Effects of epoxyeicosatrienoic acids on the cardiac sodium channels in isolated rat ventricular myocytes

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1. Whole-cell Na^+ currents (holding potential, −80 mV; test potential, −30 mV) in rat myocytes were inhibited by 8,9-epoxyeicosatrienoic acid (8,9-EET) in a dose-dependent manner with 22 ± 4% inhibition at 0·5 μM, 48 ± 5% at 1 μM, and 73 ± 5% at 5 μM (mean ± S.E.M., $n = 10$, $P < 0{\cdot}05$ for each dose *vs.* control). Similar results were obtained with 5,6-, 11,12-, and 14,15-EETs, while 8,9-dihydroxyeicosatrienoic acid (DHET) was 3-fold less potent and arachidonic acid was 10- to 20-fold less potent.

2. 8,9-EET produced a dose-dependent, hyperpolarized shift in the steady-state membrane potential at half-maximum inactivation ($V_{½}$), without changing the slope factor. 8,9-EET had no effect on the steady-state activation of Na^+ currents.

3. Inhibition of Na^+ currents by 8,9-EET was use dependent, and channel recovery was slowed. The effects of 8,9-EET were greater at depolarized potentials.

4. Single channel recordings showed 8,9-EET did not change the conductance or the number of active Na^+ channels, but markedly decreased the probability of Na^+ channel opening. These results were associated with a decrease in the channel open time and an increase in the channel closed times.

5. Incubation of cultured cardiac myocytes with 1 μM [^{3}H]8,9-EET showed that 25% of the radioactivity was taken up by the cells over a 2 h period, and most of the uptake was incorporated into phospholipids, principally phosphatidylcholine. Analysis of the medium after a 2 h incubation indicated that 86% of the radioactivity remained as [^{3}H]8,9-EET while 13% was converted into [^{3}H]8,9-DHET. After a 30 min incubation, 1–2% of the [^{3}H]8,9-EET uptake by cells remained as unesterified EET.

6. These results demonstrate that cardiac cells have a high capacity to take up and metabolize 8,9-EET. 8,9-EET is a potent use- and voltage-dependent inhibitor of the cardiac Na^+ channels through modulation of the channel gating behaviour.

Arachidonic acid is a precursor of many bioactive lipids that are involved in signal transduction and cellular regulatory mechanisms. In addition to the well-known and well-established cyclo-oxygenase and lipoxygenase pathways which generate important bio-mediators such as prostaglandins, thromboxanes and leukotrienes, the cytochrome P450 monoxygenase pathway has also emerged as an important source of bioactive arachidonic acid derivatives (McGiff, 1991). Cytochrome P450 mono-oxygenases convert arachidonic acid to four epoxy-eicosatrienoic acid (EET) regioisomers: 5,6-, 8,9-, 11,12- and 14,15-EET, as well as to 19- and 20-hydroxyeicosatetra-enoic acids (Oliw, 1994). EETs are potent endothelium-derived vasodilators that modulate vascular tone via enhancement of Ca^{2+}-activated K^+ channels in vascular smooth muscle, suggesting that these compounds are endothelium-derived hyperpolarizing factors (Gebremedhin *et al.* 1992; Hu & Kim, 1993; Campbell *et al.* 1996; Zou *et al.* 1996; Baron *et al.* 1997; Li *et al.* 1997; Li & Campbell, 1997). Studies in cultured vascular endothelial and smooth muscle cells indicate that EETs are avidly taken up and incorporated into cellular phospholipids, as well as being converted to their dihydroxyeicosatrienoic acid (DHET) derivatives (Fang *et al.* 1996; Weintraub *et al.* 1997). Thus, vascular cells are important biochemical sources and physiological targets of EETs.

Cytochrome P450 epoxygenase activity is present in rat, guinea-pig, rabbit and pig hearts (Comte & Gautheron, 1978; Guengerich & Mason, 1979; Abraham *et al.* 1987; McCallum *et al.* 1993). A human cytochrome P450 arachidonic acid epoxygenase (CYP2J2) was recently cloned and found to be constitutively expressed in the human heart (Wu *et al.* 1996). The same group subsequently reported the cloning and cDNA-directed expression of a rat P450 (CYP2J3) that is highly expressed in the heart, predominantly localized to atrial and ventricular cardiac myocytes and active in the mono-oxygenation of arachidonic acid (Wu *et al.* 1997). There are substantial amounts of EETs in the rat heart. 8,9-EET, the major regioisomer, accounts for 39% of the total (Wu *et al.* 1997). Furthermore, 11,12-EET has been shown to enhance the recovery of cardiac function following global ischaemia (Wu *et al.* 1997). The cytochrome P450 pathway is therefore a potentially important component of the cardiac arachidonic acid cascade and may play a role in regulating the response of the heart to ischaemia. The direct effects of EETs on cardiac myocytes, however, remain to be established.

In the present study, we investigated the effects of EETs on cardiac ion channels. Na^+ channels play a crucial role in cardiac electrogenesis and are a major determinant of impulse conduction. Na^+ channel blocking antiarrhythmic drugs are the most commonly used pharmacological agents for the treatment of arrhythmias. Since the effects of EETs may be particularly important during ischaemia, and since the voltage-dependent Na^+ channels are significantly modulated during cardiac ischaemia, we examined the effects of 8,9-EET on the Na^+ channels in isolated rat cardiac myocytes. We found that 8,9-EET significantly inhibits the cardiac Na^+ currents (I_{Na}) and that this inhibition is dose, voltage and frequency dependent by modulating channel gating behaviour. The other EET regioisomers are also potent I_{Na} inhibitors but 8,9-DHET is less effective. In addition, we found that cardiac myocytes have a high capacity for taking up 8,9-EET and incorporating it into phospholipids. These results demonstrate that the heart can metabolize EETs, which in turn may play an important role in modulating the electrophysiological properties of the heart.

METHODS

Solutions

(1) Nominally Ca^{2+}-free Tyrode solution contained (mM): NaCl, 138; KCl, 4·5; $MgCl_2$, 0·5; Na_2HPO_4, 0·33; glucose, 5·5; Hepes, 10; pH 7·38. The solution was vigorously oxygenated for at least 30 min prior to use.

(2) KB solution contained (mM): KOH, 70; KCl, 40; L-glutamic acid, 50; taurine, 20; $MgCl_2$, 0·5; K_2HPO_4, 1·0; EGTA, 0·5; creatine, 5; pyruvic acid, 5; Na_2ATP, 5; Hepes, 10; pH 7·38.

(3) The bath solution for whole-cell Na^+ channel recordings contained (mM): NaCl, 20; choline chloride, 130; $CaCl_2$, 1; $CoCl_2$, 2; $MgCl_2$, 2; KCl, 4·5; glucose, 5·5; Hepes, 10; pH 7·38.

(4) The pipette solution for whole-cell Na^+ channel recordings contained (mM): CsCl, 130; $CaCl_2$, 0·5; $MgCl_2$, 2; Na_2ATP, 5; GTP, 0·5; EGTA, 0·5; Hepes, 10; pH 7·25.

(5) The bath solution for single Na^+ channel recordings contained (mM): KCl, 140; $MgCl_2$, 2; EGTA, 10; Hepes, 10; pH 7·38.

(6) The pipette solution for single Na^+ channel recordings contained (mM): NaCl, 250; $CaCl_2$, 1; $MgCl_2$, 2; Hepes, 10; pH 7·38.

(7) The culture medium for neonatal rat cardiac myocytes consisted of Eagle's minimal essential medium (MEM) with 5·8 mM Hepes, 16 mM $NaHCO_3$ and 2 mM L-glutamine. Unless stated otherwise, the medium was supplemented with 15% horse serum plus 0·02 mg ml^{-1} gentamicin.

(8) The potassium glutamate (KG) solution for neonatal rat cardiac myocyte culture contained (mM): potassium glutamate, 140; $NaHCO_3$, 16; NaH_2PO_4, 0·5; Hepes, 25; glucose, 10·5; Phenol Red, 0·014.

(9) EETs, 8,9-DHET and arachidonic acid were prepared in absolute ethanol as 5 mM stock solutions and were stored under nitrogen at −20 °C. EETs and arachidonic acid were diluted to the various concentrations with buffer immediately prior to being used. The final concentration of ethanol was 0·1% or less and had no effect on the cardiac I_{Na}.

Isolation of rat ventricular myocytes

Single ventricular myocytes from rat hearts were isolated by established techniques (Isenberg & Klockner, 1982). Briefly, Sprague–Dawley rats (200–250 g body weight) were anaesthetized with methoxyfluorane. The rat heart was rapidly excised and placed in ice-cold nominally Ca^{2+}-free Tyrode solution. After the aorta was cannulated, the heart was perfused using a modified Langendorff apparatus with nominally Ca^{2+}-free Tyrode solution containing 0·1% (w/v) bovine serum albumin (BSA) for 5 min at 37 °C. The perfusate was then changed to nominally Ca^{2+}-free Tyrode solution containing 0·6 mg ml^{-1} collagenase (Worthington, CLS-2, 347 units mg^{-1}) and 0·1% (w/v) BSA for 7 min at 37 °C. The ventricles were dissected and placed in 25 ml of fresh collagenase solution (0·6 mg ml^{-1}) for 5 min. The myocardium was then cut into small pieces (approximately 1 mm cubes) and filtered through a medium mesh. After washing twice with nominally Ca^{2+}-free Tyrode solution, single ventricular myocytes were maintained in KB solution until the time of the experiment.

Whole-cell Na^+ current recordings

Voltage-dependent I_{Na} in isolated rat ventricular myocytes were recorded using patch-clamp techniques as previously described (Hamill *et al.* 1981; Lee *et al.* 1993). Bath solutions were superfused at 1–2 ml min^{-1} using a direct current-powered pump (Instech Laboratories, Inc., Plymouth Meeting, PA, USA) and solution exchanges were complete within 30–60 s. Whole-cell I_{Na} was recorded with an Axopatch 200 integrating amplifier (Axon Instruments), filtered with an eight-pole low-pass Bessel filter with a bandwidth (−3 dB) of 5 kHz and sampled at 50 kHz (12-bit resolution). Borosilicate glass capillaries (Corning 7052, Warner Instruments, Inc.) were used to make pipettes. Electrode resistance in the whole-cell pipette solution ranged from 0·5 to 1 MΩ, and seal resistance was 1–5 GΩ. Whole-cell series resistance was compensated more than 80% of the uncompensated value. pCLAMP software (Axon Instruments) was used for generating voltage-clamp protocols and for the acquisition and analysis of I_{Na}. All cellular electrophysiology experiments were performed at room temperature (21–23 °C). Spontaneous time-dependent changes of

I_{Na} typically occurred in the first 10–20 min after rupture of the sealed membrane patch. Whole-cell I_{Na} recordings were started only after the current was stable.

