0026-895X/08/7304-1185–1194$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 73:1185–1194, 2008

Vol. 73, No. 4
43067/3320957
Printed in U.S.A.

Arachidonic Acid Activates Kir2.3 Channels by Enhancing Channel–Phosphatidyl-inositol 4,5-bisphosphate Interactions

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Received October 30, 2007; accepted January 15, 2008

ABSTRACT

Kir2.0 channels play a significant role in setting the resting membrane potential, modulating action potential wave form, and buffering extracellular potassium. One member of this family, Kir2.3, is highly expressed in the heart and brain and is modulated by a variety of factors, including arachidonic acid (AA). Using two-electrode voltage clamp and inside-out patch clamp recordings from *Xenopus laevis* oocytes expressing Kir2.3 channels, we found that AA selectively activated Kir2.3 channels with an EC_{50} of 0.59 μM and that this activation required phosphatidyl inositol 4,5-bisphosphate (PIP_2). We found that AA activated Kir2.3 by enhancing channel-PIP_2 interactions as demonstrated by a shift in PIP_2 activation curve. EC_{50} for channel activation by PIP_2 were 36 and 12 μM in the absence and presence of AA, respectively. A single point mutation on the channel C terminus that enhanced basal channel–PIP_2 interactions reduced the sensitivity of the channel to AA. Effects of AA are mediated through cytoplasmic sites on the channel by increasing the open probability, mainly due to more frequent bursts of opening in the presence of PIP_2. Therefore, enhanced interaction with PIP_2 is the molecular mechanism for Kir2.3 channel activation by AA.

Inwardly rectifying K^+ (Kir) channels are essential in the control of the resting membrane potential, coupling of the metabolic cellular state with membrane excitability, and maintenance of potassium homeostasis (Doupnik et al., 1995; Nichols and Lopatin, 1997). Kir2.0 channels, also known as IRK channels, play a significant role in setting the resting membrane potential, buffering extracellular potassium, and modulating the action potential wave form (Jan and Jan, 1997). Four members of the Kir2.0 subfamily have been described: 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). At the molecular level, the Kir2.0 channels share 50 to 70% amino acid identity. Viewed from a functional perspective, all four channels of this subfamily are constitutively active and exhibit strong inward rectification. Kir2.3, which is highly expressed in the heart and brain (Périer et al., 1994), 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), pH (Coulter et al., 1995; Zhu et al., 1999a), arachidonic acid (AA) (Liu et al., 2001) and the membrane phospholipid phosphatidyl inositol 4,5-bisphosphate (PIP_2) (Du et al., 2004).

In cardiac and nervous tissues, regulation of cellular excitability is critical in homeostasis and calcium handling and can affect the response to ischemia and injury. Hence, ion channels that regulate cellular excitability, such as inwardly rectifying potassium channels, can play a pivotal role in response to inflammation and injury. The role of the polyunsaturated fatty acid AA in ischemia and inflammation in diverse tissues such as cardiac and nervous system is well established (Van der Vusse et al., 1997; Kang et al., 1995); therefore, AA effects on Kir and other channels in these tissues can have critical consequences. It has been reported that AA and its amide anandamide modulate two-pore domain K^+ channels (Fink et al., 1998) and TRP channels (Watanabe et al., 2003) and are key determinants in inactivation of delayed rectifiers K^+ channels (Oliver et al., 2004).

A common feature of Kir channels that has emerged re-

This work was supported by National Natural Science Foundation of China grant 30730031 and by National Basic Research Program grant 2007CB512100. H.Z. is a beneficiary of the National Science Fund for Distinguished Young Scholars of China grant 30325038. T.M. is supported by Beginning Grant-in-Aid 0765275U from the American Heart Association.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.107.043067.

ABBREVIATIONS: Kir, inwardly rectifying potassium channel; PKC, protein kinase C; AA, arachidonic acid; PIP_2, phosphatidyl inositol 4,5-bisphosphate; MS-222, ethyl 3-aminobenzoate; HK, high-potassium solution; LK, low-potassium solution; LC-PIP_2, long-chain (arachidonyl-stearoyl) PIP_2; BSA, bovine serum albumin; diC_8PIP_2, dioctanoyl-PIP_2.

cently is that they all require PIP_2 to maintain their activity (Huang et al., 1998; Shyng and Nichols, 1998; Zhang et al., 1999; Du et al., 2004). Furthermore, recent studies indicate that PIP_2 may play an important role in modulation of these channels by several factors, including pH, receptor stimulation, PKC, and Mg^{2+} (Du et al., 2004). PIP_2 interaction with Kir channels controls gating primarily through electrostatic interactions with positively charged residues on the channel N and C termini. Separate, noncharged structural elements in the channel may play a secondary effect on channel interaction with PIP_2 (Rosenhouse-Dantsker and Logothetis, 2007). Specific modulators may affect one or both of these interactions to control channel activity (Logothetis et al., 2007).

Among the four members of Kir2.0 family, only Kir2.3 is sensitive to regulation by AA (Liu et al., 2001). Although a direct action on Kir2.3 and channel inward rectification has been proposed (Liu et al., 2002), the molecular mechanism for these actions remains unclear. There is also a general lack of information regarding mechanisms of AA modulation of other channels mentioned above. In the present study, we demonstrate that AA interacts with intracellular sites and activates Kir2.3 channels by enhancing channel-PIP_2 interactions.

Materials and Methods

Molecular Biology. All cDNA constructs were subcloned into the pGEMHE plasmid vector and used as described. Point mutants were produced by Pfu mutagenesis with a QuikChange kit (Stratagene, La Jolla, CA). Sequences were confirmed by DNA sequencing. Recombinant mouse Kir2.3 channels (gift of L. Jan, University of California, San Francisco, CA) and human M1 receptors (gift of X. Huang, Weill Cornell Medical College, New York, NY) were expressed in *Xenopus laevis* oocytes as described previously (Du et al., 2004). In brief, ovaries were extracted from frogs anesthetized with 0.25% MS-222, and oocytes were dissociated by collagenase digestion and mechanical agitation in calcium free OR-2 media. cRNA was produced with T7 RNA polymerase using a RiboMax in vitro transcription kit (Promega, Madison, WI). cRNA of the various Kir channels and their mutants and of receptors were injected in the range of 0.5 to 2 ng/oocyte depending on the functional expression level of the given construct.

Electrophysiology. Recordings in *X. laevis* oocytes were performed 2 to 4 days after cRNA injection. Whole-oocyte currents were measured by conventional two-microelectrode voltage clamp with a GeneClamp 500 amplifier (Molecular Devices, Sunnyvale, CA). Electrodes were filled with 3 M KCl dissolved in 1% agarose to prevent the leakage of KCl into the oocytes. The electrodes had resistances less than 1 MΩ. Oocytes were constantly perfused with either a high-potassium solution (HK) containing 96 mM KCl, 1 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4, or a low-potassium solution (LK) containing 96 mM NaCl, 1 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4. Oocytes membranes were held at 0 mV and current amplitudes were measured using a ramp protocol from −140 to +40 mV or alternating voltage steps to −80 and +80 mV repeated once every second. Data acquisition and analysis were achieved using pClamp9 (Molecular Devices) and Origin 7.5 (OriginLab Corp., Northampton, MA) software.

