The Journal of Neuroscience, February 1, 1996, 16(3):1016–1025

Inhibition of the Kv4 (*Shal*) Family of Transient K^+ Currents by Arachidonic Acid

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We have found that transient A-type currents expressed in *Xenopus* oocytes from members of the Kv4 family are suppressed by arachidonic acid. Currents from members of the Kv1, Kv2, and Kv3 families showed little or no inhibition by fatty acids in this expression system, although *Shaker* currents showed a modest increase in peak amplitude. The inhibition of Kv4 channels was not prevented by cyclo-oxygenase, lipoxygenase, or cytochrome P-450 inhibitors and was mimicked by 5,8,11,14-eicosatetraynoic acid, an arachidonic acid analog that is not metabolized by these pathways. Other unsaturated *cis* fatty acids with more than two double bonds produced a similar effect. In inside-out macropatches, the current was reversibly reduced >50% by 2 mM arachidonic acid, and the inhibition developed in <40 sec. These results suggest that, at concentrations that are likely to be physiologically relevant, arachidonic acid interacts directly with the channel or with a closely associated component. Preliminary mutagenesis of Kv4.2 channels indicates that the N terminal is not required for arachidonic acid action but that the S4–S5 loop may influence the effect.

Key words: potassium channel; A-current; arachidonic acid; Shal; Kv4; modulation; fatty acids

Arachidonic acid can regulate neuronal excitability and synaptic transmission (Palmer et al., 1980; Vacher et al., 1989; Fraser et al., 1993). Ion channels, including Na^+ (Linden and Routtenberg, 1989), Ca^{2+} (Keyser and Alger, 1990), Cl^- (Anderson and Welsh, 1990; Hwang et al., 1990), K^+ (see below), and agonist-operated channels (Schwartz et al., 1988; Miller et al., 1992; Vijayaraghavan et al., 1995), are each targets of arachidonic acid action (for review, see Bevan and Wood, 1987; Clapham, 1990; Piomelli and Greengard, 1990; Ordway et al., 1991). These effects on channels may be parts of physiological pathways for modulation. In response to transmitters and hormones, arachidonic acid can be released from membranes by the activation of phospholipase A_2 by G-proteins or elevated intracellular Ca^{2+} (Chang et al., 1987; Burch, 1989) (for review, see Axelrod et al., 1988; Kim et al., 1989). Arachidonic acid can also be produced by the sequential participation of phospholipases C and D or diacylglycerol lipase (Bell et al., 1979) (for review, see Loffelholz, 1989). There is also an increase in free arachidonic acid concentration under pathological conditions such as ischemia, epilepsy, or stroke (Shimizu and Wolfe, 1990). Once produced within a cell, arachidonic acid can activate additional second-messenger pathways [e.g., Ca^{2+} (Knepel et al., 1988) and protein kinase C (PKC) (McPhail et al., 1984)] and can be transformed via several routes (cyclo-oxygenase, lipoxygenase, and epoxygenase) into additional active metabolites (for review, see Needleman et al., 1986).

Modulation of K^+ channels by arachidonic acid is widespread and diverse. The enhancement of the S-current in *Aplysia* sensory neurons by the peptide FMRF-amide (Piomelli et al., 1987), the enhancement of the M-current in rat CA1 hippocampal neurons by somatostatin (Schweitzer et al., 1990), and the modulation of the inward rectifier in cardiac myocytes by angiotensin (Kurachi et al., 1989a,b) or platelet-activating factor (Nakajima et al., 1991) are mediated by lipoxygenase metabolites of arachidonic acid. Metabolites of the epoxygenase pathway induce the opening of K^+ channels in epithelial cells (Hu and Kim, 1993). The modulation of ATP-sensitive K^+ channels in insulinoma cells by arachidonic acid likely is mediated by PKC (Müller et al., 1992); additionally, arachidonic acid itself causes the opening of K^+ channels in smooth muscle (Ordway et al., 1989), cardiac myocytes (Kim and Clapham, 1989), and cultured mesencephalic and hypothalamic neurons (Kim et al., 1995) and activates large-conductance Ca^{2+}-activated K^+ channels in vascular smooth muscle (Kirber et al., 1992). Arachidonic acid also potently suppresses the A-current in sympathetic neurons (Blair and Suprenant, 1993; Villarroel, 1993).

Little is known, however, about the molecular basis of arachidonic acid action on ion channels, although many of them have been cloned and expressed. Most of the cloned voltage-dependent K^+ channels appear to be encoded by four gene families. These families can be identified by their *Drosophila* gene homolog (*Shaker*, *Shab*, *Shaw*, and *Shal*) or by an alternative nomenclature (Kv1, Kv2, Kv3, and Kv4) (Wei et al., 1990). This classification has physiological consequences because different subunits of the same family can combine to form a functional tetrameric channel in oocytes, whereas members of different families do not (Covarrubias et al., 1991; Salkoff et al., 1992). Excitable cells may take advantage of the great diversity of K^+ channels by selectively modulating the properties of some channels and not others in response to stimuli.

Received Aug. 22, 1995; revised Oct. 30, 1995; accepted Oct. 31, 1995.

This work was supported by National Institutes of Health Grants HL48636 and GM42376. We thank Drs. Timothy Baldwin, Paul Bennet, Arthur Brown, Manuel Covarrubias, Lily Jan, Huai-Yu Mi, Bernardo Rudy, Lawrence Salkoff, and Richard Swanson for K^+ channel clones, and we thank Dr. Terry Snutch and the Genetics Institute for the pMT2 plasmid. We also thank Drs. Richard Aldrich and Catherine Smith-Maxwell for the *Shaker* chimera. We are grateful to Ross Vickery for his help during this project and to Dr. Ji-Fang Zhang for his careful reading of this manuscript. A.V. is a fellow of the Multiple Sclerosis Society.

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We have studied the effects of arachidonic acid on voltage-dependent K^+ channels expressed in *Xenopus* oocytes. We have screened several channels and have found that the members of the Kv4 family are inhibited by arachidonic acid. The arachidonic acid effect appears to be direct, and the modulation does not involve changes in the voltage dependence of inactivation or in the rate of activation or inactivation. These are also characteristics of the inhibition by muscarine and arachidonic acid of the A-current in sympathetic neurons (Blair and Suprenant, 1993; Villarroel, 1993, 1994). Thus, the effect we observe in oocytes on Kv4-family channels may underlie the muscarinic regulation of I_A in sympathetic neurons as well as inhibition of I_A in other cells. In addition, the modulation observed in oocytes can provide a system for the detailed study of fatty-acid effects on an ion channel.

