0026-895X/99/020288-08$3.00/0

MOLECULAR PHARMACOLOGY, **55:**288–295 (1999).

Inhibition of Cardiac L-Type Calcium Channels by Epoxyeicosatrienoic Acids

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Received August 26, 1998; accepted October 29, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

Epoxyeicosatrienoic acids (EETs), products of the cytochrome P-450 monooxygenase metabolism of arachidonic acid, can regulate the activity of ion channels. We examined the effects of EETs on cardiac L-type Ca^{2+} channels that play important roles in regulating cardiac contractility, controlling heart rate, and mediating slow conduction in normal nodal cells and ischemic myocardium. Our experimental approach was to reconstitute porcine L-type Ca^{2+} channels into planar lipid bilayers where we could control the aqueous and lipid environments of the channels and the regulatory pathways that change channel properties. We found that 20 to 125 nM EETs inhibited the open probability of reconstituted L-type Ca^{2+} channels, accelerated the inactivation of the channels, and reduced the unitary current amplitude of open channels. There was no selectivity among different EET regioisomers or stereoisomers. When 11,12-EET was esterified to the *sn*-2 position of phosphatidylcholine, restricting it to the hydrophobic phase of the planar lipid bilayer, the reconstituted channels were similarly inhibited, suggesting that the EET interacts directly with Ca^{2+} channels through the lipid phase. The inhibitory effects of EET persisted in the presence of microcystin, an inhibitor of protein phosphatases 1 and 2A, suggesting that dephosphorylation was not the mechanism through which these eicosanoids down-regulate channel activity. This inhibition may be an important protective mechanism in the setting of cardiac ischemia where arachidonic acid levels are dramatically increased and EETs have been shown to manifest preconditioning-like effects.

L-type Ca^{2+} channels are important for setting the strength and rate of the heartbeat. Ca^{2+} influx through L-type Ca^{2+} channels triggers the release of Ca^{2+} from sarcoplasmic reticulum, initiating and regulating the force of muscular contraction. Thus, L-type Ca^{2+} channels play a key role in excitation-contraction coupling in cardiac muscle, and regulation of their activity is important in controlling cardiac contractility (Fabiato and Fabiato, 1979). L-type Ca^{2+} channels also play a role in modulating normal and abnormal cardiac pacemaker activity and controlling heart rate (Brown, 1982). In addition, Ca^{2+} channels are responsible for slow conduction velocities in nodal cardiac cells and ischemic myocardium where fast Na^{+} channels remain chronically inactivated due to depolarization of diastolic membrane potentials (Gettes and Reuter, 1974). The abnormally slow conduction in ischemic myocardium can permit the formation of reentry pathways in and around the ischemic zone, leading to ventricular tachycardia, ventricular fibrillation, and sudden cardiac death in myocardial ischemia (Fleet et al., 1994).

In ischemic myocardium, there are changes in ionic concentrations (especially extracellular K^{+} and intracellular Ca^{2+}) resulting in changes in membrane potential, reductions in intracellular and extracellular pH, increases in catecholamine release resulting in downstream increases in intracellular Ca^{2+} and protein phosphorylation (Gettes and Cascio, 1992), and changes in lipid metabolism, including increases in the levels of arachidonic acid and its metabolites (Bend and Karmazyn, 1996). Arachidonic acid is metabolized by several pathways, including the lipoxygenase pathway (producing leukotrienes and hydroxyeicosatetraenoic acids) and the cyclooxygenase pathway (producing prostaglandins, thromboxanes, and prostacyclin). In addition, cytochrome P-450 monooxygenases metabolize arachidonic acid to four regioisomeric epoxyeicosatrienoic acids (EETs) (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET; Capdevila et al., 1995). An

This work was supported by National Institutes of Health Grants HL49449 and HL27430 (R.L.R.) and GM37922 (J.H.C.) and by the Division of Intramural Research, National Institute of Environmental Health Sciences (D.C.Z).

A preliminary account of these results appeared in abstract form [Chen J, Zeldin DC and Rosenberg RL (1998) Inhibition of cardiac L-type Calcium channels by epoxyeicosatrienoic acids. *Biophys J* **74:**A105].

ABBREVIATIONS: EET, epoxyeicosatrienoic acid; 11,12-EET-PC, 1-palmitoyl-2-epoxyeicosatrienoyl phosphatidylcholine; AA-PC, 1-palmitoyl-2-arachidonoyl phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; POPE, 1-palmitoyl-2-oleoyl phosphatidylethanolamine; POPS, 1-palmitoyl-2-oleoyl phosphatidylserine; P_o, open probability; HPLC, high-performance liquid chromatography; PFB, pentafluorobenzyl; ANOVA, analysis of variance; APD, action potential duration.

isoform of cytochrome P-450 (CYP2J2) is expressed at high levels in heart (Wu et al., 1996) and produces all four epoxy fatty acids. Each EET regioisomer is biosynthesized as either the *R, S* or *S, R* stereoisomer (Capdevila et al., 1995), and some of the biological actions of EETs are stereoselective (e.g., Zou et al., 1996).

