0026-895X/02/6205-1076–1083$7.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 62:1076–1083, 2002

Vol. 62, No. 5
1689/1015192
Printed in U.S.A.

Stereospecific Activation of Cardiac ATP-Sensitive K^+ Channels by Epoxyeicosatrienoic Acids: A Structural Determinant Study

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Received February 21, 2002; accepted July 16, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The heart is richly endowed with K_{ATP} channels, which function as biological sensors, regulating membrane potentials and electrical excitability in response to metabolic alterations. We recently reported that the cytochrome P450 metabolites of arachidonic acid, epoxyeicosatrienoic acids (EETs), potently activate cardiac K_{ATP} channels by reducing channel sensitivity to ATP. In the present study, we further demonstrate that 11(*S*),12(*R*)-EET activated the cardiac K_{ATP} channels with an EC_{50} of 39.5 nM, whereas 11(*R*),12(*S*)-EET was totally inactive. In addition, 11(*S*),12(*R*)-EET but not 11(*R*),12(*S*)-EET hyperpolarized the resting membrane potentials and shortened the duration of cardiomyocyte action potentials. By studying homologs and analogs of 11,12-EET, we also found that all four EET regioisomers are equipotent activators of the K_{ATP} channels, reducing the ATP sensitivity by more than 10-fold; however, neither altered chain length, double bond number, epoxide position, nor methylation of the carboxyl group affected channel inhibitions by ATP. All the fatty epoxides studied are potent K_{ATP} channel activators, but the ω-3 homolog was particularly potent, reducing ATP sensitivity 27-fold. Together, the results indicate that the presence of an epoxide group in a particular three-dimensional configuration is a critical determinant for K_{ATP} channel activation, and its effect is augmented by a double bond at ω-3 position. The results also suggest that fatty epoxides are important modulators of cardiac electrical excitability.

ATP-sensitive K^+ (K_{ATP}) channels are densely populated in the heart and play an important role in regulating the membrane potential in response to intracellular metabolic oscillations (Yokoshiki et al., 1998). K_{ATP} channels are thought to be activated during cardiac ischemia and hypoxia when cytoplasmic ATP is depleted, which would lead to shortened cardiac action potentials, reducing Ca^{2+} influx and minimizing intracellular Ca^{2+} overload (Noma, 1983; Nichols and Lederer, 1990; Tung and Kurachi, 1991). However, the function of the cardiac K_{ATP} channels under normal conditions is not well understood.

Arachidonic acid is an important precursor of many bioactive lipids (Brash, 2001; Funk, 2001; Roman, 2002). The cytochrome P450 (P450) epoxygenase metabolites of arachidonate have emerged as an important source of signaling agents (Zeldin, 2001; Roman, 2002). The CYP2J epoxygenases, abundant in human and rat hearts (Zeldin, 2001), convert arachidonic acid into four epoxyeicosatrienoic acid (EET) regioisomers: 5,6-, 8,9-, 11,12-, and 14,15-EET, each of which can be formed as the *R*,*S* or *S*,*R* enantiomer (McGiff, 1991; Zeldin, 2001). EET products are endogenous constituents of rat cardiac tissue, occurring at 69 ± 7 ng of total EETs/g of heart (Wu et al., 1997). EETs can be further hydrolyzed to dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolase (Zeldin et al., 1993). In coronary vascular endothelial cells, inhibition of soluble epoxide hydrolase results in the accumulation of both chain elongation and chain shortened homologs of EET (Fang et al., 2001).

There is very limited information on the specific binding of EETs in cells and an EET receptor has not yet been identified. Most of the physiological effects of EETs do not appear to involve regio- or stereospecificity (Oltman et al., 1998; Lee et al., 1999; Lu et al., 2001b; Zhang et al., 2001). However, specific high affinity binding sites for 14,15-EET are present in U-937 cells, which may be associated with a receptor (Wong et al., 1997), and stereospecific effects of 11,12-EET have been reported on renal vascular tone and K^+ channel activity (Zou et al., 1996). Nevertheless, stereospecific effects

This work was supported by a Merit Review Award from the Department of Veterans, by grants from the National Institute of Health (R01-HL-63754 to H. Lee and R01-HL-56670 to M. VanRollins) and from the American Heart Association (Grant-in-aid award 0051311Z to H. Lee and 96012380 to M. VanRollins).

ABBREVIATIONS: K_{ATP} channels, ATP-sensitive K^+ channels; P450, cytochrome P450; EET, epoxyeicosatrienoic acid; DHET, dihydroxyeicosatrienoic acid; EDT, epoxydocosatrienoic acid; EEQ, epoxyeicosaquatraenoic acid; APD_{90}, action potential duration at 90% repolarization; RP, resting membrane potential; BK channels, large conductance Ca^{2+}-activated potassium channels.

of EETs have not been previously demonstrated in cardiac tissue.

Recently, we reported that EETs potently activated the cardiac K_{ATP} channels by reducing channel sensitivity to ATP, allowing the channel to remain open in the presence of physiological concentrations of cytoplasmic ATP (5 mM) (Lu et al., 2001a). In the present study, we tried to determine whether EETs stereospecifically activated K_{ATP} channels and stereospecifically altered the membrane potentials of cardiac myocytes. In addition, to assess the role of other structural determinants, we examined the effects of various EET homologs and analogs on the K_{ATP} channel activity (Fig. 1). Although epoxide stereochemistry was critical for K_{ATP} channel activation and membrane potential hyperpolarization, the position of the epoxide group and the presence of an anionic carboxylic group were not. These results suggest that endogenous EETs may activate cardiac K_{ATP} channels by binding at specific sites in cardiomyocytes, thereby modulating cardiac electrophysiology.

Materials and Methods

Preparation of Single Ventricular Myocytes. Ventricular myocytes were isolated from male Sprague-Dawley rats by enzymatic dissociation (Lee et al., 1999). Briefly, animals were anesthetized with methoxyfluorane and the hearts were rapidly excised. A modified Langendorff apparatus was used to perfuse hearts for 5 min at 37°C with nominally Ca^{2+}-free Tyrode's solution containing 138 mM NaCl, 4.5 mM KCl, 0.5 mM $MgCl_2$, 0.33 mM Na_2HPO_4, 5.5 mM glucose, 10 mM HEPES, and 0.1% (w/v) bovine serum albumin, adjusted to pH 7.4 with NaOH. The perfusate was switched to a nominally Ca^{2+}-free Tyrode's solution containing 0.6 mg/ml collagenase (CLS-2, 347 units/mg; Worthington Chemicals, Freehold, NJ) for 10 min. The ventricle was transferred to a fresh 0.6 mg/ml collagenase solution, cut into small pieces (~2 mm^3) and incubated at room temperature (22°C) for another 5 min. Dissociated cells were filtered through a medium mesh, centrifuged, and used within 24 h while stored at 22°C in 70 mM KOH, 40 mM KCl, 50 mM L-glutamic acid, 20 mM taurine, 0.5 mM $MgCl_2$, 1.0 mM K_2HPO_4, 0.5 mM EGTA, 10 mM HEPES, 5 mM creatine, 5 mM pyruvic acid, and 5 mM Na_2ATP, adjusted to pH 7.38 with KOH.

