Arachidonic acid potentiates the feedback response of mesangial BK$_{Ca}$ channels to angiotensin II

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Departments of Medicine and Integrative Biology, Pharmacology and Physiology, University of Texas Medical School at Houston, Houston, Texas 77030; and Department of Physiology and Biophysics, University of Nebraska Medical Center, Omaha, Nebraska 68198-4575.

Stockand, James D., Meredith Silverman, David Hall, Thomas Derr, Brian Kubacak, and Steven C. Sansom. Arachidonic acid potentiates the feedback response of mesangial BK$_{Ca}$ channels to angiotensin II. Am. J. Physiol. 274 (Renal Physiol. 43): F658–F664, 1998.—The influence of arachidonic acid (AA) on the feedback regulation of mesangial contraction by large Ca$_{2+}$-activated K$^{+}$ channels (BK$_{Ca}$) was determined through single-channel analysis using the patch clamp method. The mesangial BK$_{Ca}$ is a low-gain negative feedback inhibitor of contraction that is activated in response to agonist-induced Ca$_{2+}$ and membrane depolarization. AA activated BK$_{Ca}$ in cell-attached patches in a dose-dependent manner with a maximal effect at 400 nM and a half-maximal response at 49 nM. In inside-out patches, AA directly activated BK$_{Ca}$ with a maximal effect at 400 nM. BK$_{Ca}$ was activated significantly in response to addition of 100 nM ANG II in the presence but not the absence of AA. Since it was shown previously that fatty acids stimulated both soluble and membrane-bound guanylyl cyclase, we determined whether AA activated BK$_{Ca}$ by interfering with cGMP-mediated signal transduction pathways. It was previously shown that 10 µM cGMP, via cGMP-dependent protein kinase, activated BK$_{Ca}$ in a biphasic manner with an early increase in probability of a channel existing in an open state (P$_{o}$) and a subsequent inactivation mediated by protein phosphatase 2A (PP2A). We found that 10 µM dibutyryl-cGMP enhanced BK$_{Ca}$ activity in an additive manner with saturating concentrations (400 nM) of AA. Moreover, the inactivation phase mediated by PP2A was not abolished. Thus AA does not affect the phosphorylation/dephosphorylation regulatory cycle for BK$_{Ca}$. It is concluded that AA potentiates the ANG II feedback response of BK$_{Ca}$ by a mechanism that is independent of the phosphorylation cycle. BK$_{Ca}$ is a calcium-activated potassium channel; guanosine 3',5'-cyclic monophosphate; fatty acids

GLOMERULAR MESANGIAL CELLS (MC) are smooth-muscle-like contractile cells that are important regulators of renal hemodynamics. By surrounding the glomerular capillaries, MC regulate filtrate surface area and maintain filtration rates within physiological limits. Like vascular smooth muscle, the contractile tone of MC is governed by the influences of multiple systemic and local hormone agonists. Contractile agonists, such as ANG II and endothelin-1, and relaxants, such as atrial natriuretic peptide (ANP) and nitric oxide (NO), respectively, contract and relax MC by influencing the activities of plasmalemmal ion-selective channels.

Our laboratory has recently described one mechanism by which ANP and NO regulate MC tone. Through cGMP-signal transduction, these vasodilating agents activate large, Ca$_{2+}$-activated K$^{+}$-selective ion channels (BK$_{Ca}$) (26). In both vascular smooth muscle (3) and glomerular MC (21), BK$_{Ca}$ is a feedback regulator of contraction. Agonist-induced elevations in intracellular Ca$_{2+}$ and depolarization of membrane potential activate BK$_{Ca}$, which hyperpolarizes the plasma membrane and subsequently inactivates or blocks activation of voltage-gated Ca$_{2+}$-entry channels. Thus cGMP signal transduction increases the feedback gain and potentiates the attenuation of agonist-induced Ca$_{2+}$ entry.

Arachidonic acid (AA), a saturated, long-chain fatty acid, is a ubiquitous modulator of several types of ion channels (16) and may have an important role in cell signaling mechanisms. Several studies have shown that K$^{+}$-selective channels other than BK$_{Ca}$ were modulated directly by AA (12, 13, 15–17, 29). That BK$_{Ca}$ are activated directly by AA was demonstrated in vascular smooth muscle (14) and brain cells (5). The indirect activation of BK$_{Ca}$ (by metabolites) was demonstrated in brain (6) and small renal arteries (35).