The EET precursor, arachidonic acid, has been shown to regulate a number of ion channels, including L-type Ca^{2+} channels (Petit-Jacques and Hartzell, 1996). In addition, eicosanoids derived from lipoxygenase and cyclooxygenase metabolism of arachidonic acid regulate several ion channels (Needleman et al., 1986). More recently, it has been shown that the EETs modulate intracellular ionic homeostasis. For example, 11(*R*),12(*S*)-EET increases the open probability (P_o) of Ca^{2+}-activated potassium channels in vascular smooth muscle cells (Zou et al., 1996). EETs also cause increases in intracellular Ca^{2+} in endothelial cells, renal glomerular mesangial cells, renal proximal tubule and collecting duct epithelial cells, and anterior pituitary cells (Snyder et al., 1986; Force et al., 1991; Madhun et al., 1991; Graier et al., 1995; Sakairi et al., 1995). However, 14,15-EET inhibits thrombin- and thapsigargin-dependent Ca^{2+} entry into platelets (Malcolm and Fitzpatrick, 1992). In the heart, 5,6- and 11,12-EET cause an increase in cardiomyocyte shortening and an increase in intracellular Ca^{2+} concentration (Moffat et al., 1993). Recently, Xiao et al. (1998) showed that inhibitors of cytochrome P-450 suppress cardiac L-type Ca^{2+} current. In addition, they provided evidence that one of the P-450-derived eicosanoids stimulates cardiac Ca^{2+} channels via an indirect mechanism involving increases in cAMP- and cAMP-dependent phosphorylation. However, EETs also cause a reduction in cardiac action potential duration, suggesting that they may inhibit myocardial L-type Ca^{2+} channels (Lane et al., 1998).

Because L-type Ca^{2+} channels are highly regulated by many cellular factors, including intracellular pH, intracellular Ca^{2+}, phosphorylation and dephosphorylation, $G_{s\alpha}$, and multiple lipid metabolites (McDonald et al., 1994), it is often difficult to determine whether a particular compound has a direct or an indirect effect on the channels. Our experiments test the hypothesis that EETs modulate cardiac L-type Ca^{2+} channels by a direct mechanism. Our experimental approach was to reconstitute porcine L-type Ca^{2+} channels into lipid bilayers where we can control the aqueous and lipid environments surrounding the channel molecules and can study drug effects in the absence of changes in intracellular cAMP or protein phosphorylation (Rosenberg et al., 1988). We found that EETs have an inhibitory effect on L-type Ca^{2+} channels that is partially due to an increase in the rate of channel inactivation. We observed no regiospecificity or stereospecificity of these direct effects and found evidence that EETs interact with a site on the channels that is embedded in the lipid bilayer.

Materials and Methods

Sarcolemma and Planar Lipid Bilayers. Cell surface membranes from porcine cardiac muscle, enriched in L-type Ca^{2+} channels and relatively devoid of mitochondrial and sarcoplasmic reticulum markers, were prepared by homogenization, differential centrifugation, and sucrose gradient fractionation as described previously (Rosenberg et al., 1988). Planar lipid bilayers (150–200-μm diameter) were usually formed from a mixture of 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE; 15 mg/ml) and 1-palmitoyl-2-oleoyl phosphatidylserine (POPS; 5 mg/ml) in *n*-decane (Rosenberg et al., 1988). In some experiments, 1 mg/ml 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), 1-palmitoyl-2-epoxyeicosatrienoyl-phosphatidylcholine (11,12-EET-PC), or 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (AA-PC) replaced 1 mg/ml of the POPE (see below). POPE, POPS, POPC, and AA-PC were obtained from Avanti Polar Lipids (Pelham, AL).

Fusion of Cardiac Sarcolemma with Planar Lipid Bilayers. At the time of channel incorporation, the aqueous solution on both sides of the bilayer contained 50 mM NaCl and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-NaOH, pH 7.0. The extracellular solution (in the *cis* chamber) also contained 100 mM $BaCl_2$ to provide Ba^{2+} as the current-carrying ion, to create an osmotic gradient favoring vesicle fusion, and to provide divalent cations to enhance fusion rates. The intracellular solution (in the *trans* chamber) contained recombinant activated G protein α-subunit ($G_{s\alpha}$) to reduce Ca^{2+} channel "rundown" (Wang et al., 1993). The Ca^{2+} channel agonist (+)-202-791 (0.5 μM; Sandoz) was added to both solutions to increase channel open times (Rosenberg et al., 1988). Cardiac surface membranes were added to the *cis* chamber, and incorporation occurred spontaneously, usually within 5 min.

Single-Channel Recordings. Voltage-clamp of the planar lipid bilayers and recordings of the unitary currents of reconstituted channels used modified patch-clamp electronics (Rosenberg and Chen, 1991) interfaced with a Digidata 1200 data acquisition system and controlled by software written in AxoBASIC (Axon Instruments). Voltages were defined as *trans* minus *cis*, so the *trans* chamber represents the intracellular side of the channels, as predicted for the conventional incorporation of outside-out membrane vesicles added to the *cis* chamber. Any channels that were incorporated with the reverse orientation would experience a large positive holding potential and become inactivated. The membrane voltage was held at −60 mV for 5.2 s. Channel activity was evoked by 800-ms depolarizations to 0 mV (or other depolarized voltages as noted). Data were low-pass filtered at 100 Hz (−8 dB, Bessel filter) and digitally sampled at 1 kHz. Channels were identified as L-type Ca^{2+} channels based on their conductance (~25 pS in 100 mM extracellular Ba^{2+}, giving a unitary current of ~1.2 pA at 0 mV test potential), voltage dependence of activation (requiring depolarizations to greater than −20 mV for activation), single-channel kinetics (mean open times of 20–50 ms in the presence of 0.5 μM 202-791), and slow inactivation during test pulses.

Analysis of Single-Channel Recordings. Open probability, ensemble averages, and current-voltage plots were created from single-channel analysis using analysis programs written in AxoBASIC. Leak and capacitative currents were eliminated by subtracting averages of "null" depolarizations that contained no channel openings. Current amplitudes were measured with computer-drawn lines that were fit to the closed and open levels by eye. Open probability was determined for each depolarization from the normalized time integral of the leak-subtracted recording. The number of channels in each bilayer was determined by carefully evaluating each recording and determining the maximal number of unitary steps during periods of high channel activity. Channels with an average P_o of <.1 at the beginning of the experiment were eliminated from the analysis. Ensemble averages were formed from summation of the leak-subtracted recordings and were scaled arbitrarily.

