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Decrease of inward rectification as a mechanism for arachidonic acid-induced potentiation of hKir2.3

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Abstract Previously, we showed that arachidonic acid (AA) potentiates currents flowing through a cloned human inwardly rectifying K^+ channel, hKir2.3. The mechanism by which this potentiation occurs is not understood. Here, we report that this potentiation is mediated by multiple mechanisms and that one of them, which we studied in more detail, is consistent with AA-induced decrease of inward rectification. AA (10 μM) potentiation of hKir2.3 whole-cell current increased with depolarization (40% greater at –47 mV than at –127 mV) and decreased with elevated extracellular [K^+] (158 ± 21%, 56 ± 8% and 38 ± 9% in 5.4, 70 and 135 mM K^+, respectively). Hyperpolarization elicited inward currents consisting of an instantaneous and two time-dependent components with time constants (at –97 mV) of 6.4 ± 1.1 ms and 27.8 ± 4.1 ms, respectively. AA (10 μM) significantly decreased the slow time constant (14.1 ± 0.7 ms). Consistent with the kinetic changes, AA (10 μM) right-shifted the voltage dependence of the chord conductance (mid-point shifted by + 9 mV). In inside-out patches where inward rectification was minimal, AA potentiation (38 ± 3%) was smaller than in whole-cell recording and was not voltage dependent. These results are consistent with the idea that AA potentiates hKir2.3 in part by decreasing inward rectification of the channel.

Keywords Inwardly rectifying potassium channel · Kir2.3 · Arachidonic acid · Potentiation · Inward rectification

Abbreviations *AA*: arachidonic acid · *CHO*: Chinese hamster ovary · *DMSO*: dimethyl sulfoxide · *Kir*: inwardly rectifying potassium channel · PIP_2: phosphatidylinositol 4,5-bisphosphate

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Introduction

Inwardly rectifying potassium channels (Kirs) are characterized by a subunit topology of two membrane-spanning segments that surround a pore region (Kubo et al. 1993). These channels share a common property of allowing K^+ ions to flow into the cell more readily than out of the cell. This inward rectification is largely attributed to voltage- and $[K^+]_o$-dependent channel block by intracellular Mg^{2+} and polyamines (Matsuda et al. 1987; Vandenberg 1987; Ficker et al. 1994; Lopatin et al. 1994; Stanfield et al. 1994; Fakler et al. 1995; Shyng et al. 1996). Upon depolarization, these positively charged molecules are driven into the channel pore and obstruct the flow of K^+ ions through the channel (Lopatin et al. 1995). Conversely, hyperpolarization relieves the block, giving rise to channel "activation", which manifests as time-dependent inward current. Both the kinetic and steady-state parameters of channel activation depend on $[K^+]_o$, such that higher $[K^+]_o$ causes faster activation and less rectification at a given membrane potential (Hagiwara et al. 1976; Leech and Stanfield 1981; Lopatin and Nichols 1996).

Based on sequence homology as well as functional and pharmacological properties, Kir channels are grouped into seven subfamilies, Kir1.0–Kir7.0. The constitutively active Kir2.3 belongs to a subfamily of Kirs (Kir2.0) with strong inward rectification. It is highly expressed in the human heart and brain (Périer et al. 1994) and is thought to play an important role in maintaining the cell resting membrane potential (Hille 1992). Modulation of Kir2.3 has been demonstrated for a number of signaling molecules (Coulter et al. 1995; Cohen et al. 1996; Collins et al. 1996; Henry et al. 1996; Chuang et al. 1997; Zhu et al. 1999), including phosphatidylinositol 4,5-bisphosphate (PIP_2), which appears to be essential for maintaining the constitutive activity of the channel (Zhang et al. 2001). Recently, we reported that this constitutive activity is enhanced by arachidonic acid (AA) and other long-chain fatty acids (Liu et al.

2001). In this study, we sought to understand the mechanism of this potentiation. In particular, we investigated the possible involvement of inward rectification. Our results are consistent with the idea that AA potentiates human Kir2.3 (hKir2.3) in part by decreasing inward rectification of the channel.

