

β-Subunit–Dependent Modulation of hSlo BK Current by Arachidonic Acid

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Submitted 6 July 2006; accepted in final form 28 September 2006

Sun X, Zhou D, Zhang P, Moczydlowski EG, Haddad GG. β-Subunit–dependent modulation of hSlo BK current by arachidonic acid. J Neurophysiol 97: 62–69, 2007. First published October 4, 2006; doi:10.1152/jn.00700.2006. In this study, we examined the effect of arachidonic acid (AA) on the BK α-subunit with or without β-subunits expressed in Xenopus oocytes. In excised patches, AA potentiated the hSlo-α current and slowed inactivation only when β2/3 subunit was co-expressed. The β2-subunit–dependent modulation by AA persisted in the presence of either superoxide dismutase or inhibitors of AA metabolism such as nordihydroguaiaretic acid and eicosatetraynoic acid, suggesting that AA acts directly rather than through its metabolites. Other cis unsaturated fatty acids (docosahexaenoic and oleic acid) also enhanced hSlo-α + β2 currents and slowed inactivation, whereas saturated fatty acids (palmitic, stearic, and caprylic acid) were without effect. Pretreatment with trypsin to remove the cytosolic inactivation domain largely occluded AA action. Intracellularly applied free synthetic β2-ball peptide induced inactivation of the hSlo-α current, and AA failed to enhance this current and slow the inactivation. These results suggest that AA removes inactivation by interacting, possibly through conformational changes, with β2 to prevent the inactivation ball from reaching its receptor. Our data reveal a novel mechanism of β-subunit–dependent modulation of BK channels by AA. In freshly dissociated mouse neocortical neurons, AA eliminated a transient component of whole cell K⁺ currents. BK channel inactivation may be a specific mechanism by which AA and other unsaturated fatty acids influence neuronal death/survival in neuropathological conditions.

INTRODUCTION
Polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA) and docosahexaenoic acid (DHA) are highly concentrated in the phospholipids of brain tissues and neuronal synapses. The absolute PUFAs levels and their relative concentrations are strictly controlled in mammalian neurons, and significant alterations in brain PUFAs have been found in patients with various neurological disorders and neurodegenerative diseases (Berger et al. 2006; Bois et al. 2005; Markesbery 1997). PUFAs have been implicated as substrates in peroxidative damage because of the abundance of double bonds, which constitute ideal sites for reactive oxygen species attack and free radical generation (Phillis and O’Regan 2004).

In ischemia/reperfusion, alterations in lipid metabolism, such as activation of phospholipases and release of fatty acids, are key events in a cascade that leads to neuronal death (Arai et al. 2001; Lipton 1999). PUFA treatments have been shown to exert strong neuroprotective effects because of modulation of ion channels and synaptic glutamatergic transmission in hippocampal neurons in both in vitro and in vivo ischemic models (Lauritzen et al. 2000; Strokin et al. 2006).

AA modulates the activities of various ion channels through direct or indirect pathways (Meves 1994). The direct effects are mediated by the interaction between AA and ion channel proteins or through perturbation of plasma membranes. The indirect actions on ion channels are mediated by metabolites of AA through oxygenases or cellular signal transduction pathways. The effects of AA actions during ischemia/reperfusion could be either beneficial or detrimental to neurons. For example, prostaglandins, which are produced from AA by the action of cyclooxygenase, are considered damaging in brain ischemia (Nogawa et al. 1997). Nonenzymatic oxidative metabolism of AA also generates reactive oxygen radicals that contribute to oxidative injury (Phillis and O’Regan 2004).

Large-conductance, calcium-activated potassium (BK) channels are expressed throughout the vertebrate nervous system. Because BK channel activation depends on depolarization and an increased intracellular calcium concentration, they are implicated for negative feedback regulation of Ca²⁺ influx through voltage-gated Ca²⁺ channels and regulation of cellular excitability and neurotransmitter release (Hu et al. 2001; Raffaelli et al. 2004; Sah 1996). On the one hand, therefore, activation of BK channels could serve to protect neurons and reduce the pathological consequences of ischemia and other conditions characterized by accumulation of intracellular calcium and excessive depolarization (Gribkoff et al. 2001; Runden-Pran et al. 2002). On the other hand, activation of BK channels can usher the initiation of K⁺ leakage during ischemia, which can trigger cellular dehydration and potentially programmed cell death (Lang et al. 2005). Although AA has been shown to activate BK channels in various types of native cells (Bringmann et al. 1998; Clarke et al. 2002; Denson et al. 2000), the molecular interaction between BK and AA has not yet been clarified. For example, it is not known whether AA modulates directly the pore-forming α-subunit or alters modulatory accessory subunits or other associated components.