Stimulus protocols

To measure the voltage-dependent activation of the Na^+ channels, currents were elicited from a holding potential of −80 mV to a series of 20 ms test pulses from −80 to +35 mV in 5 mV increments and at 5 s intervals. The peak I_{Na} ($I_{Na,max}$) at different conditioning pulse voltages (V_m) was measured and the peak $I_{Na,max}$–V_m relationship was fitted with the following equation:

$$I_{Na,max} = G_{Na}(V_m - E_{rev}),$$

where E_{rev} is the reversal potential. The voltage-dependent Na^+ channel conductance (G_{Na}) was fitted with a Boltzmann distribution equation:

$$G_{Na}/G_{Na,max} = 1/(1 + \exp((V_{½} - V_m)/k)),$$

where $G_{Na,max}$ is the maximum conductance, $V_{½}$ is the membrane potential at half-maximal conductance and k is the slope factor.

Voltage-dependent steady-state inactivation was determined using a two-pulse protocol consisting of a 500 ms conditioning pulse from −160 to −10 mV in 10 mV increments, followed by a test pulse of −20 mV. The steady-state inactivation curves obtained by normalizing currents to the maximal I_{Na} at −160 mV were fitted using a Boltzmann distribution equation:

$$I_{Na} = I_{Na,max}/(1 + \exp((V_m - V_{½})/k)).$$

where V_m is the conditioning pulse voltage, $V_{½}$ is the voltage at half-inactivation and k is the slope factor. To examine the use-dependent block of I_{Na} by EET, trains of 20 depolarizing pulses (holding potential, −80 mV; test potential, −30 mV; 20 ms duration) at cycle lengths of 200 ms were used. Peak current amplitudes were plotted against time.

Recovery of I_{Na} from inactivation was determined using a two-pulse protocol. A 500 ms conditioning pulse from −80 to 0 mV was followed by a recovery period ranging from 1 ms to 2 s at −80 mV. A test pulse of 20 ms duration to −30 mV was then elicited. The amplitude of the peak I_{Na} during the test pulse was normalized to the value of I_{Na} after complete recovery from inactivation, $I_{Na,max}$, and this ratio was plotted against the recovery interval. Recovery curves were analysed using a two-exponential fit as previously reported (Lee *et al.* 1993):

$$f = Amp_1(1 - e^{-t/\tau_1}) + Amp_2(1 - e^{-t/\tau_2}),$$

where Amp_1 and Amp_2 represent the relative contributions of the fast (τ_1) and slow (τ_2) time constants of recovery and $Amp_1 + Amp_2 = 1$. Curve fitting was performed using a Marquardt–Levenberg least squares fitting procedure from Origin (MicroCal Software, Inc., Northampton, MA, USA) or Igor (WaveMetrics Inc., Lake Oswego, OR, USA) software.

Single Na^+ channel recordings

Single channel recordings were performed with an improved patch clamp technique (Benndorf, 1995) in the cell-attached configuration. The patch pipettes were pulled from thick-walled borosilicate glass (Glass type 7740, Garner Glass Company). The pipette was coated with Sylgard 184 before being fire polished. The pipette resistance was 5–10 MΩ when filled with the pipette solution and the typical seal resistance was > 10 GΩ. Single channel Na^+ currents were recorded with an Axopatch 200 amplifier and an eight-pole low-pass Bessel filter (902LPF, Frequency Devices) with bandwidth of 10 kHz and sampling rate of 100 kHz (12-bit resolution). The peak current amplitude of the single Na^+ channel is greater than 2 pA at membrane potentials of −40 mV with 250 mM extracellular Na^+. Events were easily distinguished from noise. Single channel Na^+ current was identified by the amplitude and fast time course of the mean current. The capacitive transients and leak current were removed by subtraction of a sliding averaged blank formed from 10 empty traces out of the neighbourhood of the actual record. The subtracted traces were idealized at 10 kHz.

Opening and closing transitions were detected according to the conventional 50 % threshold of event amplitude. Computer-detected openings, confirmed by visual observation, were used to generate idealized records from which histograms of amplitude and closed- and open-time distributions were constructed. The closed states of the channel were determined by the last channel opening in order to separate them from inactivation states. The closed dwell time of events was fitted with a two-exponential equation. The open dwell time of events was fitted with an exponential equation. The channel open probability (P_o) was calculated with the equation:

$$P_o = I/N \times i,$$

where I is the macroscopic Na^+ current averaged from single channel recording, N is the number of functional channels in the patch, i is single channel current. Here i is identified by generation of amplitude histograms, which were fitted with Gaussian curves using a χ^2 non-linear regression routine.

Isolation and culture of neonatal rat cardiac myocytes

Neonatal rat cardiac myocytes were prepared and cultured as previously described (Atkins *et al.* 1992). Healthy rat pups aged 1–3 days old were decapitated. The hearts were isolated and placed in modified Eagle's MEM. After excess blood was rinsed off, the hearts were placed in KG solution, minced into 1 mm^3 sized pieces and incubated with collagenase (3 mg ml^{-1}) at 37 °C for 20 min. Cells were isolated by multiple-cycle trypsinization. The cardiac fragments were digested with trypsin (1 mg ml^{-1}) at 37 °C with gentle mechanical stirring for 15 min $cycle^{-1}$ for three to six cycles. The dispersed cells were pooled and placed in modified Eagle's MEM. Fibroblasts and other non-myocardial cells were allowed to settle and attach to the bottom of a tissue culture flask by incubation for 60 min at 37 °C. The unattached cells were recovered, an aliquot was counted and the remaining cells were seeded into 6-well plates in culture medium at 37 °C. Histological staining confirmed that greater than 96 % of the cultured cells were cardiac myocytes.

Synthesis of [^{3}H]8,9-EET

[^{3}H]8,9-EET was synthesized from [^{3}H]arachidonic acid as previously described (VanRollins *et al.* 1995). In brief, [^{3}H]arachidonic acid was esterified with ethereal diazomethane, and the [^{3}H]methyl arachidonate was purified by reverse-phase high-performance liquid chromatography (HPLC) and extracted into CH_2Cl_2. Methyl arachidonate was then epoxygenated using meta-chloroperoxybenzoic acid. The individual [^{3}H]EET regioisomers were isolated as methyl esters by preparative, normal-phase HPLC. Shortly before use, the methyl ester of [^{3}H]8,9-EET was saponified, extracted into water-saturated ethyl acetate, and [^{3}H]8,9-EET was isolated using semi-preparative normal-phase HPLC. The specific activity of [^{3}H]8,9-EET was 929 d.p.m. ng^{-1}.

Incubation of neonatal rat cardiac myocytes with radiolabelled 8,9-EET

Twenty-four hours after isolation of neonatal rat cardiac myocytes, the medium was removed and replaced with 1 ml of fresh modified

Eagle's MEM containing 1 μM [^{3}H]8,9-EET and supplemented with 1 % horse serum. After 30 min to 4 h, the medium was removed and saved for analysis (see below). The cells were washed with 2 ml of Dulbecco's phosphate-buffered saline (4 °C), detached by scraping gently with a rubber policeman and transferred to a test tube. Eight millilitres of $CHCl_3$–CH_3OH (2 : 1, v/v) and 1·75 ml of 4 mM HCl–154 mM NaCl were added, and the cell suspension was vortex-mixed. The phases were allowed to separate overnight at 4 °C. After transferring the lower phase to a new test tube, 1 ml of $CHCl_3$–CH_3OH–154 mM NaCl (86 : 14 : 1, v/v/v) was added to the top phase and the contents were mixed. After phase separation, the lower phase was transferred to the original $CHCl_3$ extract. The $CHCl_3$ was evaporated from the combined extractant using N_2 at 37 °C, and the lipids were resuspended in 200 μl of $CHCl_3$–CH_3OH (2:1, v/v). A 10 μl aliquot was removed for determination of radioactivity by liquid scintillation counting, and the remainder was stored for analysis (see below).

An aliquot of the incubation medium was removed for determination of radioactivity by liquid scintillation counting, and the remainder of the medium was acidified to ~pH 5 with H_3PO_4–H_2O (1 : 50, v/v). Lipids were extracted twice using 4 ml of ice-cold water-saturated ethyl acetate, followed by centrifugation and phase separation. After evaporating the ethyl acetate under a stream of N_2, the extract was resuspended in 250 μl of CH_3CN.