Macropatch channel activity was recorded from devitellinized oocytes under the inside-out mode of standard patch-clamp methods using an Axon 200B patch-clamp amplifier and pClamp9 data-acquisition software (Molecular Devices). Electrodes were made from borosilicate glass (WPI, Sarasota, FL) using a Sutter P-97 microelectrode puller and gave a tip diameter of 5 to 15 μm that had a resistance of 0.5 to 1 MΩ when filled with an electrode solution containing HK. Three bath solutions were used: 1) a fluoride, vanadate, and pyrophosphate (FVPP)-sodium solution containing 96 mM KCl, 5 mM EDTA, 10 mM HEPES, 5 mM NaF, 0.1 mM Na_3VO_4, and 10 mM Na_2PO_7, pH 7.4, to prevent current rundown (Huang et al., 1998; Zhang et al., 1999); 2) an HK solution containing 96 mM KCl, 5 mM EGTA, 1 mM Mg^{2+}, and 10 mM HEPES, pH 7.4; and 3) Mg^{2+}-free HK. Current amplitudes were measured at −80 mV with a sampling rate of 100 Hz.

Single channel activity was recorded from devitellinized oocytes under the cell-attached and inside-out modes of standard patch-clamp methods using an Axon 200B patch-clamp amplifier and pClamp9 data-acquisition software (Molecular Devices). Electrodes with resistances of 5 to 8 MΩ were used, and the data were collected at 10 kHz and filtered at 5 kHz before analysis for kinetic parameters. For the AA experiments in the cell-attached mode, oocytes were preincubated with AA, and AA was included in both the bath and the pipette solutions for the duration of the recording. Burst probability and duration were analyzed with a burst delimiter determined using pSTAT (Molecular Devices). Four separate burst delimiters (15, 100, 300, and 500ms) were used to initially analyze the data and a 300-ms burst delimiter was chosen for the final analysis according to the manufacturer's instructions (pClamp 6 Users' Manual, page 607). For calculations of the burst durations, openings separated by closed events shorter than the burst delimiters were concatenated, and then mean durations were calculated for each record as the arithmetic mean for the burst durations of the same record. Other kinetic parameters were determined as described elsewhere (Rohács et al., 2002) using two separate custom made software generously provided by Drs. Taihao Jin (University of California, San Francisco, CA) and László Csanády (Semmelweis University, Budapest, Hungary).

Chemicals. diC_8PIP_2 (sodium salt), a water-soluble derivative of PIP_2, was purchased from Cayman Chemical (Ann Arbor, MI). It was dissolved in water at a concentration of 25 mM, aliquoted, and stored at −80°C. Before experiments, a new aliquot was thawed and diluted in the bath solution, which was used only on that day. Long-chain (arachidonyl-stearoyl) PIP_2 (LC-PIP_2) was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA), dissolved in dimethyl sulfoxide at a concentration of 25 mM, aliquoted, and stored at −20°C. Before experiments, a new aliquot was thawed, diluted in the bath solution, and sonicated for 30 min on ice before use, and this aliquot was used only on that day. AA was purchased from MP Biomedicals (Irvine, CA), dissolved in dimethyl sulfoxide, and frozen in aliquots, which were thawed immediately before use. Polylysine was purchased from Sigma (St. Louis, MO), dissolved in water (90 mg/ml), aliquoted, and kept at −20°C.

Statistics. Dose-response curves were generated using nonlinear regression analysis (Prism 4.0; GraphPad Software, San Diego CA). Error bars in the figures represent S.E.M. A minimum of two to three batches of oocytes were tested for each experiment shown. Paired or unpaired *t*-tests or one-way analysis of variance with Dunnet's post hoc test were used to assess statistical significance where appropriate. Dose-responses and shifts in EC_{50} were examined for significance using the *F* test, considering the 95% confidence limit for each curve.

Results

AA Activated Kir2.3 Channels Expressed in *X. laevis* Oocytes. It has been shown previously that AA could activate Kir2.3 channels expressed in CHO cells (Liu et al., 2001). To gain insight into the mechanism of this activation, we expressed Kir2.3 channels in *X. laevis* oocytes and tested the effects of AA on these currents. Figure 1A shows the inward current trace recorded by two-electrode voltage clamp at −130 mV in LK (the dotted line indicates the zero current level). Application of AA (10 μM) to the bath solution in-

creased substantially the inward current. The current amplitude recovered to the pre-AA level after washout. Barium, a specific blocker of inwardly rectifying potassium currents, totally abolished the inward current (Fig. 1A). The effect of AA on current-voltage relationship is shown in Fig. 1B. The Kir2.3 currents show characteristic inward rectification in the LK solution, and this property was maintained even when AA increased the current amplitude. No voltage dependence of AA's effects was observed. Barium inhibited AA-activated and control currents; the three current-voltage relationships intersect around the calculated reversal potential for the potassium currents. Thus, in agreement with previous studies from channels expressed in CHO cells (Liu et al., 2001), AA activates the Kir2.3 currents expressed in *X. laevis* oocytes.

The effectiveness of AA in activating Kir2.3 was determined by measuring the dose response for channel activation (Fig. 1C). The data were fitted using nonlinear regression. The concentration needed for the half-maximal potentiation of Kir2.3 currents (EC_{50}) by AA is 0.59 μM.

AA Selectively Activated Kir2.3 Depending on the Channel's Characteristic Interaction with PIP$_2$. AA selectively activates Kir2.3 without significant effects on other members of Kir2.0 family, such as Kir2.1, Kir2.2, and Kir2.4 (Liu et al., 2001). We have shown previously that one of the significant differences among these channels is that PIP$_2$ interacts more weakly with Kir2.3 than with other Kir2.0 family members (Du et al., 2004). This weaker interaction makes Kir2.3 more susceptible to regulation by factors like PKC, membrane receptors, and pH (Du et al., 2004). In light of these findings, we reason here that the specificity seen for AA-induced activation may also require the characteristic weaker interaction between Kir2.3 and PIP$_2$. To test this hypothesis, we made the point mutant I213L in Kir2.3 and studied AA-induced action on this mutant. Our previous work has shown that the critical amino acid at this position (either an isoleucine or leucine) determines the strength of Kir channel-PIP$_2$ interaction (Zhang et al., 1999; Du et al., 2004). An isoleucine-to-leucine mutation in Kir2.3 (I213L) strengthened channel-PIP$_2$ interactions [EC_{50} for PIP$_2$ was ~29 μM for Kir2.3 and ~8 μM for Kir2.3(I213L)] (Du et al., 2004). Kir 2.1 has a leucine at this position naturally and interacts with PIP$_2$ strongly (Zhang et al., 1999; Du et al., 2004). Thus, we compared AA effects on whole-cell currents in oocytes expressing Kir2.3 or Kir2.3(I213L) (Fig. 1D). AA (10 μM)significantly increased Kir2.3 currents (130 ± 30% of basal current, n = 10), and only slightly, but not significantly, increased Kir2.3 (I213L) currents (14 ± 4% of basal, n = 6). To test whether weak PIP$_2$ interaction in any Kir2 channel was sufficient to sensitize them to AA activation, we tested the effects of AA on Kir2.1 and the mutant Kir2.1(R312Q), which has a weaker interaction with PIP$_2$ compared with wild-type Kir2.1(Du et al., 2004). We previously showed that in contrast with wild-type channel, Kir2.1(R312Q) was sensitive to receptor- and PKC-mediated modulation (Du et al., 2004). When expressed in oocytes, neither Kir2.1 nor Kir2.1(R312Q) was activated by 10 μM AA. The changes in these currents were 0.3 ± 4% of basal for Kir2.1 (n = 5) and 0.3 ± 2% of basal for Kir2.1(R312Q) (n = 6), neither of which was significant. These data indicated that weak PIP$_2$ interactions are necessary but not sufficient to confer AA sensitivity to Kir2 channels. These results further suggested that specific AA-interacting site(s) are present in Kir2.3 but absent in Kir2.1.