Inside-Out Patch Clamp Recording. Single K_{ATP} channel currents were recorded with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), filtered at 5 kHz (8-pole Bessel filter unit; Frequency Devices, Haverhill, MA) and digitized at 50 kHz (Digidata; Axon Instruments). Data were acquired, stored in a Pentium-based personal computer, and later analyzed off-line using pClamp 7.0 (Axon Instruments). The K_{ATP} channels were identified by measuring conductance and ATP sensitivity as described (Lu et al., 2001a) and channel opening probability was determined by

$$P_o = (\sum_{j=1}^{N} t_j j)/TN$$

where P_o is the single channel open probability, T is the duration of recording, t_j is the time spent with $j = 1,2,\ldots N$ channel openings and N is the maximal number of simultaneous channel openings observed when the bath solution contained no ATP. Only membrane patches containing not more than 5 K_{ATP} channels were used for P_o analysis. Channel detection levels were set according to channel amplitude and pClamp uses 50% of the channel amplitude as the threshold for detection between open and closed states. The detection levels were used to construct interlevel transition thresholds, and each threshold is halfway between its detection levels.

The patch pipettes (Corning 7056; Warner Instrument, Hamden, CT) were coated with Sylgard 184 (Dow Corning, Midland, MI) and had a typical tip resistance between 2 and 5 MΩ when filled with 140 mM KCl, 1.0 mM EGTA, 5 mM HEPES, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, pH to 7.4 with KOH. Isolated ventricular myocytes were placed in a 0.5-ml chamber, and positioned on the stage of an inverted microscope (Olympus, IX70, Japan) before being superfused at 2 ml/min with a solution containing 70 mM KCl, 70 mM L-aspartic acid monopotassium salt, 2 mM EGTA, 5 mM HEPES, 7 mM *N*-methyl-D-glucamine, pH to 7.35 with *N*-methyl-D-glucamine. To generate an ATP inhibition curve, various amounts of ATP were added to the perfusate. When high concentrations of ATP (>1 mM) were used, the pH was readjusted with KOH. Dose-response curves were fitted using a Hill equation of the following form

$$P_o = P_{o,max}/[1 + (S/EC_{50})^H]$$

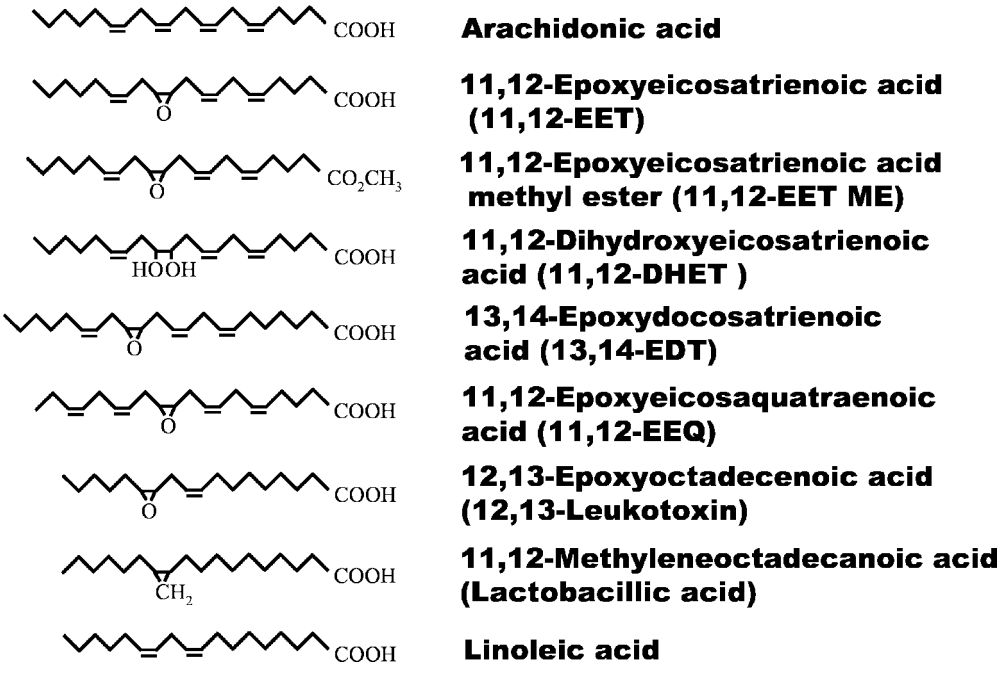

Fig. 1. Structures of 11,12-EET and its homologs/analogs.

where S represents the concentration of the chemical, EC_{50} is the concentration at half-maximal effect, and H is the Hill coefficient. Curve fittings were performed using Igor 3.16 software (WaveMetrics Inc., Lake Oswego, OR).

Whole Cell Current Clamp Recordings. Action potentials in rat ventricular myocytes were recorded at 37°C using the whole cell current clamp technique. Action potentials were elicited using 1 nA stimuli at 0.5 Hz and pCLAMP software. The signals were recorded with an Axopatch 200B amplifier, filtered at 5 kHz, and digitized at 20 kHz. The effects of 11(R),12(S)-EET, 11(S),12(R)-EET, and 11(S),12(R)-EET plus glyburide on the resting membrane potential (RP), action potential amplitude, and action potential duration at 90% repolarization (APD_{90}) were measured. For these measurements, the cells were superfused with a Ca^{2+} Tyrode's solution containing 138 mM NaCl, 4.5 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.33 mM Na_2HPO_4, 5.5 mM glucose, 10 mM HEPES, pH adjusted to 7.4 with NaOH. The pipette solution contained 130 mM KCl, 1.0 mM EGTA, 10 mM HEPES, 1.0 mM $MgCl_2$, 5 mM ATP, 1.0 mM GTP, 0.018 mM $CaCl_2$ (~200 nM free Ca^{2+}), pH to 7.38 with KOH.