To analyse the medium-associated lipids, reverse-phase high-performance liquid chromatography (HPLC) was performed using a Gilson System equipped with an automatic sample injector and a 4·6 mm × 150 mm column containing 3 μm spherical particles of EQC C_{18} (Alltech) as previously described (Fang *et al.* 1996; Weintraub *et al.* 1997). A mobile phase system was employed, consisting of water adjusted to pH 3·4 with phosphoric acid and an acetonitrile gradient increasing from 30–100 % over 60 min at a flow rate of 0·7 ml min^{-1}. Radioactivity was measured by combining the column effluent with Budget Solve scintillation

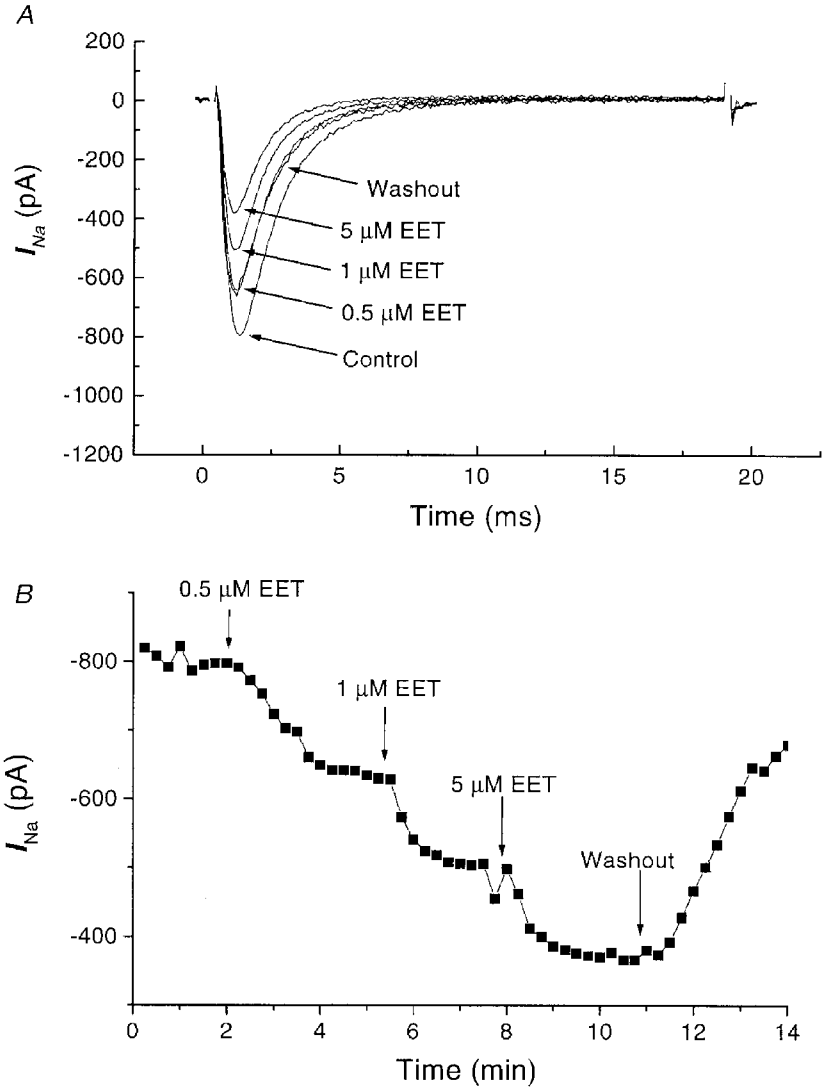

Figure 1. Effect of 8,9-EET on the Na^+ currents in isolated rat cardiac myocytes

Whole-cell I_{Na} was elicited from a holding potential of −80 mV to a test potential of −30 mV in the presence of 20 mM $[Na^+]_o$ at room temperature. *A* represents raw tracings of Na^+ currents before (control) and after application of 0·5, 1·0 and 5·0 μM 8,9-EET, followed by the partial reversal of the EET effect upon washout. The capacitive transients are eliminated for cosmetic reasons. *B* represents the peak whole-cell Na^+ current in the same cell plotted *versus* time with pulses elicited at 15 s intervals. The arrows indicate the onsets of the interventions.

solution (0·7 : 2·5, v/v) and passing the mixture through a Radiomatic Flo-One Beta isotope detector.

The cell lipid extracts were separated by thin layer chromatography (TLC) as previously described (Fang *et al.* 1996; Weintraub *et al.* 1997). Phospholipids were separated with chloroform–methanol–40% methylamine (60 : 36 : 5) or with chloroform–methanol–water (60 : 35 : 8), a solvent system that separates unesterified 8,9-EET from phospholipids and glycerides. The distribution of radioactivity on the TLC plate was determined with a gas flow proportional scanner (Radiomatic model R), using radioactive lipid standards applied to each plate as described previously (Fang *et al.* 1996).

Materials

EETs and 8,9-DHET were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA), Eagle's MEM was from Gibco, horse serum was from HyClone Laboratories (Logan, UT, USA), TLC plates were from Alltech Associates Inc. (Deerfield, IL, USA) and the rest of the chemicals were from Sigma Chemical Co.

Statistical analysis

All data were expressed as means ± S.E.M. Student's paired t test was used to compare data obtained before and after intervention. A one-way ANOVA followed by contrast testing was used to compare data from multiple groups. Statistically significant differences are defined as $P < 0·05$.

RESULTS

Effect of 8,9-EET on whole-cell I_{Na} in isolated rat ventricular myocytes

Dose-dependent effects

Whole-cell I_{Na} in isolated rat ventricular myocytes was measured with reduced external Na^+ to ensure adequate voltage control as previously described (Matsuda *et al.* 1992; Lee *et al.* 1993). Cardiac I_{Na} was rapidly inhibited by 8,9-EET in a dose-dependent manner. Figure 1 shows the results of a typical experiment. Exposure to 0·5, 1·0 and 5·0 μM 8,9-EET produced rapid inhibition of I_{Na} (holding potential = −80 mV; test potential = −30 mV), which reached a new steady state in 2–3 min at each dose. The effect of 8,9-EET was at least partially reversible upon washout. Reversibility of the effects of 8,9-EET was not enhanced by washout with buffer containing 0·1% BSA. Figure 1*A* shows the representative current tracings before and after application of 0·5, 1·0 and 5 μM 8,9-EET. Figure 1*B* shows the time course of the peak I_{Na} amplitudes. Composite data (Fig. 2*A*) showed that whole-cell I_{Na} in rat cardiac myocytes was inhibited by 22 ± 4, 48 ± 5 and 73 ± 5% with 0·5, 1·0 and 5·0 μM 8,9-EET, respectively (mean ± S.E.M., $n = 10$, $P < 0·05$ *vs.* control for all interventions). At 0·01 μM, 8,9-EET had no effect on the I_{Na}. The other EET regioisomers produced similar effects in isolated rat ventricular myocytes. I_{Na} was inhibited by 18 ± 10, 35 ± 5 and 49 ± 5% with 0·5, 1·0 and 5·0 μM 5,6-EET, respectively ($n = 4$); by 19 ± 5, 40 ± 5 and 65 ± 6% with 0·5, 1·0 and 5·0 μM 11,12-EET, respectively ($n = 4$); and by 17 ± 5, 35 ± 4 and 49 ± 5% with 0·5, 1·0 and 5·0 μM 14,15-EET, respectively ($n = 5$) ($P < 0·05$ *vs.* control for all interventions). We found that 8,9-DHET (1 μM) was also an inhibitor of the cardiac I_{Na} (15 ± 3%, $n = 3$, $P < 0·05$ *vs.* control), but was not as effective as its epoxide precursor. Further assessment of the effects of EET was performed using mainly the 8,9-regioisomer.

The effects of 8,9-EET on the rat cardiac I_{Na} were much more potent than those of its parent compound, arachidonic acid (Fig. 2). Arachidonic acid inhibited the rat cardiac I_{Na} by 1 ± 2, 8 ± 2, 21 ± 3 and 38 ± 3% with 0·5, 1·0, 5·0 and 10·0 μM, respectivley ($n = 11$, P = n.s. for 0·5 and 1 μM *vs.* control; $P < 0·05$ for 5·0 and 10·0 μM *vs.* control). These results suggest that the epoxy groups in EETs enhance their properties as Na^+ channel inhibitors, since the IC_{50} for 8,9-EET was around 1 μM, whereas the IC_{50} for arachidonic acid was greater than 10 μM.

Voltage-dependent effects

Figure 3*A* shows the effects of 0·5, 1·0 and 5·0 μM 8,9-EET on the current–voltage (I–V) relationship of whole-cell I_{Na}

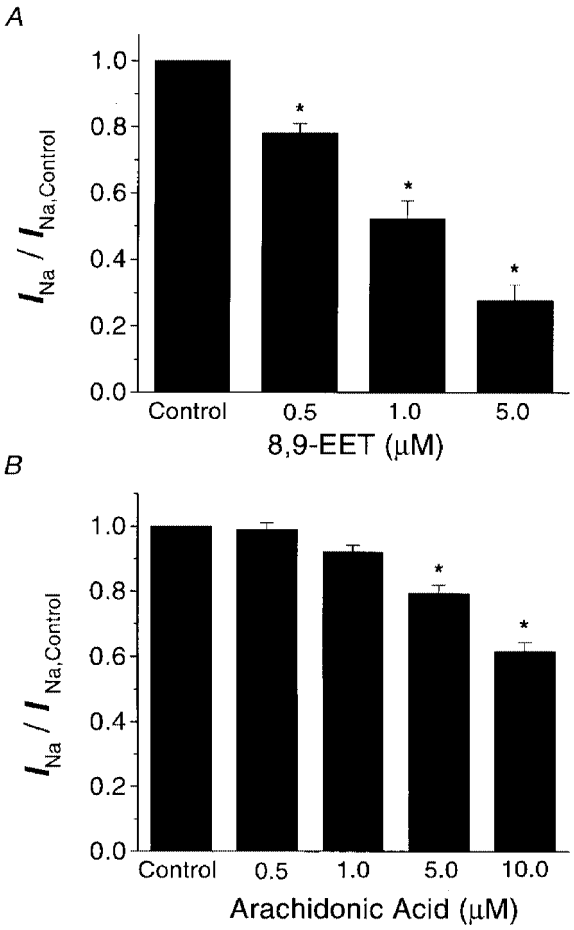

Figure 2. Dose-dependent inhibition of the Na^+ currents by 8,9-EET (*A*) and arachidonic acid (*B*)

A shows the magnitude of Na^+ current inhibition by 0·5, 1·0 and 5·0 μM 8,9-EET as a ratio of Na^+ currents under control conditions. Na^+ currents were evoked from a resting potential of −80 mV to a test potential of −30 mV ($n = 10$, * $P < 0·05$ *vs.* control). *B* shows the magnitude of Na^+ current inhibition by 0·5, 1·0, 5·0 and 10·0 μM arachidonic acid as a ratio of control Na^+ currents under conditions similar to those shown in *A* ($n = 11$, * $P < 0·05$).

normalized to the peak amplitude in the I–V relationship under control conditions. These results show that inhibition of I_{Na} by 8,9-EET occurred throughout the I_{Na} activation range and in a dose-dependent manner. There was significant reduction of I_{Na} by 1·0 and 5·0 μM 8,9-EET at membrane potentials between −50 and +20 mV ($n = 5$, $P < 0{\cdot}05$ *vs.* control).