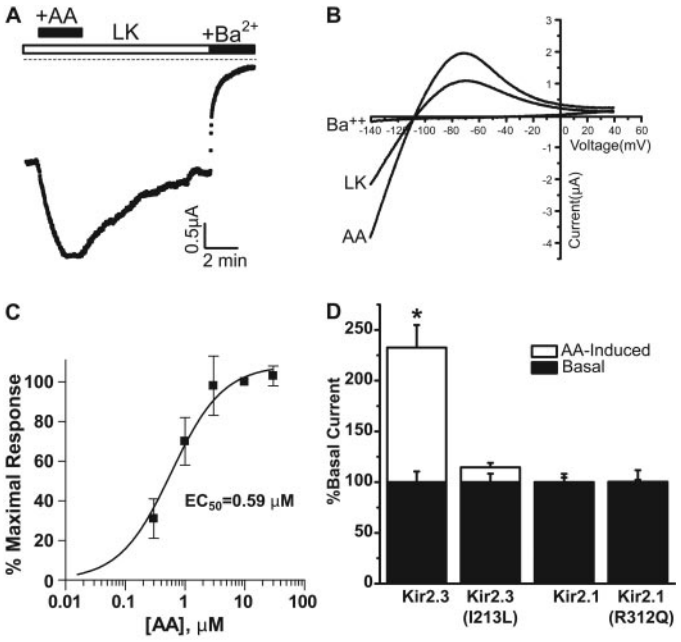

Fig. 1. AA specifically activates Kir2.3 currents expressed in *X. laevis* oocytes. Kir2.3 currents were recorded using two-electrode voltage clamp. A, time course of AA (10 μM) action on Kir2.3 currents recorded at −130 mV in LK solution. The dotted line indicates the zero current level. AA activated Kir2.3 currents in a reversible manner. B, the I-V curves of Kir2.3 currents induced by a voltage ramp from −140 to +40. AA induced-currents were completely blocked by $BaCl_2$ (3 mM); the latter was used to subtract Kir currents from leak currents. C, concentration-effect relationship curve constructed for AA-induced enhancement of Kir2.3 currents. Current enhancement data from 10 cells were individually normalized to the control value (obtained using 10 μM AA) and fitted using nonlinear regression. The EC_{50} value was 0.59 μM. D, the effects of AA (10 μM) on Kir2.3, Kir2.3(I213L), Kir2.1, and Kir2.1(R312Q) currents. All currents were normalized to corresponding basal current (before AA application). AA induced significant currents only in the Kir2.3 group. (n = 5–10; *, $p < 0.01$ compared with basal current, paired t test.)

AA Required PIP$_2$ for Channel Activation. We next tested whether AA could directly activate Kir2.3 currents and whether this activation was dependent on PIP$_2$. Data shown in Fig. 2 are from inside-out macropatches recorded at −80 mV in oocytes expressing Kir2.3 (e.g., Fig. 2A). When a patch was excised (inside-out) into a solution that contains FVPP to inhibit lipid phosphatases and thus prevent breakdown of PIP$_2$ (Huang et al., 1998), the channel ran up, the current stabilized, and AA (3 μM) induced a robust activation of Kir2.3 current. The same patch was then run down by exposing to polylysine in HK solution, which removed PIP$_2$ from the patch and the channel (Lopes C.M. et al., 2002). Application of AA (3 μM) induced a significantly smaller current compared with before channel rundown. Summary of these data are shown in Fig. 2B, where currents are normalized to the on-cell current for the same patch for comparison and analysis. These data suggest that AA cannot readily reactivate a channel that has rundown as a result of loss of PIP$_2$; hence, AA requires PIP$_2$ to activate Kir2.3 channels.

AA and PIP$_2$ Synergistically Activated Kir2.3 Currents by Increasing the Channel's Sensitivity to PIP$_2$. Our data so far suggest that AA activation of the Kir2.3 channels requires PIP$_2$. To test the interdependence of AA and PIP$_2$ for channel activation, we compared their effects on

Kir2.3 when applied separately or in combination. For this purpose, a water-soluble PIP_2, diC_8PIP_2, which produces a reversible activation of Kir channels (Zhang et al., 1999; Rohács et al., 2003), and subsaturating concentrations of AA (0.3 μM) were used. After Kir2.3 currents ran down in inside-out patches in HK solution, multiple application of 0.3 μM AA failed to activate significant Kir2.3 currents (Fig. 2C). Application of diC_8PIP_2 alone induced robust Kir2.3 currents. Simultaneous application of AA and PIP_2 activated Kir2.3 currents to a level that is larger than the additive effects induced when AA and PIP_2 were applied separately (Fig. 2C). Summary data from these experiments shown in Fig. 2D clearly indicate that AA activates Kir2.3 only in the presence of PIP_2, and perhaps this activation occurs through an enhanced channel-PIP_2 interaction.

AA Accelerated Channel Activation by PIP_2. Given the increased PIP_2 effects on channel in the presence of AA, we speculated that AA would alter the kinetics of PIP_2 interaction with the channel. To test this possibility, we analyzed both the activation and deactivation kinetics of channel by PIP_2 in the presence and absence of AA. Figure 3A shows representative tracings from two inside-out patches where Kir2.3 channels were activated using LC-PIP_2 in the presence and absence of AA. The amplitude for the traces was normalized to facilitate easier comparison. The channel activated more rapidly in the presence of AA. The bar graph below shows time to half-maximal activation (T_{50}) for several such recordings. PIP_2 activated Kir2.3 with and without AA with T_{50} values of 31 ± 2 and 80 ± 4 s, respectively, which were significantly different from one another ($p < 0.05$, unpaired t test).