MATERIALS AND METHODS

In vitro *transcription and oocyte injection.* cDNAs were linearized and RNA was synthesized *in vitro* with the following enzymes: fly *Shaker* (*Eco*RI/T3) (Timpe et al., 1988), rat Kv1.1 [RCK1] (*Kpn*I/T3) (Swanson et al., 1990), bovine Kv1.2 [BEK5] (*Xho*I/T3) (H.-Y. Mi, unpublished observations), rat Kv1.3 [Kv3] (*Hin*dIII/T7) (Swanson et al., 1990), human Kv1.4 [RCK4] (*Eco*RI/SP6) (Po et al., 1992), rat Kv1.5 [Kv1] (*Not*I/T7) (Swanson et al., 1990), rat Kv2.1 [DRK1] (*Sac*I/T3) (Frech et al., 1989), mouse Kv2.1 [*mShab*] (*Sac*II/T3) (Pak et al., 1991b), mouse Kv4.1 (*Sac*I/T3) (Pak et al., 1991a), rat Kv4.2 (*Not*I/T7) (Baldwin et al., 1991), fly *Shal2* (*Sac*II/T3) (Wei et al., 1990), rat Kv3.1 [NGK1] (*Not*I/T7) (Hartmann et al., 1991), human Kv3.4 (*Sal*I/T3) (Rudy et al., 1991). Rat Kv4.2 was subcloned in the pMT2 vector (Swick et al., 1992) for nuclear injection. This method was preferred for Kv4.2 because comparable levels of expression (peak currents of 4–9 mA at +40 mV, from a holding potential of −90 mV) were difficult to obtain even with very high concentrations of cRNA.

Stage V or VI oocytes were defolliculated enzymatically with collagenase [1.5 mg/ml in Ca^{2+}-free OR2, made with (in mM): NaCl 82.5, KCl 2.5, $MgCl_2$ 1, HEPES 5, pH 7.6] for 2 hr at room temperature. The oocytes were injected with 50 nl of cRNA or with 10–30 nl of cDNA (10 ng/ml) for nuclear injection of Kv4.2. Oocytes were kept in ND96 buffer [(in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, pH 7.6] at 18°C and used for recording 1–4 d after injection. All recordings were done at room temperature (~22°C).

Electrophysiology. Whole-cell currents were recorded in oocytes under two-electrode voltage clamp, by using a virtual-ground Warner oocyte clamp OC-725A amplifier (180 V compliance; Warner Instruments, Hamden, CT). To increase the clamping performance, the capacitor on the DC gain was substituted by a series of 10 selectable capacitors (from 1 mF to 10 pF). We were able to clamp wild-type *Shaker* currents of 70 mA evoked from −80 mV during a step to +60 mV in <0.5 msec with the capacitor set to the lowest value. Most experiments were done with the 10 pF capacitor setting. The electrodes were filled with 3 M KCl and had resistances of 0.3–1 MΩ. Linear capacitative and leakage currents were subtracted on-line by a P/4 or P/5 pulse protocol, except where indicated. The oocytes were bathed in "*Xenopus* saline" made with (in mM): NaCl 100, KCl 2.5, $MgCl_2$ 1, $MnCl_2$ 2, HEPES 5, pH-adjusted to 7.2 with NaOH. Mn^{2+} was included in the solution to block Ca^{2+} currents and to prevent the activation of the endogenous Ca^{2+}-activated Cl^- current. The presence of 2 mM Mn^{2+} instead of Ca^{2+} caused a shift in the half-inactivation voltage on the inactivating currents tested. The shift was 6.0 ± 0.3 mV (n = 5) for Kv1.4 currents (from −52.1 ± 3.0 to −46.1 ± 3.1 mV), 12.2 mV (n = 1) for Kv3.2 currents (from −10.3 to 1.8 mV), and 14.7 ± 1.0 mV (n = 7) for Kv4.2 currents (from −70.9 ± 2.2 to −56.2 ± 2.1 mV). Similar shifts in the voltage dependence of inactivating K^+ channels have been described previously (Mayer and Sugiyama, 1988; Agus et al., 1991). The currents were filtered at 2500 Hz with an 8-pole Bessel filter (Frequency Devices 902, Haverhill, MA).

The vitellin layer was stripped for patch recording using a hypertonic solution composed of 0.1 gm/ml sucrose plus (in mM): NaCl 100, KCl 32.5, $MgCl_2$ 1, $MnCl_2$ 0.5, HEPES 5, pH 7.2. The oocytes were bathed in "patch-formation saline" made of (in mM): KCl 100, $MgCl_2$ 0.5, $MnCl_2$ 0.5, HEPES 5, pH 7.2 (adjusted with NaOH). The electrode was made from micropipettes (Drumond, Broomall, PA), fire-polished, and filled with *Xenopus* saline; the resulting resistances were on the order of 4–5 MΩ. After seal formation, the electrode was pushed into the oocyte to establish the inside-out configuration; then it was retracted rapidly and transferred to "inside-out saline," which had a composition similar to patch formation saline, except that it contained 1 mM EGTA and no $MnCl_2$. The currents were recorded with an Axopatch-1C amplifier (Axon Instruments, Foster City, CA) and filtered at 500 Hz with the incorporated 3-pole Bessel filter. The external salines were perfused continuously at 2.5 ml/min in a small chamber of 1.7 × 1.7 mm^2 cross-section. All drugs were stored as a 100 mM stock solution in dimethylsulfoxide (DMSO) in sealed amber vials under N_2 at −80°C. DMSO was dehydrated with Sigma molecular sieves (3 Å nominal pore diameter; St. Louis, MO). For recordings, the stocks were diluted into saline containing 0.1% DMSO. Thus, when the final concentration of a drug was 50 mM, the DMSO concentration was 0.15%. DMSO at concentrations as high as 0.25% did not produce any significant effect on *Shaker* or rat Kv4.2 currents. Arachidonic acid and 5,8,11,14-eicosatetraynoic acid (ETYA) did not have an appreciable effect on uninjected oocytes or on the holding and leakage currents of oocytes expressing K^+ channels.

Drugs. Arachidonic acid was from Calbiochem (La Jolla, CA); 5,8,11-eicosatriynoic acid (ETI) was from Cayman Chemical (Ann Arbor, MI); ETYA, 8,11-eicosadiynoic acid (EDYA), linolenic acid, γ-linolenic acid, caffeic acid, methoxsalen, proadifem (SKF-525A), and esculetin were from BioMol Research Labs (Plymouth Meeting, PA). All other drugs were from Sigma.

Mutagenesis. The amino acids at position 3–28 of Kv4.2 were deleted by PCR-based mutagenesis. The 5′-end primer was a 27 mer in which the sequences encoding amino acids 29–32 were preceded by a sequence that encoded amino acids 1–3 and introduced an *Nco*I site at the position of the start methionine (C GGG CCC ATG GCA GCT CCC CCA AGG CA). The 3′-end oligo was complementary to a region on the S1–S2 extracellular loop. The amplified product contained an additional *Nco*I site found at position T182 in Kv4.2. The Kv4.2 clone contained an *Nco*I site at the start methionine. Both Kv4.2 and the PCR product were digested with *Nco*I, gel-purified, and ligated together; clones with the proper orientation were selected. The deletion was found to contain an unintended R → P mutation at position 32; however, because the action of arachidonic acid was preserved in these deleted channels (see below), this additional mutation did not pose a problem for the interpretation of the results.