BK channels are composed of four homologous pore-forming α-subunits, along with optional β accessory subunits that regulate many important aspects of BK channel function. To date, four mammalian β-subunits have been identified in humans (KCNMβ1-4, Brenner et al. 2000; Knaus et al. 1994; Meera et al. 2000; Uebele et al. 2000; Wallner et al. 1999; Weiger et al. 2000; Xia et al. 2000), each of which confers unique functional properties on the resulting BK channels. In particular, both β2- and β3-subunits result in kinetically distinct inactivating BK channels (Wallner et al. 1999; Xia et al. 2000). In the brain, there seems to be a widespread expression of Slo α-subunit and hβ4. Expression of β2/3 is less robust and...
probably more specific to certain cell types, making them an intriguing target with respect to selective neuronal function. Virtually no hβ1 mRNA can be detected in the CNS.

In this study, we used the *Xenopus* oocyte expression system and expressed the hSlo-α-subunit alone or in conjunction with the β2–4 subunits that have been shown to be present in the brain. We show that AA alters β-subunit modulation of BK channel inactivation and potentiates steady-state conductance in the presence of β2/β3. AA does not induce a significant response of BK channels in the absence of β or in the presence of β4. The β-subunit–dependent modulation of BK channels by AA may have important implications on neuronal function under pathophysiological conditions.

Methods

All animals used in this study were handled in compliance with the United States public service policy on care and use of laboratory animals and National Institutes of Health guide for the care and use of laboratory animals, as well as institutional animal care and use committee guidelines.

Preparation of oocytes

Female *Xenopus laevis*, the African clawed frog, were purchased from Nasco. Frogs were anesthetized with 0.2% tricaine methanesulfonate (MS 222). Clumps of oocytes were surgically removed and washed in Ca2+–free ND96 medium containing (in mM) 82.5 NaCl, 2 KCl, 1.8 MgCl2, and 5 HEPES, pH 7.4. Single oocytes were dissociated with 1.5 mg/ml type II collagenase (Sigma, St. Louis, MO) in Ca2+–free ND96 solution at 18°C for 2 h to remove follicle cells. The oocytes were stored in ND96 incubation medium containing (in mM) 96 NaCl, 2 KCl, 1.8 CaCl2, 1.8 MgCl2, and 5 HEPES, pH 7.4, and kept at 18°C for ≥2 h before RNA injection.

Synthesis of cRNA

pcDNA3-hSlo was used for synthesis of cRNA. pBF-hβ2 and pBF-hβ3 were provided by Dr. Christopher Lingle, and pcDNA3-hβ4 was from Dr. Ligia Toro. The plasmids were first linearized with an appropriate restriction enzyme: XbaI for pcDNA3-hSlo, MluI for pBF-hβ2 and hβ3, and Ncol for pcDNA3-hβ4. In vitro transcription of each individual cRNA was performed using mMESSAGE mMACHINE T7 (hSlo and β4 constructs) or SP6 (β2 and β3 constructs) kit (Ambion, Austin, TX) in the presence of the cap analog m7G(5')G according to the manufacturer’s instructions. The cRNA products were purified with RNeasy affinity column (Qiagen, Valencia, CA), and aliquots were kept at −80°C.

Microinjection into oocytes

hSlo-α-subunit was injected at 10–15 ng/oocyte, and αβ was injected at 1:2 ratios by weight to ensure a molar excess of β-subunits over α subunits. Oocytes were injected with 50 nl cRNA encoding for 1) α-subunits alone; 2) α + β2–4; and 3) β-subunits alone. Control oocytes were injected with the same volume of water. Injected oocytes were incubated at 18°C in ND96 incubation medium and were used for electrophysiological experiments 3–7 days after injection. During this period, *Xenopus* oocytes after mRNA injection gave us enough channel expression in healthy oocytes.

Preparation of neocortical neurons

Mice were used between 9 and 15 days of age. The method for cell isolation was similar to that described previously (Sun et al. 2003). In brief, mice were killed by decapitation. The brain was removed rapidly and chilled in 0–4°C oxygenated isolation buffer containing (in mM) 120 NaCl, 5 KCl, 1 CaCl2, 10 HEPES, and 25 glucose (pH adjusted to 7.0 with NaOH). Neocortical slices were incubated for 20 min with oxygenated isolation buffer containing Protease (type XIV from *Streptomyces griseus*, Sigma P-5147, 0.5 mg/ml, at 32°C). Slices were washed in oxygenated buffer and maintained for ≤3 h. Immediately before recording, a cortical slice was dissociated by gentle trituration with fire-polished Pasteur pipettes. The cells were collected and placed into a plastic petri dish and were allowed to settle for several minutes and adhere to the petri dish before superfusion was initiated. Recordings were obtained only from pyramidal-shaped neurons that had a single thick proximal dendrite and did not show any visible evidence of injury.