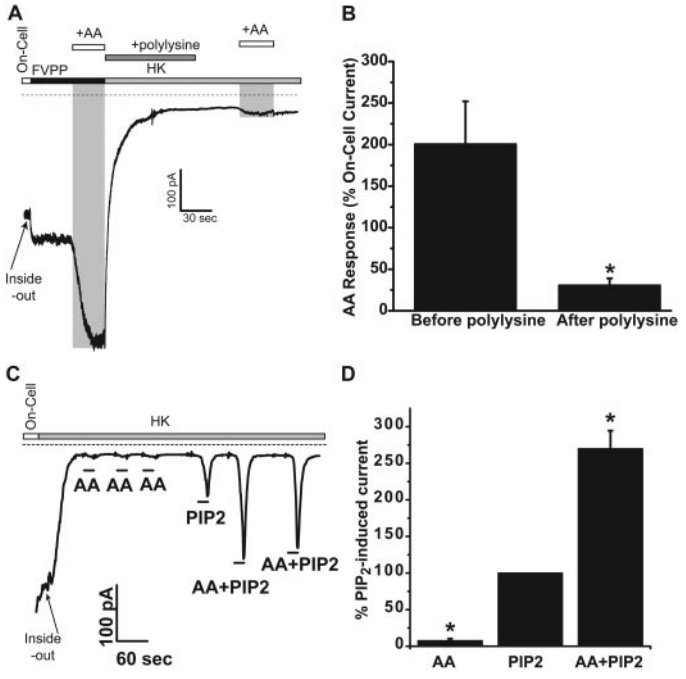

Fig. 2. AA directly activates Kir2.3 currents and requires PIP_2 for its effect. A, inside-out macropatch from an oocyte expressing Kir2.3 held at −80 mV. The patch was excised into FVPP solution, the channel "ran up," and then stabilized. Application of AA (3 μM) in FVPP via the bath enhanced Kir2.3 currents. The dotted line indicates zero current level. The channel was then run down using polylysine in HK. Application of AA (3 μM) in HK induced only small currents after channel rundown. B, summary data for Kir2.3 current induction by AA. Data were normalized to the corresponding on-cell currents for each patch. The AA response after rundown was significantly smaller than the response before rundown ($n = 4$; *, $p < 0.01$ paired t test). C, AA requires PIP_2 for Kir2.3 channel activation and they activate currents in a synergistic manner. AA (0.3 μM), or diC_8PIP_2 (10 μM) were applied alone or in combination to inside-out macropatches from oocytes expressing Kir2.3 held at −80 mV. Kir2.3 currents were run down and AA, diC_8PIP_2, and AA+diC_8PIP_2 were applied to the patch as indicated. AA alone has minimal effects on currents that had run down (also see Fig. 2A), diC_8PIP_2 alone activated the currents in a reversible manner, and application of AA with diC_8PIP_2 induced a larger-than-additive activation. D, summary data from repeated experiments ($n = 5$) as shown in C. (*, $p < 0.01$ compared with PIP_2 alone, paired t test.)

AA slowed Inhibition of Kir2.3 Current Due to PIP_2 Removal. To test the effects of AA on the kinetics of channel deactivation, we used the polylysine block assay. The speed with which Kir channels in inside-out patches are blocked by polylysine or PIP_2 antibody reflects channel deactivation as a result of loss of PIP_2 interactions (Zhang et al., 1999; Lopes et al., 2002). Figure 3B, top, shows current traces of Kir2.3 with and without AA (0.3 μM) during the process of polylysine inhibition. Summary data of the half-time (T_{50}) for polylysine inhibitions (Fig. 3B, bottom) show that AA slowed the polylysine block of Kir2.3 currents, suggesting enhanced channel-PIP_2 interactions. Polylysine blocked Kir2.3 with T_{50} values of 14.7 ± 0.8 and 5.0 ± 0.4 s with and without AA, respectively, which were significantly different from one another ($p < 0.05$, unpaired t test).

AA Effects on Kinetics of Fast-Acting diC_8PIP_2. LC-PIP_2 is most likely incorporated into the membrane, which is reflected in the slow kinetics with which it activates Kir2.3 channels. To test the effects of AA on kinetics of the PIP_2-induced channel activation more directly, we used the water-soluble short-chain PIP_2 (diC_8PIP_2) that washes on and off the patch rapidly and facilitates better measurement of activation and deactivation kinetics. Sample traces from two inside-out macro-patches where diC_8PIP_2 was applied in the presence or absence of AA are shown in Fig. 3C. In the presence of AA, diC_8PIP_2 application activated the channels more rapidly. Furthermore, the channel deactivated more slowly upon removal of diC_8PIP_2. Fig. 3D shows normalized currents for easier comparison. Summary data for activation and deactivation T_{50} values in the absence or presence of AA are shown in Fig. 3, E and F, respectively. Together, the data from Fig. 3 suggest that the presence of AA may facilitate enhanced channel-PIP_2 interactions.

AA Enhanced Apparent Potency of PIP_2 for Channel Activation. Noncontiguous regions in the N- and C-termini of Kir channels facilitate PIP_2 interactions; therefore, direct PIP_2 binding experiments to the intact channel are not feasible. However, these interactions can be assayed functionally using the water-soluble diC_8PIP_2. To more directly test whether the AA effects seen above are due to enhanced interactions of PIP_2 with Kir2.3, we performed concentration-response experiments for PIP_2 activation of Kir2.3 in the presence or absence of AA. We used two different concentrations of AA (0.3 and 3 μM) and diC_8PIP_2 that produces a reversible activation of K channels (Zhang et al., 1999; Rohács et al., 2003) for these experiments. Kir2.3 currents activated by various concentrations of diC_8PIP_2 in the absence or presence of 3 μM AA are shown in Fig. 4, A and B, respectively. Concentration-response relationship curves constructed from several such recordings are shown in Fig. 4C. Short chain diC_8PIP_2 activates Kir2.3 with half-maximal concentration (EC_{50}) of 36.3 μM [−1.5 ± 2.3 (S.E.M.); 95%

confidence limits, 21–62 μM] in the absence of AA, which is consistent with our previously published report (Du et al., 2004). In the presence of 0.3 and 3 μM AA, the diC$_8$PIP$_2$ EC$_{50}$ was reduced to 18.7 μM [−1.3 ± 1.4 (S.E.M.); 95% confidence limits, 12–27 μM] and 11.8 μM [−0.4 ± 0.6 (S.E.M.); 95% confidence limits, 10 to 13 μM], respectively. The EC$_{50}$ with 3 μM AA was significantly different from the EC$_{50}$ without AA ($p < 0.01$, F test). In addition, AA did not increase the maximal currents induced by saturating concentrations of diC$_8$PIP$_2$ (300 μM). Without AA, maximal channel activation was 28 ± 6% of the corresponding on-cell current ($n = 6$), whereas with AA, the maximal level was 35 ± 5% of on cell current ($n = 4$), which was not significantly different. Thus, AA indeed increases the apparent potency of PIP$_2$ for Kir2.3 activation without a change in maximal activation.

Effects of AA on Kir2.3 Single-Channel Activity. PIP$_2$ is the common requirement for activation of all Kir channels (Du et al., 2004; Guy-David and Reuveny, 2007). Although the role of PIP$_2$ on channel gating has not been examined explicitly, in the case of Kir2.3, PIP$_2$ may act as a gating molecule because it is the only required signal for channel activation. To gain insight into the mechanism by which AA influences PIP$_2$ interaction with Kir2.3, we tested the effects of AA on properties of Kir2.3 single channels expressed in oocytes. We recorded single channel activity at −80 mV for extended periods from oocytes in the cell-attached mode. Figure 5, A and B, shows sample cell attached recordings from two oocytes with and without AA. The oocytes were incubated with AA (5 μM) for 1 to 2 min before patch formation, and AA was included in the pipette and was present for