Large, Ca$_{2+}$-activated K$^{+}$ channels from different cell types, such as brain, vascular smooth muscle, and MC, are differentially regulated by cell signaling pathways. For example, BK$_{Ca}$ from rat brain are regulated by cAMP-activated kinase (19). BK$_{Ca}$ from pituitary tumor cells (GH4C1) (30, 31) and some vascular smooth muscle cells (34) are activated when dephosphorylated by cGMP-dependent protein phosphatase. However, BK$_{Ca}$ from MC are activated by cGMP-dependent protein kinase (25) and inactivated by protein phosphatase 2A (PP2A) (22). Thus the pathways for regulating BK$_{Ca}$ are cell specific.

Since MC are important regulators of glomerular filtration rate, it is important to determine the signal transduction pathways for regulating the ion channels that govern contraction. Studies have shown that both the membrane-bound (28) and particulate (2) forms of guanylyl cyclase are stimulated by free fatty acids (FFA). Thus these studies were performed to determine whether low concentrations of AA could enhance the feedback response of BK$_{Ca}$ to ANG II and whether cGMP-activated protein kinase or PP2A was an intermediary for this effect.

METHODS

MC cultures. Human MC, originally isolated by the laboratory of Hanna Abboud, were obtained at the fifth generation and subcultured by standard methods previously described.
The medium (Waymouth, pH 7.4) was supplemented with 10 mM HEPES, 2.0 mM glutamine, 0.66 U/ml insulin, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, antibiotic solution (100 U/ml penicillin and 100 µg/ml streptomycin), and 17% fetal bovine serum. For single-channel analysis, cells were grown on 22 mm cover glasses, type (no. 1 type; Fisher, Pittsburgh, PA) and maintained in a humidified tissue culture incubator at 37°C and 5% CO2 (IR Autoflow; Nuaire, Plymouth, MN). Only subpassages 5–10 were used, as MC maintain both the phenotypic shape that is characteristic of smooth muscle cells and exhibit BKCa during these generations.

**Patch-clamp procedure.** The bathing solution for inside-out experiments and the pipette solution for all experiments contained (in mM) 140 KCl, 10 HEPES, 2 MgCl₂, and 0.001 CaCl₂, pH 7.4. For cell-attached patches, the bath solution (physiological saline) contained (in mM) 135 NaCl, 5 KCl, 10 HEPES, 2 MgCl₂, and 1 CaCl₂. The free Ca²⁺ concentration of the bath was adjusted from 1 mM to 1.0 µM by buffering with 1.08 mM EGTA. The amount of added EGTA was calculated using the calcium concentration program by MTK Software. Single-channel analysis was made at 23°C using standard patch-clamp techniques (9). The pipette was inserted into a polycarbonate holder, connected to the head stage of the patch amplifier (L/M-EPC 7; Adams and List, Great Neck, NY) by a Ag-AgCl wire, and then lowered onto the cell. A high resistance (> 5 GΩ) seal between the pipette and MC membrane was obtained by applying suction.

Upon obtaining a seal, current recordings of BKCa were made with the pipette attached to the membrane (cell attached) or after withdrawing the patch of membrane from the cell (excised, inside-out). BKCa were immediately identified by the characteristic magnitude of single-channel current and voltage-gating properties as previously described in this laboratory (24). The unitary current (i), defined as zero for the closed state (C), was determined as the mean of the best-fit Gaussian distribution of the amplitude histograms. Channels were considered to be in an open state (S) when the current was \( \geq (n - \frac{1}{2})i \) and \( \leq (n + \frac{1}{2})i \), where \( n \) is the maximum number of current levels observed. The probability of a...
channel existing in an open state ($P_o$) is defined as the time spent in a conducting state ($S$) divided by the total time of the recording. The time in a conducting state was calculated using the best-fit Gaussian distribution of the amplitude histogram. When more than one channel occupied a patch, open probability was calculated by dividing by 1 minus the number of current levels the total of the sum of the frequency of observing a given current state times the appropriate current level (conducting state). To enable accurate calculation of open probability in cell-attached patches, the number of channels was determined after excising the patch into a bathing solution containing 1 mM Ca$^{2+}$. At this concentration of Ca$^{2+}$ and a command potential of 40 mV, the open probability is greater than 0.80 (24). If the number of channels could not be determined, then the channel activity was calculated as $NP_o = \Sigma nP_{n}$, where $P_n$ is the probability of finding n channels open. To obtain a dose-response curve, the data were fit to the Michaelis-Menten equation, $\Delta P_{o} = \Delta P_{o_{\text{max}}} \times [AA/(K_m + AA)]$, where $\Delta P_{o_{\text{max}}}$ is the maximum change in open probability, and the $K_m$ is the concentration of AA that results in a half-maximal increase in $NP_o$.