The Kv4.2 chimera containing the S4–S5 loop from *Shaker* was made by using a multistep PCR protocol that exchanged the sequence LRILGGYTLKSCASE from Kv4.2 with LQILGRYTLKASMRE from *Shaker* (nonconserved amino acids are underlined). The first PCR amplified a segment of a *Shaker* construct that previously had introduced silent *Stu*I site at position G379 (a gift from Dr. R. Aldrich, Stanford University). There is a *Stu*I site at the equivalent position in Kv4.2 (G309). The 3′-primer (primer B1) was a hybrid between *Shaker* and Kv4.2 sequences (GAG CTC CCG CAT TGA AGC TTT CAG AGT TCG TCC), and the 5′-primer (primer A1) flanked the *Stu*I site. Primer B1 introduced a *Hin*dIII site that was used for the screening of positive clones. The second PCR amplified the adjacent region of Kv4.2. The 5′-primer for the second PCR (primer A2: GCT TCA ATG CGG GAG CTC GGC TTC TTG CTC TTT TCC) partially overlapped primer B1, and the 3′-primer (primer B2) flanked the *Kpn*I site of Kv4.2. The third PCR was performed by mixing 1/100 of the product of PCR 1 and 2, and amplifying with primers A1 and B2. The product was subcloned between the *Stu*I and *Kpn*I sites of Kv4.2. The sequence of this region was verified.

The *Shaker* chimera containing the S4–S5 loop from Kv4 was generously provided by Drs. C. Smith-Maxwell and R. Aldrich, Stanford University.

RESULTS

Kv4 currents are selectively inhibited when expressed in *Xenopus* oocytes

To establish a system for studying the effects of arachidonic acid on a molecularly defined K^+ channel, we expressed 12 channels in *Xenopus* oocytes, including representatives of each of the four main families of voltage-dependent K^+ channels. Figure 1 shows the effect of 25 mM arachidonic acid on several of these. The most dramatic effect observed was the 57.8 ± 1.7% (n = 5) (mean ± SD, n = number of experiments) reduction of rat Kv4.2 current, which encodes an A-type transient K^+ current. A similar potent reduction was observed with other members of this family: mouse

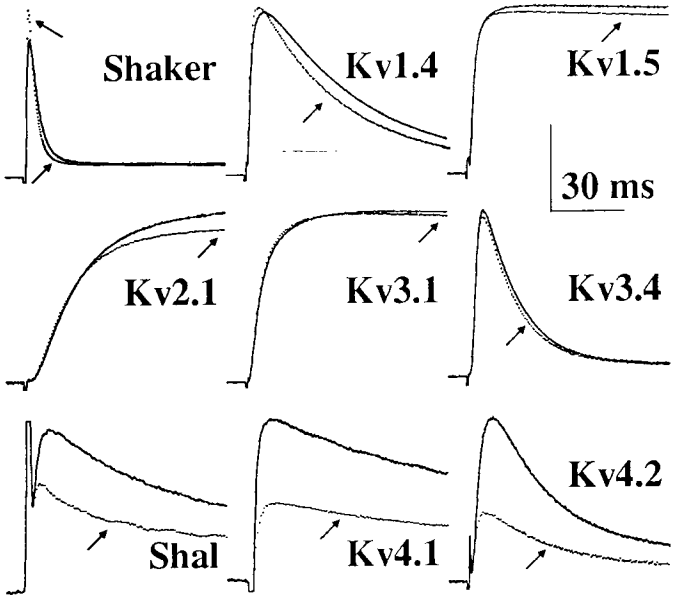

Figure 1. Arachidonic acid selectively inhibits Kv4-family channels expressed in *Xenopus* oocytes. Currents from nine cloned K^+ channels expressed in *Xenopus* oocytes were examined in standard solution (*solid lines*) and in the presence of 25 mM arachidonic acid (*dotted lines* and *arrows*). The currents were evoked by stepping the membrane potential to +40 mV from a holding potential of −80 mV, except for Kv1.4, which was held at −100 mV. The *horizontal calibration bar* is 30 msec and the *vertical bar* (from top to bottom, left to right) is (in mA) 8.1, 7.7, 5.7, 2.5, 8.0, 4.2, 0.6, 1.2, and 0.8. The traces were leak-subtracted on-line using a P/4 or P/5 protocol.

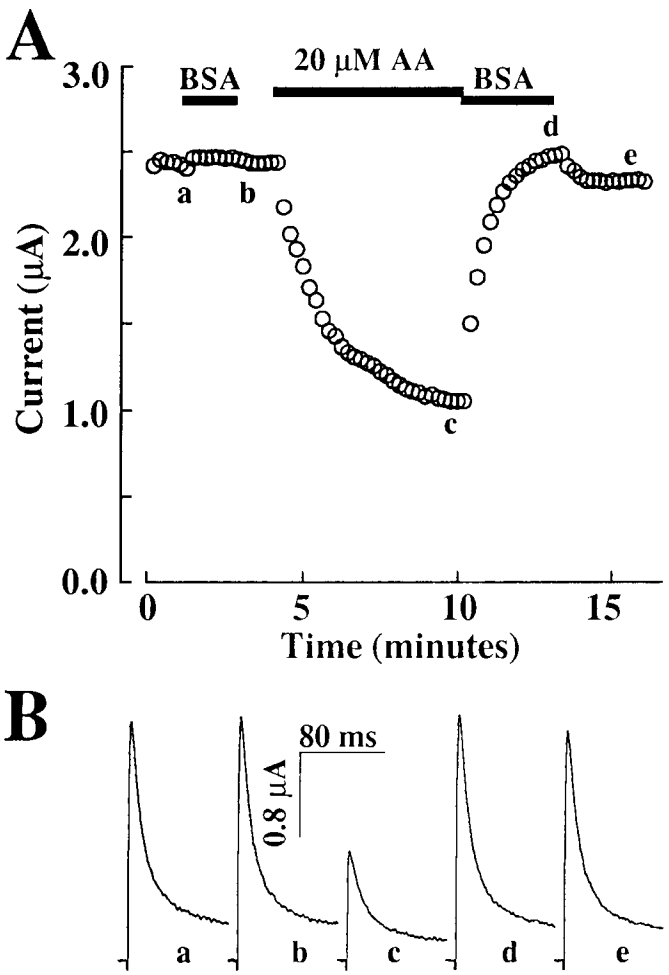

Figure 2. Time course of Kv4.2 inhibition by arachidonic acid. *A*, Peak current evoked from a holding potential of −90 mV during a step to +40 mV. The times of application of *BSA* (0.5 mg/ml) and arachidonic acid (*AA*) are indicated by the *bars*. Because of its ability to bind fatty acids, BSA was used to hasten the washout of arachidonic acid. BSA alone caused a very slight augmentation of the current (compare *d* and *e*). *B*, Representative traces of Kv4.2 currents taken at time points *a–e* indicated in *A*.

Kv4.1 and fly *Shal*. In contrast, the peak *Shaker* current was enhanced by 20.5 ± 4.1% (n = 4; see also Fig. 3*D*). Only minor effects were observed in the other channels tested (bovine Kv1.2, rat Kv1.3, human Kv1.4, rat Kv1.5, rat Kv2.1, mouse Kv2.1, rat Kv3.1, and rat Kv3.4).

The response to arachidonic acid was studied in more detail in rat Kv4.2, because the effect was robust. In addition, a similar reduction had been observed for the A-current in sympathetic neurons in response to muscarine (which is known to mobilize arachidonic acid) and to externally applied arachidonic acid (Blair and Suprenant, 1993; Villarroel, 1993, 1994).