Materials and methods

Channel expression and cell culture

Human Kir2.3 was sub-cloned in the expression vector pcDNA3.1 (Invitrogen, Carlsbad, Calif.) and stably expressed in a mutant line of Chinese hamster ovary (CHO) cells (Steglich and Scheffler 1982) as previously described (Liu et al. 2001). Cells were grown in low glucose Dulbecco's modified Eagle's medium supplemented with 5% heat inactivated fetal bovine serum, non-essential amino acids, G-418 (400 mg/L) and putrescine (500 μM) in a humidified, 37 °C incubator with 10% CO_2. Whole-cell and macro patch clamp recordings were performed 1–4 days after cells were plated on uncoated glass cover slips.

Electrophysiology

Standard whole-cell as well as macro patch clamp recording techniques were used (Hamill et al. 1981). The series resistance (typically 2–5 MΩ) was compensated (≥85%) in all experiments. Recording pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, Fla.) using a Sutter micropipette puller (Sutter Instrument, Novato, Calif.) and fire polished with a Narishige microforge (Narishige Scientific Instrument Lab, Tokyo, Japan). Solution-filled pipettes typically had a resistance of 1–2 MΩ. Currents were amplified, filtered and digitized with an AXOPATCH 200B patch-clamp amplifier and 1200 series Digi-Data digitizer (Axon Instruments, Foster City, Calif.). Data were acquired at room temperature (20–22 °C).

Solutions

For whole-cell recording, cells were perfused with a modified Earle's balanced salt solution (MEBSS) containing (mM): 132 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 10 glucose and 10 HEPES, pH = 7.4. Two other extracellular solutions containing either (mM) 70/67 KCl/NaCl or 135/0 KCl/NaCl (otherwise identical to MEBSS) were also used in some experiments to elevate $[K^+]_o$. Experiments involving these two solutions are indicated accordingly. The intracellular solution contained (mM): 5 NaCl, 40 KCl, 100 KF, 5 EGTA, 3 EDTA, 10 HEPES and 5 glucose, pH = 7.4. For inside-out patches, the pipette solution contained 70 mM KCl and 67 mM NaCl and was otherwise identical to MEBSS. The patch-perfusing solution was the same as the intracellular solution for whole-cell recording.

The stock solution of AA (10 mM in DMSO) was stored in small aliquots at –80 °C and freshly thawed and diluted into the perfusing solution each time before use. AA was purchased from Sigma (St. Louis, Mo.).

Data analysis

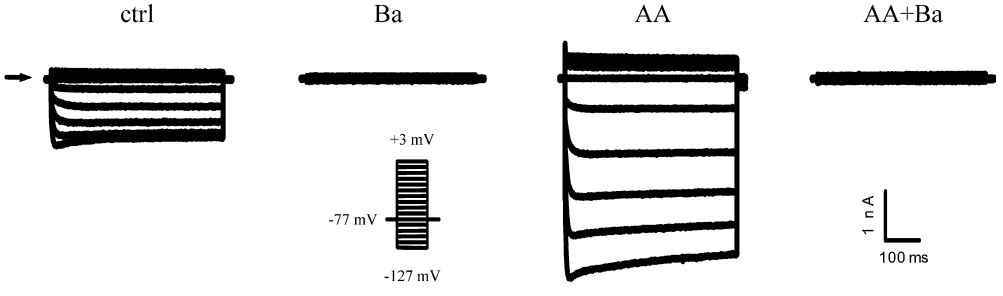
Percent (%) potentiation by AA was calculated as $(I_{AA}/I_{ctrl}–1) \times 100$, where I_{ctrl} and I_{AA} are current amplitudes in the absence and presence of AA. Fold potentiation by AA was calculated as I_{AA}/I_{ctrl}. The chord conductance was calculated as $I_V/(V–E_K)$, where I_V is the steady-state current amplitude at membrane potential V, and E_K is the equilibrium potential for potassium. The chord conductance was then normalized to the value at –127 mV for each cell. Records in the presence of 3 mM Ba^{2+} were used to subtract Kir current from leak current in whole-cell recordings. For inside-out patch experiments, a solution containing 135 mM CsCl was perfused to completely block the Kir current. Such records were used for leak current subtraction. Data were expressed as mean ± SEM, and the number of independent experiments (n) was given. The normalized chord conductance was fitted with a double Boltzmann function given by $g_c = A/(1+\exp\{(V–V1_{1/2})/dx_1\}) + (1–A)/(1+\exp\{(V–V2_{1/2})/dx_2\})$, where g_c is the normalized chord conductance, V is membrane voltage, A and $1–A$ are fractional amplitudes of the two components, $V1_{1/2}$ and $V2_{1/2}$ are voltages at which 50% of the maximum responses for each component (i.e. 50% of A and $1–A$, respectively) are reached, and dx_1 and dx_2 are the slope factors for the two components. Where appropriate, two-tailed student's t-tests were performed to determine the statistical significance of AA effects. Statistical significance is denoted by * ($P < 0.05$). The junction potentials in whole-cell recording were corrected. Data were analyzed off-line using pCLAMP6 (Axon Instruments) and Microcal Origin (version 5.0, Microcal Software, Northampton, Mass.).