Currents were recorded onto the hard drive of a Compaq Presario Pentium computer using the Axoscope acquisition program. Analysis was performed using pClamp program set 6.0 (Axon Instruments, Foster City, CA).

Protocol. To determine the effects of AA on BK$_{Ca}$, concentrations of 4 nM to 4 µM AA were added to the bathing solution, and the $P_o$ was monitored in cell-attached and excised patches. To determine the effects of AA on BK$_{Ca}$ feedback, ANG II (100 nM) was added to the bathing solution in the presence or absence of 400 nM or 4 µM AA. The interaction of AA with cGMP was determined by monitoring the $P_o$ of BK$_{Ca}$ after adding dibutyryl-cGMP (DB-cGMP, 10 µM) to the bathing solution in the absence and presence of 400 nM AA.

AA, purchased from Sigma Chemical, was dissolved in 1% methanol and stored at 0°C as 100 mg/ml stock.

RESULTS

Effects of AA on BK$_{Ca}$. Figure 1 shows dose-response relations for the activation of BK$_{Ca}$ by AA in cell-attached patches. As shown in the tracings of the first patch (Fig. 1A), the activity of BK$_{Ca}$ increased from a control value ($NP_o$) of 0.001 to 0.01, 0.15, and 0.30 after subsequent additions of 4, 40, and 400 nM AA, respectively. The $NP_o$ reversed to 0.010 after flushing AA. In the second patch (Fig. 1B), $NP_o$ increased from 0.002 in control to 0.033 after addition of 40 nM and to 1.02 after addition of 400 nM AA. After flushing AA, the baseline $NP_o$ was 0.62. Addition of 4 µM AA increased $NP_o$ to 0.97. The data are summarized in the dose-response curve (Fig. 1C). After fitting the curve to the Michaelis-Menten equation, the maximal response to AA is 0.24, and the concentration of AA resulting in a half-maximal response ($K_m$) is 49 nM.

Figure 2 shows the response of BK$_{Ca}$ to 4 µM AA in inside-out patches. In this patch, the $P_o$ of BK$_{Ca}$ was increased from 0.05 to 0.20 by 4 µM AA. For the inside-out patches in which the $P_o$ of BK$_{Ca}$ could be determined, it was found that the $P_o$ values for the effects of 400 nM (0.17 ± 0.02, n = 3), 4 µM (0.15 ± 0.04, n = 7), and 40 µM (0.18 ± 0.04, n = 3) AA were not significantly different. Thus the effects of AA are saturating at 400 nM in excised patches.

Effects of AA on the feedback response to ANG II. The tracings of the cell-attached patch in Fig. 3 show the effects of 100 nM ANG II on BK$_{Ca}$ in the absence and presence of either 400 nM or 4 µM AA. As shown in Fig. 3A, the $P_o$ increased minimally from 0.004 to 0.009 upon addition of 100 nM ANG II, indicating a low-gain feedback mechanism. After flushing ANG II and then adding 400 nM AA, $P_o$ increased to 0.073 within 60 s (Fig. 3B). In the continued presence of 400 nM AA, ANG II activated BK$_{Ca}$ to 0.190. As shown in Fig. 3C, after exposure to 4 µM AA, the $P_o$ increased from 0.005 to 0.084. In the continued presence of 4 µM AA, BK$_{Ca}$ was activated by 100 nM ANG II to a $P_o$ of 0.202.

The bar graph of Fig. 4 shows a summary of the effects of AA (400 nM or 4 µM AA) on the response of BK$_{Ca}$ to ANG II. In control, the $P_o$ was 0.039 ± 0.0014 (n = 5) and 0.0041 ± 0.0010 after addition of 100 nM ANG II (n = 3; not significant). The $P_o$ increased to 0.058 ± 0.02 after addition of 400 nM or 4 µM AA (n = 6). In the continued presence of AA, addition of ANG II increased the $P_o$ to 0.27 ± 0.04 (n = 5). This value was significantly greater (P < 0.05) than the individual values for control, ANG II, and AA groups using the ANOVA plus the Student-Newman-Keuls test. However, there was no significant difference among the values for control and ANG II. These results suggest that the feedback response to ANG II is potentiated by saturating concentrations of AA.