The reduction in the current induced by arachidonic acid developed slowly, reaching a near-steady-state value in ~5 min (Fig. 2). After arachidonic acid washout, the current recovered very slowly, probably because of the difficulty of removing arachidonic acid from the interior of the oocyte. To accelerate the recovery, bovine serum albumin (BSA), which binds fatty acids, was added to the bathing solution, and the current recovered to ~90% of the control level in 5 min. BSA by itself occasionally caused a very slight increase in the peak current (no more than 5%).

In sympathetic neurons, the arachidonic acid-mediated I_A inhibition is mimicked by the nonhydrolyzable arachidonic acid analog ETYA. This is true for the reduction of Kv4.2 current in oocytes as well (10 mM ETYA; Fig. 3*A*). To compare the effects of the two fatty acids (and other fatty acids used in this study), cumulative dose–response curves were made by bath-applying a series of increasing concentrations of a drug, doubling the drug concentration every 3 min. This protocol was selected as a way to compare a full range of concentrations on individual oocytes, while circumventing the lengthy times required for the washout of fatty acids and the baseline changes in the oocytes caused by protracted recordings. Although 3 min was not always sufficient to reach a complete steady state of blockade (Fig. 2*A*), extending the application to 9 min caused only a minor shift in the dose–response curves (data not shown).

Figure 3*C* (*open circles*) shows a cumulative dose response for arachidonic acid. The maximal reduction obtained with this cumulative protocol was estimated to be 67.9 ± 2.0% (n = 5), and the IC_{50} was ~8 mM. Under these assay conditions, ETYA was more potent than arachidonic acid (Fig. 3*C*, *filled circles*). The maximal reduction was 73.0 ± 3.9% (n = 9), and the IC_{50} was ~2 mM. Fly *Shal* and mouse Kv4.1 also were potently suppressed by ETYA (data not shown). In contrast, ETYA did not mimic the effect of arachidonic acid on *Shaker* currents, causing little or no enhancement (Fig. 3*B,D*).

The marked effect of arachidonic acid and ETYA on Kv4.2 currents in oocytes allowed us to investigate the mechanism by

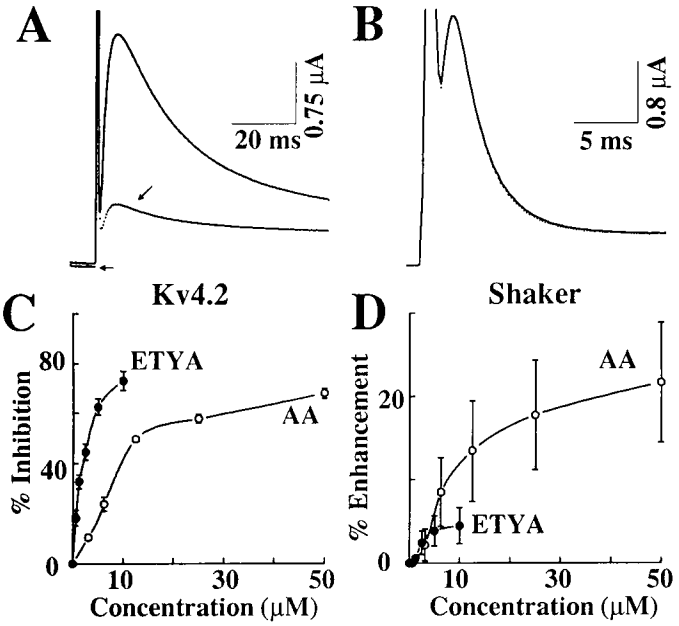

Figure 3. ETYA mimics the effects of arachidonic acid on Kv4.2 but not on *Shaker*. *A*, Effect of 10 mM ETYA on Kv4.2 (*dotted line* and *arrows*). The current was evoked by a voltage step from −90 to +40 mV. The traces were not leak-subtracted. The small increase in holding current was not related to the application of ETYA. *B*, Effect of 10 mM ETYA on *Shaker* (*dotted line*). The current was evoked by a voltage step from −80 to +40 mV. The traces were not leak-subtracted. *C*, Cumulative dose–response relationship of Kv4.2 to ETYA (*filled circles*; $n = 9$) and arachidonic acid (*open circles*; $n = 5$). The cumulative dose–response relationship was determined by exposing the oocytes to a series of bath solutions such that the drug concentration was doubled every 3 min, and the amplitude of the Kv4.2 current was monitored with repeated voltage steps. Generally similar dose–response curves (data not shown) were obtained from 9 min incubations, which allowed the fatty acid effects to come more fully to equilibrium. *Bars* represent the SEM. *D*, Cumulative dose response of *Shaker* to ETYA (*filled circles*; $n = 3$) and arachidonic acid (*open circles*; $n = 4$).

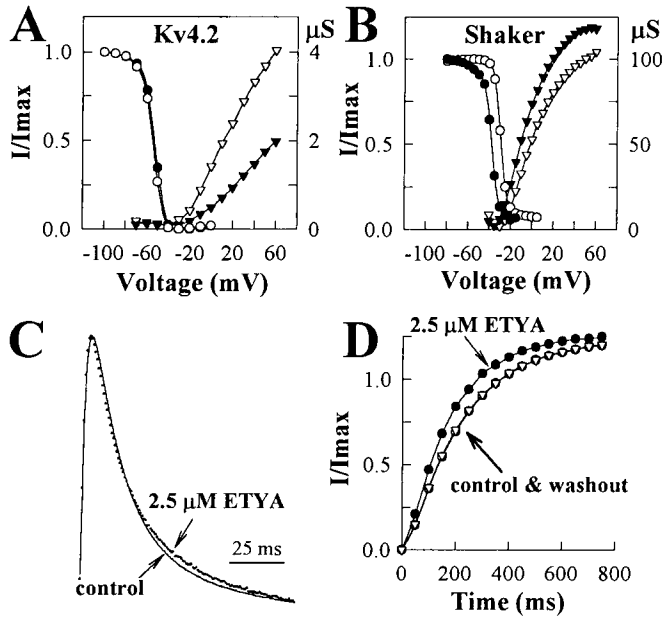

Figure 4. Effect of arachidonic acid and ETYA on biophysical parameters of Kv4.2 and *Shaker*. *A*, Kv4.2 inactivation (*circles*) and activation (*triangles*) in the presence (*filled symbols*) and absence (*open symbols*) of 2.5 mM ETYA. Inactivation was determined by measuring the peak outward current at +40 mV after 5 sec at the holding potential. After the test pulse, the oocyte was shifted to the next holding potential. The inactivation curves were then scaled by normalizing them as I/I_{max}. The activation curves are given as conductances (in mS). *B*, *Shaker* inactivation and activation in the presence (*filled symbols*) and absence (*open symbols*) of 25 mM arachidonic acid. The protocol and normalization of the inactivation curves are the same as those in *A*. *C*, Comparison of the activation and inactivation kinetics of Kv4.2 in the presence (*dotted trace*) and absence (*solid trace*) of 2.5 mM ETYA. The current in the presence of ETYA was reduced by >50% and has been scaled for comparison. *D*, Comparison of the kinetics of recovery from inactivation of Kv4.2 with (*filled circles*), without (*open circles*), and after washout (*open triangles*) of 2.5 mM ETYA. Peak currents during 20 msec pulses to +40 mV have been plotted versus the elapsed time interval from an inactivating pulse (1 sec at +40 mV). The membrane potential was held at −90 mV.