Materials. All chemicals used were racemic mixtures unless mentioned otherwise. The 1-^{14}C-labeled EET regioisomers, 13,14-EDT, 11,12-EEQ, and the 11,12-EET enantiomers were freshly synthesized as previously described (Zhang et al., 2001). Arachidonic acid was purchased from Nu-Chek-Prep (Elysian, MN). 11,12-EET methyl ester, 11,12-DHET, 12,13-leukotoxin, and linoleic acid were obtained from Cayman Chemical (Ann Arbor, MI). Lactobacillic acid was purchased from Matreya Inc. (State College, PA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Arachidonic acid, EETs, 11,12-EET methyl ester, 12,13-leukotoxin, 13,14-EDT, linoleic acid, and lactobacillic acid were prepared in 100% ethanol (5 mM) and stored under nitrogen at −20°C. Glyburide was dissolved in dimethylsulfoxide and stored at −20°C. The chemical stocks were used at greater than or equal to 1:10^3 dilutions in the experiments, and the final concentrations of ethanol or dimethylsulfoxide were less than 0.1%.

Statistical Methods. Data are presented as mean ± S.E.M. Student's t test or paired t test was used to compare data between two groups. One-way analysis of variance with Tukey's test was used to compare data from multiple groups using Sigma Stat 2.0 software (Jandel Co., San Rafael, CA). Statistical significance was assumed at $p < 0.05$.

Results

Because 5 μM 11,12-EET activates the K_{ATP} channels by reducing the channel sensitivity to ATP (Lu et al., 2001a), we compared the effects of 5 μM of various EET homologs and analogs. The results on EET regioselectivity are shown in Fig. 2. Figure 2A shows recordings of K_{ATP} channels at −60 mV in the presence of 1 mM cytoplasmic ATP, with or without 5 μM 5,6-, 8,9-, 11,12-, and 14,15-EET. ATP (1 mM) was used because it almost totally (99%) suppressed K_{ATP} channel activities. However, in the presence of 5 μM EETs, appreciable K_{ATP} channel activity was evident. The K_{ATP} channel P_o versus ATP concentration relationships in the presence and absence of 5 μM EET regioisomers are plotted in Fig. 2B. All EET regioisomers were potent in reducing ATP sensitivity and increased the ATP IC_{50} from 31.5 ± 7.3 ($n = 7$) to 306 ± 52 μM for 5,6-EET ($n = 7$, $p < 0.05$ versus control), to 315 ± 36 μM for 8,9-EET ($n = 6$, $p < 0.05$ versus control), to 366 ± 72 μM for 11,12-EET ($n = 7$, $p < 0.05$ versus control), to 498 ± 83 for 14,15-EET ($n = 6$, $p < 0.05$ versus control), and without significant changes in the Hill coefficient of 1 to 1.2. Thus, the position of the epoxide group was not critical for K_{ATP} activation. The ATP IC_{50} values for the EETs in this study were two-fold lower than previously reported (Lu et al., 2001a). We believe this difference could be accounted for by the source of EETs. In our previous study, we obtained 11,12-EET from a commercial source, whereas in the present study, [1-^{14}C]EETs were synthesized and the final concentrations were carefully checked by liquid scintillation techniques.

EET homologs and analogs (Fig. 1) were also tested to delineate whether other structural groups were important for EET activation of the K_{ATP} channels (Fig. 3). Arachidonate and 11,12-DHET, the respective precursor and product

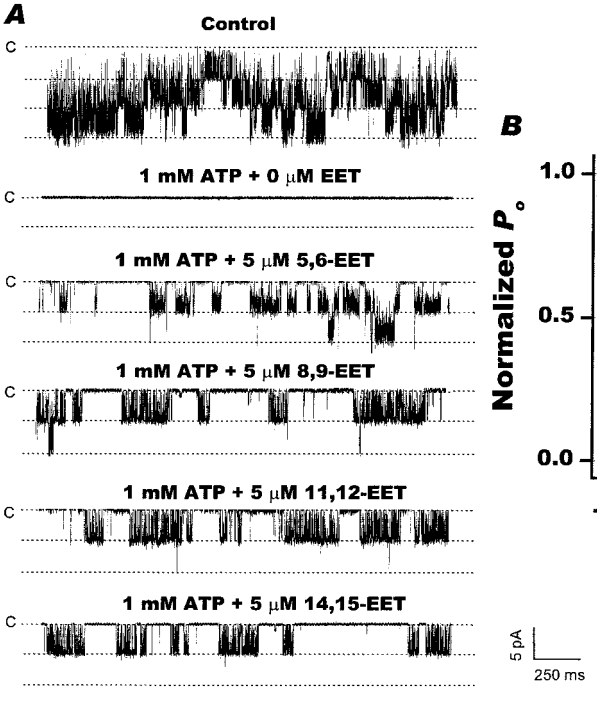

Fig. 2. Effects of EET regioisomers on cardiac K_{ATP} channel activity. A, typical tracings of K_{ATP} channels recorded at a membrane potential of −60 mV with 1 mM cytoplasmic ATP in the absence or presence of 5 μM 5,6-, 8,9-, 11,12-, or 14,15-EET. B, normalized P_o-ATP concentration relationships showing the effects of no EET (open circles), 5 μM 5,6-EET (filled diamonds), 5 μM 8,9-EET (filled squares), 5 μM 11,12-EET (filled circles), and 5 μM 14,15-EET (filled triangles) on ATP inhibition of K_{ATP} channels. K_{ATP} currents were measured at −60 mV with varying cytoplasmic ATP concentrations. Normalized P_o was obtained by dividing the measured P_o with maximum P_o observed at 10^{-7} M ATP.

of 11,12-EET, had no effect on the K_{ATP} channel activity; the ATP IC_{50} remained at 43.7 ± 10.1 μM ($n = 6, p$ = N.S. versus control) and 39.5 ± 11.3 μM ($n = 7, p$ = N.S. versus control), respectively (Fig. 3A). These results suggest that EET activation of the K_{ATP} channels in cardiac myocytes are not due to nonspecific fatty acid effects. In contrast, the methyl ester of 11,12-EET produced an effect similar to that of unesterified 11,12-EET; the ATP IC_{50} was shifted to 453 ± 110 μM ($n = 6, p$ = N.S. versus 11,12-EET), and the Hill coefficient (1.0) remained unaltered (Fig. 3B). Thus, the presence of the epoxide group but not that of the anionic carboxylic group is crucial for K_{ATP} channel activation.

EET homologs and analogs were also used to test the effects of chain length on K_{ATP} channel activation. 12,13-Leukotoxin (9*E*,12,13-*cis*-epoxy-18:1), an 18-carbon epoxide with two less double bonds than the EETs, activated the K_{ATP} channel and increased the ATP IC_{50} of the channel to 398 ± 79 μM ($n = 6, p < 0.05$ versus control), similar to those of EETs (Fig. 3C). In contrast, lactobacillic acid (*cis*-11,12-methylene 18:0), an analog of 12,13-leukotoxin, which contains a *cis* methylene ring instead of a *cis* oxirane ring and no double bond, had no effect on the ATP IC_{50} (34.3 ± 13.1 μM, $n = 7, p$ = N.S. versus control). Furthermore, the unsubstituted fatty acid analog, linoleic acid (9*E*,12*E*-18:2), had no effect on the K_{ATP} channel activation with ATP IC_{50} at 34.1 ± 12.5 μM ($n = 6, p$ = N.S. versus control). These results confirmed that the epoxide group is critical for K_{ATP} channel activation, whereas chain shortening by two carbons or reducing the number of double bonds did not alter the epoxide effect.