Synthesis and Purification of Eicosanoids and Phospholipids. Racemic 11,12- and 14,15-EET were prepared as described previously (Corey et al., 1980; Falck and Manna, 1982) and purified by reverse-phase high-performance liquid chromatography (HPLC) on a 5-μm Microsorb C18 column (4.6 × 250 mm; Rainin Instruments Co., Woburn, MA) using a linear gradient from $CH_3CO_2H/H_2O/CH_3CN$ (0.1:49.95:49.95) to CH_3CO_2H/CH_3CN (0.1:99.9) over 40 min at 1 ml/min (Capdevila et al., 1990). To isolate 11,12-EET enantiomers, 11,12-EET was first derivatized to the corresponding pentafluorobenzyl (PFB) ester by reaction with PFB bromide as described pre-

viously (Capdevila et al., 1990). The enantiomers of 11,12-EET-PFB were then resolved by chiral-phase HPLC on a Chiralcel OD column (4.6 × 250 mm; J. T. Baker Chemical Co., Phillipsburg, NJ) using hexane/isopropanol (0.15:99.85) at 1 ml/min (Hammonds et al., 1989). The corresponding free acids were regenerated by saponification in KOH/methanol and purified by reverse-phase HPLC. 11,12-EET-PC was synthesized from AA-PC by nonselective epoxidation as described previously (Karara et al., 1991). The corresponding epoxidized phospholipids were purified by reverse-phase HPLC on a Bondapak C18 column (3.9 × 300 mm; Waters Associates, Marlborough, MA) using a linear solvent gradient from 20 mM choline in $CH_3OH/CH_3CN/H_2O$ (90.5:4.08:5.42) to 20 mM choline in $CH_3OH/CH_3CN/H_2O$ (90.5:8.8:0.7) over 30 min at 1.5 ml/min (Karara et al., 1991).

Statistical Analysis. Statistical analyses of experimental data was made using paired or unpaired Student's *t* test (for before/after comparisons and comparisons between two experimental groups, respectively) or one-way analysis of variance (ANOVA) (Tukey's test; SigmaStat, SPSS, Chicago, IL). All data are reported as mean ± S.E.M. A statistically significant difference was assigned to a value of $p < .05$.

Results

Figure 1 shows the effects of 11,12-EET, added to the intracellular chamber, on a single reconstituted L-type Ca^{2+} channel. During the period of recording before 11,12-EET addition (Fig. 1A), we observed robust channel activity evoked by the membrane depolarization. In this example, the channel showed very little inactivation during the sustained 800-ms depolarization (Haack and Rosenberg, 1994). After a 3-min recording to evaluate the baseline activity of the channel, the addition of 125 nM 11,12-EET to the *trans* (intracellular) chamber caused a number of effects, including a decrease in P_o, an acceleration of channel inactivation, and a decrease in channel unitary current amplitude (Fig. 1B).

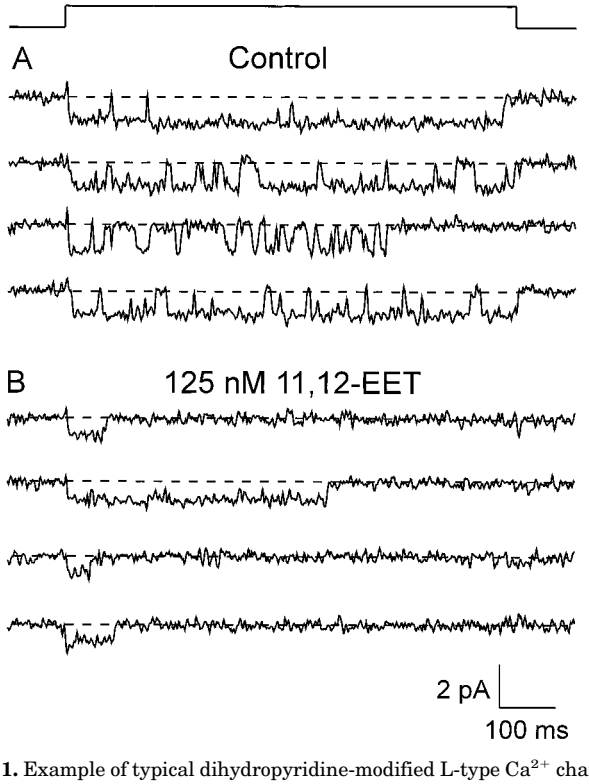

Fig. 1. Example of typical dihydropyridine-modified L-type Ca^{2+} channel activity in a planar lipid bilayer and effect of 125 nM 11,12-EET added to the intracellular chamber. A, sequential recordings obtained before the addition of the 11,12-EET. Downward transitions represent the opening of an L-type Ca^{2+} channel evoked by the membrane depolarization from a holding potential of −60 mV to the test potential of 0 mV as indicated by the period at the top of the figure. B, sequential recordings from the same channel obtained after addition of 125 nM 11,12-EET to the *trans* (intracellular) chamber.