which a fatty acid can alter channel function. Analysis of voltage-clamped currents in the presence and absence of these agents revealed that neither arachidonic acid nor ETYA shifted the voltage dependence of inactivation (Fig. 4*A*). The effect on the voltage dependence of activation could not be examined accurately because a maximum conductance was not reached. In addition, neither arachidonic acid nor ETYA substantially changed the activation or inactivation kinetics as shown in Figure 4*C*, in which the current trace in the presence of ETYA (*dotted line*) has been scaled to the peak of the control current (*solid line*). In this context, the inhibition in oocytes is similar to the effect of arachidonic acid on I_A in sympathetic neurons (Villarroel, 1993). In contrast, the arachidonic acid-mediated enhancement of *Shaker* current was accompanied by a −6.3 ± 2.7 mV ($n = 4$) shift in the voltage dependence of inactivation (Fig. 4*B*), and by an acceleration of the activation and inactivation kinetics (see Fig. 1). ETYA had little effect on the current–voltage relationship (data not shown) or on the activation and inactivation kinetics of *Shaker* currents (Fig. 3*B*). No major effects of ETYA were observed in any of the other channels tested (see above and Fig. 1), except that the closely related Kv4.1 and fly *Shal* channels were inhibited in a manner very similar to that described for Kv4.2 (data not shown).

The inhibition of Kv4.2 currents was accompanied by a modest, but significant ($p < 0.05$, paired t test), acceleration of the rate of recovery from inactivation (Fig. 4*D*). In the presence of 2.5 mM ETYA, the time constant decreased from 188.3 ± 16.8 to 152 ± 14.4 msec at −90 mV ($n = 3$), and from 280.0 ± 35.9 to 201.2 ± 23.3 msec at −80 mV. Similar results were obtained with 25 mM arachidonic acid.

The similarity of action of ETYA and arachidonic acid action with respect to biophysical parameters of Kv4.2 suggests that both fatty acids use the same mechanism to suppress the current. Experiments described below with injected BSA and mutant chimeric channels support this hypothesis. In subsequent experiments, we often used ETYA instead of arachidonic acid because of its greater potency, stability, and immunity to metabolism.

Pharmacology of Kv4.2 inhibition

To define further the means by which these fatty acids altered Kv4.2 currents, we initiated a series of pharmacological experiments. Although these compounds were applied in the bath, they are membrane-permeable and may be acting in the plane of the membrane or intracellularly. To investigate the site of action, we injected oocytes with BSA which, because of its ability to bind fatty acids, can be regarded as a fatty acid chelator (Spector et al.,

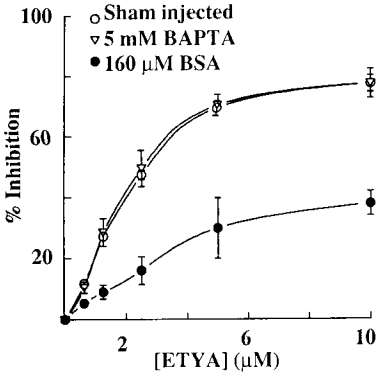

Figure 5. Injection of BSA, but not of BAPTA, decreased ETYA inhibition. Cumulative dose response to ETYA of Kv4.2-expressing oocytes injected with 50 nl of saline (*open circles*; $n = 4$), 100 mM BAPTA (*open triangles*; $n = 4$), or 300 mg/ml BSA (*filled circles*; $n = 5$). *Bars* indicate the SEM. The volume of an oocyte was estimated to be 1 ml; therefore, internal concentrations were ~5 mM BAPTA and 160 mM BSA.

1971). In the injected cells, ETYA inhibition was partially prevented (Fig. 5, *filled circles*) and, therefore, it is unlikely that the inhibition of the current was attributable to an extracellular site of ETYA action. In addition, internal BSA precluded the acceleration in the rate of recovery from inhibition (data not shown).

A hypothetical path by which internal arachidonic acid might act is by causing an increase in intracellular Ca^{2+}. The A-current of hippocampal neurons, for example, is very sensitive to intracellular Ca^{2+} (Chen and Wong, 1991), and arachidonic acid, presumably after reaching the cell interior, can cause an increase in intracellular Ca^{2+} in some cells (Oike et al., 1994). Indeed, because Kv4.2 is present in the dendrites and soma of hippocampal neurons (Sheng et al., 1992), it likely is a major contributor to this Ca^{2+}-sensitive A-current. To test the possibility that Ca^{2+} mediates this effect in oocytes, we injected oocytes with the potent Ca^{2+} chelator bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid (BAPTA). We confirmed the efficacy of the injected BAPTA by monitoring the Ca^{2+}-activated Cl^- current that is endogenous to the oocyte during pulses to +40 mV (in 10 mM Ca^{2+} and no added Mn^{2+}). BAPTA injection blocked this current (data not shown) and, therefore, intracellular Ca^{2+} could be effectively chelated. Figure 5 shows that the cumulative dose response of ETYA in cells injected with BAPTA (*triangles*) was similar to the dose response in cells injected with buffer (*open circles*); the effect of the fatty acid is unlikely to be mediated by Ca^{2+}.

Arachidonic acid that enters the oocyte could be subject to metabolism by the cyclo-oxygenase, lipoxygenase (Hawkins and Brash, 1989), or cytochrome P-450 (epoxygenase) pathways, and one of the metabolic derivatives of arachidonic acid could be active on the channel. Although ETYA inhibits many of these enzymes and is not thought to be metabolizable, it remained possible that arachidonic acid was converted to a more potent metabolite. We attempted to block each of these pathways with several of the commonly used agents: 5 mM indomethacin (a cyclo-oxygenase blocker), 50 mM nordihydroguaiaretic acid (NDGA), or 50 mM caffeic acid (lipoxygenase blockers), and 10 mM proadifem or 10 mM methoxsalen (epoxygenase blockers). None of these inhibitors was effective in preventing the effect of arachidonic acid. Esculetin (50 mM), NDGA (10 mM), and clotrimazole (1–10 mM) each reduced the magnitude of the current in the absence of arachidonic acid and, therefore, could not be used as blockers of arachidonic acid metabolism. Although the efficacy of the blockers we used is not known in the oocyte, a requisite metabolism of arachidonic acid appears to be unlikely.

Another mechanism by which internal arachidonic acid might inhibit Kv4.2 is by activating a PKC (McPhail et al., 1984). To address this possibility, oocytes were incubated with the kinase blockers sphingosine (10 mM) and staurosporine (1 mM). No effect was observed on the inhibition by arachidonic acid or ETYA and, thus, PKC likely does not mediate this effect.

The suppression of Kv4.2 by arachidonic acid in inside-out patches is fast, potent, and partially reversible

The effect of arachidonic acid was studied in inside-out patches, which held two advantages for this investigation. First, in the absence of nucleotides, phosphorylation events cannot take place; the cytochrome P-450 pathway is not active because the cofactors nicotinamide-adenine dinucleotide phosphate (NADP) and reduced NADP (or NADPH) are not present, and changes in intracellular Ca^{2+} cannot take place. Thus, the patch provides an alternative to the pharmacological investigation described above. In addition, the large volume of yolk in the oocyte may serve as a powerful sink for applied fatty acids. It can distort, therefore, the efficacy of bath applied arachidonic acid either by binding it or by metabolizing it. Inside-out patches may reveal the potency of arachidonic acid on the channel more accurately.