To determine whether these effects of 8,9-EET were due to changes in the I_{Na} activation, we analysed the voltage activation of I_{Na} by determining channel conductance from the current elicited and the Na^+ electrochemical driving force using Ohm's law as described in Methods. The conductance–voltage (G–V) relationships under control conditions and in the presence of 0·5, 1·0 and 5·0 μM of 8,9-EET are shown in Fig. 3*B*. The half-activation values ($V_{½}$) were −34·9 ± 1·5, −36·5 ± 3·0, −35·6 ± 0·6 and −36·6 ± 3·6 mV for control, 0·5, 1·0 and 5·0 μM 8,9-EET, respectively ($n = 5$, n.s.) The slope factors (k) were

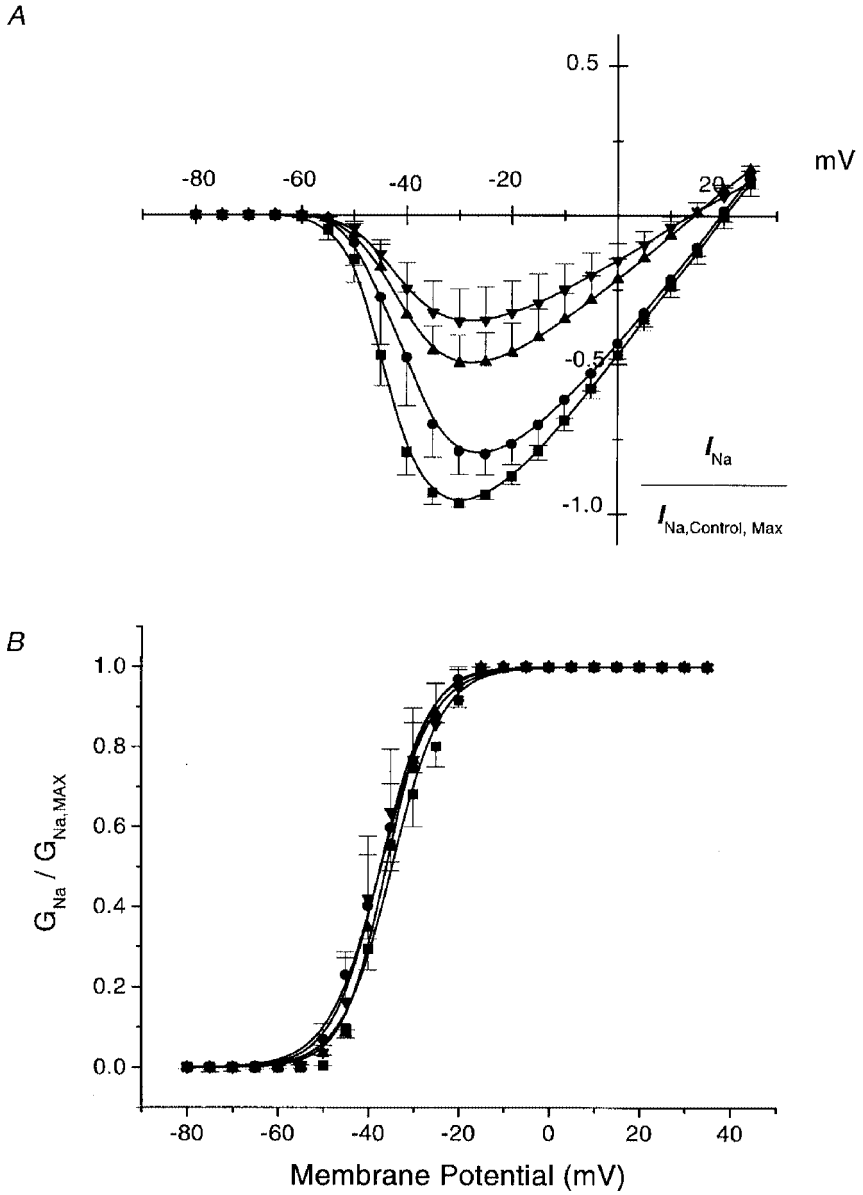

Figure 3. Effect of 8,9-EET on whole-cell (*A*) Na^+ current–voltage relations and activation curves (*B*)

A represents the current–voltage relations in five cells, in the presence of 0 (control, ■), 0·5 μM (●), 1·0 μM (▲) and 5·0 μM (▼) 8,9-EET. Holding potentials are at −80 mV with pulse durations of 20 ms. Pulses are repeated at 5 mV increments at 5 s intervals. Values are normalized to the peak I_{Na} amplitude under control conditions and are expressed as means ± S.E.M. *B* represents the whole-cell Na^+ current activation curve showing the conductance–voltage relations at baseline (control, ■) and in the presence of 0·5 μM (●), 1·0 μM (▲) and 5·0 μM (▼) 8,9-EET. Values are normalized to maximal conductance ($G_{Na,max}$) and are expressed as means ± S.E.M. The data are curve-fitted using a Boltzmann equation. $V_{½}$ is the half-activation value and k is the slope factor. Differences between control values and those with the various doses of 8,9-EET are not statistically significant.

5·0 ± 0·4, 4·8 ± 0·7, 4·3 ± 0·2 and 4·2 ± 0·6 for control 0·5, 1·0 and 5·0 μM 8,9-EET, respectively ($n = 5$, n.s.). These results suggest that 8,9-EET had no significant effect on the activation of I_{Na} in rat cardiac myocytes.

To determine whether the effects of EET were due to changes in I_{Na} inactivation, we examined the effects of prepulse potential on the suppression of the cardiac I_{Na} by 8,9-EET (Fig. 4). Figure 4*A* shows that 8,9-EET reduced the steady-state Na^+ channel availability in a dose-dependent manner. In Fig. 4*B*, which shows the normalized steady-state inactivation curves, there was a dose-dependent shift in the half-inactivation value ($V_{½}$) from −76·1 ± 2·0 mV for control, to −77·8 ± 1·5 mV with

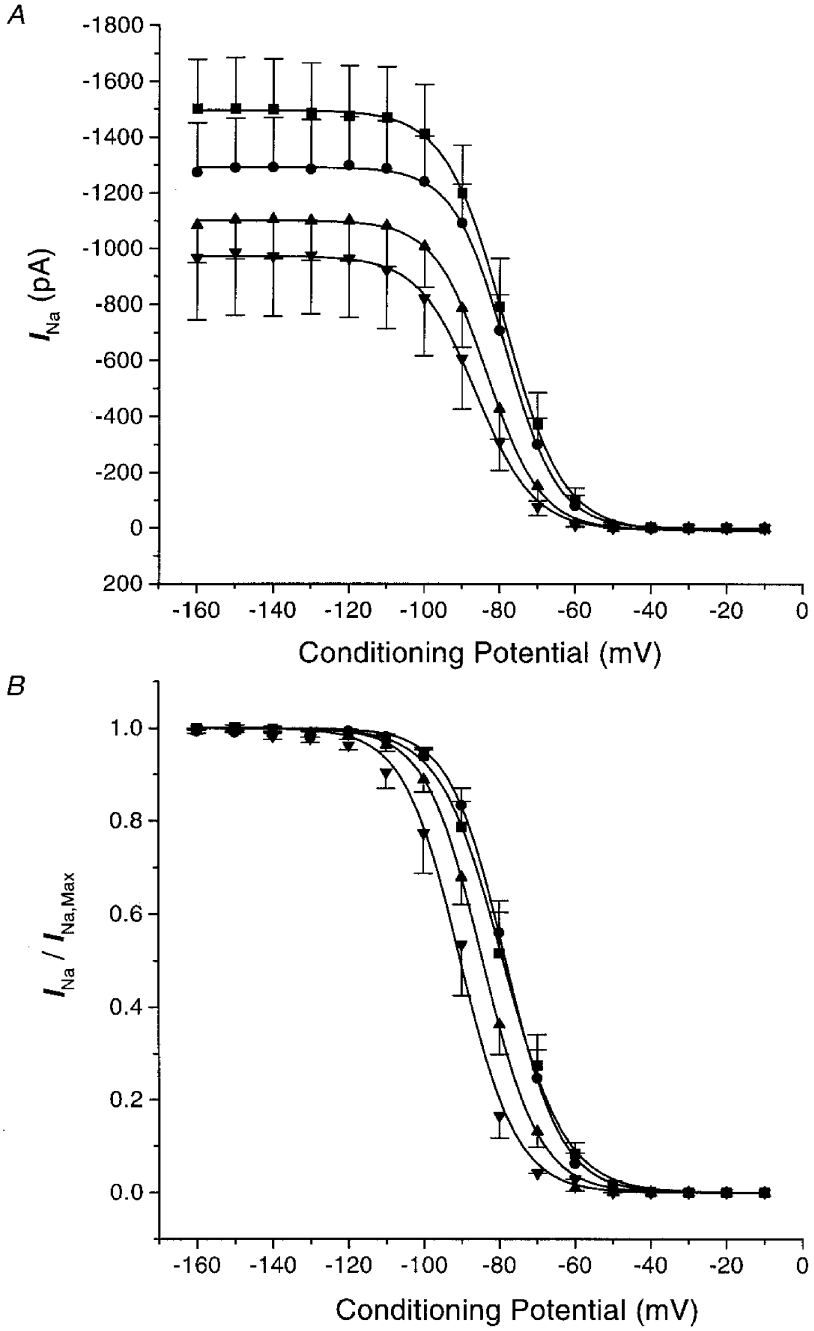

Figure 4. Effect of prepulse potential on the inhibition of Na^+ current by 8,9-EET

A shows the amplitudes of the Na^+ currents (test potentials of −20 mV) plotted against the indicated prepulse potentials (500 ms) under control conditions (■) and after application of 0·5 μM (●), 1·0 μM (▲) and 5·0 μM (▼) 8,9-EET. Each point represents the means ± S.E.M. of nine experiments. *B* shows the effects of 8,9-EET on the whole-cell Na^+ current steady-state inactivation curves. The data in *A* are normalized to maximal Na^+ current and plotted *versus* prepulse potentials for control (■) and after application of 0·5 μM (●), 1·0 μM (▲) and 5·0 μM (▼) 8,9-EET. Data are expressed as means ± S.E.M. The data are fitted with a Boltzmann equation where $V_{½}$ represents the half-inactivation potential and k is the slope factor. The $V_{½}$ values are significantly different for 1·0 and 5·0 μM 8,9-EET *versus* control ($P < 0·05$). The differences in k values are not statistically significant.