To more fully characterize these effects, we first determined P_o during each depolarization before and after the addition of 11,12-EET. Figure 2A shows a plot of P_o for the experiment illustrated in Fig 1, in which each bar represents the P_o from a single depolarization. During the 2 min before the addition of 11,12-EET, channel P_o averaged around .278 ± .050. Within a few seconds after the addition of 125 nM 11,12-EET, P_o decreased to an average of .043 ± .018 (averaged over 3 min), and remained low for the remainder of the experiment. Efforts to wash out the EET and recover high P_o were unsuccessful, probably because the hydrophobic EET molecules partitioned into the bilayers and dissociated slowly. Similar results were obtained in seven independent experiments with 125 nM EET, with an average reduction in P_o from .309 ± .091 to .069 ± .019 (representing a 78% decrease in P_o; $n = 7$, $p < .05$, paired *t* test). A 55% decrease in P_o also occurred at 20 nM 11,12-EET (.144 ± .033 to .064 ± .013; $n = 4$), indicating that these effects were demonstrable within the physiologically relevant concentration range. The

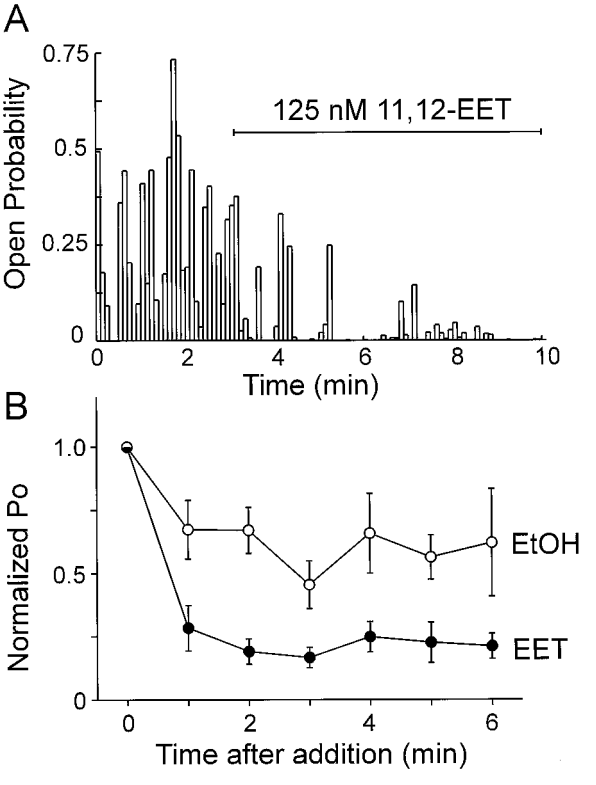

Fig. 2. Effect of 11,12-EET on L-type Ca^{2+} channel P_o. A, plot of P_o versus time for the experiment illustrated in Fig 1. Each vertical bar registers the P_o for each depolarization of the bilayer. After the addition of 125 nM 11,12-EET to the intracellular chamber (indicated by the horizontal bar), the P_o was substantially reduced. B, change in P_o after the addition of 60 nM 11,12-EET (●) or 0.057% ethanol vehicle (EtOH) (○). Data from 10 different experiments were combined. In each, the P_o during each minute after the addition (calculated as the average P_o from 10 depolarizations) was normalized to the P_o recorded during the 1 min immediately before addition. Error bars indicate the S.E.M. Although there was some run-down of P_o recorded in the presence of ethanol, the P_o was significantly lower at all time points in the presence of 60 nM 11,12-EET ($p < .05$, unpaired *t* test).

large variability in P_o between individual Ca^{2+} channels and the small number of experiments performed with 20 nM 11,12-EET were responsible for the apparent difference in initial P_o between these two groups. In addition, the large variability in P_o between individual channels made it difficult to evaluate the dose-response behavior below 20 nM. The overall effects of 60 nM, 125 nM, and higher concentrations of 11,12-EET on Ca^{2+} channel P_o were similar.

Reconstituted L-type Ca^{2+} channels show a tendency to "run down" even when stimulated by dihydropyridine agonists and activated $G_{s\alpha}$ (Wang et al., 1993). Thus, we determined whether the inhibitory effects of 11,12-EET were significantly greater than the decrease in P_o that normally occurs during prolonged recordings due to channel rundown. Figure 2B shows the time course of the effect of 60 nM 11,12-EET added to the *trans* (intracellular) chamber, compared with the effect of 0.057% (v/v) ethanol that was used as the vehicle for EET addition. Data from 10 individual channels in each experimental group were combined. At all time points, EET caused a significant decrease in channel P_o compared with the ethanol addition ($p < .05$, unpaired t test). The addition of ethanol did not cause a significant decrease in P_o compared with experiments in which no addition was made (data not shown). Thus, 60 nM intracellular 11,12-EET caused a significant decrease in L-type Ca^{2+} channel P_o, even when the natural tendency of the channel to run down was accounted for.

After the addition of 11,12-EET, Ca^{2+} channels tended to enter a long-lived closed state earlier in each depolarization than before the addition (compare Fig. 1, B and A), indicating that one of the effects of 11,12-EET was to accelerate the inactivation of the L-type Ca^{2+} channels during the depolarization. To more completely characterize the effect on Ca^{2+} channel inactivation rates, we prepared ensemble averages of the single-channel activity before and after the addition of EET to the intracellular chamber. As shown in Fig. 3, 60 nM 11,12-EET caused a substantial increase in the rate of channel inactivation. Both the initial rate of inactivation and the overall extent of inactivation during the 800-ms depolarization were increased by the EET. Similar increases in the rate and extent of inactivation were seen in 9 of 10 experiments at this concentration of 11,12-EET. Thus, the decrease in P_o shown in Fig. 1 and characterized in detail in Fig. 2 was due in part to an increase in the rate of channel inactivation during the depolarization period.