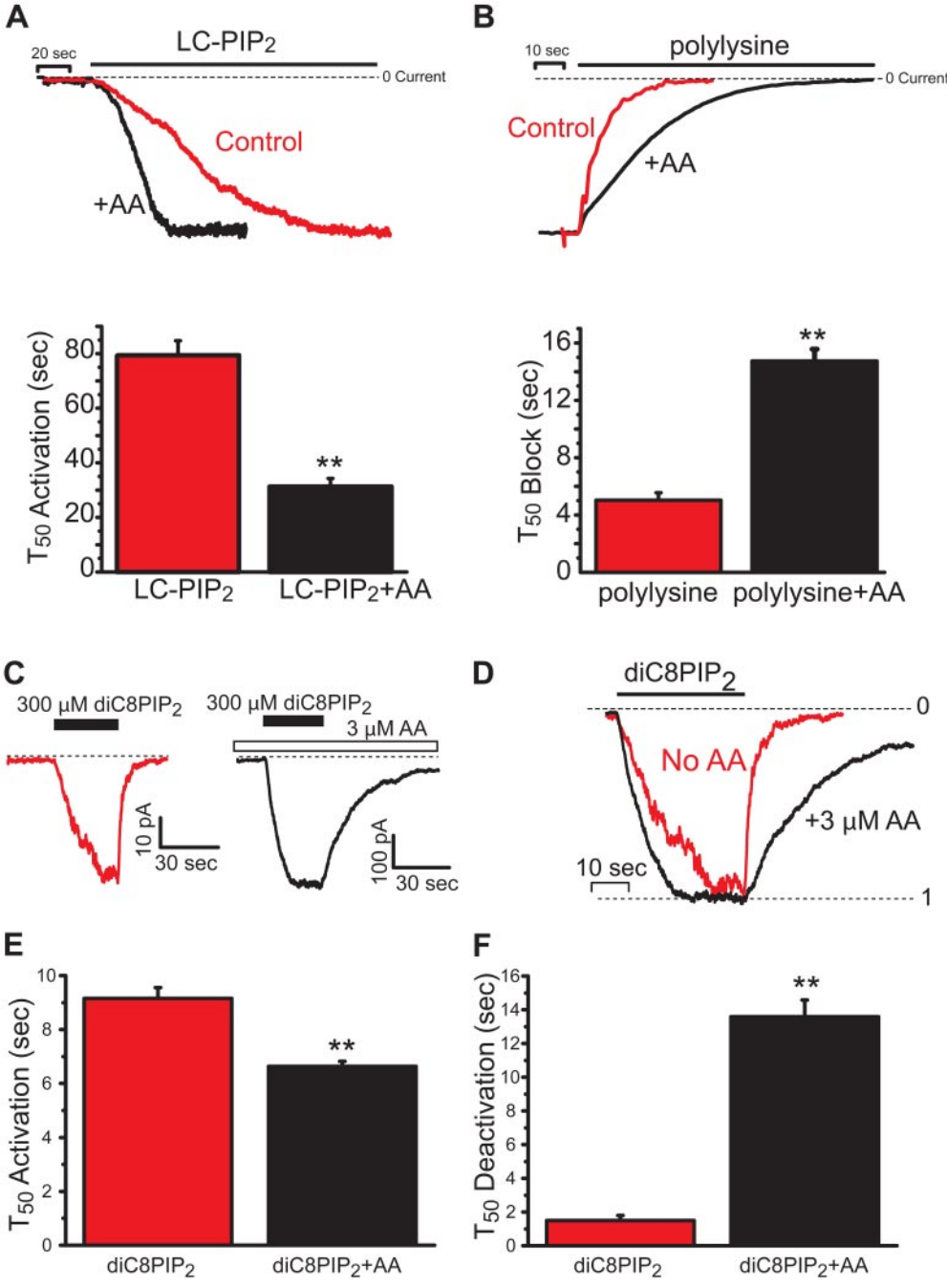

Fig. 3. AA alters the time course for PIP$_2$-induced activation and deactivation of Kir2.3 channels. A, Kir2.3 currents from inside-out patches were first run down and then activated by 10 μM LC-PIP$_2$ in the absence (red trace) or presence (black trace) of 3 μM AA. The current traces are normalized to facilitate side-by-side comparison. Summary data for activation T_{50} by LC-PIP$_2$ is shown below the traces. Presence of AA resulted in faster kinetics of Kir2.3 activation by LC-PIP$_2$, indicating that AA significantly accelerated channel activation ($n = 5$; **, $p < 0.05$, compared with no AA, unpaired t test). B, polylysine (90 μg/ml) was applied as indicated to inhibit the Kir2.3 currents in inside-out patches in the presence or absence of AA (0.3 μM). Current amplitudes were normalized to compare the rundown kinetics. AA significantly slows down the polylysine-induced current rundown. Summary data for T_{50} for polylysine-induced inhibition of Kir2.3 currents are shown below the traces. ($n = 6$; **, $p < 0.01$ compared with no AA, unpaired t test.) C, activation of Kir2.3 by water-soluble diC$_8$PIP$_2$ in the absence or presence of 3 μM AA in shown in two representative macropatches. AA was present for the duration of the recording in the right, as indicated by the white bar. PIP$_2$ was applied for the same length of time for all patches tested using a fast perfusion system (black bar). Channels activated faster and deactivated more slowly in the presence of AA. D, normalized traces for easier comparison of activation and deactivation kinetics. E and F, summary data for T_{50} of activation and deactivation in the presence ($n = 4$) and absence ($n = 5$) of AA. AA reduced T_{50} for activation and increased the T_{50} for deactivation consistent with data above for LC-PIP2 and polylysine (**, $p < 0.05$; unpaired t test).

the duration of the recording. In the control patch, the channels show typical bursting behavior that lasts for several minutes, then the channels close and re-open later during the recording. In the patches containing AA, after similar initial bursting and closure, channels reopen with bursting behavior. The single channel conductance is slightly reduced in the presence of AA. Recordings were performed for fixed time periods to ensure similar analysis parameters. Several kinetic parameters were determined from five individual recordings for each condition on two different batches of oocytes. Burst delimiters of 15, 100, 300, and 500 ms were used for the analysis with similar results. Results using 300-ms delimiter are presented in Fig. 5C. AA increased total open probability, and this was due to increased bursting probability and reduced interburst closed times, because open probability within the bursts was not changed.

Because endogenous PIP_2 levels in the oocytes may vary and AA access to the channel may be limiting in cell-attached recordings, we tested the effects of AA on single channel parameters in inside-out patches where solution changes and PIP_2 levels can be precisely controlled. Sample tracings from these are shown in Fig. 5D. In the control experiments, the patch was bathed in HK solution. Application of diC_8PIP_2 increased channel activity, which manifested mainly as more frequent bursts of opening. Application of AA on top of diC_8PIP_2 further increased the bursting behavior. Summary data for these parameters in several single-channel inside-out patches are shown in Fig. 5E. These data are in close agreement with those from cell-attached patches, collectively pointing to increased bursting behavior, and therefore, total open probability in the presence of AA. It is noteworthy that AA (or exogenous PIP_2) did not significantly increase the open probability within the burst. It has been suggested that Kir channel bursting is due to PIP_2 binding and occupancy of channel (Jin et al., 2006). Hence, AA-induced increase in bursting suggests that PIP_2 more readily can "hop on" the channel in the presence of AA. Furthermore, that AA does not increase open probability during the burst suggests that AA itself cannot gate the channel. Given that PIP_2 is needed for AA-induced activation (see Fig. 2), we conclude that AA is not a gating molecule but rather a facilitator for PIP_2-mediated gating.