Electrophysiological recordings

Recording pipettes were made from filament borosilicate capillary glass (1.2 mm OD, 0.69 mm ID; Warner, Hamden, CT), using a Flaming/Brown micropipette puller (model P-87, Sutter Instrument, Novato, CA). The pipettes were fire-polished and had resistances of 5–8 MΩ when filled with solutions listed below. Experiments were conducted at room temperature (22–25°C).

Voltage-clamp experiments were performed in *Xenopus* oocytes from which the vitelline membrane had been manually removed after shrinkage in a hyperosmotic medium containing (in mM) 200 K-glucuronate, 20 KCl, 1.0 MgCl2, 10 EGTA, and 10 HEPES, pH 7.4. Currents were recorded in either inside-out or outside-out patches. Both the pipette and the bathing solution contained (in mM) 116 K-glucuronate, 4 KCl, and 10 HEPES, pH 7.2. Extracellular solutions contained 1 mM MgCl2. Test solutions bathing the cytoplasmic face of the plasma membrane contained 0 (5 mM EGTA and no added Ca2+), 25, or 100 μM CaCl2.

Whole cell K+ currents in mice neocortical neurons were recorded using the same methods as previous studies (Sun et al. 2003). Briefly, pipette solutions contained (in mM) 110 K-glucuronate, 10 KCl, 5 NaCl, 2 MgCl2, 10 HEPES, 0.5 EGTA, 1 ATP, 0.2 GTP, and 0.1 leupeptin, with pH adjusted to 7.4. To record calcium-activated potassium channel currents, cells were superfused with a HEPS solution containing (in mM) 140 NaCl, 3 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, 0.0005 TTX, and 1 4-AP, 0.005 glibenclamide, and 10 glucose (pH 7.4). TTX, 4-AP, and glibenclamide were routinely included in the K+ current recording.

Membrane potential and membrane currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Current signals were filtered at 2–5 kHz and digitized at 20–50 kHz. Stimulus generation and data acquisition were controlled with the Clampex program of the pClamp8 software package (Axon Instruments).

Drugs and solutions

All salts and chemicals were obtained from Sigma (St. Louis, MO). Stock solutions of AA were 100 mM either in dimethyl sulfoxide (DMSO), or water (sodium salt). Stock solutions of DHA, oleic acid, palmitic acid, elaidic acid, stearic acid, caprylic acid, nordihydroguaiaretic acid (NDGA), and eicosatetraynoic acid (ETYA) were all 100 mM in DMSO. Lysophosphatidylinositol (sodium salt) stock solution was 20 mM in methanol, and sphingosine was 50 mM in DMSO. Superoxide dismutase (SOD) stock solution was dissolved in medium at 25,000 U/ml; aliquots of stock solutions were kept at −80°C and were later diluted with bath solution before use. Final concentration of DMSO was <1:2,000, a concentration that was found to have no effect in control studies without drugs.

β2 “ball” peptides consisting of 26 N-terminal amino acids (MF- WTSGRTSSSYRHEDEKNIYQKIR) were synthesized (Invitrogen). The peptides were dissolved to a concentration of 10 mM in 50 mM DMSO.
Tris · Cl (pH 7; Wallner et al. 1999). Aliquots of stock solution were kept at −20°C and were further diluted in the bath solution before experiments.

**Data analysis**

Whole cell K⁺ currents were recorded using the same protocol as we have done previously (Sun et al. 2003). Currents were evoked by depolarizing steps (−60 to +50 mV) from a holding potential of −70 mV for 200 ms in 10-mV steps, with 5-s intervals between sweeps.

Oocyte inside-out and outside-out patches were held at a potential of −70 mV before hyperpolarizing to −140 mV for ≤150 ms to remove inactivation. Membrane patches were depolarized stepwise from −100 to +120 mV in 20-mV steps for the duration of 200 ms with 5-s intervals between sweeps (unless otherwise indicated). In some cases, the hyperpolarizing prepulses were omitted for recording noninactivating currents (α alone or α + β4), and the data were grouped because no difference was found in the data obtained. In a few cases, short hyperpolarizing prepulses after the depolarizing steps were applied before stepping back to −70 mV. Conductance-voltage (G-V) relations were generated by measuring values at the end of the voltage steps for the sustained current and at the peak value during voltage steps for the peak current at a given activation potential. G-V curves for activation were fit with a Boltzmann equation using Origin program. Steady-state inactivation curves were generated by stepping to potentials between −140 and +40 mV (in 20-mV increments) for 2 s before a 200-ms test step to +100 mV. Peak current activated at +100 mV is plotted as a function of conditioning potential. Data are presented as means ± SE. Student’s t-test is used for statistical significance.