EET homologs and analogs were also used to test the effects of chain elongation or increasing the number of double bonds (Fig. 3D). 13,14-EDT is an 11,12-EET chain-elongation homolog with two additional carbons inserted at the COOH end. 11,12-EEQ is an ω-3 analog of 11,12-EET with an additional double bond between 17-C and 18-C (Fig. 1). 13,14-EDT was as effective as 11,12-EET in reducing the K_{ATP} channel sensitivity to ATP; the IC_{50} shifted to 321 ± 64 μM ($n = 6, p < 0.05$ versus control; p = N.S. versus 11,12-EET). Surprisingly, 11,12-EEQ was more potent than 11,12-EET in reducing ATP sensitivity in the K_{ATP} channel; yet, although 11,12-EEQ shifted the IC_{50} to 1071 ± 261 μM ($n = 5, p < 0.05$ versus 11,12-EET), the Hill coefficient remained unaltered at 1.1. These results confirmed that small (2 carbon) changes in chain-length were not critical; however, an additional double bond at the ω-3 position enhanced the ability of the epoxide to activate the K_{ATP} channels.

To determine whether 11,12-EET stereospecifically activated K_{ATP} channels, we synthesized and tested both of the 11(*R*),12(*S*)-EET and 11(*S*),12(*R*)-EET enantiomers. Figure 4A shows recordings of multiple K_{ATP} channels in the presence of 5 μM 11(*S*),12(*R*)-EET (left) and 11(*R*),12(*S*)-EET (right). In the presence of 1.0 mM cytoplasmic ATP, K_{ATP} channel activity was significantly enhanced by 11(*S*),12(*R*)-EET but not by 11(*R*),12(*S*)-EET. 11(*S*),12(*R*)-EET shifted the ATP inhibitory curve to the right. The IC_{50} of 329 ± 124 μM ($n = 5, p < 0.05$ versus control) and Hill coefficient of 1.2 matched that of racemic 11,12-EET. In contrast, 11(*R*),12(*S*)-EET had no effect; the IC_{50} remained at 32.3 ± 11.2 μM ($n = 5, p$ = N.S. versus control; $p < 0.05$ versus11(*S*),12(*R*)-EET) (Fig. 4B). Thus, the three-dimensional presentation of the epoxide was critical for activating K_{ATP} channels.

The potency of 11(*S*),12(*R*)-EET in activating K_{ATP} channels was estimated by measuring the EC_{50} for channel activation. Channel activity was determined at −60 mV and with 100 μM cytosolic ATP because submaximal (only 80%) K_{ATP} channel activity was inhibited under these conditions. 11(*S*),12(*R*)-EET dose dependently enhanced the K_{ATP} channel activities (Fig. 5A. left column), whereas, 11(*R*),12(*S*)-EET was inactive (Fig. 5A, right column). 11(*S*),12(*R*)-EET

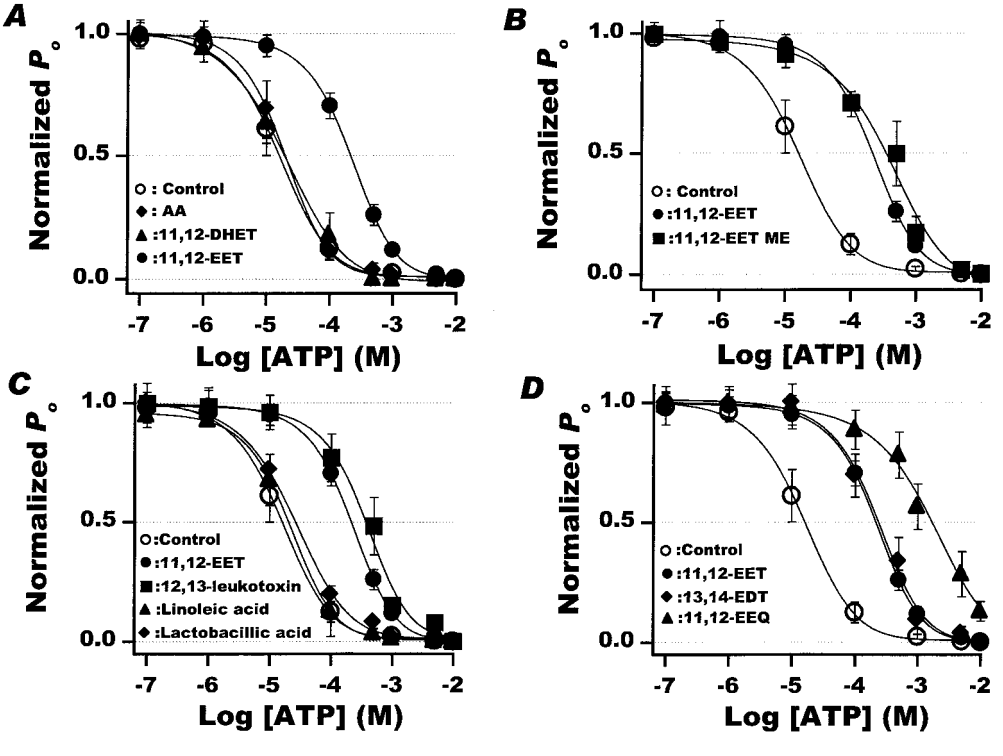

Fig. 3. Effects of 11,12-EET and its homologs/analogs on ATP inhibition of the cardiac K_{ATP} channel activity. Experiments were conducted and P_o normalized as described in Fig. 2B. Normalized P_o-ATP relationships showing the effects of the following compounds on ATP inhibition of K_{ATP} channels: in the absence (open circles) and presence (filled diamonds) of 5 μM arachidonic acid, 5 μM 11,12-DHET (filled triangles), and 5 μM 11,12-EET (filled circles) (A); in the absence (open circles) and presence (filled circles) of 5 μM 11,12-EET and 5 μM 11,12-EET methyl ester (filled squares) (B); in the absence (open circles) and presence (filled triangles) of 5 μM linoleic acid, 5 μM lactobacillic acid (filled diamonds), 5 μM 12,13-leukotoxin (filled squares), and 5 μM 11,12-EET (filled circles) (C); in the absence (open circles) and presence (filled circles) of 5 μM 11,12-EET, 5 μM 13,14-EDT (filled diamonds), and 5 μM 11,12-EEQ (filled triangles) (D). The P_o-ATP curve for 11,12-EET was reproduced in B, C, and D for comparison purposes.

potently activates the K_{ATP} channel with an EC_{50} of 39.5 ± 12.4 nM (n = 5), and this was about 2-fold higher than that previously reported for 11,12-EET (Lu et al., 2001a). We believe the differences in the source of EETs may account for the difference in results. In contrast, 11(R),12(S)-EET did not change channel activity at any concentration tested (Fig. 5B).