Results

AA potently and reversibly potentiated hKir2.3 currents

In Fig. 1, a series of step voltage pulses (–127 to + 3 mV) elicited whole-cell hKir2.3 currents with strong inward rectification. Bath application of 10 μM AA significantly enhanced the current amplitude throughout this voltage range. The AA-induced current was fully sensitive to Ba^{2+}, suggesting that it flowed through hKir2.3 channels.

Fig. 1 AA potentiates whole-cell hKir2.3 currents heterologously expressed in CHO cells. Currents were elicited by 500 ms voltage steps from –127 mV to + 3 mV (in 10 mV increments; also see inset for protocol) in control (*left*), 3 mM Ba^{2+} (*second from left*), 10 μM AA (*second from right*) and 10 μM AA + 3 mM Ba^{2+} (*right*). Inter-pulse intervals were 3 s. The *arrow* on the *left* indicates zero current level. The holding potential was –77 mV



Previously, we have shown that this potentiation is potently dependent on the AA concentration with an EC_{50} value of 447 nM at –97 mV (Liu et al. 2001).

AA potentiation of hKir2.3 was dependent on membrane potential

The time course of AA potentiation of whole-cell hKir2.3 currents is shown in Fig. 2A for two membrane potentials, –97 mV and –57 mV. Increases in the current amplitude were observable within 30 s of AA application. The control current amplitude at –57 mV (~25 mV above E_K) was much smaller than that at –97 mV (~15 mV below E_K), as would be expected of K^+ currents with strong inward rectification. However, the relative current increase in the presence of AA (10 μM) was significantly higher at –57 mV than at –97 mV. This effect is illustrated more clearly in Fig. 2B, in which the fold potentiation by AA was plotted over a wider range of membrane potentials. Here, AA potentiation was steeply voltage dependent near E_K and was ~40% greater at –47 mV than at –127 mV. The voltage dependence of AA potentiation could be described by a Boltzmann function with a $V_{1/2}$ value of –90.7 mV.

The effect of AA was also manifest in Fig. 3, where the relative chord conductances in the absence and presence of 10 μM AA were plotted as a function of membrane potential. Both sets of data were well fitted with a double Boltzmann function. AA shifted the chord conductance curve to more positive potentials. The voltage at which the chord conductance was decreased by 50% was right shifted by 9 mV in the presence of 10 μM AA, from –86 mV to –77 mV.

It is interesting to note that significant AA potentiation was still present at very hyperpolarized potentials, between –127 mV and –107 mV (see Figs. 1 and 2B). Potentiation in this voltage range was distinguishable from that described above by its lack of voltage dependence (Fig. 2B).