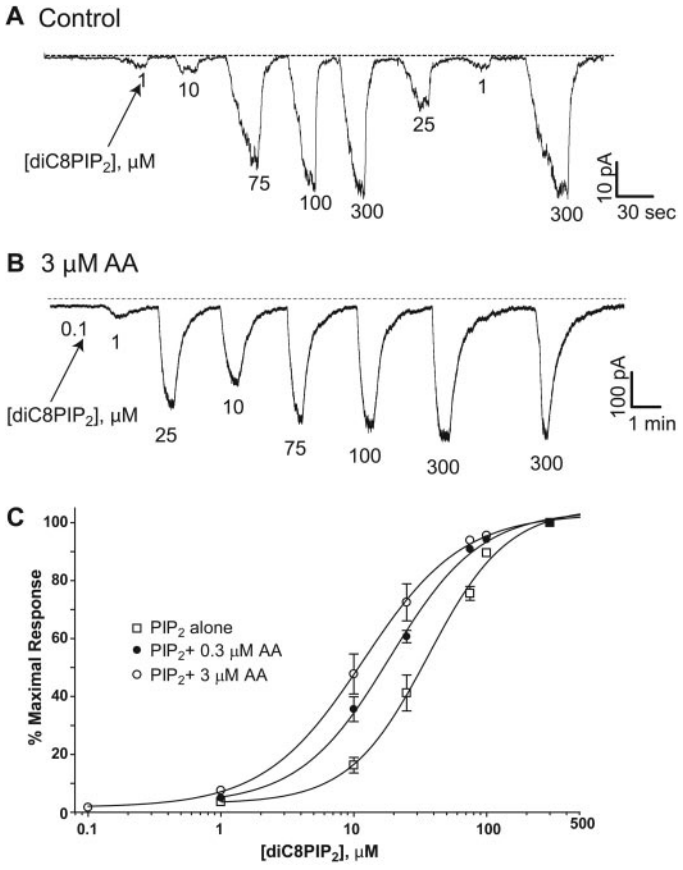

Fig. 4. AA sensitizes PIP_2 activation of Kir2.3. A and B, Kir2.3 currents were activated by multiple concentrations of diC_8PIP_2 in the absence or presence of AA (3 μM); concentrations were randomized to avoid possible cumulative effects. C, the concentration-response curve for diC_8PIP_2 in the absence of AA (□, $n = 5$) and presence of 0.3 μM AA (●, $n = 4$) and 3 μM AA (○, $n = 4$). The curves were fitted by nonlinear regression analysis; the EC_{50} value for diC_8PIP_2 activation was 36.3 μM, which was reduced to 18.7 and 11.8 μM, respectively, in the presence of 0.3 and 3 μM AA (see text for details on S.E.M. and confidence limits). The leftward shift in the dose-response curve was significant in the presence of 3 μM AA ($p < 0.01$, F test).

AA Activated Kir2.3 Currents from Internal Side of the Membrane. Because PIP_2 is located in the inner leaflet of the lipid membrane, it would follow that the site of action for AA is on the cytoplasmic side of the channel. We proceeded to test this by using bovine serum albumin (BSA) to block the action of AA from either side of the membrane. Figure 6A shows an experiment in which BSA (10 μM) was included in the recording pipette before forming the patch; thus, BSA would be present on the outside of the membrane for the duration of the recording. The patch was subsequently excised, exposing the inside of the membrane to the bath. Application of AA in the bath clearly activated Kir2.3 in the presence of BSA in the pipette. This effect of AA was blocked in a reversible manner only when BSA was applied to the inside of the membrane (in the bath). Summary data from patches in which BSA was applied either inside or outside the membrane are shown in Fig. 6B. It is clear that BSA blocked AA action only when applied on the cytoplasmic side of the membrane, suggesting that AA works on the cytoplasmic sites on the channel.

AA Attenuated Inhibition of Kir2.3 Currents Induced by M1 Receptor Activation. Our previous study has shown that Kir2.3 currents are inhibited by activation of type 1 muscarinic acetylcholine receptor (M1) (Du et al., 2004). This inhibition is induced by M1-mediated PIP_2 hydrolysis. Because AA enhances channel-PIP_2 interactions, it should therefore reduce its receptor-mediated inhibition. We tested this hypothesis by coexpressing M1 receptor and Kir2.3 in oocytes by determining the effect of AA on M1-induced inhibition of Kir2.3 currents. Figure 7A shows M1-induced inhibition of Kir2.3 currents in the absence and presence of AA (10 μM). In these experiments, a 20 mM K^+ solution was used, and current traces from holding potentials of +80 mV (above the dotted line) and −80 mV (below the dotted line) were recorded by two-electrode voltage clamp. At the end of the experiments, a barium solution (3 mM) was applied to block the K^+ current to set the baseline for the measurements. M1 was activated by acetylcholine (ACh, 10 μM), applied for a period indicated by the bar. An endogenous transient Ca^{2+}-activated Cl^- current was visible immediately upon ACh application, an indication of M1-induced PIP_2 hydrolysis and subsequent Ca^{2+} release. ACh induced a