Effects of AA on the activation of BK$_{Ca}$ by cGMP. Since it was shown previously that BK$_{Ca}$ are activated by cGMP-dependent protein kinase and inhibited by PP2A, the following experiments were performed to determine whether AA affected the phosphorylation cycle of BK$_{Ca}$. The tracings of Fig. 5 show the effects of DB-cGMP in the absence and presence of 400 nM AA. As shown previously, BK$_{Ca}$ are activated transiently by DB-cGMP from 0.001 to 0.46 (peak activity) after 10 s. The rundown phase was previously shown to be the result of subsequent inhibition by PP2A. In the presence of 400 nM AA, the basal $P_o$ was 0.04. Addition of DB-cGMP activated BK$_{Ca}$ to 0.21 after 10 s, and the $P_o$ returned to baseline after 20 s.
Figure 6 shows a summary of the effects of DB-cGMP in the absence and presence of 400 nM AA. In the absence of AA, DB-cGMP activated BKCa from 0.004 ± 0.002 to 0.31 ± 0.07 (n = 5; P < 0.0001). After 20 s, the Po decreased to 0.016 ± 0.005. In the presence of AA, the basal activity increased to 0.021 ± 0.005 (n = 4; P < 0.001). After addition of DB-cGMP, the Po increased to 0.30 ± 0.10 (n = 4; P < 0.001) after 10 s and ran down to 0.060 ± 0.029 after an additional 20 s. Thus, although the baseline Po increased after addition of AA, neither the peak response to DB-cGMP nor the rundown phase was significantly affected.

DISCUSSION

The BKCa channels of both vascular smooth muscle (3) and MC (24) serve as feedback regulators of contraction. In vascular smooth muscle, BKCa activate in response to depolarization and elevated intracellular Ca2+ concentration, bringing the membrane potential back to resting values (3). In MC, the gain in this feedback response is minimal in the absence of relaxing agents, such as NO and ANP (26). The results of the present study showed that the gain in the feedback response of BKCa can be potentiated by low concentrations of AA. The effects of AA are independent of phosphorylation by cGMP-activated kinase and dephosphorylation by PP2A and are consistent with a direct activation of BKCa.

Mechanism of action of AA. There are several possible mechanisms for the enhancement by AA of the BKCa-mediated feedback control of contraction. One possibility is that AA increases the fluidity of the membrane, which changes the selectivity to divalent cations of the Ca2+ binding site on BKCa. Bregestovski et al. (4) found that the fatty acid 2-decanoic acid, shown previously to enhance membrane fluidity, increased the activity of BKCa in human aortic smooth muscle by increasing its sensitivity to Mg2+. Since the cytoplasmic solutions of the present study contained Mg2+, it is possible that AA increased the sensitivity of BKCa to Mg2+, which could serve as a better activator of BKCa than Ca2+.

It is also possible that AA affects the Ca2+ activity in close proximity to BKCa. In support of this theory, some investigators have suggested that AA increases intracellular release of Ca2+ from intracellular stores and augments Ca2+ influx (20). However, the direct activa-
tion of BKCa by AA in inside-out patches does not support this theory. Moreover, Ordway et al. (18) found that FFA activated smooth muscle K1 channels in the complete absence of Ca2+ (5 mM EGTA).

AA also may influence a membrane-associated regulator of BKCa. Our laboratory has shown that BKCa is activated by protein kinase G (25) and inactivated by PP2A (22). Both enzymes are likely closely associated with the membrane. Because some studies have shown that AA activates guanylyl cyclase (7, 10) and inhibits myosin light chain phosphatase (8), we investigated the possibility that AA activates BKCa by interacting with cGMP-activated kinase or PP2A. The finding that DB-cGMP activated BKCa transiently and to the same magnitude in the presence and absence of AA suggests that the mechanism does not involve guanylyl cyclase, cGMP, cGMP-activated protein kinase, and PP2A.

