40 min after formation of the whole-cell configuration) before acquiring data. Similar precautions were also taken whenever a compound was added to the pipette solution.

absence of intracellular BSA. These results suggested that the potentiation observed in Fig. 2B was not due to intracellular AA.

Second, we addressed whether there might be a separate, intracellular site to which AA might also bind to potentiate Kir2.3. To test for this possibility, we included AA (10 μM) in the recording pipette. If AA potentiated Kir2.3 by binding to an intracellular site, internal application of AA should increase Kir2.3 current upon formation of the whole-cell configuration, independently of external AA. Furthermore, this potentiation would diminish the effect of subsequent application of extracellular AA. Although the whole-cell current did typically increase moderately within the first few minutes of whole-cell formation, it was not an effect of intracellular AA because the same phenomenon also occurred in recordings in which no intracellular AA was applied[4] (data not shown). The effect of extracellular AA in the presence of intracellular AA is illustrated in Fig. 5, B and C. Here, 40 min after formation of the whole-cell configuration, perfusion of extracellular AA (10 μM) still gave rise to a large current increase similar to that seen without intracellularly applied AA, further indicating that intracellular AA was ineffective in increasing Kir2.3 current.

We further investigated the sidedness of the AA action using cell-free macro-patches (Fig. 6), where perfusion of AA and BSA to either side of the membrane could be more easily manipulated. Interestingly, bath application of AA (10 μM) to inside-out patches increased the current amplitude.[5] However, this increase was completely abolished by adding BSA (10 μM) to the pipette (i.e., to the extracellular side of the membrane). In contrast, in outside-out patches, BSA (10 μM) in the pipette did not prevent potentiation by extracellular application of AA (10 μM), in good agreement with the whole-cell experiments shown in Fig. 5. These results argue that the potentiation observed in inside-out patches in the absence of extracellular BSA was induced not by intracellular AA, but by AA that permeated through the membrane to the extracellular side.

Transmembrane and/or Intracellular Domains of Kir2.3 Are Essential for AA Potentiation. The striking difference between the effects of AA on Kir2.3 and on the other Kir2.0 channels (Fig. 3) suggests a specific interaction between AA and the Kir2.3 channel protein. To better understand the nature of this interaction, we constructed chimeras using different regions of Kir2.1 and Kir2.3 (Fig. 7A). As shown in Fig. 7B, a chimera consisting of the N-terminal half of Kir2.3 and the C-terminal half of Kir2.1 (Kir2.3–2.1) did not confer AA potentiation; nor did the reverse chimera, Kir2.1–2.3. The AA effect was only marginally recovered with a chimera consisting of the entire extracellular region of Kir2.3 (including the pore) and the intracellular plus transmembrane segments of Kir2.1 (Kir2.1–2.3–2.1). Interestingly, the reverse chimera, Kir2.3–2.1–2.3 (i.e., the extracel-

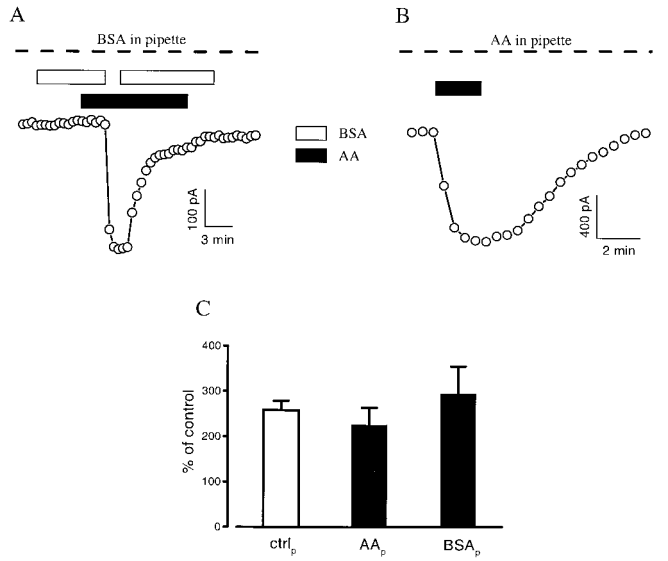

Fig. 5. Potentiation of whole-cell Kir2.3 current in the presence of intracellular BSA or AA. BSA (A; 10 μM) or AA (B; 10 μM) was included in the recording pipette. Extracellular AA (10 μM), BSA (10 μM), or AA + BSA (10 μM each) was perfused as indicated by the horizontal bars above the ○. Dashed lines represent the zero current level. Same voltage protocol as in Fig. 2. ○, average current amplitudes during the last 50 ms of the responses at −97 mV. C, average AA potentiation in five cells in the presence of BSA$_p$ and in seven cells in the presence of AA$_p$. AA$_p$, AA in pipette; BSA$_p$, BSA in pipette; ctrl$_p$, control solution in pipette.