0·5 μM, to −82·1 ± 1·4 mV with 1·0 μM and to −89·2 ± 2·4 mV with 5·0 μM 8,9-EET ($n = 9$, $P < 0·05$ for 1·0 and 5·0 μM 8,9-EET *vs.* control). On the other hand, the slope factor was not affected by 8,9-EET (6·06 ± 0·39 for control, 6·07 ± 0·42 for 0·5 μM, 6·52 ± 0·46 for 1·0 μM and 6·50 ± 0·53 for 5·0 μM 8,9-EET; $n = 9$, n.s. *vs.* control). The inhibition of I_{Na} in rat cardiac myocytes by 8,9-EET was voltage dependent. At a prepulse potential of −120 mV, whole-cell I_{Na} values were 81·7 ± 1·3, 67·1 ± 3·1 and 62·0 ± 8·8% of control values in the presence of 0·5, 1·0 and 5·0 μM 8,9-EET, respectively. At a prepulse potential of −90 mV, whole-cell I_{Na} values were 79·0 ± 2·2, 54·7 ± 5·8 and 37·9 ± 10·8% of control values in the presence of 0·5, 1·0 and 5·0 μM 8,9-EET, respectively. At a prepulse potential of −70 mV, whole-cell I_{Na} values were 56·9 ± 11·9, 31·1 ± 10·4 and 12·7 ± 8·0% of control values in the presence of 0·5, 1·0 and 5·0 μM 8,9-EET, respectively. Taken together, these results indicated that 8,9-EET is a potent inhibitor of the cardiac I_{Na} at both physiological and depolarized membrane potentials.

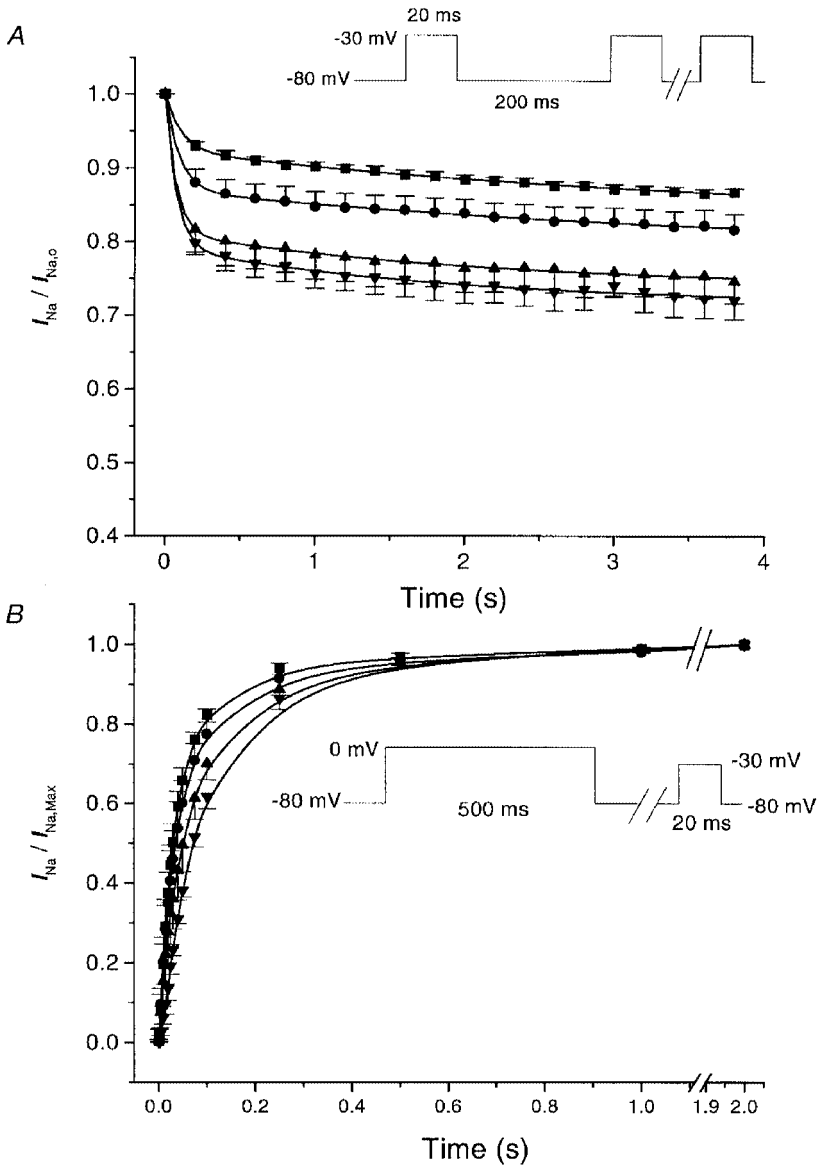

Figure 5. Effect of 8,9-EET on the frequency-dependent inhibition of the cardiac Na^+ currents (*A*) and the recovery of the Na^+ currents from inactivation (*B*)

A shows the peak whole-cell Na^+ current amplitudes during trains of 20 depolarizing current pulses at a cycle length of 200 ms with holding potentials of −80 mV and test potentials of −30 mV (inset). Results are normalized against the amplitude of the first pulse and are expressed as means ± S.E.M. Responses in the presence of 0 (control, ■), 0·5 μM (●), 1·0 μM (▲) and 5·0 μM (▼) 8,9-EET are plotted against time ($n = 6$). *B* shows the effect of 8,9-EET on the recovery of the Na^+ current from inactivation using a two-pulse protocol. Conditioning pulses at 0 mV of 500 ms duration are followed by test pulses at −30 mV of 20 ms duration with an intertrain interval of 5 s (inset). Holding potentials are −80 mV with interpulse recovery intervals between 1 and 2000 ms. The results are expressed as means ± S.E.M. for 0 (control, ■), 0·5 μM (●), 1·0 μM (▲) and 5·0 μM (▼) 8,9-EET ($n = 6$).

Table 1. Parameters of sodium channel recovery from inactivation

	Amp_1	τ_1 (ms)	Amp_2	τ_2 (ms)
Control	0·87 ± 0·03	41·4 ± 7·4	0·13 ± 0·03	128·1 ± 20·2
0·5 μM 8,9-EET	0·80 ± 0·03	46·3 ± 11·6	0·20 ± 0·03	203·5 ± 35·8
1·0 μM 8,9-EET	0·76 ± 0·04	60·4 ± 13·2	0·25 ± 0·04	264·1 ± 45·6*
5·0 μM 8,9-EET	0·80 ± 0·06	80·9 ± 12·8*	0·20 ± 0·05	401·3 ± 64·5*

Data from Fig. 5*B* were analysed using a two-exponential fit with an equation of the following form: $f = Amp_1(1 - e^{-t/\tau_1}) + Amp_2(1 - e^{-t/\tau_2})$. Amp_1 and Amp_2 represent the relative contribution (as a ratio) and τ_1 and τ_2 represent the time constants (in ms) of the fast and slow components of I_{Na} recovery. $n = 6$, *$P < 0·05$ compared with control values.

Frequency-dependent effects

To characterize further the cardiac I_{Na} inhibition by 8,9-EET, we determined use-dependent I_{Na} block using trains of depolarizing pulses. Under control conditions, a train of 20 depolarizing pulses at 200 ms interpulse intervals with a holding potential of −80 mV and a test potential of −30 mV produced a modest amount of accumulation of channel inactivation. The current amplitude of the last pulse of the train was 86·8 ± 0·5% of that of the first pulse of the train. In the presence of 8,9-EET, the same pulse protocol resulted in the development of significantly greater amounts of use-dependent I_{Na} block (Fig. 5*A*). The current amplitudes of the last pulse were 81·7 ± 2·2, 74·7 ± 2·9 and 72·1 ± 2·6% of that of the first pulse with 0·5, 1·0 and 5·0 μM 8,9-EET respectively ($n = 6$, $P < 0·05$ *vs.* control for 0·5, 1·0 and 5·0 μM 8,9-EET). The mechanism of frequency-dependent reduction of I_{Na} under control conditions and in the presence of 8,9-EET can be explained by the kinetics of Na^+ channel repriming as previously reported (Lee *et al.* 1993). We examined the effects of 8,9-EET on the recovery of steady-state inactivation of I_{Na} by using the two-pulse protocol described in Fig. 5*B*. 8,9-EET produced dose-dependent slowing of the I_{Na} recovery from inactivation, resulting in the development of further frequency-dependent inhibition of I_{Na}. The time course of I_{Na} recovery showed two exponential components: a fast component with an amplitude (Amp_1) of 0·87 ± 0·03 and a time constant (τ_1) of 41·4 ± 7·4 ms, and a slow component with an amplitude (Amp_2) of 0·13 ± 0·03 and a time constant (τ_2) of 128·1 ± 20·2 ms (Table 1). Both τ_1 and the τ_2 of I_{Na} recovery were prolonged by 8,9-EET in a dose-dependent manner. At 5·0 μM 8,9-EET, τ_1 was 2-fold (80·9 ± 0·06 ms) and τ_2 was 3-fold (401·3 ± 64·5 ms) greater than the control values ($n = 6$, $P < 0·05$ *vs.* control for both).

Effects of 8,9-EET on single channel I_{Na} in isolated rat cardiac myocytes

To examine the effects of 8,9-EET on the single channel Na^+ currents in isolated rat cardiac myocytes, experiments were conducted in the cell-attached configuration. In these experiments, the pipette contained 250 mM of Na^+ to enhance the size of unitary current and to improve signal-to-noise ratios. Figure 6 shows the raw current tracings of cell-attached patches that contained a single Na^+ channel elicited from a holding potential of −100 mV to various test potentials. The unitary Na^+ current amplitudes were plotted against membrane potentials and the results were fitted with a linear regression equation. The single Na^+ channel conductance (γ) was calculated to be 27 pS, under the conditions of our recordings, and 5 μM 8,9-EET did not alter γ.

Figure 7*A* shows characteristic raw tracings of single Na^+ channel recordings, with a holding potential of −100 mV and a test potential of −40 mV, obtained under control conditions and after the application of 5 μM 8,9-EET. The number of active Na^+ channels in each membrane was not altered by 8,9-EET. However, the probability of Na^+ channel opening was dramatically reduced by 8,9-EET. Figure 7*B* shows the ensemble-averaged current from an experiment with 500 sweeps in the presence and absence of 8,9-EET. Figure 7*C* shows the collective data in bar graphs. Under control conditions, the probability of Na^+ channel opening (P_o) was 0·270 ± 0·027 ($n = 7$). However, exposure to 5 μM 8,9-EET markedly reduced P_o to 0·048 ± 0·026 ($P < 0·05$ *vs.* control). Thus, 8,9-EET did not reduce the number of Na^+ channels, but instead reduced the probability that the Na^+ channel would be open.