To determine whether activation of the K_{ATP} channel altered the membrane potential in whole cells, we examined the effects of the 11,12-EET enantiomers on action potentials recorded from isolated ventricular myocytes. One typical experiment is shown in Fig. 6A. Group data are shown in Fig. 6, B and C. Superfusion of 5 μM 11(S),12(R)-EET hyperpolarized the RP from −79.3 ± 2.3 mV to −83.4 ± 2.6 mV (n = 8, p = 0.008) and shortened the APD_{90} from 41.6 ± 5.0 ms to 35.5 ± 4.8 ms (n = 8, p = 0.006). The amplitude of the action potential at baseline (104.1 ± 5.5 mV) was not changed by 11(S),12(R)-EET (102.2 ± 5.7 mV, n = 8, p = N.S.). Both the changes in RP and APD_{90} were blocked by 2 μM glyburide, suggesting that the 11(S),12(R)-EET effects were due to K_{ATP} channel activation (Fig. 6B). However, superfusion of 5 μM 11(R),12(S)-EET did not alter the RP (−84.5 ± 1.4 mV versus −83.5 ± 1.9 mV at baseline, n = 8, p = N.S.), the APD_{90} (42.7 ± 3.7 ms versus 42.2 ± 2.3 ms at baseline, n = 8, p = N.S.), or the action potential amplitude (101.6 ± 3.6 mV versus 101.0 ± 2.3 mV at baseline, n = 8, p = N.S.) (Fig. 6C). These results indicate that modulation of the cardiac action potential by 11,12-EET is stereospecific.

Discussion

In the present study, we have made four important and novel observations. First, we have demonstrated that activation of the K_{ATP} channel by EET involves absolute stereospecificity; only the 11(S),12(R)-EET enantiomer is active. Second, the epoxide group on EET is a critical structural determinant for activation of the K_{ATP} channels. Third, an ω-3 double bond enhances the potency of the epoxide for K_{ATP} channel activation. Fourth, 11(S),12(R)-EET hyperpolarizes resting membrane potentials and shortens action potentials. These results suggest that endogenous EETs are specific activators of the cardiac K_{ATP} channels and may play an important role in regulating cardiac electrical excitability.

EETs regulate vital physiological functions including vasoreactivity (Campbell et al., 1996; Oltman et al., 1998; Zhang et al., 2001), inflammatory responses (Node et al., 1999), and cell proliferation (Fleming et al., 2001). Ion channels constitute an important class of EET effectors. In blood vessels, EETs cause vasodilation by activating the large con-

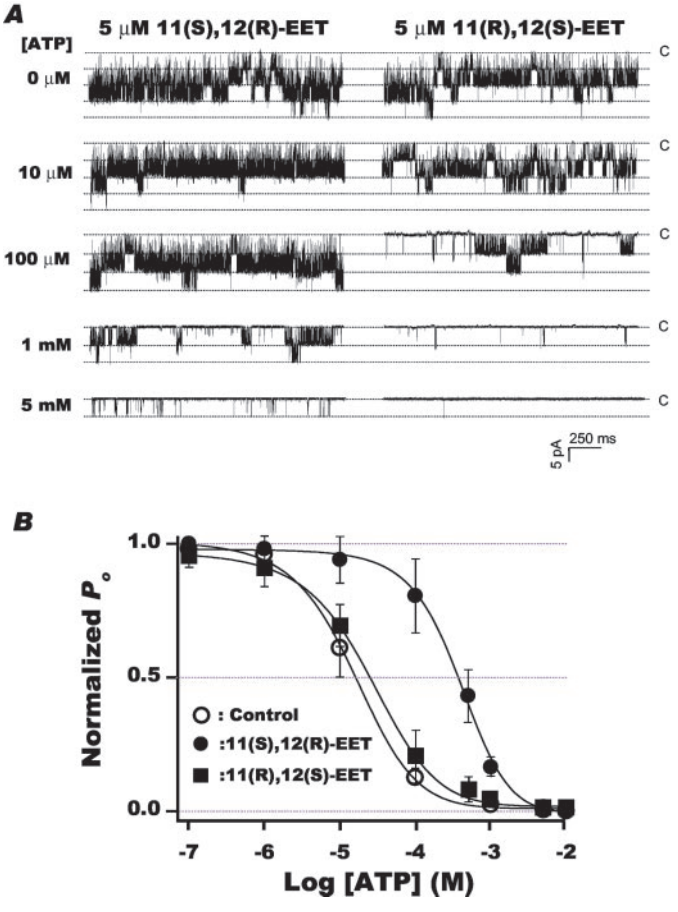

Fig. 4. Stereospecific effect of 11,12-EET on K_{ATP} channel activity. Experiments were performed and P_o normalized as described in Fig. 2B. A, current tracings of K_{ATP} channels in the presence of 5 μM 11(S),12(R)-EET (left) or 5 μM 11(R),12(S)-EET (right) and varying concentrations of ATP. B, relationships of normalized P_o plotted against cytoplasmic ATP concentrations in the absence (open circles) and presence (filled circles) of 5 μM 11(S),12(R)-EET or 5 μM 11(R),12(S)-EET (filled squares). n = 5 for both groups.