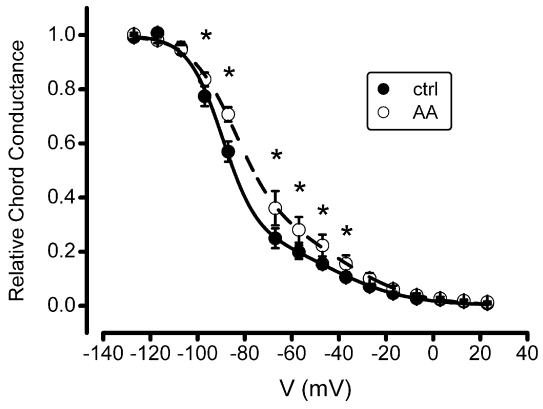
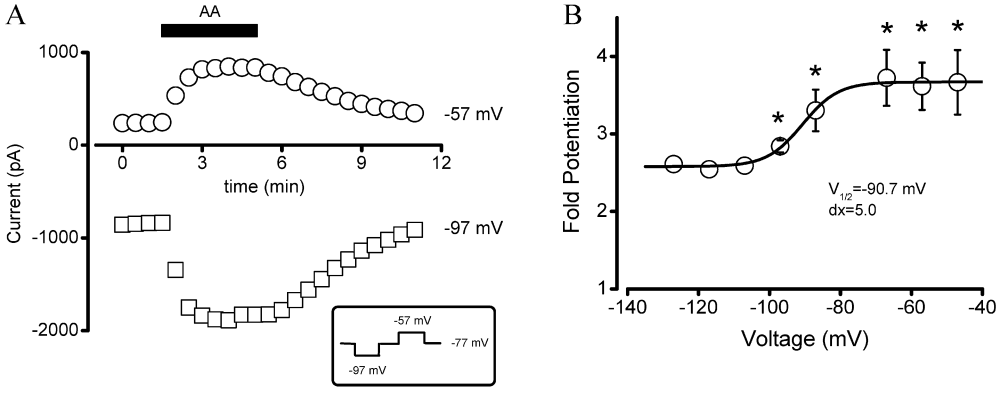


Fig. 3 AA right shifts the voltage dependence of the chord conductance. Same voltage protocol as in Fig. 1. The average current amplitude during the last 50 ms of the response to each test potential was used in the calculations. The chord conductances in control (*solid circles*) and in 10 μM AA (*open circles*) were individually normalized to the respective values at –127 mV for each cell ($n=7$). Both sets of data were fitted to a double Boltzmann function (*solid curve*: control; *dashed curve*: 10 μM AA). The results of the fits are as follows. For control, $V_{1/2}$ = –89.4 mV (relative amplitude = 72.8%) and –43.4 mV; dx = 7.3 and 16.5, respectively. For 10 μM AA, $V_{1/2}$ = –84.2 mV (relative amplitude = 68.3%) and –38.3 mV; dx = 9.4 and 15.8, respectively. *Asterisks* (*) indicate statistically significant differences ($P<0.05$) between the control and AA values at the corresponding voltages

Fig. 2A, B AA potentiation of whole-cell hKir2.3 currents is voltage dependent. **A** Time course of AA (10 μM) potentiation and wash at –57 mV (*circles*) and –97 mV (*squares*). Once every 30 s the cell was stepped from a holding potential of –77 mV to –97 mV or –57 mV for 250 ms each (see *inset* for voltage protocol). The average current amplitude during the last 50 ms of the response to each test potential was used in the calculations. $[K^+]_o$ was 5.4 mM. **B** Voltage dependence of AA (10 μM) potentiation. *Solid curve* was the best fit of the data ($n=7$) to a Boltzmann function with a $V_{1/2}$ value of –90.7 mV and slope factor (dx) of 5.0. *Asterisks* (*) denote statistically significant differences ($P<0.05$) between the value at –127 mV and those at the indicated voltages. Where the error bars are omitted, they are smaller than the size of the symbol. Voltage protocol was the same as that in Fig. 1 except for the fewer voltage steps here