Patch recordings were difficult because none of the channels of the Kv4 family expresses robustly in oocytes and the single-channel current of these channels is small (Baldwin et al., 1991). Attempts were made to obtain macropatches from the N-deleted mutant (noninactivating) as well as wild-type. The levels of expression of the N-deleted mutant were higher than for the wild-type, and we only obtained macropatch records from the former. This deletion did not affect the sensitivity of the channel to arachidonic acid (see below).

Figure 6*A* shows the effect of 4 mM arachidonic acid in an inside-out macropatch. The current was inhibited almost completely in the presence of arachidonic acid (*trace b*) and recovered nearly to control levels after the drug was removed (*trace c*). The time course of the response is illustrated in Figure 6*B*. The response was much faster and more potent than in the intact oocyte, presumably because several barriers were removed under inside-out recording conditions and the internal yolk of the oocyte was not trapping arachidonic acid. Thus, in Figure 6*B*, 4 mM arachidonic acid acted within 20 sec of application, whereas in the intact oocyte (see Fig. 2*A*) 20 mM arachidonic acid took >5 min to reach a steady state of block. Figure 6*C* compares a plot of the extent of inhibition at different concentrations measured 40 sec after applying the drug. The current was suppressed completely at 8 mM and was reduced >50% at 2 mM. After washout, the current recovered to 80% of control after challenging with 2 mM and to >65% after applying 4 or 8 mM arachidonic acid. These results exclude the participation of a kinase, cytochrome P-450, or Ca^{2+}. The participation of a phosphatase is also very unlikely, because recovery would require the action of a kinase.

The efficacy of related fatty acids does not correlate well with their predicted effects on membrane fluidity

To assess the specificity of the inhibitory response, cumulative dose–response curves were constructed under two-electrode voltage clamp for an array of structurally related fatty acids. Because fatty acids may cause changes in the fluidity of the membrane, we

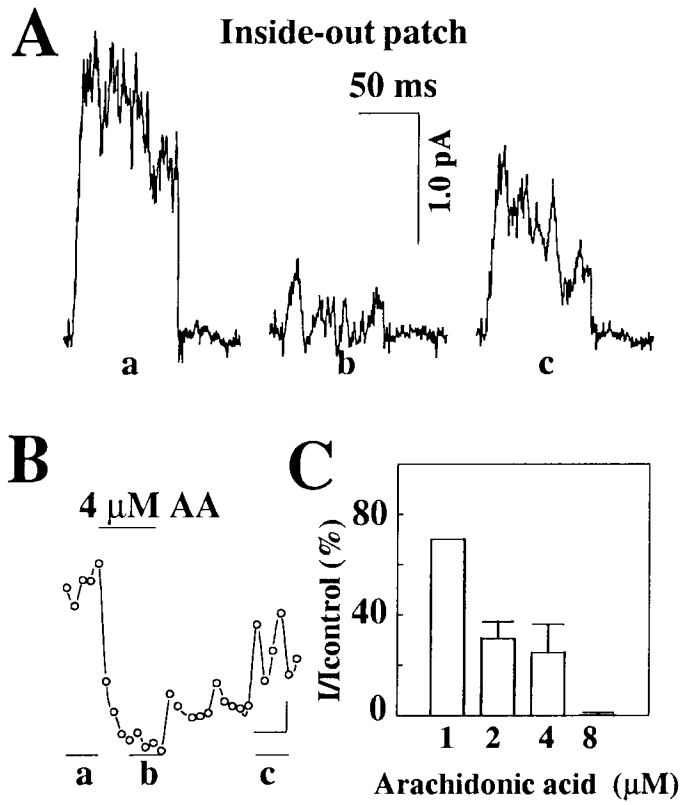

Figure 6. Inhibition of Kv4.2 by arachidonic acid in inside-out patches is fast, potent, and reversible. *A*, Currents from the N-deleted mutant were evoked in the inside-out configuration during a 90 msec step to +80 mV from a holding potential of −80 mV. The figure shows an ensemble average of five depolarizations (selected traces are indicated in *B*) in control conditions (*a*), in the presence of 4 mM arachidonic acid (*b*), and after recovery (*c*). Fat-free BSA (25 mM) was used to help remove arachidonic acid (BSA alone did not have any appreciable effect on channel activity). *B*, Plot of the total integrated current versus time. *Bars a*, *b*, and *c* indicate the records used to generate the ensemble averages in *A* and are positioned at zero current level. The *bar* at the *top* indicates the time of arachidonic acid application. The *horizontal* and *vertical scale bars* are 20 sec and 250 pA/msec, respectively. *C*, Current amplitude as a percent of control at four concentrations of arachidonic acid. *Vertical bars* represent the SD. The data were obtained (from left to right) from *1*, *2*, *5*, and *8* patches.

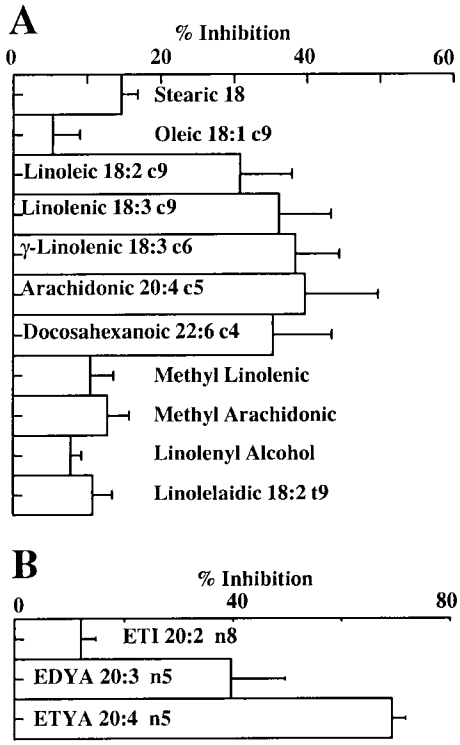

Figure 7. Pharmacology of Kv4.2 inhibition. *A*, The percent inhibition produced by different fatty acids at 25 mM is compared. The name of the fatty acid is followed by the number of carbon atoms, then the number of double bonds, and then by the nature and position of the first double bond (*c*, *cis*; *t*, *trans*). *B*, The inhibitory effects (at 5 mM) of fatty acids with triple, rather than double, bonds. Descriptive numbers are as above; however, the letter *n* is followed by a number indicating the position of the first triple bond.

sought to determine whether their potency to inhibit the channel correlated with their predicted action on membrane fluidity. The extent of inhibition of different fatty acids at 25 mM is illustrated in Figure 7*A*.