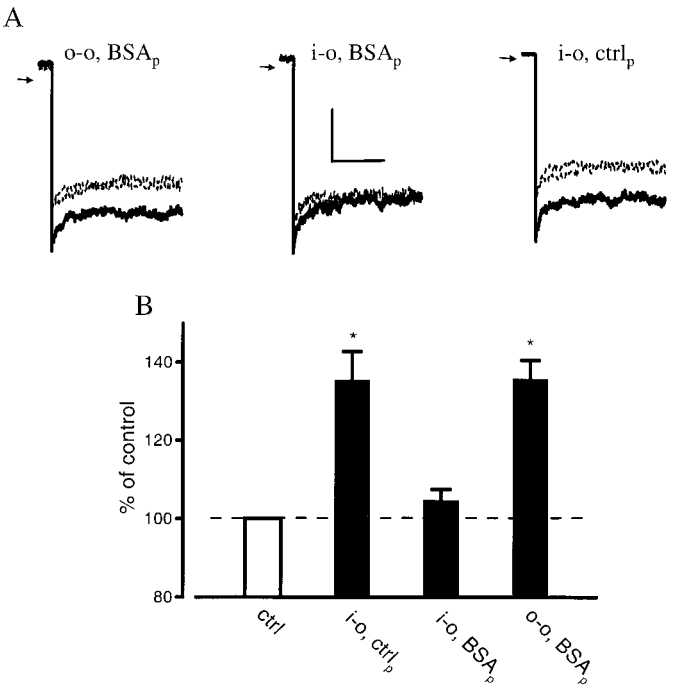

Fig. 6. Effects of AA on Kir2.3 current in excised macro-patches. A, AA (10 μM) was perfused onto outside-out patches with BSA (10 μM) in the pipette (o-o, BSA$_p$; left) or inside-out patches (i-o) either with (BSA$_p$; middle) or without (ctrl$_p$; right) BSA (10 μM) in the pipette. All effects were reversible. Thin dashed traces: control and wash. Thick solid traces: 10 μM AA. Arrows represent zero current level. K^+ concentrations bathing the extracellular and intracellular sides of the membrane were 70 mM and 135 mM, respectively. Patches were held at 0 mV and hyperpolarized to −120 mV for 250 ms once every 10 s. Horizontal bar, 100 ms; vertical bar, 50 pA, 30 pA, and 80 pA (left, middle, and right, respectively). B, average responses to bath application of 10 μM AA to outside-out patches with BSA (10 μM) in the pipette (n = 3) or to inside-out patches either with (n = 3) or without (n = 3) BSA (10 μM) in the pipette. Dashed line represents the control level (no AA).

[4] This initial current run-up was probably due to relief of Kir2.3 inactivation by intracellular Mg^{2+} (Chuang et al., 1997), because there was 3 mM EDTA and 5 mM EGTA in the pipette. All the data were acquired after this current run-up.

[5] Although significant, the amount of AA potentiation in excised patches was substantially smaller than that in whole-cell recordings. This can be largely accounted for by the higher extracellular K^+ concentration (70 mM) and stronger hyperpolarization (−120 mV) applied to the patches, both of which significantly reduce AA potentiation in whole-cell recording as well (Y. Liu et al., unpublished results).

lular domains of Kir2.1 plus the intracellular and transmembrane domains of Kir2.3), was as sensitive to AA as the wild-type Kir2.3 channel. These results suggest that the differential effects of AA on Kir2.1 and Kir2.3 do not arise from the amino acid differences in the extracellular region of these channels. Instead, differences in transmembrane and/or intracellular domains of these channels are responsible for the disparity in their responses to AA.

Discussion

Arachidonic acid can modulate ion channels either directly or indirectly. A major mechanism of indirect modulation involves metabolic hydrolysis of AA via COX, LOX, or P450 pathways (Piomelli et al., 1987; Scherer and Breitwieser, 1990; Hu and Kim, 1993). Other indirect effects have been reported through formation of oxygen radicals (Keyser and Alger, 1990) and activation of PKC (Keyser and Alger, 1990; Schmitt and Meves, 1993). Direct actions of AA itself have been demonstrated for block of gap junctions (Giaume et al., 1989) and activation of smooth muscle K^+ channels (Ordway et al., 1989), as well as two-pore (Fink et al., 1998) and transient receptor potential Ca^{2+} channels (Chyb et al., 1999). A putative FA-binding domain was identified for the *N*-methyl-D-aspartic acid receptor (Petrou et al., 1993), which is subject to AA modulation (Miller et al., 1992). Here, we demonstrated for the first time that AA modulates a human inwardly rectifying K^+ channel, Kir2.3, and that this modulation is probably a direct action on the channel by AA itself.