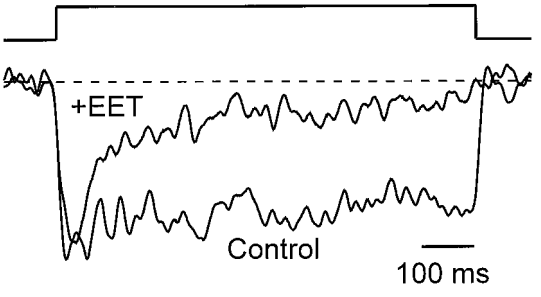

Fig. 3. EET increases inactivation rates of L-type Ca^{2+} channels. Ensemble average of a single-channel experiment before and after the addition of 60 nM 11,12-EET to the intracellular chamber. The P_o after the addition of EET was lower than before the addition, but the ensemble averages were scaled to the same peak inward current to permit comparison of inactivation rates during the depolarization. After the addition of EET, the rate and extent of inactivation were increased. Ensemble averages were digitally filtered at 20 Hz.

Figure 1B also suggests that the unitary conductance of L-type Ca^{2+} channels was reduced by 11,12-EET because the current amplitude at a test potential of 0 mV was reduced compared with that during the control period. To more fully characterize this effect, we measured unitary current amplitudes at a number of test depolarizations and calculated the slope conductance before and after 11,12-EET addition. Figure 4 shows a representative I-V plot from a single L-type Ca^{2+} channel. In this example, the unitary conductance decreased from 26 to 19 pS after the addition of 20 nM EET to the *trans* (intracellular) chamber. Similar effects were observed in five of six channels tested (at 20 and 60 nM 11,12-EET), with an average reduction of conductance from 23.8 ± 1.0 pS to 17.8 ± 1.5 pS ($p < .05$, unpaired t test, $n = 6$). Because of the low-pass filtering of the recordings, it is possible that the observed decrease in conductance was due to unresolved closing or blocking events with mean dwell times of <0.5 ms. There was no significant change in the extrapolated reversal potential, indicating that the 11,12-EET had no effect on the ability of the L-type Ca^{2+} channel to select for divalent cations (in this case, Ba^{2+}) over monovalent cations (e.g., Na^+).

Thus, 11,12-EET, added to the intracellular chamber at concentrations of 20 to 125 nM, caused a decrease in channel P_o, an acceleration of channel inactivation rates, and a decrease in channel unitary conductance. To determine whether the effects of EET depended on the side of the channel to which it was applied and to determine whether there was any regiospecificity or stereospecificity of the effects, we performed the set of experiments summarized in Fig. 5. In these experiments, the P_o of a number of single L-type Ca^{2+} channels was averaged before and after the addition of EETs (or the ethanol vehicle) to either the *cis* (extracellular) or *trans* (intracellular) chamber. The figure shows the percent decrease in P_o after EET or ethanol addition, relative to the P_o during the control period. The addition of ethanol (0.057%) was associated with a 36 ± 9% decrease in P_o, due to the run-down of Ca^{2+} channel activity. A similar decrease in P_o was observed even when no additions were made (data not shown). The addition of 11,12-EET to the *trans* (intracellular) chamber caused a 76 ± 6% decrease in P_o that was significantly greater than the effect of run-down

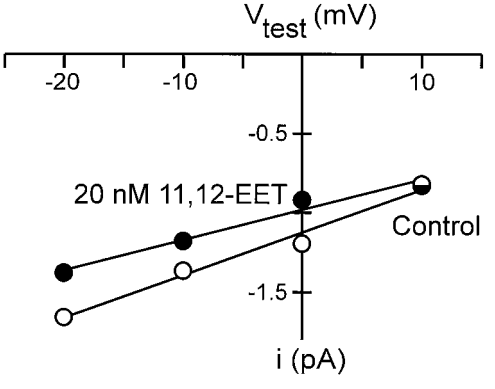

Fig. 4. EET reduces the conductance of L-type Ca^{2+} channels. Unitary current amplitudes before (○) and after (●) the addition of 20 nM 11,12-EET to the intracellular chamber were measured by eye with a computer-drawn horizontal vector and plotted versus test potential. The lines shown represent a linear regression fits of the data sets, yielding a slope conductance of 26 pS before the addition and 19 pS after the addition of EET.

($p < .05$ versus ethanol, ANOVA). Similarly, the addition of 11,12-EET to the *cis* (extracellular) chamber caused a decrease in channel P_o of 67 ± 16%. Another regioisomer of EET (14,15-EET), added to either the *trans* (intracellular) or *cis* (extracellular) chamber, also caused a similar reduction in Ca^{2+} channel P_o (69 ± 17% and 81 ± 10%, respectively). All of these EET additions caused decreases in Ca^{2+} channel P_o that were not significantly different from each other. We also compared the effects of 11(*R*),12(*S*)-EET and 11(*S*),12(*R*)-EET on L-type Ca^{2+} channel P_o. As shown in Fig. 5, 11(*S*),12(*R*)-EET (added to the *trans* side) had a slightly greater inhibitory effect on L-type Ca^{2+} channel P_o than did the 11(*R*),12(*S*)-EET or racemic 11,12-EET, but these differences were not significant. 11(*S*),12(*R*)-EET was also slightly more effective from the *trans* than the *cis* side, but again, the difference was not significant (data not shown). In summary, we found that 11,12-EET, 14,15-EET, and the enantiomers of 11,12-EET all caused a similar decrease in Ca^{2+} channel P_o and were approximately equally effective from either the intracellular or extracellular chambers.