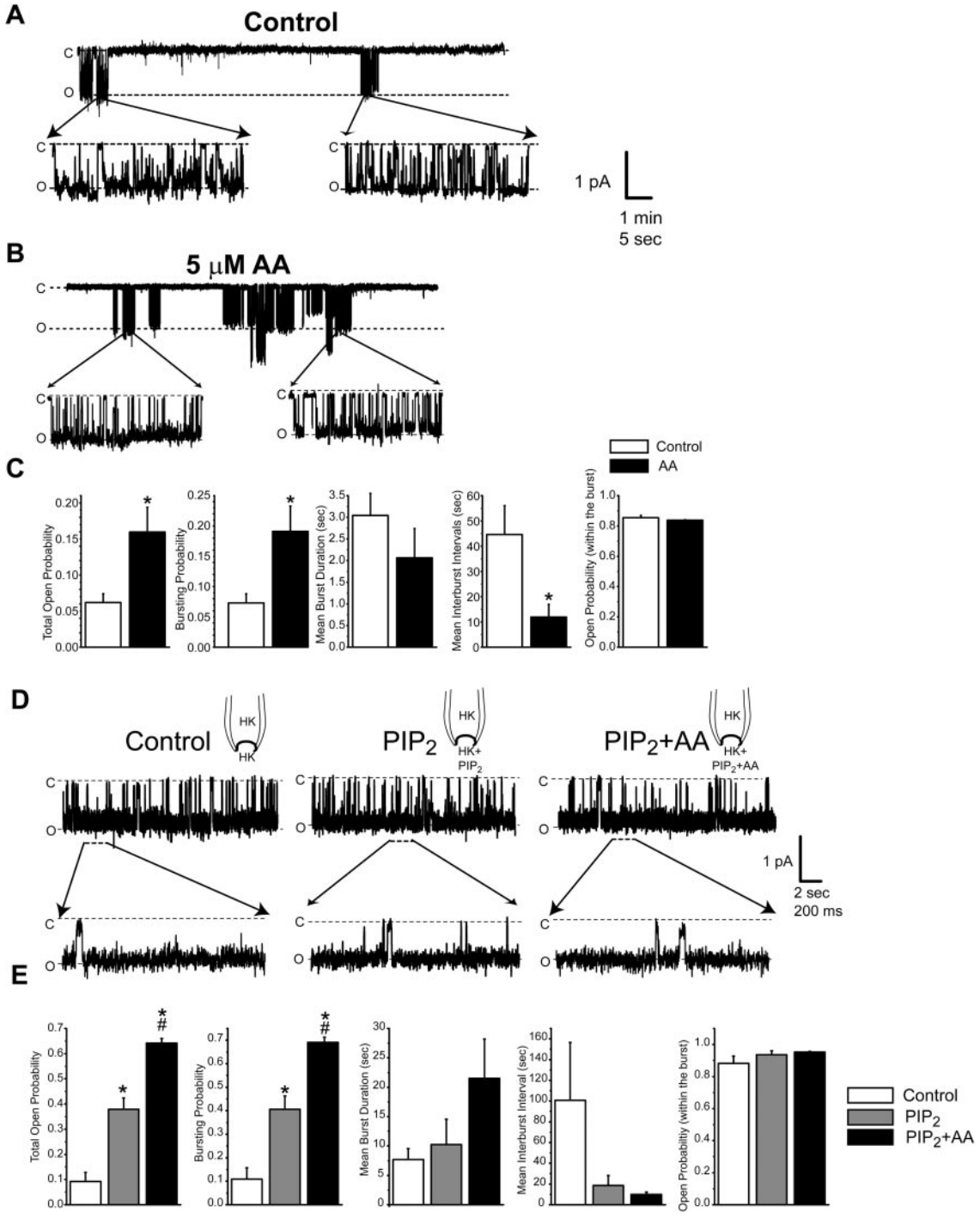

Fig. 5. AA increases Kir2.3 open probability as a result of more frequent bursting and reduced interburst closed times. A, cell-attached recording from an oocyte expressing Kir2.3 in the absence of AA. Gadolinium (100 μM) was included in the pipette to block endogenous stretch-sensitive channels. Kir2.3 channels show bursting opening and go silent before bursting further several minutes later. Channel openings during the bursts are seen in the expanded traces below. B, cell-attached recordings in the presence of 5 μM AA. The oocyte was incubated with AA for 1 to 2 min before patch formation, and AA was included in the pipette. Because AA can traverse the membrane, we assume it is present inside the cells at the beginning of the recording. The channels here again show bursting with increased frequency. Channel openings during the bursts are seen in the expanded traces below. C, summary of single-channel parameter from cell attached single-channel patches in the presence and absence of AA. Total open probability and bursting probability were significantly increased by AA, whereas interburst closed times were reduced ($n = 5$; $*$, $p < 0.05$ unpaired t test). D, inside-out recordings from oocytes expressing Kir2.3 under control condition, in the presence of exogenous diC$_8$PIP$_2$, and in the presence of diC$_8$PIP$_2$ with AA. Channels show typical bursting behavior again; PIP$_2$ or AA did not alter intraburst characteristics. The bursting in the control group is likely from endogenous PIP$_2$ that remained in the patch immediately after patch excision and ran down shortly after. Expanded traces are shown below. E, summary for single channel parameter under the three different conditions (control, PIP$_2$ alone, PIP$_2$+AA). PIP$_2$, as well as AA, significantly increased open probability and bursting probability ($n = 4$; $*$, $p < 0.05$, compared with control; #, $p < 0.05$ compared with PIP$_2$ alone, one-way analysis of variance with Dunnett post hoc test). There was also a trend toward increased burst duration and reduced interburst closings, which did not reach significance because of the stringency of the statistical analysis and limited number of experiments. However, the overall data are consistent with those in cell-attached patches. It is noteworthy that neither exogenous PIP$_2$ nor AA increased intraburst opening probability.

large inhibition of inward Kir2.3 current held at −80 mV (Fig. 7A, left). In the presence of AA, ACh-induced inhibition of Kir2.3 currents was significantly smaller compared with that observed in the absence of AA (Fig. 7, A, right, and B, summary data). Because the extent of inhibition on Kir2.3 induced by ACh depends on channel-PIP_2 interaction, these results confirm that AA enhances this interaction.

Discussion

In the present study, we showed that PIP_2 was the molecular mediator of AA-induced activation of Kir2.3 channels. AA required PIP_2 for its effects on Kir2.3 because it failed to activate channels that had run down as a result of PIP_2 depletion. Furthermore, it increased the channel sensitivity to PIP_2 in a measurable and dose-dependent manner and increased channel bursting behavior. Finally, AA acted through cytoplasmic sites and reduced receptor-mediated inhibition of Kir2.3 channels.

Previous works have shown that AA can activate Kir2.3 (Liu et al., 2001), a two-pore-domain K^+ channel (Fink et al., 1998), and TRPV4 channel (Watanabe et al., 2003); however, mechanism of these activations remains unclear. Note that activity of all these channels is sensitive to PIP_2. In a recent study, Oliver et al. (2004) presented evidence that AA and anandamide cause rapid voltage-dependent inactivation of otherwise noninactivating Kv channels. Furthermore, it was shown in this study that PIP_2 could convert A-type channels into delayed rectifiers. Thus, PIP_2 and AA seem to exert bidirectional control of Kv channel gating (Oliver et al., 2004), indicating a functional interaction between PIP_2 and AA.

Our new findings provide further support for the notion that PIP_2 serves as the common moderator of Kir function by various physiological factors (Du et al., 2004) and that PIP_2 is the master key of functional modulation of PIP_2-dependent channels (Guy-David and Reuveny, 2007). In a previous study, we showed that the characteristic interactions with PIP_2 determine regulation of Kir channels by diverse modulators such as PLC-coupled receptors, PKC, intracellular Mg^{2+}, and pH. We concluded that the strength of channel-PIP_2 interactions determines the sensitivity of Kir channels to regulation by these modulators (Du et al., 2004). Accordingly, a channel with weak interactions with PIP_2, such as Kir2.3, would be a suitable target for regulation, whereas a channel with strong interactions with PIP_2, such as Kir2.1, would not (Du et al., 2004). Although regulation by several modulators could be generalized across several channels, AA is unique in positively modulating Kir2.3 channel. Our data strongly support the notion that AA indeed enhanced interaction between Kir2.3 and PIP_2, because 1) PIP_2 activation of Kir2.3 currents was potentiated by AA, 2) AA increased apparent potency of PIP_2 in activating Kir2.3, 3) AA slowed polylysine-induced inhibition of Kir2.3 currents, 4) AA accelerated PIP_2-induced channel activation and decelerated channel deactivation upon PIP_2 removal, and 5) AA removed M1 receptor-mediated inhibition of Kir2.3 channels. Furthermore, a single point mutant of Kir2.3 (Iso213 to Leu) that strengthened the interaction between Kir2.3 and PIP_2 significantly reduced the AA-induced activation of the channel. Using single-channel recordings in the cell-attached and inside-out modes, we found that presence of AA affected channel opening by increasing channel bursting behavior. Because PIP_2 is likely to be a gating molecule for these channels, the bursting behavior reflects channel occupancy by PIP_2. Short intraburst closings are more likely to reflect changes at the selectivity filter, as has been suggested for other channels (Blunck et al., 2006). Therefore, increased bursting, and reduced interburst closed times without accompanying changes in intraburst parameters in the presence of AA reflect its effects on PIP_2 occupancy. One can imagine a scenario in which, in the presence of AA, PIP_2 can more easily interact with the channel, as also suggested by the shift in the concentration-response curve; once PIP_2 binds the channel, its open times and bursting duration are minimally affected. Thus, the main conclusion drawn from the results of the present study is that AA activates Kir2.3 by increasing its interaction with PIP_2. This is in contrast to Kir3 channels whose activity is reduced by AA, presumably through a PIP_2-dependent mechanism (Rogalski and Chavkin, 2001). It is noteworthy that the AA effects on Kir2.3 are more robust and at approximately one tenth the concentration needed for Kir3 channel inhibition. Kir members with weak PIP_2 interactions (e.g., Kir3.x and Kir2.3) are also modulated by membrane receptors (Kobrinsky et al., 2000; Du et al., 2004), PKC (Henry et al., 1996; Zhu et al., 1999b), Mg^{2+}(Chuang et al., 1997), and pH (Qu et al., 1999; Zhu et al., 1999a). However, Kir members that interact strongly with PIP_2 (e.g., Kir2.1)