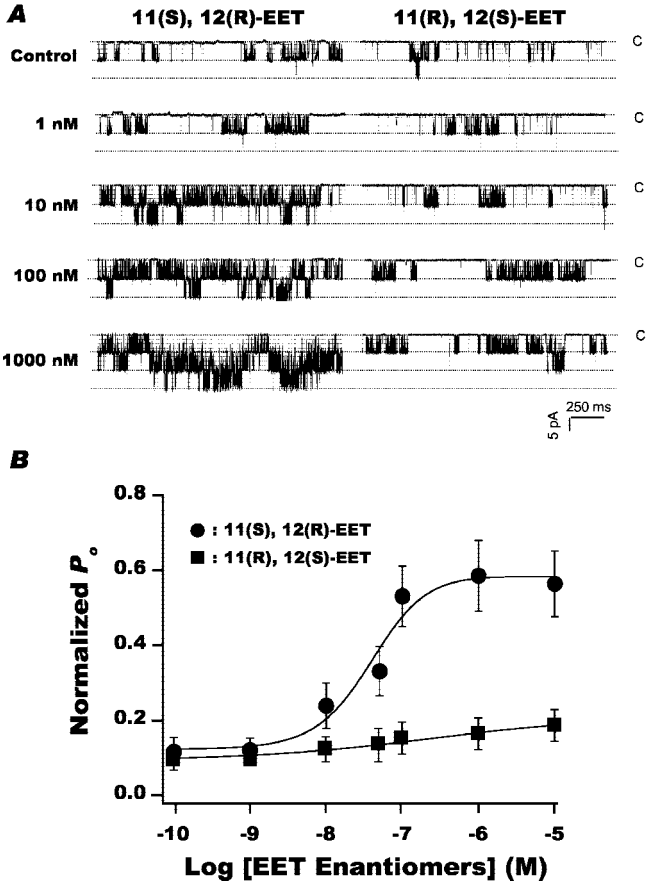

Fig. 5. Dose-dependent effect of 11(S),12(R)-EET on K_{ATP} channel activation. A, raw tracings of K_{ATP} channel currents were recorded at −60 mV with 0.1 mM ATP, 1 μM Ca^{2+}, and in the presence of various concentrations of 11(S),12(R)-EET (left) and 11(R),12(S)-EET (right). B, 11(S),12(R)-EET activated cardiac K_{ATP} channel with an EC_{50} of 39.5 ± 12.4 nM (filled circles), whereas 11(R),12(S)-EET was totally inactive (filled squares). n = 5 for both groups.

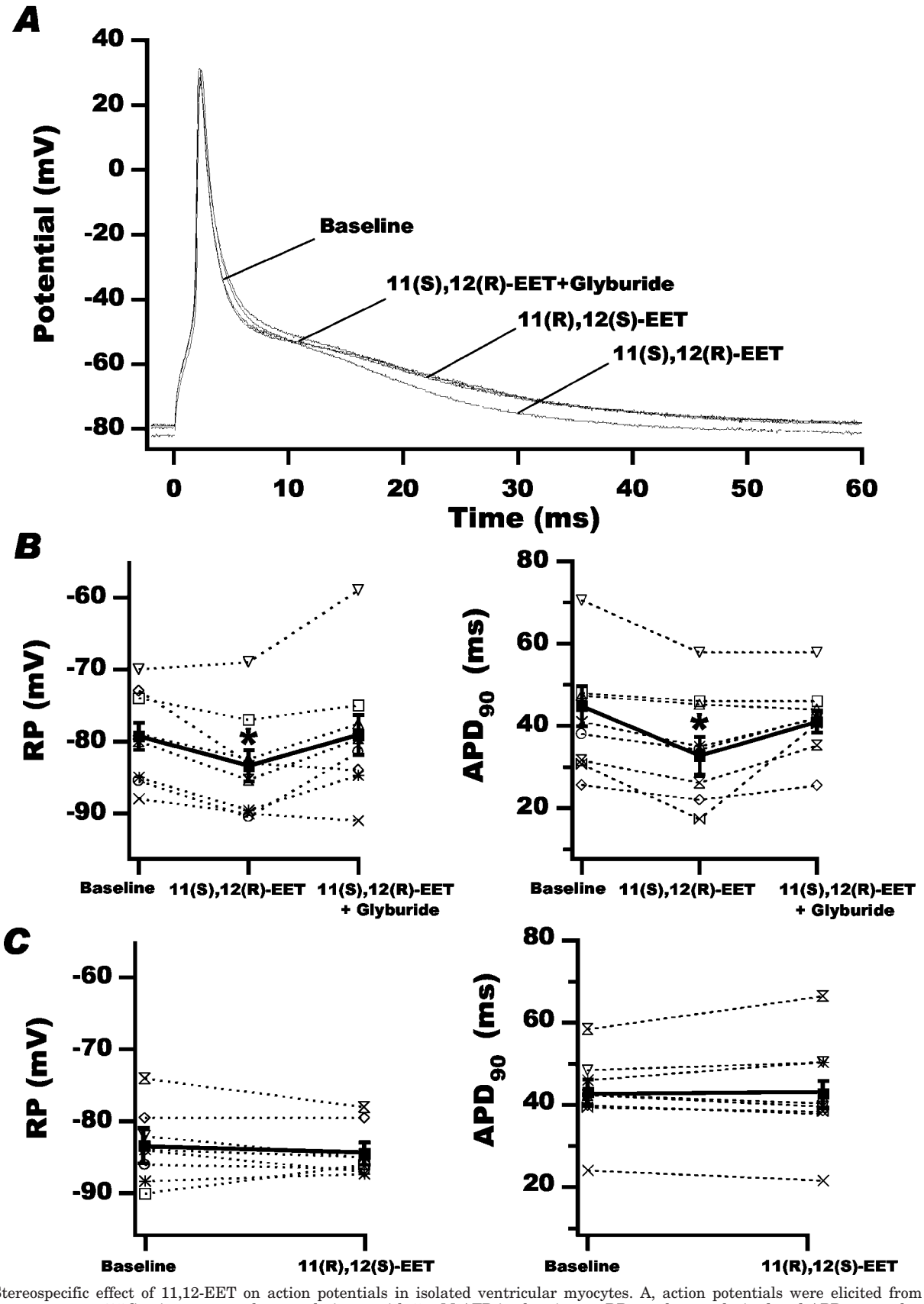

Fig. 6. Stereospecific effect of 11,12-EET on action potentials in isolated ventricular myocytes. A, action potentials were elicited from a single ventricular myocyte at 37°C using current clamp techniques with 5 mM ATP in the pipette. RP was hyperpolarized and APD_{90} was shortened on exposure to 5 μM 11(*S*),12(*R*)-EET and these effects were reversed by superfusion with 2 μM glyburide. 11(*R*),12(*S*)-EET had no effect. Scatter plots of group data are shown for 11(*S*),12(*R*)-EET ($n = 8$) (B) and for 11(*R*),12(*S*)-EET ($n = 8$) (C). The dark solid symbols and lines represent the means ± S.E.M. $*$, $p < 0.05$ versus baseline.