Because the EETs seemed effective regardless of the side to which they were added, we hypothesized that the EETs partition into the lipid bilayer and interact with a site on the L-type Ca^{2+} channel that is embedded in the bilayer. To test this hypothesis, we evaluated the effects of 11,12-EET-PC, a derivative of phosphatidylcholine in which 11,12-EET is esterified to the *sn*-2 position of the phospholipid. In these experiments, the lipid mixtures used to form the planar lipid bilayers included either 5% (w/w) 11,12-EET-PC or 5% POPC in the mixture of 70% POPE and 25% POPS. As shown in Fig. 6, Ca^{2+} channels reconstituted into bilayers with 5% 11,12-EET-PC had significantly lower P_o than those reconstituted into bilayers with 5% POPC ($p < .05$, ANOVA). The effect of the 11,12-EET-PC depended on the presence of the epoxy moiety because channels incorporated into bilayers containing 5% arachidonyl-PC (with arachidonic acid esterified to the *sn*-2 position) displayed similar P_o values as those reconstituted into bilayers containing POPC. The channels incorporated into bilayers containing 11,12-EET-PC inactivated faster than did those incorporated into bilayers containing POPC (data not shown), similar to the effect seen when 11,12-EET was added to the aqueous solutions (Fig. 3). Thus, when the location of the EET was restricted to the lipid bilayer, the P_o of the Ca^{2+} channels was lower and inactivation rates were faster than in control experiments. This observation, combined with the observation that 11,12-EET was equally effective when added to either side of the channels, indicates that the site of action for the decrease in P_o is within the hydrophobic phase of the lipid bilayer. In none of the experiments with 11,12-EET-PC, AA-PC, and POPC did the unitary conductance of the channels change, suggesting that the effects of EETs on gating (i.e., P_o and inactivation) may arise from interactions with a site that is different from that which causes the decrease in unitary current amplitudes.

Dephosphorylation of L-type Ca^{2+} channels is known to decrease channel P_o (McDonald et al., 1994), so we wanted to determine whether dephosphorylation was necessary for the EET-dependent decrease in P_o of reconstituted channels. This is a reasonable mechanism because coreconstitution of phosphatases has been observed (Wang et al., 1993), and it is possible that the EETs could inhibit Ca^{2+} channels by stimulating the activity of phosphatases that are associated with the sarcolemma and are reconstituted into the bilayers along

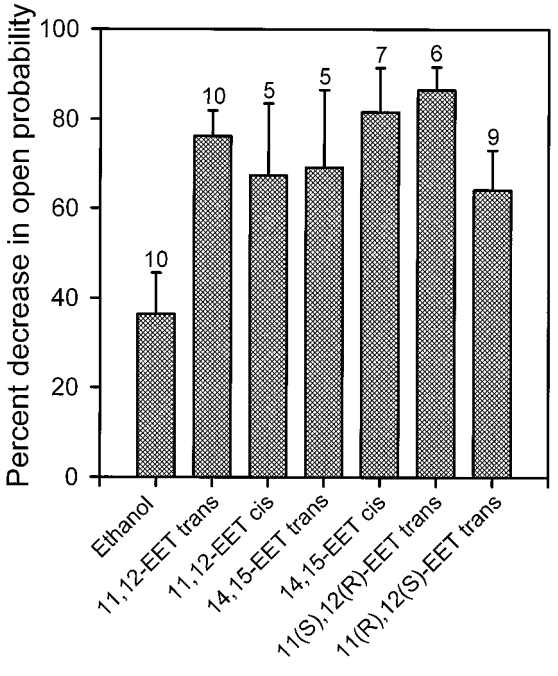

Fig. 5. Comparison of the effects of different EET regioisomers and stereoisomers added to either the intracellular or extracellular chambers. Each bar represents the percent decrease in P_o measured during the 3 min after the addition of 60 nM EET or 0.057% (v/v) ethanol compared with the P_o averaged over the 2 min before the addition. The number of experiments in each group is indicated above each bar. Error bars indicate S.E.M. There was no significant difference between intracellular or extracellular additions, between 11,12-EET and 14,15-EET, or between 11(*S*),12(*R*)-EET and 11(*R*),12(*S*)-EET.

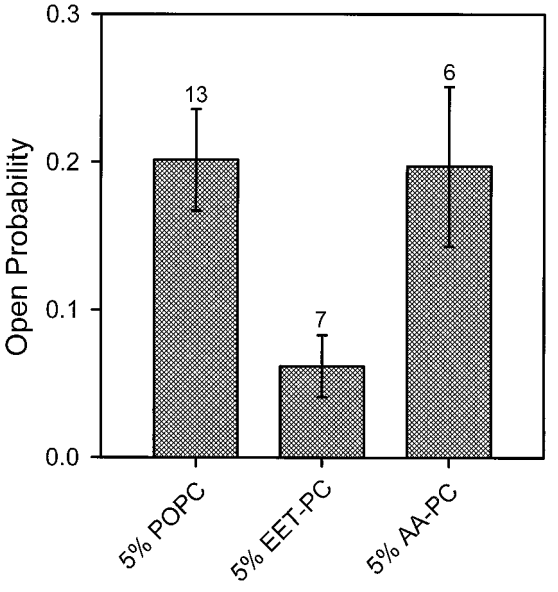

Fig. 6. 11,12-EET esterified to the *sn*-2 position of phosphatidylcholine (11,12-EET-PC) inhibits Ca^{2+} channel P_o, whereas arachidonic acid similarly esterified to PC (AA-PC) does not. Planar lipid bilayers were formed from 14 mg/ml POPE, 5 mg/ml POPS, and 1 mg/ml POPC, 11,12-EET-PC, or AA-PC. Ca^{2+} channels were incorporated in the bilayers, and activity was recorded for 3 to 5 min. P_o was measured and plotted for each of the bilayer conditions. The number of experiments in each group is indicated above each bar. The P_o in bilayers containing 5% EET-PC was significantly different ($p < .05$) from that in bilayers with 5% POPC or 5% AA-PC (one-way ANOVA, Tukey test).