**RESULTS**

**Effects of AA on hSlo-α channels with or without β subunits expressed in Xenopus oocytes**

Inside-out or outside-out patches taken from *Xenopus* oocytes expressing the hSlo-α-subunit alone, or the α-subunit plus one of the β2-4-subunits, were examined to determine the steady-state and kinetic properties of BK channels. Patches were exposed to 25 μM intracellular Ca²⁺ (unless otherwise indicated), a value that was sufficient for adequate activation of the BK channels. Patches pulled from oocytes that were not injected or injected with either H₂O or β-subunits alone showed no evidence of channel activity under our recording conditions and were used as negative controls (n = 33 patches). Currents obtained from channels composed of only the α-subunit displayed no inactivation over the course of the pulses (n = 44 patches; Fig. 1A). However, channels composed of either α + β2 (n = 120 patches; Fig. 1B) or α + β3 (n = 30 patches; Fig. 1C) inactivated to varying degrees throughout the depolarization protocols. Currents obtained from channels composed of α + β4 did not inactivate (n = 22 patches; Fig. 1D). Application of AA (10–30 μM) did not significantly alter hSlo-α channel activity in the absence of β-subunits (n = 7 patches; Fig. 1A), but markedly increased both the peak and steady-state currents in the presence of β2 (n = 14 patches; Fig. 2B) or β3 subunits (n = 9 patches; Fig. 1C). AA did not significantly alter maximal conductance of the steady-state current produced by co-expression of hSlo-α channels with β4 subunits (n = 10 patches; Fig. 1D).

Our data show that AA enhanced hSlo-α currents only when β2 or β3 subunits are co-expressed. Because both β2 and β3 subunits confer inactivation to BK channels, AA might enhance hSlo-α currents by slowing or removing inactivation. We thus concentrated on the hSlo-α + β2 for further studies. AA reversibly enhanced the peak current amplitude and prolonged inactivation of hSlo-α + β2 currents. AA caused an increase in single-channel opening probability as well as an increase in the number of open channels (Fig. 2A). The effects of AA on peak current amplitude and inactivation kinetics of hSlo-α + β2 currents were dose-dependent in the range of 1–50 μM (Fig. 2B). Higher concentrations of AA resulted in complete loss of the inactivating component.

A prepulse potential of −140 mV was used in our study to relieve inactivation of hSlo-α + β2 currents and give maximal currents. The full steady-state inactivation curves were shown in Fig. 3. Patches were held at 0 mV before stepping to different conditional potentials (−140 to +40 mV) for 2 s.

**FIG. 1.** Effects of arachidonic acid (AA) on hSlo-α channels with or without β-subunit. Current recordings were obtained in symmetrical K⁺ gluconate containing 25 μM Ca²⁺ (intracellular). Patches were held at −70 mV before undergoing a hyperpolarizing step to −140 mV to remove inactivation, immediately followed by 200-ms depolarizing steps from a range of potentials (−100 to +120 mV) in 20-mV steps. A: representative noninactivating hSlo-α current traces were taken before and during AA (30 μM). G-V curves of the steady-state hSlo-α currents were measured at the end of command steps (200 ms) before and during AA (10–30 μM, n = 7 patches). B: representative current traces from co-expression of α + β2-subunits were recorded before and during AA (30 μM). G-V curves were measured at the peak of the current before and during application of AA (10–30 μM, n = 14 patches). C: representative current traces of hSlo-α + β3 currents were recorded before and during application of 10 μM AA. G-V curves were measured at the end of command steps (n = 9 patches). D: representative current traces show currents resulting from co-expression of α and β4, taken before and during application of 10 μM AA. G-V curves were measured at the end of command steps (n = 10 patches). Values are expressed as means ± SE.
Peak current elicited at +100 mV from each conditioning potential was normalized to the peak current elicited from −140 mV. AA (20 μM) increased the maximal available current (Fig. 3A) and shifted the fractional availability of hSlo-α + β2 currents to more positive potentials (V_{0.5} from −93.5 ± 2.0 to −56.7 ± 1.6 mV, n = 8 patches; Fig. 3B).