AA potentiation of hKir2.3 was dependent on extracellular $[K^+]$

Because AA potentiation of hKir2.3 was a function of the membrane potential on which the degree of inward rectification steeply depends, we wanted to see whether changes in $[K^+]_o$, another factor that alters inward rectification, might also affect AA potentiation. We first



showed that, as reported for other inwardly rectifying K^+ channels (Hagiwara and Takahashi 1974; Sakmann and Trube 1984; Ho et al. 1993; Kubo et al. 1993; Périer et al. 1994), the membrane conductance of hKir2.3 increased with the square root of $[K^+]_o$ (Fig. 4A, inset). As a result, the steady-state current amplitude (Fig. 4A, B) in 70 mM K^+ was much bigger than that in 5.4 mM K^+ at voltages 15 mV negative to their respective E_K (–97 mV and –32 mV, respectively). However, the relative increase in current amplitude by AA was the opposite: much smaller in 70 mM K^+ than in 5.4 mM K^+. It was decreased even further in 135 mM K^+. The average AA potentiation was 158 ± 21%, 56 ± 8% and 38 ± 9% in 5.4, 70 and 135 mM K^+, respectively (Fig. 4C).

Fig. 4A–C Dependence of AA potentiation on $[K^+]_o$. **A** Whole-cell hKir2.3 current traces from a single cell in the absence (*solid lines*) and presence (*dashed lines*) of 10 μM AA for 5.4 mM K^+ (*left panel*; holding and test potentials were –77 mV and –97 mV, respectively) and 70 mM K^+ (*right panel*; holding and test potentials were –12 mV and –32 mV, respectively). *Arrows* represent the zero current level. *Inset*: dependence of the channel chord conductance on $[K^+]_o$. *Open circles* represent steady-state conductance values at –97, –32 and –15 mV for 5.4 ($n = 8$), 70 ($n = 6$) and 135 ($n = 5$) mM K^+, respectively. These voltages are 15 mV below the respective E_K values for the three K^+ concentrations. Conductance values were normalized for each cell to that in 5.4 mM K^+. Error bars are smaller than the size of the symbols. The linear fit to the data yielded a slope factor of 0.49. **B** Time course of AA (10 μM) potentiation in 5.4 mM (–97 mV; *black bar*) and 70 mM (–32 mV; *open bar*) extracellular K^+. **C** Plot of fold AA (10 μM) potentiation in 5.4 ($n = 12$), 70 ($n = 6$) and 135 ($n = 3$) mM $[K^+]_o$. Cells were hyperpolarized to the respective potentials (–97 mV, –32 mV and –15 mV for 5.4, 70 and 135 mM K^+, respectively) for 250 ms once every 30 s from a holding potential of –77 mV (5.4 mM K^+), –12 mV (70 mM K^+) and 5 mV (135 mM K^+), respectively. The average current amplitudes during the last 50 ms of the responses to these hyperpolarizing steps were used in the calculations

AA accelerated the activation kinetics of hKir2.3

The kinetics of activation of whole-cell hKir2.3 currents in response to hyperpolarization consisted of three components: instantaneous, fast and slow (Table 1), in agreement with the results from studies of Kir2.3 expressed in *Xenopus* oocytes (Lopatin et al. 1995). The instantaneous component is thought to correspond to channel block by putrescine and/or Mg^{2+}, whereas the slower, time-dependent components reflect block by spermine and spermindine (Ficker et al. 1994; Lopatin et al. 1995). The time constants of the two time-dependent components at –97 mV were 6.4 ± 1.1 ms (τ_f) and 27.8 ± 4.1 ms (τ_s), respectively. The time constant of the slow component was decreased by ~50% in the presence of 10 μM AA, to 14.1 ± 0.7 ms (Fig. 5 and Table 1). Accordingly, the relative amplitude of this component at –77 mV was also decreased in 10 μM AA, from 41 ± 3% (control) to 29 ± 4% (10 μM AA).

AA potentiation of hKir2.3 in inside-out membrane patches was smaller and voltage independent

To further evaluate the relation between channel rectification and AA potentiation, we studied the AA effect in inside-out macro patches, in which inward rectification was greatly diminished due to diffusion of cytosolic blocking particles (e.g., polyamines) away from their site(s) of action (Fig. 6A). Although AA (10 μM)