with the L-type Ca^{2+} channels. Indeed, Xiao et al. (1998) suggest that EETs stimulate L-type Ca^{2+} channels by increasing the concentration of cAMP, increasing the cAMP-dependent phosphorylation of L-type Ca^{2+} channels and decreasing their dephosphorylation. To test the role for dephosphorylation in the EET-induced inhibition of reconstituted Ca^{2+} channels, we tested the effect of 11,12-EET in the presence of 10 nM microcystin. This concentration of microcystin is sufficient to inhibit any protein phosphatases 1 and 2A that might be present with the channels in the bilayers (with IC_{50} values of 1.7 and 0.04 nM for type 1 and 2A protein phosphatases, respectively; Honkanen et al., 1990). As shown in Fig. 7, 60 nM 11,12-EET caused an inhibition of the channels even in the presence of the microcystin, arguing against a role for protein dephosphorylation in the inhibitory effects of EETs on L-type Ca^{2+} channels.

Discussion

Our results show that EETs, products of the cytochrome P-450 metabolism of arachidonic acid, can directly inhibit the activity of cardiac L-type Ca^{2+} channels. The inhibition is manifested as 1) a decrease in channel P_o, 2) an acceleration of channel inactivation during depolarizations, and 3) a decrease in the unitary current amplitude of open channels. We found little regioselectivity or stereoselectivity in the effects and showed that EETs are likely to act on channel gating via interaction with a hydrophobic domain of the Ca^{2+} channel that is in contact with the bilayer lipid. The inhibitory effects of EETs persisted in the presence of an inhibitor of phosphoprotein phosphatases, indicating that the inhibition is not due to protein dephosphorylation.

The direct inhibitory effects of EET on L-type Ca^{2+} channels differs from results recently reported by Xiao et al. (1998). In that report, Ca^{2+} channels in rat ventricular myocytes were studied with whole-cell patch-clamp techniques, and inhibitors of cytochrome P-450 suppressed the Ca^{2+} current. The suppression was blocked by dialysis with intracellular cAMP and by dialysis with hydrolysis-resistant ATP, suggesting that the effects were due to a decrease in intracellular cAMP and cAMP-dependent phosphorylation. These investigators also observed an increase in Ca^{2+} current after extracellular addition of 11,12-EET. The authors concluded that P-450-derived eicosanoids caused an increase in cAMP and an increase in Ca^{2+} current via an increase in cAMP-dependent phosphorylation of the channels. Our results indicate that EETs directly inhibit Ca^{2+} channels, independent of changes in cAMP or protein phosphorylation. Thus, the EETs may have multiple effects in the heart that can either increase or decrease Ca^{2+} channel activity depending on the metabolic and regulatory state of the cells. Under basal conditions, where changes in cAMP and resultant protein phosphorylation may be a dominant regulator of Ca^{2+} channel activity, EETs may increase Ca^{2+} channel activity by stimulating cAMP-dependent protein kinase. On the other hand, under conditions in which the cells are already stimulated and cAMP levels are near maximal (including ischemic conditions in which sympathetic nerves innervating ventricular muscle are highly activated, causing the activation of *beta* adrenergic receptors and stimulation of cardiac adenylyl cyclase), EETs may cause an inhibition of Ca^{2+} channels via the direct pathway.

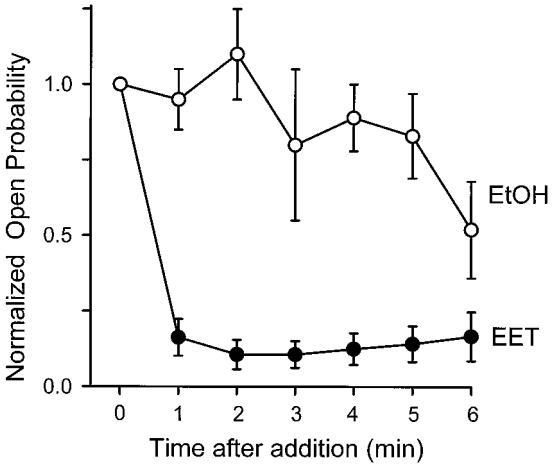

Fig. 7. Microcystin, an inhibitor of phosphoprotein phosphatases 1 and 2A, did not inhibit the effect of 60 nM 11,12-EET in the intracellular chamber (n = 8). Microcystin (10 nM) was present in the *trans* chamber to inhibit endogenous phosphatase activity that may be present in the cardiac sarcolemma. After Ca^{2+} channel incorporation and recording of baseline activity for 3 min, the addition of 60 nM 11,12-EET to the *trans* (intracellular) chamber (●) caused a rapid decrease in P_o relative to addition of 0.057% ethanol vehicle (EtOH) (○), similar to that recorded in the absence of microcystin (Fig. 2B).

Our results showing inhibitory effects of EETs on L-type Ca^{2+} channels are consistent with recent observations that EETs cause a decrease in action potential duration (APD) and preconditioning-like effects in the heart (Lane et al., 1998). In that study, 11,12-EET caused a significant 7- to 8-ms shortening of the APD that was even more pronounced after washout. Calcium influx through L-type Ca^{2+} channels is the predominant ionic mechanism for the plateau of the cardiac action potential, and inhibition of Ca^{2+} influx with Ca^{2+} channel blockers has been shown to shorten cardiac APD, suggesting that the decrease in APD observed with EETs is due, at least in part, to an inhibition of Ca^{2+} channels. Lane et al. (1998) also observed that 11,12-EET preserved myocardial contractility and delayed cell-to-cell uncoupling during ischemia. These findings were consistent with previous results showing that 11,12-EET improves functional recovery in the heart after prolonged global ischemia (Wu et al., 1997). Such effects can be explained, in part, by inhibition of L-type Ca^{2+} channels resulting in decreased $[Ca^{2+}]_i$ and the concomitant decrease in activity of Ca^{2+}-dependent enzymes (e.g., calpain) that have deleterious effects on myocardial contractile proteins.