Because BK channels are highly sensitive to intracellular Ca^{2+}, a reduction in free Ca^{2+} or in Ca^{2+} sensitivity might result in reduced steady-state inactivation, which would lead to an increase in current amplitude and slowing of inactivation. To study whether Ca^{2+} plays a role in AA action, AA was tested in Ca^{2+}-free intracellular solution. Ca^{2+} was removed by adding EGTA (5 mM) to solutions with 0 CaCl_{2}.

Under these conditions, AA had no effect on hSlo-α currents alone (n = 3 patches; Fig. 4A), but markedly enhanced hSlo-α + β2 currents (n = 4 patches; Fig. 4B). We also tested AA action to enhance hSlo-α + β2 currents at higher intracellular Ca^{2+} (100 μM) and found it qualitatively similar to that in the presence of lower intracellular Ca^{2+} or without Ca^{2+} (n = 3 patches, data not shown). These data suggest that AA action does not require the presence of Ca^{2+} and that AA action is not a result of decreased free Ca^{2+} or Ca^{2+} sensitivity.

### Potential mechanisms of AA action

AA ACTS DIRECTLY AND NOT THROUGH ITS METABOLITES. Some ion channels are activated by the oxidation products of fatty acids rather than by the parent fatty acid itself. This can happen nonenzymatically through the action of oxygenases. Because we used excised patches in this study, soluble cytoplasmic signaling components are unlikely to be involved in AA action. To determine whether superoxide anions generated from AA metabolism play a role in the activation of BK channels by AA, we examined the effect of AA in the presence of SOD, a known scavenger of superoxide free radicals. SOD alone did not significantly alter hSlo-α + β2 currents, and bath application of AA, in the presence of SOD, induced a potentiation of peak current and slowing of inactivation (n = 6 patches; Fig. 5). These data suggest that superoxide radicals are not critically involved in the AA action.

Lipoxygenases, one of the three types of oxygenases that metabolize AA, can translocate to the plasma membrane (Hagmann et al. 1993; Rouzer and Kargman 1988); thus they may potentially be present in the membrane patches used. Thus we tested a cyclooxygenase and lipoxygenase inhibitor eicosatetraynoic acid (ETYA). Because ETYA can also react with oxygen to produce superoxide radicals, SOD (50–100 U/ml) was co-applied with ETYA. AA enhanced current amplitude and slowed inactivation in the presence of ETYA (n = 4 patches; Fig. 5). Similarly, AA action persisted in the presence of another inhibitor of AA metabolism, nordihydroguaiaretic acid (NDGA, a nonspecific lipoxygenase inhibitor; Fig. 5, C and D). These results indicate that the AA action is not mediated by any of its metabolites and that AA acts directly to alter BK channel activity in the presence of β2-subunit.

### Figures

**Fig. 2.** AA enhances hSlo-α + β2 currents and slows inactivation. Macroscopic hSlo-α + β2 currents were recorded from either inside-out or outside-out patches. Currents were evoked by 200-ms voltage steps to +80 mV. A: superimposed traces were taken before (1), during (2), and after bath application of 30 μM AA (3) at the time-points indicated in plots of time-course of peak current (I_{max}) and inactivation time constant (τ). Inset: channel activities were recorded before (top) and during (bottom) AA (10 μM) application. B: percent change in I_{peak} and ratios of inactivation time constants (τ_{inact}/τ_{control}) were plotted as a function of AA concentrations (1–50 μM, n = 3–16 patches). Values are expressed as means ± SE.

**Fig. 3.** Effects of AA on steady-state inactivation properties of hSlo-α + β2 currents. Macroscopic hSlo-α + β2 currents were recorded from inside-out patches. From a holding potential of 0 mV, patches were stepped to potentials between −140 and +40 mV for 2 s before a 200-ms test step to +100 mV. A: representative traces were taken before and during bath application of AA (20 μM). B: normalized fractional availability is plotted as a function of conditioning potential. AA, 20 μM, n = 8 patches. Values are means ± SE.