Analysis of the single Na^+ channel kinetics is presented in Fig. 8. The courses of both channel open dwell time and channel closed dwell time were fitted with exponential curves. Figure 8*A* shows the Na^+ channel open-duration histogram and the distribution could be fitted with a single exponential equation. 8,9-EET shortened the channel open time constant from 163 μs under control conditions to 100 μs. The Na^+ channel closed-duration histograms are shown in Fig. 8*B*, where the distribution was described by a two-exponential equation. 8,9-EET prolonged both time constants; τ_1 was increased from 22 μs to 108 μs, and τ_2 from 1·4 ms to 1·95 ms in the presence of 5 μM 8,9-EET. These results indicate that 8,9-EET inhibits the cardiac Na^+ channels by shortening the duration of channel opening and prolonging the duration of channel closing. Thus, 8,9-EET alters the gating behaviour of the Na^+ channel.

Incubation of cultured cardiac myocytes with [^{3}H]8,9-EET

To examine the capacity of neonatal rat cardiac myocytes to take up and metabolize 8,9-EET, cells were incubated with 1 μM [^{3}H]8,9-EET for 30 min to 4 h, after which the medium- and cell-associated lipids were individually extracted and analysed. Following a 2 h incubation with [^{3}H]8,9-EET, about 75% of the applied radioactivity was present in the medium, while 25% was associated with the cells (Fig. 9*A*). During a 4 h incubation, the cells took up more radioactivity (234 $\pm$ 18 pmol well^{-1} (4 h incubation) *vs.* 178 $\pm$ 16 pmol well^{-1} (2 h incubation), $n = 3$, $P < 0{\cdot}05$). Following incubations of 2–4 h duration, analysis of cell lipids by thin-layer chromatography (TLC) indicated that virtually all the radioactivity was contained in phospholipids, principally phosphatidylcholine (not shown). After a 30 min incubation, most of the uptake was also in phospholipids, but approximately 1–2% of the radioactivity associated with the cells remained as unesterified 8,9-EET (Fig. 9*B*).

Analysis of the lipids extracted from the medium following a 2 h incubation with [^{3}H]8,9-EET indicated that 86% of the radioactivity remained as [^{3}H]8,9-EET, while 13% was converted to a more polar metabolite that co-migrated with radiolabelled 8,9-DHET (Fig. 10*A*). After a 4 h incubation, 56% of the medium-associated radioactivity was in the form of [^{3}H]8,9-EET, while 32% was present as [^{3}H]8,9-DHET (Fig. 10*B*). In addition, another metabolite was detected which eluted from the column before [^{3}H]8,9-

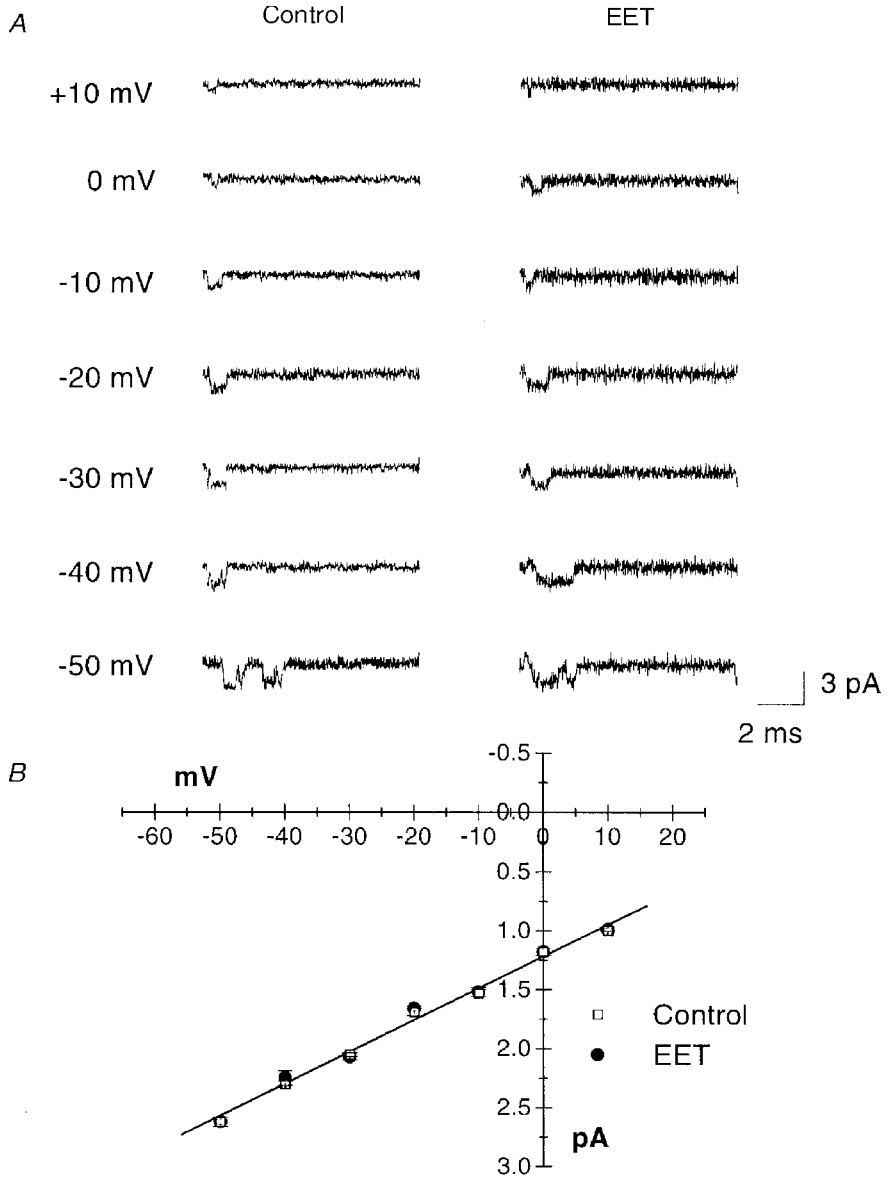

Figure 6. Effect of 8,9-EET on single Na$^+$ channel conductance

Currents from single Na$^+$ channels in rat ventricular myocytes are recorded in cell-attached configuration from a holding potential of -100 mV to various test potentials as indicated. The raw current tracings under control conditions or after exposure to 5 μM 8,9-EET are shown in *A*. In *B*, the unitary Na$^+$ current amplitudes are plotted against membrane potentials and the results are fitted with a linear regression equation. The single Na$^+$ channel conductance (γ) is 27 pS and 5 μM 8,9-EET does not alter γ ($n = 4$).

DHET and accounted for about 13% of the medium-associated radioactivity. The chemical identity of this metabolite remains to be determined.

DISCUSSION

In this study, we have demonstrated that EETs are potent inhibitors of the voltage-dependent Na^+ channels in rat cardiac myocytes. The inhibition of I_{Na} by 8,9-EET is dose, voltage and use dependent through modulation of the Na^+ channel gating behaviour. In addition, we have demonstrated that cardiac myocytes can avidly take up 8,9-EET and incorporate it into cellular phospholipids. These results suggest that EETs may play an important role in the modulation of cardiac electrophysiology.

The cytochrome P450-derived EETs are potent vasodilators in multiple vascular beds, including the coronary (Gebremedhin *et al.* 1992; Campbell *et al.* 1996; Chataigneau *et al.* 1998; Miura & Gutterman, 1998; Oltman