The mechanism of activation of BKCa by AA can be determined, in part, by studying the actions of a variety of other fatty acids. Although the present study has not focused on the specific molecular requirements for activation of BKCa by fatty acids, other investigators have studied the effects of varying the length and the number of double bonds of fatty acids. Ordway et al. (17) found that BKCa could be activated by polyunsaturated fatty acids such as AA and also by monounsaturated and saturated FFA, such as linoelaidic and myristic acid, respectively. It is significant that myristic acid activated BKCa, since it is not a substrate for cyclooxygenase and lipoxygenase enzymes. Moreover, the finding that K+ channels can be activated in the presence of eicosatetraenoic acid (18), an inhibitor of AA metabolic pathways, suggests that AA, and not the metabolites of AA, is directly activating BKCa in MC. On the other hand, BKCa of small renal arteries of the rat were directly activated by the epoxygenase metabolite, 11,12-epoxyeicosatrienoic acid (35), and BKCa of bovine adrenal medullary chromaffin cells were activated by the lipoxygenase metabolite 15-hydroxyperoxyeicosatetraenoic acid (27). Thus more studies are needed to determine the effects of AA metabolites on the mesangial BKCa.

Fig. 5. Effects of DB-cGMP on BKCa in absence and presence of 400 nM AA. Basal P0 was 0.001 (first tracing). In this cell-attached patch, BKCa are activated transiently by DB-cGMP from 0.005 to 0.46 at peak and back to 0.01 (second tracing). AA (400 nM) activated BKCa to P0 = 0.04 (third tracing). Addition of DB-cGMP activated BKCa to 0.21 after 10 s (fourth tracing). Channel activity ran down to 0.05 after 90 s. All conditions are same as in Fig. 1.

Fig. 6. Summary of effects of DB-cGMP in cell-attached patches in presence and absence of 400 nM AA. In absence of AA (blank bars), dibutyryl-cGMP (DB-cGMP) activated BKCa from 0.004 ± 0.002 to 0.31 ± 0.07. P0 decreased to 0.016 ± 0.005 after 20 s. In presence of AA (hatched bars), basal activity increased to 0.021 ± 0.005. After addition of DB-cGMP, P0 increased to 0.30 ± 0.10 after 10 s and ran down to 0.060 ± 0.029 after an additional 20 s.
The data from the present and other studies suggest that AA activates BK<sub>Ca</sub> by directly interacting with the channel protein. It is not known whether K<sup>+</sup> channels have binding sites for AA; however, it is known that other proteins such as albumin contain specific binding sites for FFA (23).

Physiological and pathophysiological significance of AA-activated BK<sub>Ca</sub>. Although several studies have shown that ion channels are regulated by AA (12, 13, 15–17, 29), the physiological significance of this signaling pathway is still speculative. Some investigators suggest that AA is elevated in the serum and cytosol during cardiac ischemia (16). Activation of K<sup>+</sup> channels would reduce the action potential and load on the heart. Similarly, glomerular ischemia would elevate AA resulting in an attenuated contractile response to ANG II and an enhancement of the relaxing effects of ANP and NO. The reduced tension in MC would conserve ATP.

Several studies have documented an increase in fatty acid metabolism in cells grown under the simulated pathological conditions of diabetes (32, 33). Williams and Schrier (32) have shown that AA release is significantly elevated when cultured rat MC are grown in high ambient glucose concentrations. High intracellular glucose directly metabolizes to diacylglycerol, an activator of protein kinase C, which, in turn, activates phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and releases AA. PLA<sub>2</sub> activity activates protein kinase C, which, in turn, activates phospholipase A<sub>2</sub> and releases AA. PLA<sub>2</sub> activity and AA levels are elevated under conditions of glomerular damage and high blood glucose. The pathological modulation of the mesangial BK<sub>Ca</sub> and subsequent elevation of filtration rate by AA may partly explain the hyperfiltration associated with the early stages of diabetes mellitus (1).

In summary, these results show that mesangial BK<sub>Ca</sub> like that of brain BK<sub>Ca</sub> is activated directly by low concentrations of AA. The effects of AA augment the feedback response to ANG II by a mechanism independent of phosphorylation by cGMP-dependent kinase and dephosphorylation by PP2A. These results indicate that elevated physiological or pathophysiological concentrations of AA can affect the contractility of MC and could partially explain the hyperfiltration in the early stages of diabetes mellitus.

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REFERENCES

24. Stockand, J. D., and S. C. Sansom. Large Ca<sup>2+</sup>-activated K<sup>+</sup> channels responsive to angiotensin II in cultured human mesan-