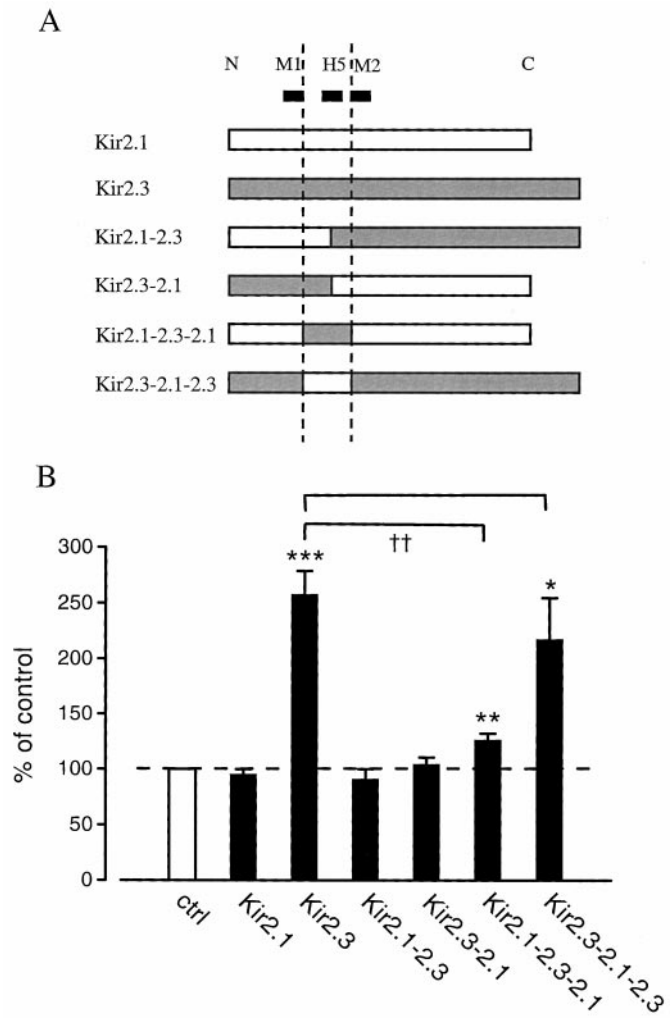

Fig. 7. A, schematic representations of Kir2.1, Kir2.3, and the Kir2.1/2.3 chimeras as labeled. The transmembrane (M1 and M2) and pore (H5) regions are indicated by solid bars at the top. The segment bracketed by the vertical dashed lines is the extracellular (including the pore) region. B, AA (10 μM) effects on whole-cell currents of Kir2.1/2.3 chimeras: Kir2.1–2.3 (n = 3), Kir2.3–2.1 (n = 4), Kir2.1–2.3–2.1 (n = 7), and Kir2.3–2.1–2.3 (n = 7). All the chimeras exhibited currents with strong inward rectification. Same voltage protocol as in Fig. 2 was used. All effects were reversible. Dashed line represents the control level (100%). Second comparisons between (A) Kir2.3 and Kir2.1–2.3–2.1 and (B) Kir2.3 and Kir2.3–2.1–2.3 are indicated by connectors above the bar graph.

Potentiation of Kir2.3 is independent of the AA metabolic cascade. This is evidenced by the observation that this effect a) was not reversed by inhibitors of COX, LOX, or P450 pathways; b) was mimicked by other FAs that are not substrates for metabolism; c) persisted in cell-free membrane patches; and d) resulted from *extracellular* AA action. PGE_2, one of the principal AA metabolites through the COX pathway, was also ineffective. We cannot rule out the possibility that some untested AA metabolites might additionally modulate the channel.

Active oxygen radicals can be generated from AA metabolism. Keyser and Alger (1990) found that AA-induced oxygen radicals suppressed Ca^{2+} current in hippocampal neurons. In contrast, we saw no change in AA potentiation of Kir2.3 current with intracellular application of SOD, a superoxide free radical scavenger. In addition, hydrogen peroxide blocked, instead of enhancing, the Kir2.3 current.

Although activation of PKC has been shown to be involved in AA modulation of ion channels, it is apparently not responsible for the enhancement of Kir2.3 current by AA. First, PKC inhibitors did not prevent the effect. Second, AA was effective in cell-free membrane patches. Finally, activation of PKC *suppressed* Kir2.3 current, as reported by Henry et al. (1996).

Our results with excised patches suggest that Ca^{2+}, nucleotides, and other water-soluble cytosolic factors are not required for AA potentiation of Kir2.3. Thus, signal transduction mechanisms that are dependent upon such factors do not seem to mediate the AA response.