**Fig. 4.** Effects of AA on hSlo-α + β2 currents in the absence of intracellular Ca^{2+}. Macroscopic hSlo-α + β2 currents were recorded from inside-out patches at Ca^{2+}-free intracellular solutions (5 mM EGTA and no added CaCl_{2}). Currents were evoked by 200-ms voltage steps ranging from −20 to +140 mV. A: representative current traces of hSlo-α currents were measured at the end of command steps (n = 3 patches). B: representative current traces of hSlo-α + β2 currents were taken before and during AA (30 μM). G-V curves were measured at the peak of current (n = 4 patches). Values are means ± SE.
DOUBLE BONDS IN FREE FATTY ACID STRUCTURE PLAY A ROLE. AA is a cis-polysaturated fatty acid (20:4, cis-5,8,11,14) with negative charge. To examine the fatty acid specificity of the activation of hSlo-α + β2 currents, we tested a range of fatty acids. Besides AA, other cis-unsatuated fatty acids tested included docosahexaenoic acid, which is a long-chain polysaturated fatty acid that is also highly enriched in the brain (DHA, 22:6, cis-4,7,10,13,16,19), and oleic acid, which is a cis-monounsatured fatty acid (OA, 18:1, cis-9). Both DHA (20 μM, n = 6 patches) and OA (5–30 μM, n = 8 patches) mimicked AA action to reversibly enhance hSlo-α + β2 currents and slow inactivation (Fig. 6). Elaidic acid (EA, 18:1, trans-9, 30 μM), which is a trans-configuratinal isomer of 9-octadecenoic acid, similarly altered the current, although to a lesser extent than OA, which is a cis-configuration of 9-octadecenoic acid (n = 5 patches, P < 0.05; Fig. 6, C and D). None of the saturated fatty acids with various acyl chain lengths [stearic acid (SA), 18:0, 30 μM; palmitic acid (PA), 16:0, 30 μM; caprylic acid (CA), 8:0, 30 μM] induced significant changes in the hSlo-α + β2 currents (n = 3–4 patches; Fig. 6, C and D). Among all the fatty acids tested, OA is the most potent to enhance hSlo-α + β2 currents (Figs. 2 and 6, A and B). We also tested other lipid compounds with different charges, including lysophosphatidylinositol (LPI, negative), lysophosphatidyicholine (LPC, neutral), and sphingosine (Sph, positive). None of these molecules mimicked AA action to enhance BK currents (n = 3–10 patches; Fig. 6, C and D). For example, sphingosine markedly inhibited BK currents both in the presence and absence of β2 subunits (77 ± 1.4 and 60 ± 4.0% reduction of peak hSlo-α + β2 and hSlo-α alone currents, respectively, n = 4 patches). Our data suggest that double bonds seem to be necessary for the action of fatty acids to activate BK channels in the presence of β2.

AA ACTION IS MEDIATED BY N-TYPE INACTIVATION. BK channels inactivate through the cytosolic N terminus of the β-subunit (Wallner et al. 1999). To determine whether the AA action is associated with N-type inactivation of BK channels, we used trypsin to remove the cytosolic inactivating domain from the β2-subunit (Solaro and Lingle 1992). In inside-out patches,
short exposure to bath applied trypsin (0.1 mg/ml, <1 min) produced a slowing of inactivation and potentiation of hSlo-α + β2 currents in all patches tested (n = 11 patches; Fig. 7). When AA (30 μM) was applied after trypsin, trypsin pretreatment seemed to occlude largely the actions of AA. The peak conductance before and after AA following trypsin did not change significantly (8.9 ± 3 vs. 9.5 ± 1.9 nS, n = 6 patches), and no obvious shift in activation was seen (Fig. 7). Thus these data suggest that the action of AA is mostly mediated by altering trypsin-sensitive inactivation and that AA has no obvious effect on activation, when inactivation is removed.

AA ACTS ON THE β-SUBUNIT TO PREVENT BALL-RECEPTOR INTERACT. From our results, we believe that AA may exert its effect either on the β2-subunit, by preventing the ball from reaching its receptor, or on the α-subunit, by rendering the receptor insensitive to the ball. To distinguish between these alternatives, the ball–receptor interaction was examined with a synthetic ball-domain peptide from β2 before and after application of AA. Peptides consisting of the first 26 amino acids of the β2-subunit were able to induce inactivating currents in channels formed by the α-subunit alone (Wallner et al. 1999). In Fig. 8, 1 μM β2 ball peptide induced inactivation of hSlo-α current similar to that of hSlo-α + β2 currents. AA at 30 μM, a concentration high enough to remove the inactivation of the β2-subunit (Fig. 2), failed to slow inactivation of the α-subunit alone induced by the ball peptide (1–3 μM, n = 11 patches; Fig. 8). These results suggest that AA action to prevent N-type inactivation is not mediated by changes in the ball receptor or a direct interaction with the inactivation ball.