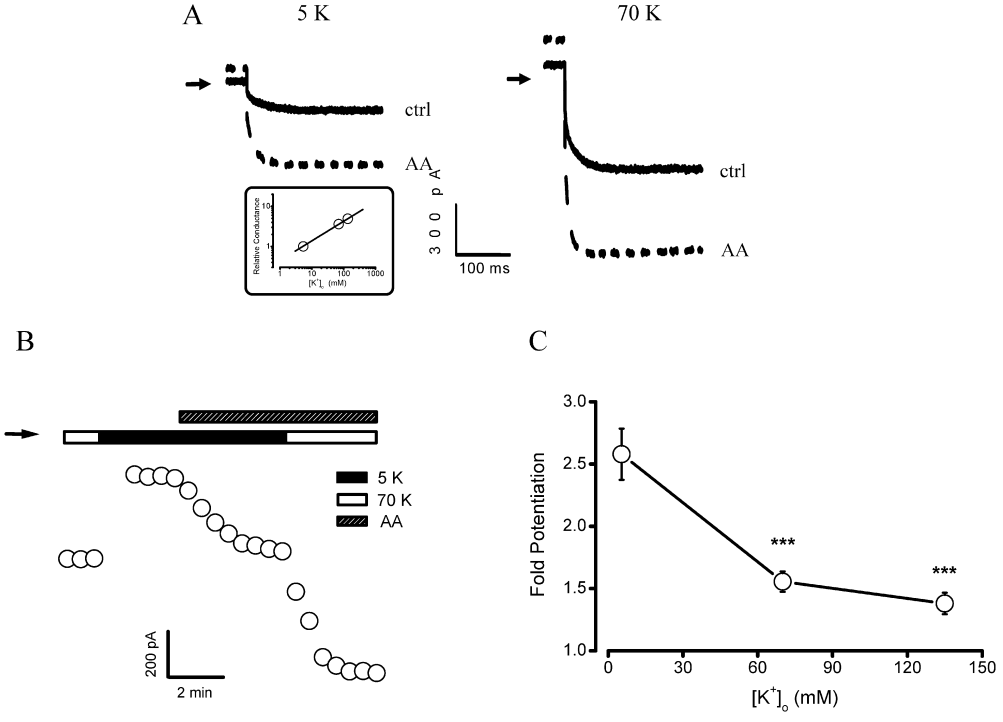


Table 1 Summary of the effect of AA on the time-dependent components of current activation of hKir2.3. Cells bathed in 5.4 mM K^+ were hyperpolarized to –97 mV from a holding potential of –77 mV. The time-dependent current was well fitted to a double exponential function in both control and 10 μM AA. τ_f and A_f: time constant and relative amplitude of the fast component; τ_s and A_s: time constant and relative amplitude of the slow component. Asterisks (*) indicate statistically significant differences ($P < 0.05$) from control values

Condition	τ_f (ms)	τ_s (ms)	A_f (% total)	A_s (% total)	$A_f + A_s$ (% total)	n
Control	6.4 ± 1.1	27.8 ± 4.1	9.8 ± 2.3	40.7 ± 2.9	50.5 ± 3.0	4
10 μM AA	5.8 ± 0.6	14.1 ± 0.7*	11.1 ± 3.3	28.5 ± 4.2*	39.5 ± 1.8*	4

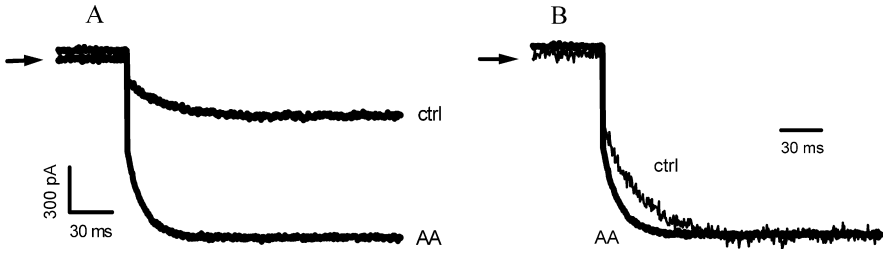


Fig. 5A, B AA accelerates the kinetics of current activation of hKir2.3. **A** Whole-cell currents in control and 10 μM AA in response to a 200 ms hyperpolarizing pulse to –97 mV (holding potential was –77 mV). **B** Normalized currents from **A**. The steady-state current amplitude at –97 mV in control was normalized (×3.1) to that in AA. *Arrows* in **A** and **B** represent the zero current level