The level of unsaturation of a fatty acid and membrane fluidity changes are intricately related. The presence of the first *cis* double bond has the most significant effect in the physical parameters related to membrane fluidity (Stubbs and Smith, 1984). However, oleic acid, which has one *cis* double bond, had little effect in Kv4.2. This contrasts with the modulation of Cl^- channels in epithelial cells that are blocked with oleic acid (and other *cis* unsaturated fatty acids) (Hwang et al., 1990). Fatty acids with more than two *cis* double bonds inhibited Kv4.2 significantly and had similar IC_{50} values. Linolenaidic acid (which has *trans* instead of *cis* double bonds) had little effect, suggesting that the *cis* double bonds are important for the effect. The carboxyl group also appears to be important for the inhibition, because the extent of inhibition produced by methyl esters of arachidonic and linolenic acids, and by linolenyl alcohol, was significantly lower than the reduction caused by the respective acids. This specificity also correlates poorly with changes in membrane fluidity; the methyl esters, alcohols, and acids are predicted to be comparable with one another in changing fluidity.

The effect of fatty acid analogs with triple instead of double bonds was examined by constructing cumulative dose–response curves from 0.625 to 10 mM. These compounds are expected to have minimal effects on membrane fluidity, because the hydrophobic carbon chain is relatively straight (the four carbon atoms around a triple bond are linear). The extent of inhibition produced by 5 mM EDYA, ETI, and ETYA, which have two, three, and four triple bonds, respectively, is illustrated in Figure 7*B*. These analogs were more potent than arachidonic acid, which is an effect that is difficult to reconcile with the idea that arachidonic acid-mediated Kv4.2 inhibition is mediated by an alteration of membrane fluidity.

Thus, in general, there is poor correlation of Kv4.2 inhibition and predicted effects on membrane fluidity. The efficacy of any individual fatty acid may have been distorted by a particular sensitivity of that compound to sequestration or metabolism: the fatty acids with triple bonds, for example, may be most potent because they are not metabolizable. Nevertheless, the lack of correlation is so universal that a more specific interaction of fatty acids with the channel seems to be the more likely hypothesis.

Arachidonic acid modulation is affected by mutations in the S4–S5 loop

Because the action of arachidonic acid on Kv4.2 appears to be direct, we have begun a mutagenesis analysis to determine

whether certain candidate domains of the channels are involved in this modulatory effect. As a starting point, we focused on the N-terminal portion and the S4–S5 loop of the channel for several reasons. (1) The acceleration of the rate of recovery from inactivation by arachidonic acid and ETYA (Fig. 4*D*) suggested that a domain involved in inactivation participates. Although inactivation has not been studied as extensively in these channels as it has in the *Shaker* channel and the Kv1 family, the N-terminal domain has been shown to influence inactivation rates (Baldwin et al., 1991; Pak et al., 1991a). This inactivation likely is analogous to the "ball and chain" model of N-inactivation in *Shaker* (Hoshi et al., 1990). The S4–S5 linker may be involved in this process as a receptor for the inactivation particle (Isacoff et al., 1991). (2) Arachidonic acid is a hydrophobic molecule, and we hypothesized that it interacts with hydrophobic regions of the channel. The N-terminal portion is very hydrophobic, and it is highly conserved between all of the members of the Kv4 family. (3) Current models of channel topology place both the N-terminal and the S4–S5 linker on the intracellular side of the membrane; an intracellular action of arachidonic acid would be compatible with our observations with injected BSA.

To test the role of the N-terminal domain (the "ball"), a highly hydrophobic stretch of 26 amino acids from the N terminus (residues 3–28) was deleted from Kv4.2. As expected, the rate of inactivation in this construct was very slow (Baldwin et al., 1991; Pak et al., 1991a), but the effects of arachidonic acid and ETYA were similar to that in the wild-type channel, as determined by cumulative dose–response curves (data not shown). Thus the N terminus plays little or no role in the modulation.

To address the role of the S4–S5 loop, which may form part of the receptor of the inactivation particle (Isacoff et al., 1991), this segment was exchanged reciprocally between Δ4–46-*Shaker* (in which the N-terminal domain was deleted to remove inactivation), and Kv4.2 channels. In Figure 8*A*, the effect of 10 mM ETYA (*dotted lines*) is illustrated for (from left to right) Kv4.2, a chimera with the S4–S5 loop from *Shaker* in Kv4.2 background (Kv4.2/S4S5Sh), a chimera with the S4–S5 loop from Kv4.2 in *Shaker* background (*Shaker*/S4S5Kv4) (a gift from Dr. Richard Aldrich), and Δ4–46-*Shaker*. Specifically, the exchanged sequences were (with nonconserved amino acids underlined): LRILGGYTLKSCASE (Kv4.2) and LQILGRYTLKASMRE (*Shaker*). The rate of inactivation of the Kv4.2/S4S5Sh chimera was altered, which is consistent with the role of the S4–S5 loop in inactivation of Kv4 channels. The effect of ETYA on both chimeras was intermediary between the insensitivity of *Shaker* (Fig. 8*A*, *right*) and the potent inhibition of Kv4.2 (Fig. 8*A*, *left*). This is illustrated in the cumulative dose response of Figure 8*B*. That the S4–S5 loop can confer sensitivity to the *Shaker* channel suggests the S4–S5 linker plays a role in fatty-acid inhibition. That exchange of this region in Kv4.2 did not abolish sensitivity, however, indicates that this is not the only site of action. That the effect of ETYA on both chimeras is partial also indicates that other parts of the channel are necessary for the full response.

Furthermore, if the S4–S5 loop were a binding site for fatty acids, an alteration might be expected in the IC_{50} values or in the rank order of potencies of various fatty acids in the chimera. We examined the effect of several fatty acids (arachidonic acid, linoleic acid, and linolenyl alcohol) in the Kv4.2/S4S5Sh chimera and found that, in comparison with their actions on Kv4.2, the cumulative dose–response curves for these compounds were reduced by a similar degree in the chimera, with no apparent change in the IC_{50} values (data not shown). Thus, the involvement of the S4–S5 loop in fatty-acid action likely is indirect, and the binding site for these modulators likely resides elsewhere.

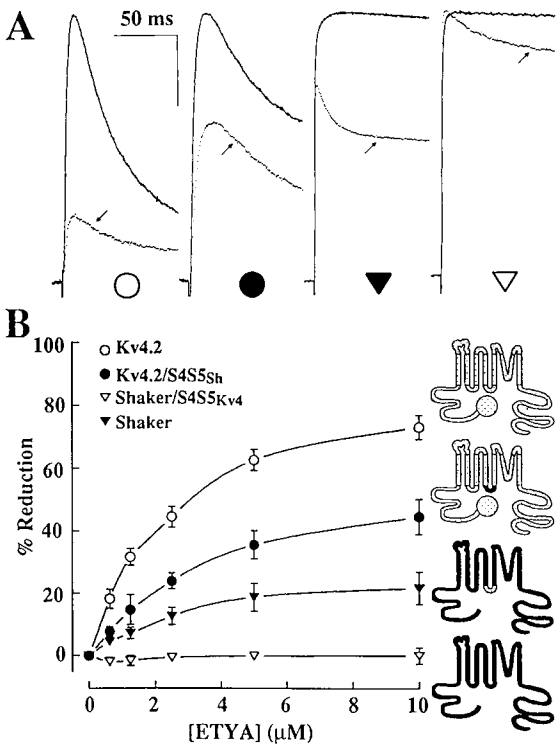

Figure 8. The S4–S5 linker may influence the actions of fatty acids. *A*, A comparison (from left to right) of Kv4.2, chimera Kv4/S4S5Sh, chimera *Shaker*/S4S5Kv4, and *Shaker*. The exchanged amino acids are listed in the text. The effect of 10 mM ETYA (*dotted traces* and *arrows*) is compared with control saline (*solid traces*) for 90 msec steps to +40 mV. A noninactivating (N-terminal-deleted) *Shaker* mutant was used in these experiments and revealed an additional action of ETYA on the *Shaker* channel, a decrease in current amplitude at the end of the pulse relative to peak amplitude. This effect is distinct from the inhibition of peak current in Kv4.2. *Horizontal scale bar*, 50 msec; *vertical bar* (from left to right), 1.1, 1.0, 4.5, and 2.4 mA. *B*, Cumulative dose–response curves for the reduction by ETYA of peak current amplitude evoked by steps to +40 mV. Each *point* is the average of at least five independent experiments. *Bars* indicate the SEM.