Pathophysiological relevance of the action of fatty acids on BK channels

Under certain pathophysiological conditions such as ischemia/reperfusion, brain levels of many fatty acids, including unsaturated fatty acid such as AA, DHA, and OA, increase rapidly (Lipton 1999). Small changes caused by any individual fatty acids may induce major changes in BK channel kinetics. For example, lower concentrations of AA (5 μM, 15 ± 4% change in Ipeak, n = 7 patches) and OA (3 μM, 27 ± 2% change in Ipeak, n = 5 patches) individually had only mild effects on hSlo-α + β2 currents (Fig. 9A). However, when coapplied, the effects they induced at these concentrations were dramatic (89 ± 26% change in Ipeak, n = 3 patches; Fig. 9A).

In freshly dissociated mouse neocortical neurons, a transient outward current, recorded in the presence of 4-AP (1 mM), TTX (0.5 μM), and glybenclamide (5 μM), was mostly mediated by BK channels (Sun et al. 2003). External application of 10 μM AA removes or prevents inactivation, resulting in the disappearance of the early inactivation phase (the dip) of the current (n = 5; Fig. 9B). These results suggest that the action of unsaturated fatty acids on BK channels may have significant influence on neuronal survival/death under pathophysiological conditions.

**Discussion**

We report here on a novel mechanism of β-subunit–dependent modulation of hSlo-α channels by AA. AA enhanced hSlo-α current and slowed inactivation only when β2/3 subunits were co-expressed. The AA action on hSlo-α + β2 currents was mediated directly by AA rather than by its metabolites and was not mediated by a direct interaction with AA with the inactivation ball. The AA action is also not mediated by interacting with the ball receptor site on the α-subunit. We suggest that AA may remove inactivation by interacting with the β2 to prevent the inactivation ball from reaching its receptor.

**Figure 7.** AA alters trypsin-sensitive inactivation of hSlo-α + β2 currents. Macroscopic hSlo-α + β2 currents were recorded from inside-out patches. A: representative current traces taken before, after trypsin exposure (0.1 mg/ml), and followed by AA (30 μM). Currents were evoked by a series of depolarizing steps (−80 to +80 mV, in 20-mV steps) from a holding potential of −70 mV followed by a hyperpolarizing −140 mV. B: I-V curves were measured at the peak of current shown in A. Inset: plot of time-course of Ipeak (pA) from the same recording as in A.

**Figure 8.** AA fails to slow inactivation of hSlo-α current induced by a free β2 ball peptide. Macroscopic currents were evoked by 200-ms voltage steps to +80 mV from inside-out patches. Representative current traces of hSlo-α (A) and plots of time-course of peak current (Ipeak) and inactivation time constant (τ) (B) were recorded before and during additive application of 1 μM β2 ball peptide and 30 μM AA.

**Figure 9.** Physiological implications of AA action on BK channels. A: macroscopic hSlo-α + β2 currents were recorded from inside-out patches. Currents were evoked by 200-ms voltage steps to +80 mV. Superimposed traces were taken before and during additive application of 5 μM AA and 3 μM OA (left) or 3 μM OA and 5 μM AA (right). B: whole cell outward currents in mouse neocortical neurons were recorded in the presence of 4-AP (1 mM), TTX (0.5 μM), and glybenclamide (5 μM). Currents were activated by depolarizing voltage steps to 0 mV from a holding potential of −70 mV. Superimposed traces were taken before and during bath application of AA (10 μM).
Modulation of a variety of voltage-dependent potassium channels by AA is well documented (Meves 1994). AA has been shown to activate BK channels in various native cell types (Bringmann et al. 1998; Clarke et al. 2002; Denson et al. 2000). However, it was not clear whether AA modulates the pore-forming α-subunits, some associated proteins, or the β-subunits. AA activation of BK channel activities could be a result of AA producing a shift in the voltage/or Ca^{2+} dependence, making it easier for channels to open, or a result of AA removing an inactivation mechanism. In this study, we showed that, in the presence of hSlo β2- or β3-subunits, AA potentiates a steady-state BK current by modulating the β-subunit–dependent inactivation. AA failed to significantly alter BK current in the absence of the β-subunit. Our data provide an explanation for the observation that BK channel is activated by AA in native cells where β2/β3 is present.

In this study, the action of AA on hSlo-α + β2 currents is membrane-delimited because our data were obtained from excised patches. Although lipoxygenases could potentially be present in the excised membrane patch (Hagmann et al. 1993; Rouzer and Kargman 1988), inhibitors of AA metabolism did not have much effect on AA action. Furthermore, oleic acid, which is not a substrate for the AA metabolic pathways that yield bioactive compounds, induced a similar response to that of AA. These observations provide evidence that metabolites are not necessary for AA action. Additional data that indicate that the effect of AA is a direct one include our observation that nonenzymatic metabolites of AA do not mediate its action. For example, the AA-induced response was not abolished by adding the superoxide free radical scavenger SOD, indicating that the action is not through the generation of reactive oxygen radicals. Thus our data indicate that AA interacts directly either with the channel protein or the lipid membrane to alter hSlo-α + β2 currents.