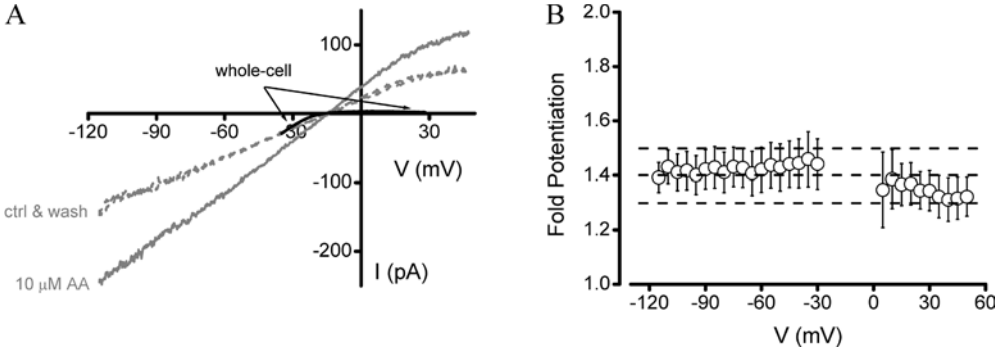
potentiation was still present under these conditions (38 ± 3%; $n = 10$; also see Fig. 5A), it was significantly smaller than that in whole-cell recording. In addition, the "residual" potentiation in inside-out patches was no longer voltage dependent (Fig. 6B), in contrast to the steep voltage dependence of AA potentiation in whole-cell recording, where polyamines were present (Fig. 2B).

Fig. 6A, B AA potentiation of hKir2.3 currents in inside-out membrane patches. **A** Current-voltage relationship for an inside-out patch in the absence (*gray dashed curves*) and presence (*gray solid curve*) of 10 μM AA. The pipette solution contained 70 mM K^+. Currents were elicited by a ramp protocol (from –115 mV to +47 mV at 425 mV/s). For comparison of rectification, the whole-cell *I-V* curve (from –35 mV to +33 mV; *black solid curve*) in 70 mM $[K^+]_o$ was superimposed. The whole-cell current was normalized to the control patch current at –35 mV. **B** Voltage dependence of AA potentiation in inside-out patches. Data ($n = 6$) were obtained from ramp *I-V* curves such as those shown in **A** and were binned (5 mV/bin). *Dashed lines* represent potentiation levels of 1.3, 1.4 and 1.5, respectively

Discussion

Previously, we reported that arachidonic acid is a potent and selective activator of the inwardly rectifying K^+ channel, hKir2.3 (Liu et al. 2001). The mechanism by which this potentiation occurs was not clear and is the subject of the current study. Several possibilities exist that may account for the AA potentiation. For example, AA may alter the channel gating or increase single channel conductance and/or the number of active channels. Alternatively, it may decrease channel inactivation induced by intracellular Mg^{2+} (Chuang et al. 1997). Here, we sought to investigate whether another process, inward rectification, might be responsible for this AA action. We studied how AA potentiation is affected by conditions that alter inward rectification, such as membrane potential, $[K^+]_o$, and cell-free membrane patches. Reciprocally, we also examined how AA affects inward rectification, as reflected by changes in the channel activation kinetics and the voltage dependence of the chord conductance. Our results support the idea that AA potentiates hKir2.3 in part by reducing inward rectification of the channel.

AA potentiation of whole-cell hKir2.3 current increases steeply with depolarization. This occurs in a voltage range in which the chord conductance is also steeply dependent on membrane potential. It is unlikely



that, in the absence of inward rectification, AA would increase the single channel conductance (if any) in a voltage-dependent manner. In addition, an increase in the number of active channels would only result in a voltage-independent current increase. Instead, a more plausible scenario for the voltage-dependent potentiation and its correlation with the voltage dependence of the chord conductance is that AA destabilizes the (voltage-dependent) channel block by intracellular polyamines. In other words, as more channels are blocked by polyamines with increasing depolarization, more channels will also be available for unblock via AA-induced dissociation of polyamines from their blocking site(s), resulting in increased potentiation. This explanation is consistent with the observation that AA increases not only the absolute, but also the relative chord conductance, resulting in a right shift of its voltage dependence. This shift is qualitatively similar to the right shift of the voltage dependence of the chord conductance caused by elevated $[K]_o$. That the rate of channel activation [i.e., the rate of polyamine dissociation from the blocking site(s)] is increased by AA lends further support to this hypothesis. The AA-mediated, faster channel activation is also reminiscent of similar effects induced by elevated $[K^+]_o$, which destabilizes polyamine block (Lopatin and Nichols 1996).