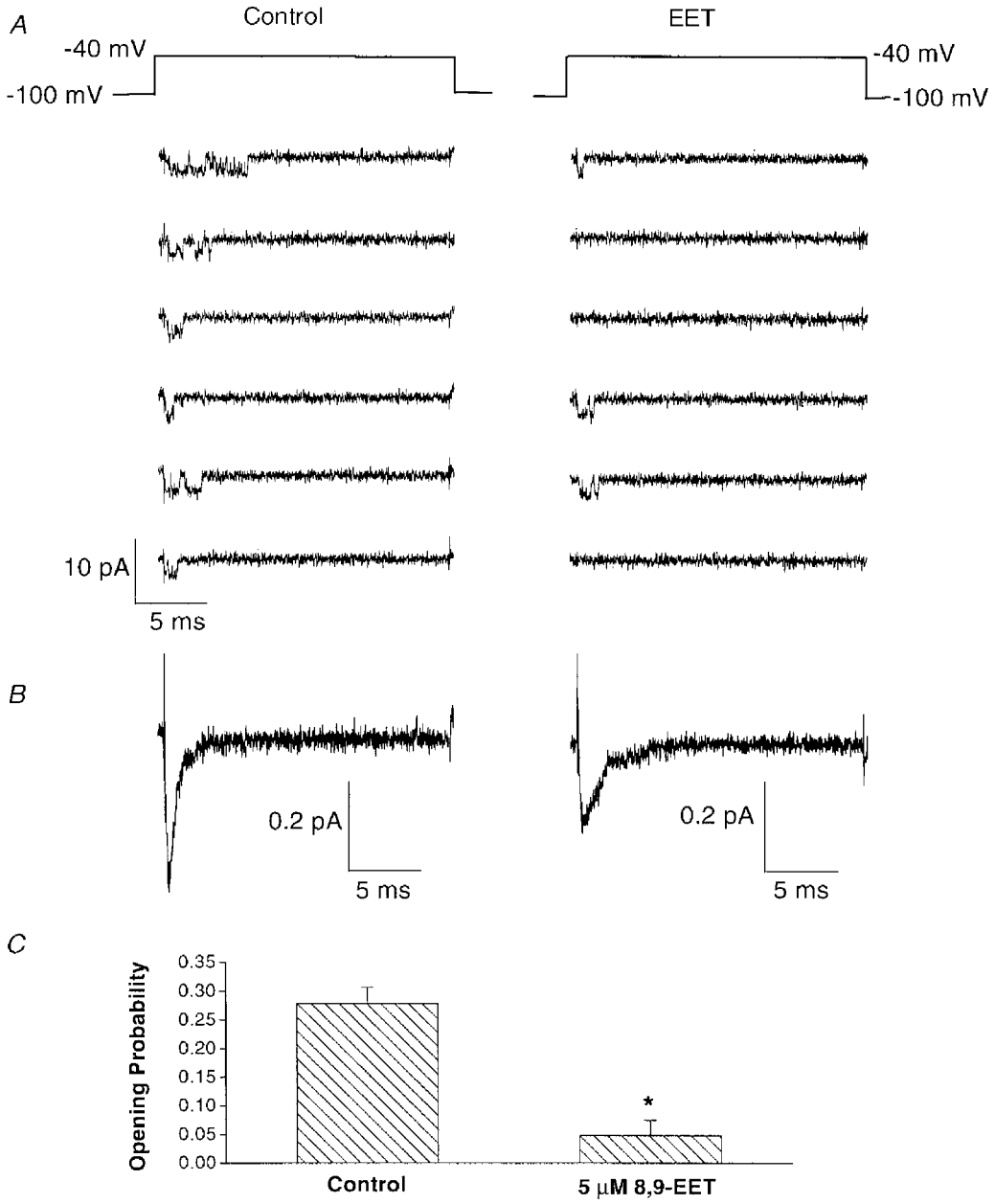

Figure 7. Effect of 8,9-EET on single Na^+ channel opening probability

Representative single channel recordings in cell-attached membrane patches of rat ventricular myocytes with a holding potential of −100 mV and a test potential of −40 mV before (control) and after application of 5 μM 8,9-EET are shown in *A*. In the presence of EET, the number of null sweeps is significantly increased. The ensemble-averaged currents from 500 sweeps before (control) and after application of 5 μM 8,9-EET are shown in *B*. *C* represents the results from seven experiments measuring single Na^+ channel opening probability (P_o) under control conditions and in the presence of 5 μM 8,9-EET. P_o is 0·270 ± 0·027 for the controls, and 8,9-EET markedly reduced P_o to 0·048 ± 0·026.

et al. 1998). Direct electrophysiology studies showed that EETs activate Ca^{2+}-dependent K^+ channels and are, by definition, endothelium-derived hyperpolarizing factors (Hu & Kim, 1993; Zou *et al.* 1996; Baron *et al.* 1997; Li *et al.* 1997; Li & Campbell, 1997). In addition, EETs are known to modulate ion channels in non-vascular smooth muscle. For example, EETs inhibit a Ca^{2+}-insensitive Cl^- channel (Salvail *et al.* 1998) and activate Ca^{2+}-activated K^+ channels in bovine tracheal smooth muscle (Dumoulin *et al.* 1998). Therefore, it is apparent that ion channels constitute major effector targets of EETs. However, the effects of EETs on cardiac ion channels have not been studied in detail.

Cytochrome P450 is ubiquitous, and cytochrome P450-dependent mono-oxygenase activity has been detected in mammalian hearts (Comte & Gautheron, 1978; Guengerich & Mason, 1979; Abraham *et al.* 1987; McCallum *et al.* 1993). The newly identified human and rat isoforms of cytochrome P450, CYP2J2 and CYP2J3, respectively (Wu *et al.* 1996, 1997), are highly expressed in hearts and are active in the mono-oxgenation of arachidonic acid. The recombinant human CYP2J2 and the rat CYP2J3 have the capacity to generate all four EET regioisomers (Wu *et al.* 1996, 1997). Moreover, direct quantification showed that the rat heart contains substantial amounts of endogenous EETs, with 8,9-EET being the major regioisomer, constituting 39% of the total. These results provide strong evidence that EETs can be actively synthesized *in situ* in the heart. Our results further suggest that cardiac myocytes may also take up unesterified EETs and incorporate them into cellular phospholipids. Cardiac myocytes have a high capacity for EET uptake, incorporating 25% into phospholipids over 2 h. The fact that cardiac myocytes continue to accumulate radioactive EET over a 4 h period suggests that the heart is a site where EETs can be stored in esterified form in phospholipids. The bound EETs could then be released upon phospholipase activation, as was recently shown for cardiac endothelial cells (Weintraub *et al.* 1997). However, even though most of the uptake is incorporated into phospholipids, the small amount of unesterified 8,9-EET (1–2%) in the cell is capable of modulating the Na^+ channel

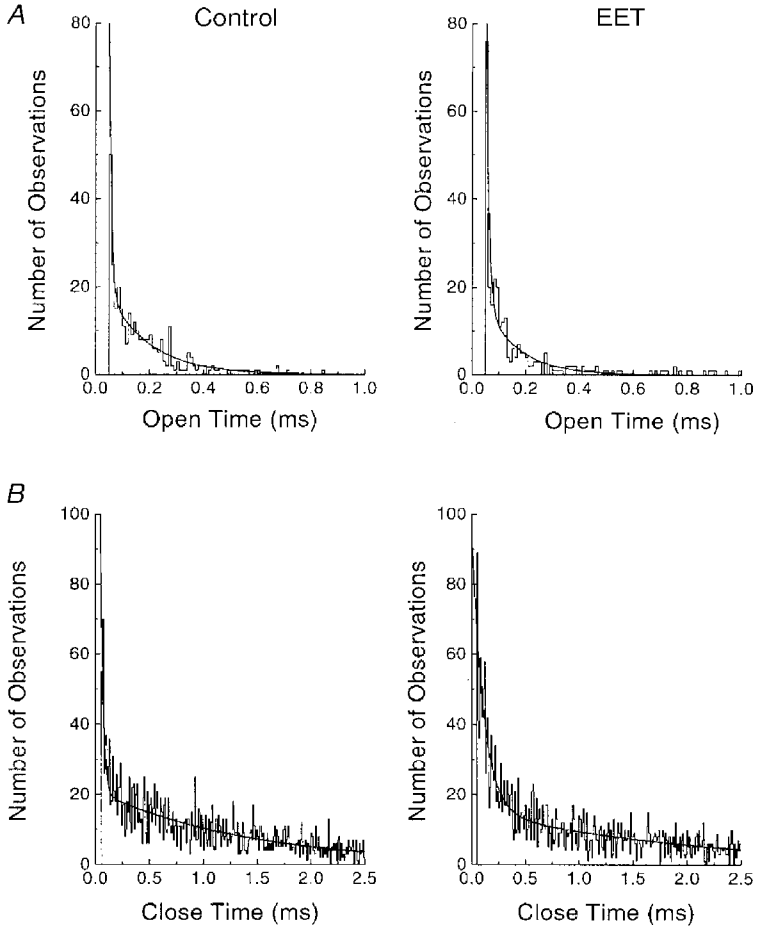

Figure 8. Effect of 8,9-EET on the kinetics of single Na^+ channels

Single Na^+ channel currents are recorded in cell-attached patches with holding potentials of −100 mV and test potentials of −40 mV. *A* shows the Na^+ channel open duration histograms for 0 (control) and 5 μM 8,9-EET. The open dwell time distributions are fitted with a single exponential equation with time constants (τ) of 163 μs for control and 100 μs for 8,9-EET. *B* shows the Na^+ channel closed-duration histograms for control and 5 μM 8,9-EET treatment. The mean closed time of the Na^+ channel recordings is fitted with a two-exponential equation. Both τ_1 and τ_2 are prolonged by 8,9-EET. τ_1 is 22 μs for control and 108 μs for EET and τ_2 is 1·4 ms for control and 1·95 ms for EET.

function by interacting with the channel protein. Our results, therefore, support the possibility that EETs are important products of arachidonic acid metabolism in the heart.

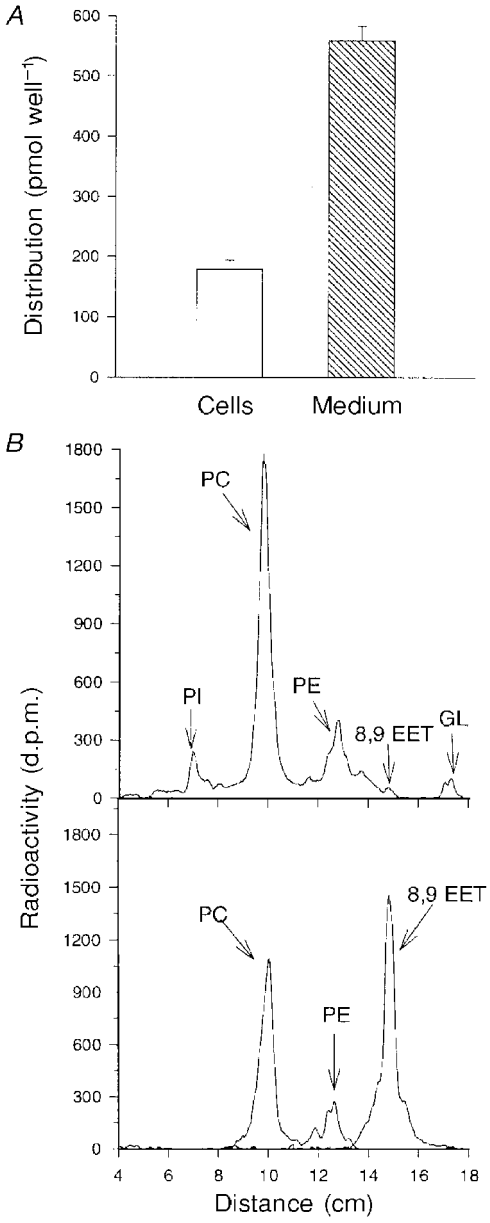

Figure 9. Distribution of radioactivity following incubation of rat neonatal myocytes with [^{3}H]8,9-EET

Cells were incubated in medium containing 1 μM [^{3}H]8,9-EET for 30 min to 4 h, after which the cell- and medium-associated lipids were extracted and analysed. The distribution of radioactivity between the cells and medium (quantified by liquid scintillation counting) following a 2 h incubation with [^{3}H]8,9-EET is shown in *A*. The values are expressed as means ± S.E.M., $n = 3$. In *B*, a representative TLC chromatogram (upper panel) shows the distribution of radioactivity in cell lipids following a 30 min incubation with [^{3}H]8,9-EET. PI , phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; GL, glycerides. Similar results were obtained from a duplicate culture. Migration of radiolabelled phospholipid standards (PC and PE) and 8,9-EET are shown in the lower panel.