DISCUSSION

In this paper, we describe the inhibition by arachidonic acid of Kv4.2 channels expressed in *Xenopus* oocytes. Sensitivity to fatty acids appears to distinguish the members of the Kv4 family from all of the other cloned K^+-channel genes that we screened in oocytes. The inhibition progressed under conditions in which the metabolism of arachidonic acid was likely to be prevented. A requirement for the participation of a kinase, a phosphatase, or Ca^{2+} also can be excluded because the inhibition took place in inside-out patches and was reversible. We have found no correlation between the potency of various fatty acids on Kv4.2 inhibition and their expected influence on membrane fluidity. Taken together, these observations are consistent with a model in which arachidonic acid interacts directly with the channel or a closely associated component and thereby reduces the amplitude of the current.

Although in the oocytes only Kv4 family members showed a substantial inhibition by arachidonic acid, the situation *in vivo* may be more complex because of greater fatty-acid metabo-

lism, the presence of other kinases and modifying enzymes, and the presence of additional channel subunits. Recently, for example, it has been shown that Kv1.5 channels expressed in Chinese hamster ovary cells are blocked by arachidonic acid (Honoré et al., 1994). We and others (Timpe and Fantl, 1994) have observed only a very small reduction of that current in oocytes (see Fig. 1). The published modulation of Kv1.5 differs from the effect reported here for Kv4.2 in several respects. In Kv1.5, arachidonic acid caused a shift in the current–voltage relationship and a clear change in kinetics, such that more current was blocked at the end than at the beginning of a voltage step. In addition, it was reported that neither linoleic acid nor linolenic acid had any significant effect on Kv1.5 (Honoré et al., 1994). Moreover, the site of action in Kv1.5 appears to be extracellular. In future experiments, these differences may be helpful in defining the structural requirements of modulation by fatty acids. Others (Gubitosi-Klug et al., 1995) recently have described an action of arachidonic acid on the Kv1.1 channel expressed in Sf9 cells. This effect, which consists of modest changes in the activation and inactivation kinetics of Kv1.1, resembles some of the actions we have observed on other members of the Kv1 family (Fig. 1). It is clearly distinct from the modulation of the Kv4-family channels that we have observed.

Does the action of arachidonic acid on Kv4 channels expressed in oocytes correspond to a modulation that occurs *in vivo*? As discussed below, there are several reported modulations of currents that are good candidates for *in vivo* correlates of this effect. However, one general issue for evaluating such a hypothesis concerns the levels of arachidonic acid effective in the present study and those that are reached in agonist-stimulated cells. Unfortunately, the latter are not known with certainty. It has been argued that the effects observed with concentrations of arachidonic acid in the low micromolar range have physiological significance (Anderson and Welsh, 1990), because the K_m values of the enzymes that metabolize arachidonic acid are 3–28 mM (Needleman et al., 1986). In addition, in cases in which the effect of a neurotransmitter has been demonstrated to be mediated by arachidonic acid, concentrations of 10 mM or greater were necessary to mimic the modulation (Piomelli et al., 1987; Kurachi et al., 1989b; Schweitzer et al., 1990). In the present study, a 69% inhibition of Kv4.2 current was observed in inside-out patches at concentrations as low as 2 mM. Therefore, the physiological significance of the effect is highly plausible.

All known members of the Kv4 family produce transient A-type currents, and even a subtle reduction of these currents by neurotransmitters can produce substantial changes in the physiology of neuronal circuits (Harris-Warrick et al., 1994). In neurons and myocytes, there are abundant examples of A-current modulations that are candidates for a phenomenon corresponding to the one we see in oocytes. Neurotransmitter modulation of I_A has been described in neostriatal (Akins et al., 1990), dorsal raphe (Aghajanian, 1985), sympathetic (Blair and Suprenant, 1993; Villarroel, 1994), hippocampal (Nakajima et al., 1986; Saint et al., 1990), and cerebellar Purkinje neurons (Wang et al., 1992), as well as in cardiac myocytes (Apkon and Nerbonne, 1988) and Purkinje cells (Nakayama and Fozzard, 1988) (where it is designated I_{to}). The second messenger involved in the response is not known for any of these cells.

Which of these effects are likely to represent fatty-acid inhibition of Kv4.2? Kv4.2 transcript and protein are present at relatively high levels in cardiac myocytes (Dixon and McKinnon, 1994; Barry et al., 1995), and it is likely, therefore, that Kv4.2 underlies much of I_{to} (Barry et al., 1995). Stimulation of α_1 receptors inhibits I_{to}, and this inhibition leads to a prolongation of the action potential (Apkon and Nerbonne, 1988). Elsewhere, α_1 receptors have been shown to mobilize arachidonic acid (Burch et al., 1986) and, indeed, arachidonic acid can reduce I_{to} in cardiac myocytes (Damron et al., 1993). It is tempting, therefore, to propose that arachidonic acid is mediating at least part of the adrenergic modulation by interacting directly with a Kv4 channel.

Similarly, in hippocampal neurons Kv4.2 is localized on the soma and dendrites (Sheng et al., 1992) and, therefore, likely contributes to I_A in these cells. Muscarinic agonists reduce I_A and thereby increase excitability (Nakajima et al., 1986). Muscarinic responses also can include production of arachidonic acid (Conklin et al., 1988; Kanterman et al., 1990) and, thus, an arachidonic acid-mediated inhibition of Kv4.2 channels also may be at work in the hippocampus. Yet it should be noted that the correspondence is not perfect: the reduction of I_A in hippocampal neurons is accompanied by a shift to more depolarized potentials in the current–voltage relationship, indicating that additional mechanisms may be taking place. The somatostatin receptor that can be found in hippocampal neurons also can trigger the release of arachidonic acid (Bito et al., 1993) and, therefore, it will be of interest to determine whether this peptide also can inhibit I_A.

In rat celiac ganglion neurons (Blair and Suprenant, 1993) and bullfrog sympathetic neurons (Villarroel, 1993, 1994), the inhibition of I_A by muscarine is mimicked by arachidonic acid. These currents and their modulations are very similar to those reported here; therefore, although in sympathetic neurons the expression of Kv4 channels has not yet been demonstrated, inhibition by arachidonic acid of a channel in this family is also a distinct possibility. Thus, the present studies of expressed Kv4 channels may provide insight into several modulatory phenomena *in vivo* and should encourage further investigation of the involvement of this signaling pathway in the regulation of transient currents.

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