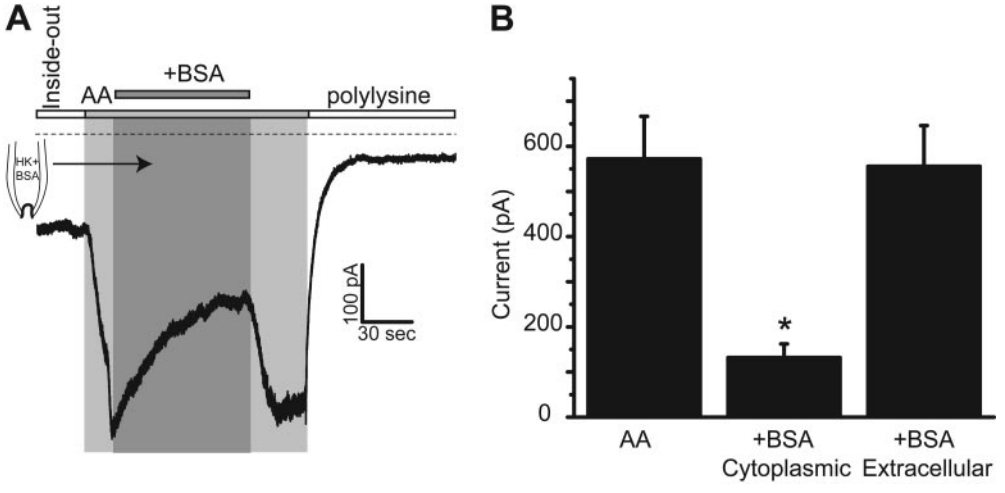

Fig. 6. BSA blocks AA activation of Kir2.3 from the cytosolic side of the membrane. A, inside-out macropatch from oocyte expressing Kir2.3 held at −80 mV. BSA (10 μM) was included in the patch pipette before forming the seal. AA (2 μM) and BSA (10 μM) were applied in the bath as indicated. Only BSA in the bath inhibited AA-induced current in a reversible manner. B, current amplitudes from several patches where BSA was present *either* in the pipette (BSA inside, extracellular) *or* applied in the bath (BSA outside, cytoplasmic) to determine its effect on AA-induced currents (*, $p < 0.05$ compared with AA alone; $n = 3$ for BSA inside and $n = 4$ for BSA outside).

are not subject to modulation by these factors (Du et al., 2004). We therefore postulate that for all these channels, PIP_2 may act as the final activating molecule. Regulation of channel function by other modulators, which may have action sites on Kir channels common to all or specific to one Kir channel, proceed through modulation of channel-PIP_2 interactions, and these interactions can be manifested as further activation or inhibition depending on the specific channel and modulator. AA interactions with Kir2.3 fit this mechanism of action, as shown throughout. AA also activates TREK-1 channels, a member of the two-pore domain K^+ channel family (Patel et al., 2001; Chemin et al., 2007). This family of K^+ channels is also modulated by other factors, such as volatile anesthetics, intracellular pH, and membrane receptors (Kim, 2003). These channels are also PIP_2-dependent (Lopes et al., 2005). It will be interesting to see whether PIP_2 plays a role in AA-induced regulation of TREK channels.

It is well established that the uptake of AA and other long-chain fatty acids by mammalian cells is rapid (for review, see Glatz et al., 2002). Therefore, it is not surprising to see AA effects from either side of the membrane (Fink et al., 1998), because AA may "flip" across the membrane bilayer to reach a putative site of action. A previous study presented a puzzling result with regard to the site of AA action (Liu et al., 2001), because it suggested that BSA blocked the effect of AA from the outside of the membrane. However, a chimera, comprising the transmembrane and intracellular domains of Kir2.3 and the extracellular region of Kir2.1 (Kir2.3−2.1−2.3), was virtually identical to Kir2.3 with regard to the response to AA (Liu et al., 2001). Our data in the present study clearly demonstrate that AA activates Kir2.3 from the internal site of the membrane (Fig. 6).

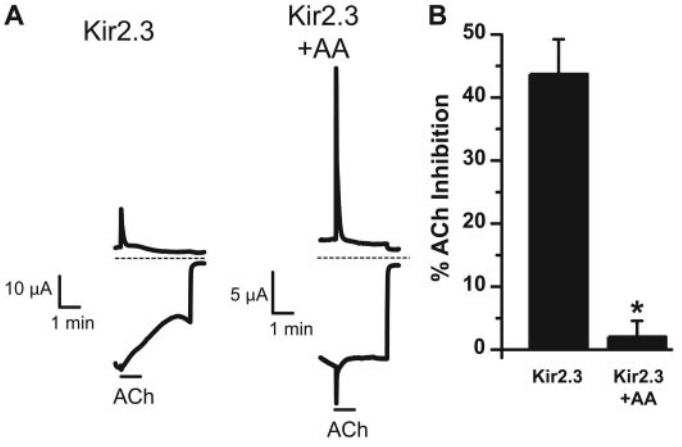

Fig. 7. AA reduces the inhibition of Kir2.3 currents induced by M1 activation. A, ACh-induced inhibition of Kir2.3 currents in the absence or the presence of AA. M1 receptor and Kir2.3 channels were expressed in oocytes, and currents were recorded at −80 mV (below the dotted line) and +80 mV (above the dotted line) in 20 mM K^+ solution. Dotted line indicates zero current level. ACh was applied to activate the M1 receptor as indicated. The transient outward spike is from an endogenous calcium-activated chloride current. Channel inhibition in the control experiment persisted after removal of ACh and started to recover at a later point; this is consistent with the Kir channel inhibition through receptor-mediated PIP_2 hydrolysis, which has been described previously (Kobrinsky et al., 2000; Du et al., 2004). Presence of AA blocked this inhibition without affecting the receptor-mediated PIP_2-hydrolysis as indicated by the robust chloride current activation. B, summary data for effects of AA on ACh-induced Kir2.3 inhibition. ACh significantly inhibited Kir2.3 currents, and AA attenuated the ACh-inhibition of Kir2.3. (*, $p < 0.01$ versus ACh-induced inhibition in the absence of AA, unpaired t test.)

Considering that neither Kir2.1 nor Kir2.1(R312Q) is sensitive to AA regulation, it is likely that there are specific interacting site(s) for AA that are only present in the cytoplasmic domains of Kir2.3. Therefore, although weak PIP_2 interactions are a necessary prerequisite for AA modulation of Kir2.3, it is not sufficient to confer sensitivity. PIP_2 is known to lie in the inner leaflet of the membrane, and all PIP_2-interacting sites found on Kir channels are localized in intracellular domains (Huang et al., 1998; Zhang et al., 1999; Lopes et al., 2002). Given the evidence presented above, an attractive scenario is that AA binds to intracellular domains of Kir2.3 to induce conformational changes that enhance channel–PIP_2 interactions. The shift in the apparent PIP_2 affinity on the channel is consistent with these enhanced interactions.

Kir2.3 is distributed in neurons and cardiac myocytes (Périer et al., 1994), where AA can hyperpolarize membrane potentials (Kang et al., 1995). During metabolic inhibition produced by ischemia and hypoxia, intracellular pH falls, the cytosolic concentrations of free fatty acids and Ca^{2+} increase, and phospholipases are activated, resulting in cell dysfunction (Lipton, 1999). Thus in both physiological and pathophysiological conditions, lipid metabolism and factors such as AA could regulate the function of Kir2.3 channels. However, the effects of AA on cardiac IK1 currents have not been tested, and the relative contribution of Kir2.3 to this current remains unclear, so the relevance of AA activation of Kir2.3 during ischemia in the cardiac tissue remains undetermined.

Acknowledgments

We thank Drs. Taihao Jin and László Csanády for single channel data analysis software and Drs. Gerda Breitwieser (Geisinger Clinic) and Taihao Jin (UCSF) for helpful comments and discussions.

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