ductance Ca^{2+}-activated K^+ (BK) channels and are putative endothelium-derived hyperpolarizing factors (Li and Campbell, 1997; Campbell and Harder, 1999). EETs are endogenous constituents of the heart (Wu et al., 1997). Moreover, EETs inhibit cardiac Na^+ channels with the characteristics of a "modulated receptor" mechanism (Lee et al., 1999). The effects of EETs on the cardiac L-type Ca^{2+} channel are more variable, and both stimulation (Xiao et al., 1998) and inhibition (Chen et al., 1999) of channel activity have been observed. Recently, we reported that EETs are potent activators of the cardiac K_{ATP} channels, capable of activating the channel in the presence of 5 mM ATP, with an EC_{50} of 10^{-8} M (Lu et al., 2001a). The present study confirms that EETs are important K_{ATP} channel activators. We believe that the stereospecific effects of 11,12-EET on the K_{ATP} channel indicate specific interaction between 11,12-EET and the K_{ATP} channel protein or a channel-associated protein. It is likely that such specificity would entail a physiologically relevant and important function. The structurally specific requirements of the EET molecule for activating myocardial K_{ATP} channels is in sharp contrast to the relative lack of structural specificity for activating BK channels in coronary blood vessels (Zhang et al., 2001). EET-induced dilation and activation of BK channels in coronary microvessels did not show stereospecificity (Zhang et al., 2001). Also, 11,12-DHET is as potent as 11,12-EET in producing coronary vasorelaxation (Oltman et al., 1998) and in activating coronary BK channels (Lu et al., 2001b). To date, the stereospecificity of EET-induced vascular effects has been restricted to platelets (Fitzpatrick et al., 1986) and renal vessels (Katoh et al., 1991; Zou et al., 1996). Interestingly, 11(*R*),12(*S*)-EET rather than 11(*S*),12(*R*)-EET activates the BK channels in renal arteriolar smooth muscle cells (Zou et al., 1996), suggesting that stereospecificity is unique for different channel proteins.

Only limited data suggest EETs bind to specific receptors; however, binding of EETs to these sites have not yet been shown to alter cellular function (Wong et al., 1997). Fatty acid epoxides interact enantioselectively with many proteins including P450 epoxygenases and epoxide hydrolases (Orru and Faber, 1999). In the present study, the epoxide is a critical functional group in EETs, which activates K_{ATP} channels. Interaction between the oxirane ring and specific residues on the K_{ATP} channel conceivably requires precise configuration and orientation, thereby producing specific conformational changes in the channel protein leading to reduced ATP sensitivity and enhanced channel activity. Because substitution of the epoxide with diols or with a methylene ring abolished the K_{ATP} effects, the oxirane ring oxygen may facilitate critical hydrogen bond bridges or form an "intermediate" with nucleophilic groups of the K_{ATP} channel. However, formation of stable intermediates is unlikely because the effects of EETs on the K_{ATP} channel were rapidly reversible upon drug washout. Perhaps more importantly, the present findings exclude the possibility that EETs activate the K_{ATP} channels via a mechanism similar to that by phosphoinositides, in which the positively charged residues at the COOH terminus of the K_{ATP} channel are tethered by the electrostatic force of the anionic groups of the phospholipids at the cytoplasmic surface of the membrane, allowing the channel to be active (Fan and Makielski, 1997). Such a mechanism would require neither stereospecificity nor the presence of an epoxide group. Epoxide-based modulation of protein function has received increasing attention, and the utilization of epoxides in drug design has been shown to be effective in a number of compounds including a novel antitumor antibiotic, Rhizosin (Tolcher et al., 2000) and an angiogenesis inhibitor, TNP-470 (Stadler et al., 1999).

ω-3 fatty acids are essential fatty acids enriched in fish oils and have been shown to be cardioprotective from their antithrombosis, anti-atherosclerosis, anti-inflammatory, anti-vasoconstriction, and anti-arrhythmic effects (Connor, 2000). Kang and Leaf (2000) suggest that the suppression of fatal cardiac arrhythmias by ω-3 polyunsaturated fatty acids may involve the inhibition of the cardiac Na^+ channels and L-type Ca^{2+} channels. The P450 epoxygenase metabolites of ω-3 fatty acids (EEQs) are potent vasodilators in coronary microvessels (Zhang et al., 2001). It is intriguing that the ω-3 fatty acid metabolite, 11,12-EEQ, is more potent than its ω-6 epoxide homolog, 11,12-EET, in activating the cardiac K_{ATP} channels. Whether this is related to the cardioprotective effects of ω-3 fatty acids is not known, but the physiological function of ω-3 fatty epoxides deserve further investigation. The ω-3 double bond, undoubtedly renders the hydrocarbon tail more rigid and nucleophilic. This appears to be the only structural determinant studied that augmented the ability of the fatty epoxide to activate K_{ATP} channels. Small changes in carbon chain length, lowered double bond numbers, altered epoxide positions, and the loss of the negative carboxylic charge did not affect the potency of the epoxide in reducing ATP sensitivity of the K_{ATP} channels in cardiac myocytes.

The physiologic importance of K_{ATP} channel activation by EET, in the presence of 5 mM ATP, is demonstrated by the hyperpolarization of resting membrane potentials and shortening of the action potentials. The effects of 11,12-EET were again stereospecific and completely reversed by glyburide, suggesting that these electrophysiological effects were mediated by the K_{ATP} channels. Whether these effects are cardioprotective or not is at present unclear, although there appears to be a greater effect on membrane potential than on APD_{90}. Hyperpolarization of membrane potential would likely suppress abnormal automaticity and is therefore antiarrhythmic in outcome. However, shortening of the action potential may promote reentry mechanisms of arrhythmia and may create dispersion of refractoriness, resulting in proarrhythmic effects. These issues will require detailed electrophysiological studies to resolve. Recently, blood in the coronary sinus of dog hearts showed significant increases in the levels of EETs and their metabolites during coronary occlusion and reperfusion (Nithipatikom et al., 2001). Under these conditions, EETs would accumulate in cardiomyocytes and ATP would be depleted, creating a situation in which significant activation of the K_{ATP} channels could occur. Indeed, using a K_{ATP} channel knockout mouse, the molecular basis of the ST segment elevation during acute myocardial infarction can be attributed to the activation of K_{ATP} channels (Li et al., 2000).

In conclusion, we have identified the structural determinants of EET for activation of the cardiac K_{ATP} channels and showed the effects of EET to be stereospecific. Our results indicate that EET can activate the K_{ATP} channels under physiological conditions and may serve to regulate cardiac electrical excitability.

References

Brash AR (2001) Arachidonic acid as a bioactive molecule. *J Clin Investig* **107:**1339–1345.

Campbell WB, Gebremedhin D, Pratt PF, and Harder DR (1996) Identification of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factors. *Circ Res* **78:**415–423.

Campbell WB and Harder DR (1999) Endothelium-derived hyperpolarizing factors and vascular cytochrome P450 metabolites of arachidonic acid in the regulation of tone. *Circ Res* **84:**484–488.

Chen J, Capdevila JH, Zeldin DC, and Rosenberg RL (1999) Inhibition of cardiac L-type calcium channels by epoxyeicosatrienoic acids. *Mol Pharmacol* **55:**288–295.

Connor WE (2000) Importance of n-3 fatty acids in health and disease. *Am J Clin Nutr* **71:**171S–175S.

Fan Z and Makielski JC (1997) Anionic phospholipids activate ATP-sensitive potassium channels. *J Biol Chem* **272:**5388–5395.