Cardiac myocytes can also metabolize 8,9-EET to 8,9-DHET, similar to the pathway observed in arterial smooth muscle (Fang *et al.* 1996). Our finding that 8,9-DHET is not as effective as 8,9-EET as a Na^+ channel inhibitor suggests the presence of a metabolic pathway for 'degrading' EET, hence providing further support that 8,9-EET is functionally active in cardiac myocytes. This could be a mechanism for removal or regulating the level of EET in the heart. After 4 h of incubation, accumulation of a polar metabolite of 8,9-EET was observed but its identity is at present unknown. This substance could be from a further step into the degradative pathway, and might be a chain-shortened product such as dihydroxyhexadecadienoic acid

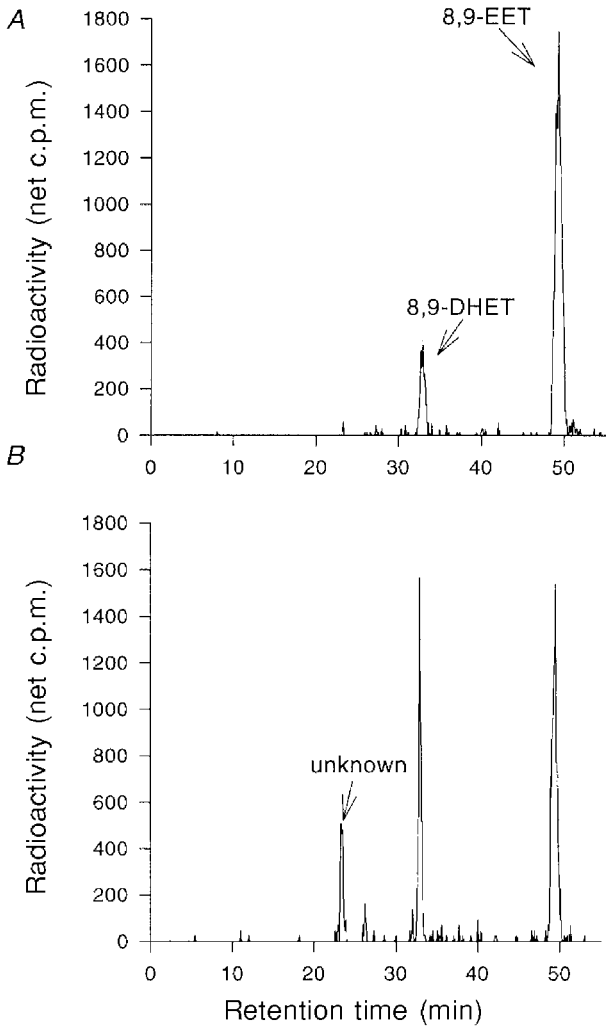

Figure 10. Metabolites of [^{3}H]8,9-EET found in the medium of cultured rat neonatal myocytes during 2 h (*A*) and 4 h (*B*) incubations

Following incubation, lipids were extracted from the medium and separated by reverse-phase HPLC, and the radioactivity was assayed with an on-line flow scintillation detector. In *A*, after a 2 h incubation, the radiolabelled components co-migrated with 8,9-EET and 8,9-DHET standards. In *B*, after a 4 h incubation, a major unidentified product formed from [^{3}H]8,9-EET is apparent. Both panels contain a chromatogram from a single culture, but similar results were obtained from a duplicate culture in each case.

(DHHD) which has been previously identified in arterial smooth muscle cells (Fang *et al.* 1996). It is currently unknown whether this polar EET metabolite has any effects on I_{Na} or other cardiac ion channels.

Moffat *et al.* (1993) examined the effects of EETs in isolated guinea-pig hearts and in ventricular myocytes. They found that EETs produced no effects on the contractility of normal isolated hearts. In hearts subjected to 60 min of low-flow ischaemia, however, 5,6- and 11,12-EET significantly delayed the recovery of myocardial function during early reperfusion. Furthermore, both 5,6- and 11,12-EET increased cell shortening and intracellular Ca^{2+} concentrations in isolated cardiac myocytes, suggesting that during ischaemia EETs may lead to intracellular Ca^{2+} overload. Recently, a calcium current (I_{Ca}) in isolated rat ventricular myocytes was shown to be augmented by 11,12-EET and suppressed by P450 inhibitors and by anti-P450 antibodies (Xiao *et al.* 1998*a*). These results suggest that endogenously produced or exogenously administered EETs augment I_{Ca} via modulation of cellular cAMP levels, because 11,12-EET significantly increased, whereas P450 inhibitors reduced, the cAMP content of rat ventricular myocytes. Yet, EETs do not affect the cAMP levels in human platelets (Fitzpatrick *et al.* 1986), suggesting that the effects of EET on cellular cAMP contents are probably tissue specific.

In the present study, we found that 8,9-EET and other EET regioisomers are potent inhibitors of the cardiac Na^+ channel. It is unlikely that the effects of 8,9-EET on I_{Na} were mediated through increased concentration of cAMP because we have previously reported that β-adrenergic stimulation and cAMP-mediated phosphorylation enhance I_{Na} (Matsuda *et al.* 1992; Lu *et al.* 1998). Moreover, long chain fatty acids have been reported to suppress I_{Na} in cardiac myocytes (Xiao *et al.* 1995) and in HEK293 cells transfected with the α-subunit of the human (Xiao *et al.* 1998*b*) or rat cardiac Na^+ channel (Bendahhou *et al.* 1997). However, the present results indicate that 8,9-EET is at least ten times more potent than arachidonic acid in inhibiting I_{Na}, suggesting the epoxide group confers an enhanced capacity to blockade the Na^+ channel. 8,9-EET produces significant inhibition of I_{Na} at 0·5 μM, with an IC_{50} around 1·0 μM, concentrations similar to those required to produce vasodilatation (Campbell *et al.* 1996; Fang *et al.* 1996; Weintraub *et al.* 1997) and to activate ion channels in smooth muscle cells (Li & Campbell, 1997; Li *et al.* 1997; Dumoulin *et al.* 1998; Salvail *et al.* 1998).

The mechanism of inhibition of the cardiac I_{Na} by 8,9-EET was determined from single channel studies. Our results suggest that the single Na^+ channel conductance and the number of active channels are not altered by the effects of 8,9-EET. Instead, the probability of channel opening was dramatically reduced, with an increase in the number of null sweeps. These findings are similar to the inhibition of I_{Na} by lidocaine (lignocaine; Nilius *et al.* 1987; Grant *et al.* 1989). 8,9-EET alters the gating behaviour of Na^+ channels by abbreviating the mean open time, as has been reported for lidocaine (Nilius *et al.* 1987). This could be interpreted as indicating that 8,9-EET directly interacts with the open conformation of the Na^+ channel protein. In addition, 8,9-EET prolongs the Na^+ channel mean closed time. This, together with the negative shift in the steady-state inactivation curve, suggests that 8,9-EET binding also results in accumulation of Na^+ channels in an absorbing inactivated state.

Inhibition of the cardiac I_{Na} by 8,9-EET exhibits both tonic and use-dependent blocks. The basic requirements for use dependence are that there is development of channel blockade with each depolarization, and that the interval between depolarizations is not sufficient to allow complete recovery from this block. Our results indicate that there is a frequency-dependent increase in accumulation of channel inactivation in the presence of 8,9-EET, which slows channel recovery from inactivation. The behaviour of 8,9-EET as a Na^+ channel inhibitor is similar to that of local anaesthetics and Class I antiarrhythmics, which are thought to interact with the Na^+ channel through a 'modulated receptor' mechanism (Hondeghem & Katzung, 1977). The basic elements of the modulated receptor hypothesis are: (i) the Na^+ channel has a specific receptor for interaction with the drug or inhibitor; (ii) the voltage-induced conformational changes in the channel result in different affinities of the receptor for the drug in each of the channel states; (iii) drug-bound channels can continue to gate; (iv) the rate of recovery from inactivation is modified due to a change or shift of inactivation kinetics for drug-bound channels; and (v) drug-bound channels may not conduct ionic current (Snyders *et al.* 1992). The effects of 8,9-EET on the cardiac I_{Na} embody these features. Therefore, it would be reasonable to infer that 8,9-EET exerts its effects through binding to a specific site on the Na^+ channel with specific binding and unbinding rate constants, rather than through mechanisms such as modulation of membrane fluidity as a result of incorporation into phosphatidylcholine. In support of this possibility, a small amount of unesterified 8,9-EET was detected in the cardiac myocyte cell lipids after a 30 min incubation. Indeed, Kang & Leaf (1996) have reported evidence that some polyunsaturated fatty acids bind directly to the Na^+ channel proteins. Whether EETs bind to the same site is unclear at present.

The effects of 8,9-EET on I_{Na} are voltage dependent, with greater inhibition of I_{Na} at more depolarized potentials. This finding has potential pathophysiological implications since the suppressive effects of EETs on cardiac impulse conduction and tissue excitability would be amplified during conditions of ischaemia or myocardial infarction (Kleber *et al.* 1995). Interestingly, ischaemia-induced phospholipolysis occurs in many tissues including the heart (Hazen & Gross, 1992), leading to release of arachidonic acid. The increase in arachidonic acid levels may promote the *in situ* formation of EETs through the cytochrome P450 mono-oxygenase pathway. But perhaps a more important source is the hydrolytic release of EETs esterified to phospholipids, the

location of 99% of the EETs present in cells and tissues (Karara *et al.* 1991, 1992; Nakamura *et al.* 1997). We have shown in this study that cardiac myocytes have a high capacity to take up unesterified 8,9-EET and incorporate it into cellular phospholipids. In addition, the formation of esterified EET in phospholipids has been shown to be dramatically increased (59-fold increase) in the presence of oxidative stress, a condition that induces free radical oxidation of membrane phospholipids (Nakamura *et al.* 1997). Hence, during conditions of ischaemia, the cellular concentrations of unesterified EETs may be enhanced through three different mechanisms: increased arachidonic acid release with subsequent cytochrome P450 epoxygenation, non-enzymatic oxidation and hydrolysis of membrane phospholipids through lipid peroxidation, and release of esterified EETs through activation of phospholipases. Indeed, the amount of EETs released from phospholipids through activation of phospholipase A_2 has been estimated to reach the micromolar range in human platelets (Zhu *et al.* 1995). These findings, together with the voltage-dependent effects on I_{Na}, strongly suggest that EETs may play an important role in the modulation of cardiac electrophysiology during ischaemia.

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