Our results from the trypsin pretreatment experiments have indicated that the action of AA is likely to be based on a trypsin-sensitive “ball-and-chain” mechanism, which involves the cytosolic N terminus of the β-subunits (Solaro and Lingle 1992; Wallner et al. 1999). The action of AA was largely occluded by prior exposure to trypsin, suggesting that AA acts to relieve the cytosolic inactivation. AA failed to slow inactivation induced by the cytosolic free ball peptide, suggesting that AA does not directly interact with the ball domain itself to relieve inactivation. Our data with various fatty acids and lipids show that there is a modest chemical specificity in the interaction between fatty acids and BK channel protein. Double bonds play a critical role in the action of fatty acids. All the unsaturated fatty acids tested in our study with different chain conformations, including the cis polyunsaturated fatty acid (AA, 20:4n-6 and DHA, 22:6n-3) and the cis mono-unsaturated fatty acid (OA, 18:1n-9), induced a similar response, whereas saturated fatty acids with different acyl chain lengths (SA, 18:0; PA, 16:0; CA, 8:0) and other lipids with different charges were not able to mimic AA action. Furthermore, OA was more potent than AA and DHA to enhance hSlo-α + β2 currents, which is consistent with their potency to promote hexagonal phase formation of fatty acid-phosphatidyethanolamine mixtures (Epand et al. 1991). Lyso phosphatidies which have a different shape (smaller head with larger tail) from unsaturated fatty acids, were not able to mimic AA action. Thus our results can be rationalized by considering the effective shape of unsaturated fatty acids in which double bonds make the cross-section of the hydrophobic region larger than that of the polar carboxyl head. When incorporated in bilayers, these molecules promote a hexagonal negative curvature (Culnis and Kruijff 1979; Epand et al. 1991; Perozo et al. 2002). We hypothesize that a conformational change of the β-subunit, as a result of membrane deformation induced by AA and other unsaturated fatty acids, may prevent the cytosolic inactivation ball from reaching its target.

Our findings may have implications in certain pathophysiological conditions. In the brain, the level of free AA and other fatty acids dramatically increases as a result of increased phospholipase A2 activity in ischemia/reperfusion (Lipton 1999; Phillis and O’Regan 2004). First, it should be noted that the concentrations of AA used are not outside the range of AA concentrations that can exist in the brain during such pathophysiologic conditions as stroke and ischemia (Lipton 1999). In addition, the action of such free fatty acids may not be based on a singular interaction with one fatty acid in such conditions. Indeed, we show in this work that small concentrations of more than one fatty acid may lead to major changes that amplify the effect of each alone. Because 1) a high BK channel density is detected in the cortex and at the axonal terminals (Knaus et al. 1996; Wanner et al. 1999; Weiger et al. 2000) and 2) BK channels in cortical neurons exhibit inactivation (Smith and Ashford 2000; Sun et al. 2003), an increased AA and other unsaturated fatty acids during ischemia/reperfusion may alter K+ efflux and release of neurotransmitters such as glutamate by activating BK channels in neocortical neurons. However, free reactive oxygen radicals from oxidative metabolism of AA can also exert opposite effects on BK channels (Tang et al. 2004), especially during reoxygenation. Therefore it is possible that AA may have opposing effects, and additional experiments are needed in this area to decipher the temporal results induced by AA and its metabolism in pathophysiologic conditions.

In summary, we discovered that β-dependent BK channel inactivation is a specific molecular target for AA and other unsaturated fatty acids. The pronounced slowing of inactivation kinetics may be a specific mechanism by which AA and unsaturated fatty acids modulate K+ efflux, neuronal excitability, and neurotransmitter release, and thus influence neuronal death/survival in various neuropathological conditions.

ACKNOWLEDGMENTS

We appreciate the technical assistance of O. Gavrialov and Drs. X. Gu and V. Verselis. The β4-subunit was provided by Dr. L. Toro and β2 and 3 were obtained from Dr. C. Lingle.

GRANTS

This work was supported by National Institute of Neurological Diseases and Strok Grant 1P01 NS-42202.

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J Neurophysiol • VOL. 97 • JANUARY 2007 • www.jn.org