Fang X, Kaduce TL, Weintraub NL, Harmon S, Teesch LM, Morisseau C, Thompson DA, Hammock BD and Spector AA (2001) Pathways of epoxyeicosatrienoic acid metabolism in endothelial cells. Implications for the vascular effects of soluble epoxide hydrolase inhibition. *J Biol Chem* **276:**14867–14874.

Fitzpatrick FA, Ennis MD, Baze ME, Wynalda MA, McGee JE, and Liggett WF (1986) Inhibition of cyclooxygenase activity and platelet aggregation by epoxyeicosatrienoic acids. Influence of stereochemistry. *J Biol Chem* **261:**15334–15338.

Fleming I, Fisslthaler B, Michaelis UR, Kiss L, Popp R, and Busse R (2001) The coronary endothelium-derived hyperpolarizing factor (EDHF) stimulates multiple signalling pathways and proliferation in vascular cells. *Pfluegers Arch* **442:**511–518.

Funk CD (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science (Wash DC)* **294:**1871–1875.

Kang JX and Leaf A (2000) Prevention of fatal cardiac arrhythmias by polyunsaturated fatty acids. *Am J Clin Nutr* **71:**202S–207S.

Katoh T, Takahashi K, Capdevila J, Karara A, Falck JR, Jacobson HR, and Badr KF (1991) Glomerular stereospecific synthesis and hemodynamic actions of 8,9-epoxyeicosatrienoic acid in rat kidney. *Am J Physiol* **261:**F578–F586.

Lee H, Lu T, Weintraub NL, VanRollins M, Spector AA, and Shibata EF (1999) Effects of epoxyeicosatrienoic acids on the cardiac sodium channels in isolated rat ventricular myocytes. *J Physiol* **519:**153–168.

Li PL and Campbell WB (1997) Epoxyeicosatrienoic acids activate K^+ channels in coronary smooth muscle through a guanine nucleotide binding protein. *Circ Res* **80:**877–884.

Li RA, Leppo M, Miki T, Seino S, and Marban E (2000) Molecular basis of electrocardiographic ST-segment elevation. *Circ Res* **87:**837–839.

Lu T, Hoshi T, Weintraub NL, Spector AA, and Lee H (2001a) Activation of ATP-sensitive K^+ channels by epoxyeicosatrienoic acids in rat cardiac ventricular myocytes. *J Physiol* **537:**811–827.

Lu T, Katakam PV, VanRollins M, Weintraub NL, Spector AA, and Lee H (2001b) Dihydroxyeicosatrienoic acids are potent activators of Ca^{2+}-activated K^+ channels in isolated rat coronary arterial myocytes. *J Physiol* **534:**651–667.

McGiff JC (1991) Cytochrome P-450 metabolism of arachidonic acid. *Annu Rev Pharmacol Toxicol* **31:**339–369.

Nichols CG and Lederer WJ (1990) The regulation of ATP-sensitive K^+ channel activity in intact and permeabilized rat ventricular myocytes. *J Physiol* **423:**91–110.

Nithipatikom K, DiCamelli RF, Kohler S, Gumina RJ, Falck JR, Campbell WB, and Gross GJ (2001) Determination of cytochrome P450 metabolites of arachidonic acid in coronary venous plasma during ischemia and reperfusion in dogs. *Anal Biochem* **292:**115–124.

Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC, and Liao JK (1999) Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science (Wash DC)* **285:**1276–1279.

Noma A (1983) ATP-regulated K^+ channels in cardiac muscle. *Nature (Lond)* **305:**147–148.

Oltman CL, Weintraub NL, VanRollins M, and Dellsperger KC (1998) Epoxyeicosatrienoic acids and dihydroxyeicosatrienoic acids are potent vasodilators in the canine coronary microcirculation. *Circ Res* **83:**932–939.

Orru RV and Faber K (1999) Stereoselectivities of microbial epoxide hydrolases. *Curr Opin Chem Biol* **3:**16–21.

Roman RJ (2002) P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol Rev* **82:**131–185.

Stadler WM, Kuzel T, Shapiro C, Sosman J, Clark J, and Vogelzang NJ (1999) Multi-institutional study of the angiogenesis inhibitor TNP-470 in metastatic renal carcinoma. *J Clin Oncol* **17:**2541–2545.

Tolcher AW, Aylesworth C, Rizzo J, Izbicka E, Campbell E, Kuhn J, Weiss G, Von Hoff DD, and Rowinsky EK (2000) A phase I study of rhizoxin (NSC 332598) by 72-hour continuous intravenous infusion in patients with advanced solid tumors. *Ann Oncol* **11:**333–338.

Tung RT and Kurachi Y (1991) On the mechanism of nucleotide diphosphate activation of the ATP- sensitive K^+ channel in ventricular cell of guinea-pig. *J Physiol* **437:**239–256.

Wong PY, Lai PS, Shen SY, Belosludtsev YY, and Falck JR (1997) Post-receptor signal transduction and regulation of 14(*R*), 15(*S*)- epoxyeicosatrienoic acid (14,15-EET) binding in U-937 cells. *J Lipid Mediat Cell Signal* **16:**155–169.

Wu S, Chen W, Murphy E, Gabel S, Tomer KB, Foley J, Steenberger C, Falck JR, Moomaw CR, and Zeldin DC (1997) Molecular cloning, expression and functional significance of a cytochrome P450 highly expressed in rat heart myocytes. *J Biol Chem* **272:**12551–12559.

Xiao YF, Huang L, and Morgan JP (1998) Cytochrome P450: a novel system modulating Ca^{2+} channels and contraction in mammalian heart cells. *J Physiol* **508:**777–792.

Yokoshiki H, Sunagawa M, Seki T, and Sperelakis N (1998) ATP-sensitive K^+ channels in pancreatic, cardiac and vascular smooth muscle cells. *Am J Physiol* **274:**C25–C37.

Zeldin DC (2001) Epoxygenase pathways of arachidonic acid metabolism. *J Biol Chem* **276:**36059–36062.

Zeldin DC, Kobayashi J, Falck JR, Winder BS, Hammock BD, Snapper JR, and Capdevila JH (1993) Regio- and enantiofacial selectivity of epoxyeicosatrienoic acid hydration by cytosolic epoxide hydrolase. *J Biol Chem* **268:**6402–6407.

Zhang Y, Oltman CL, Lu T, Lee H, Dellsperger KC, and VanRollins M (2001) EET homologs potently dilate coronary microvessels and activate BK_{Ca} channels. *Am J Physiol Heart Circ Physiol* **280:**H2430–H2440.

Zou AP, Fleming JT, Falck JR, Jacobs ER, Gebremedhin D, Harder DR, and Roman RJ (1996) Stereospecific effects of epoxyeicosatrienoic acids on renal vascular tone and K^+-channel activity. *Am J Physiol* **270:**F822–F832.

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