Many *cis*-unsaturated fatty acids, including AA, increase membrane fluidity (Meves, 1994). AA increased not only the Kir2.3 current amplitude, but also its rate of activation (Y. Liu et al., unpublished results). The latter is consistent with an effect caused by an increase in membrane fluidity (Meves, 1994). It is thus tempting to explain the AA effect by its ability to perturb the order of membrane lipids. However, several lines of evidence argue against this explanation. First, ETYA is more effective than AA in increasing membrane fluidity (Brown et al., 1992), but does not potentiate Kir2.3. Second, saturated and *trans*-unsaturated FAs do not increase membrane fluidity (Meves, 1994). Yet, both myristic acid (saturated) and linolelaidic acid (*trans*-unsaturated) potentiate Kir2.3. Finally, the AA effect is mediated by extracellular, but not intracellular, AA. Such sidedness would not be expected if perturbation of the membrane lipid structure was the underlying mechanism. Likewise, micelles or detergent effects can also be ruled out on the basis of sidedness, and the observation that the AA response is readily reversible and fully Ba^{2+}-sensitive. Taken together, our data sup-

port a mechanism by which AA acts directly to potentiate Kir2.3.

It is well established that the uptake of AA and other long-chain FAs by mammalian cells is rapid (for review, see Glatz et al., 1997). Therefore, it is not surprising to see AA effects from either side of the membrane (Ordway et al., 1989; Fink et al., 1998) because AA may "flip" across the membrane bilayer to reach a putative site of action. Using molecules with a large charged head group, Petrou et al. (1995) presented evidence that FAs bind only to an intracellular site of a smooth muscle K^+ channel, despite their ability to activate the channel from both sides of the membrane. In our study, AA potentiation was also observed in both inside-out and outside-out patches. However, intracellularly applied AA failed to reproduce the effect when BSA, a high-affinity AA-binding protein, was included on the extracellular side. In contrast, extracellularly applied AA was effective regardless of whether intracellular BSA was present. These results argue that the potentiation results from an AA action at an extracellular site. It is possible, although less likely, that a transmembrane site with a side preference for AA binding may be involved.

Of the Kir2.0 channels, only Kir2.3 is modulated by AA. This suggests an interaction between AA and Kir2.3 that is specific to this channel. Because AA seems to act at an extracellular site, we first sought to explain this specificity by extracellular differences between Kir2.3 and the other Kir2.0 channels. Alignment of the extracellular sequences of these channels revealed a 12 amino-acid insertion in Kir2.3 between M1 and H5 (the pore region) that is absent in the other channels. We hypothesized that if this stretch were responsible for the specific AA effect on Kir2.3, then a chimera consisting of the N-terminal half of Kir2.3 that encompasses this stretch and the C-terminal half of Kir2.1 (Kir2.3–2.1) might retain AA sensitivity. This did not turn out to be the case. In fact, even a chimera consisting of the entire extracellular region of Kir2.3 (Kir2.1–2.3–2.1) was unable to restore much of the AA sensitivity. In contrast, the AA effect was virtually fully recovered, with the reverse chimera comprising the transmembrane and intracellular domains of Kir2.3 and the extracellular region of Kir2.1 (Kir2.3–2.1–2.3). One explanation for these results is that AA may bind to an extracellular region that is identical in Kir2.1 and Kir2.3. Alternatively, AA may be capable of binding to both Kir2.1 and Kir2.3 despite amino acid differences at the binding site. In either scenario, the AA action does not seem to be affected by replacing the extracellular domains of Kir2.3 with those of Kir2.1. This suggests that the molecular determinants for selective potentiation of Kir2.3 lie in the transmembrane and/or intracellular region(s) of the channel. An attractive possibility is that these molecular determinants may be responsible for transduction of the AA action on the channel activity. Additional experiments will be necessary to test for this possibility and other potential mechanisms.

Inhibition of Kir2.3 has been reported for a number of endogenous signaling molecules, including ATP (Collins et al., 1996), PKC (Henry et al., 1996), G-protein $\beta\gamma$ subunits (Cohen et al., 1996), Mg^{2+} (Chuang et al., 1997), and H^+ (Coulter et al., 1995; Zhu et al., 1999). Here, we showed that AA, another endogenous molecule, *potentiates* rather than inhibits Kir2.3 current at physiological pH. This action of AA (and other FAs) may be important for regulating cellular excitability. In rat hippocampal neurons, AA causes hyperpolarization of the resting membrane potential (Carlen et al., 1989). In rat cardiomyocytes, polyunsaturated FAs, including AA, hyperpolarize the resting membrane potential, shorten the duration, and decrease the frequency of action potentials (Kang et al., 1995). These results may, in part, explain the anti-arrhythmic actions of polyunsaturated FAs (Kang et al., 1995). Given the tissue distribution of Kir2.3 (Périer et al., 1994), it is conceivable that these effects may, at least in part, be mediated by potentiation of Kir2.3.

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