If the hypothesis is correct that AA decreases inward rectification, one would expect that decreasing inward rectification by means other than hyperpolarization should also decrease AA potentiation. To this end, we explored two additional avenues of manipulating the level of inward rectification in our experiments. Specifically, we sought to decrease inward rectification by either raising $[K^+]_o$ (to destabilize polyamine block via electrostatic repulsion) or recording from cell-free membrane patches [to allow diffusion of polyamines away from their site(s) of action]. Indeed, we found that AA potentiation of hKir2.3 was compromised under both conditions. Furthermore, in inside-out membrane patches where little inward rectification remained, the AA potentiation was smaller and no longer voltage dependent, in contrast to the steep voltage-dependence of potentiation present in whole-cell recording. These results further support the idea that AA potentiation involves the removal of inward rectification.

At present, we do not know how AA decreases inward rectification. We speculate that this action may involve allosteric interactions between AA and the site(s) of polyamine block. One piece of supportive evidence comes from the intracellular nature of polyamine block (Ficker et al. 1994; Lopatin et al. 1994; Shyng et al. 1996) and our previous observation that AA acts on the extracellular side of the channel (Liu et al. 2001). It is also intriguing to note that, in contrast to hKir2.3, none of the other members of the hKir2.0 subfamily are sensitive to AA (Liu et al. 2001), despite sharing ~60% amino acid identity with hKir2.3 and exhibiting similarly strong inward rectification. Thus, it appears unlikely that AA interferes directly with the inward rectification process per se. Instead, decrease of inward rectification may be secondary to an AA-induced conformational change that is specific to Kir2.3. Previously, we have shown that intracellular and/or transmembrane domains of hKir2.3 are essential for AA potentiation (Liu et al. 2001). This suggests that the molecular determinants for selective potentiation of hKir2.3 lie in the transmembrane and/or intracellular region(s) of the channel. It is tempting to speculate that these critical determinants may serve to relay the signal of (extracellular) AA binding to the (intracellular) site(s) of polyamine block.

The existence of voltage-independent AA potentiation both in inside-out patches and at very negative potentials in whole-cell recording (see Figs. 2B and 6B) suggests that processes other than voltage-dependent inward rectification are also involved. Shyng et al. (1996) noted that inhibition of polyamine synthesis in cells heterologously expressing Kir2.1 resulted in a voltage-dependent current increase, which gave rise to a +10 mV shift in the voltage dependence of the chord conductance. Additionally, it also caused a voltage-independent current increase at very negative potentials, suggesting the possibility of voltage-independent polyamine block in the untreated cells expressing Kir2.1 [also see Lopatin et al. (1994) for possible voltage-independent polyamine block of Kir2.3]. These results bear qualitative resemblance to what we observed here with AA. Our data do not rule out the possibility of an AA-sensitive, voltage-independent component of polyamine block in hKir2.3.

Alternatively, other processes, such as channel gating, may mediate the voltage-independent potentiation. Recent discoveries of the critical role of PIP_2 in maintaining the activity of Kirs, including Kir2.3 (Zhang et al. 2001), raise the possibility that AA may potentiate hKir2.3 by changing the level of PIP_2 in the plasma membrane. Another process, Mg^{2+}-induced inactivation, has been reported to occur in Kir2.3 (Chuang et al. 1997). However, while it is conceivable that AA may destabilize this inactivated state, the presence of EGTA (5 mM) and EDTA (3 mM) in our intracellular solution makes it a less likely scenario. Additional experiments will be necessary to test for these and other potential mechanisms.

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