The effects of EETs on L-type Ca^{2+} channel activity occurred at concentrations in the nanomolar range. These concentrations are substantially lower than those required to observe effects of EETs on left ventricular developed pressure in isolated-perfused rat hearts (Wu et al., 1997) and on cardiac action potentials in perfused rabbit papillary muscle preparations (Lane et al., 1998). These differences may be due to a limited access of the EETs to the site of action in the intact, perfused models. The concentration of EETs in human heart homogenates is also in the nanomolar range (Wu et al., 1996), indicating that the observed effects of EETs on L-type Ca^{2+} channels occur at physiologically relevant concentrations. Furthermore, the biosynthesis of EETs is increased significantly in stenosed coronary arteries (Rosolowsky et al., 1990), suggesting that higher levels of the eicosanoids may be attained under pathological conditions such as ischemia.

Zou et al. (1996) found regiospecific and stereospecific effects of EETs on renal artery large-conductance Ca^{2+}-activated K^+ channel activity. They found that 11(*R*),12(*S*)-EET increased the activity of the maxi-K^+ channels, whereas 11(*S*),12(*R*)-EET and 14,15-EET were inactive. Our results here indicate the absence of regiospecificity or stereospecificity in the effects of EETs on L-type Ca^{2+} channels. Thus, the mechanisms of the effects on voltage- and Ca^{2+}-activated K^+ channels are likely to be different from the mechanisms of inhibition of voltage-gated Ca^{2+} channels. These results highlight the complexity of EET effects on ion channels and other cellular regulators and suggest that further work is necessary to understand more fully the multitude of EET actions in excitable cells.

Because we used dihydropyridine agonists and $G_{s\alpha}$ to maintain the activity of the L-type Ca^{2+} channels in planar lipid bilayers, it is possible that our observations are due to an EET-dependent inhibition of dihydropyridine binding to the Ca^{2+} channels or an inhibition of channel-$G_{s\alpha}$ interactions. EET-dependent inhibition of agonist binding is unlikely, however, because the concentration of dihydropyridine agonist used in our experiments will likely overcome any inhibitory effects of the EETs. The addition of 5 μM (+)202-791 (a 10-fold higher concentration than normally used) did not reverse the inhibition caused by 20 to 60 nM 11,12-EET in the *trans* chamber (data not shown). It remains possible that EETs affect channel-$G_{s\alpha}$ interactions, although this mechanism could not explain the effect of 11,12-EET on channel conductance or inactivation rates.

Epoxyeicosatrienoyl-phospholipids can be formed enzymatically or through autoxidation of glycerolipids that contain arachidonate at the *sn*-2 position (Karara et al., 1991). In fact, >85% of EETs found in vivo are esterified to cellular glycero-phospholipid (Karara et al., 1989, 1991). An enzymatic, stereoselective pathway for EET acylation to lysoglycerolipids has been described in rat liver (Karara et al., 1991). The presence of oxidized phospholipids in biological membranes has been shown to affect membrane physicochemical properties, including alterations in enzymatic activity of membrane-bound enzymes, changes in membrane fluidity and fusogenic properties, and alterations in membrane ion permeabilities (Frei et al., 1985; Sevanian and Hochstein, 1985; Karara et al., 1991). This report, which documents for the first time a biological effect of an EET-phospholipid on a specific ion channel, illustrates the usefulness of reconstituted channel/bilayer systems in elucidating the molecular mechanisms that underlie some of these effects.

Calcium channels play an essential role in the maintenance of the slow conduction pathways in ischemic myocardium. Thus, Ca^{2+} channels are partially responsible for the evolution of reentry pathways that degenerate into ventricular tachycardia and fibrillation (Fleet et al., 1994). To understand the pathophysiology of cardiac ischemia, it is necessary to understand the regulation of cardiac L-type Ca^{2+} channels. Ca^{2+} channels are highly regulated by cellular modulators, the levels of which are altered dramatically during cardiac ischemia. For example, ischemia is accompanied by changes in cAMP, protein kinase activity, pH, resting membrane potential, intracellular [Ca^{2+}], and levels of lipid metabolites (Gettes and Cascio, 1992). Many of these changes, including low pH, elevated intracellular [Ca^{2+}], and depolarized diastolic membrane potential, lead to inhibition of Ca^{2+} channels (McDonald et al., 1994). Under physiological and pathophysiological conditions with Ca^{2+} as the ion carried by Ca^{2+} channels, there is faster channel inactivation than when Ba^{2+} is the charge carrier, due to the Ca^{2+}-dependent inactivation (McDonald et al., 1994). Under these conditions, it is likely that EETs will cause a further increase in Ca^{2+} channel inactivation rates, although the overall effect of EETs may be smaller than when Ba^{2+} is the charge carrier. Other effects of ischemia can increase Ca^{2+} channel activity, such as increased *beta* adrenergic stimulation (McDonald et al., 1994) and increased levels of amphipathic acylcarnitine lipid derivatives (Liu and Rosenberg, 1996). Our observation that the EETs cause an inhibition of L-type Ca^{2+} channel activity suggests another cellular mechanism that may lead to protection of the myocyte from Ca^{2+}-induced injury and may provide a novel pharmacological target for preventing the formation of dangerous reentry arrhythmias and cardiomyocyte dysfunction.

Acknowledgments

We thank J. R. Falck for providing synthetic EETs and W. Cascio, T. Eling, Q.-Y. Liu, L. Lyford, and E. Murphy for helpful discussions.

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