0022-3565/97/2811-0384$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 281, No. 1
Printed in U.S.A.

JPET 281:384–392, 1997

Effects of Class III Antiarrhythmic Drugs on Transient Outward and Ultra-rapid Delayed Rectifier Currents in Human Atrial Myocytes[1]

JIANLIN FENG, ZHIGUO WANG,[2] GUI-RONG LI[3] and STANLEY NATTEL

Department of Medicine (J.F., Z.W., G.-R.L., S.N.), Montreal Heart Institute and University of Montreal, and Department of Pharmacology and Therapeutics (S.N.), McGill University, Montreal, Quebec, Canada

Accepted for publication December 30, 1996

ABSTRACT

A variety of class III antiarrhythmic agents have been shown to block the delayed rectifier current, but their effects on other K^+ currents, particularly in human tissues, are less clear. We studied the concentration-dependent actions of the class III compounds *d*-sotalol, E-4031 and ambasilide on the transient outward current (I_{to}) and the ultra-rapid delayed rectifier current (I_{Kur}) in human atrial myocytes. *d*-Sotalol and E-4031 failed to alter I_{to} or I_{Kur} at concentrations up to 500 and 50 μM, respectively. In contrast, ambasilide produced a concentration-dependent inhibition of I_{to} and I_{Kur}, with statistically significant effects at 10 μM and maximum effects at 100 μM. The 50% inhibitory concentration of ambasilide averaged 23 $\pm$ 2 μM and 34 $\pm$ 3 μM for I_{to} and I_{Kur} respectively. Ambasilide did not alter the voltage-dependence of activation or inactivation of I_{to}, or the voltage-dependence of I_{Kur}, and it did not affect I_{to} recovery from inactivation. On the other hand, ambasilide accelerated I_{to} inactivation, by introducing a more rapid component that accelerated with increasing drug concentration. Furthermore, block of both I_{to} and I_{Kur} developed over time after the onset of depolarization, with time constants of 5.8 $\pm$ 0.8 msec and 2.5 $\pm$ 0.4 msec at concentrations of 10 and 50 μM for I_{to} and 6.1 $\pm$ 0.8 msec and 2.1 $\pm$ 0.3 msec at 10 and 50 μM for I_{Kur}. We conclude that neither *d*-sotalol nor E-4031 affects I_{to} or I_{Kur}, whereas ambasilide produces efficacious open-channel block of both currents, in human atrial myocytes.

Class III antiarrhythmic agents exert their actions by prolonging the cardiac action potential and thereby increasing the refractory period, without altering phase 0 sodium current or conduction velocity. A variety of class III drugs are in current clinical use or in development, including sotalol (Singh and Nademanee, 1987), dofetilide (Rasmussen *et al.*, 1992), E-4031 (Fujiki *et al.*, 1994) and ambasilide (Takanaka *et al.*, 1992). Although data have been presented that suggest that sotalol is a highly selective antagonist of the delayed rectifier K^+ current (Carmeliet, 1985), and particularly of the rapid component I_{Kr} (Sanguinetti and Jurkiewicz, 1990), other work has suggested that sotalol potently inhibits the transient outward current (Berger *et al.*, 1989). Dofetilide and E-4031 have been characterized as specific blockers of I_{Kr} (Rasmussen *et al.*, 1992; Fujiki *et al.*, 1994; Colatsky *et al.*, 1990), largely on the basis of experiments with cells isolated from experimental animals. Ambasilide has been found to inhibit both components of the delayed rectifier: I_{Kr} and the slower component I_{Ks} (Zhang *et al.*, 1992). In an experimental canine model of AF, ambasilide was found to be a more potent antiarrhythmic agent than *d*-sotalol and to prolong refractoriness with much less reverse use-dependence than *d*-sotalol (Wang *et al.*, 1994b).

Recent work has helped to clarify the ionic currents governing human atrial repolarization. Whereas delayed rectifier K^+ currents in human atrium resemble corresponding currents in a variety of animal cells (Wang *et al.*, 1993a;

Received for publication June 17, 1996.
[1] This study was supported by the Medical Research Council of Canada, the Québec Heart Foundation, and the Fonds de Recherche de l'Institut de Cardiologie de Montréal.
[2] Supported by a Medical Research Council of Canada Postdoctoral Fellowship.
[3] Supported by a Fonds de la Recherche en Santé du Québec Research Scholarship.

ABBREVIATIONS: AF, atrial fibrillation; ANOVA, analysis of variance; 4AP, 4-aminopyridine; CP, conditioning pulse; E-4031, investigational class III drug; EGTA, ethylene glycol-bis (β-aminoethylether)-N,N,N′, N′-tetraacetic acid; I, I_{to} amplitude; I_C, current under control conditions; I_{Ca}, calcium current; I_D, current under drug conditions; I_K, delayed rectifier K^+ current; $I_{K,ACh}$, ACh-induced K^+ current; I_{K1}, inward rectifier current; I_{Kr}, rapid component of delayed rectifier K^+ current; I_{Ks}, slow component of delayed rectifier K^+ current; I_{Kur}, ultra-rapid delayed rectifier K^+ current; I_{Max}, maximum current; I_{Na}, sodium current; I_{to}, transient outward current; Kv1.5, potassium channel clone of Shaker family; nS, nanosiemens; NS, nonsignificant; O.D., outside diameter; P_1, first pulse of a paired-pulse protocol; P_2, second pulse; R_s, series resistance; *t*, time; τ_1, rapid-phase time constant; τ_2, slow-phase time constant; τ_c, capacitive time constant; TP, test potential.

Wang *et al.*, 1994a), some K^+ currents in human atrial cells show important differences from other species. For example, human atrial I_{to} has quite different kinetic properties from I_{to} in rabbit atrium (Fermini *et al.*, 1992), and a novel type of delayed rectifier with kinetic and pharmacologic properties resembling those of the cloned human K^+ channel Kv1.5 appears to be important in human atrial repolarization (Wang *et al.*, 1993b). The latter current has been designated I_{Kur}, or the ultra-rapid delayed rectifier, because its activation kinetics are two orders of magnitude faster than those of I_{Kr} (Wang *et al.*, 1993b).

Relatively little is known about the effects of antiarrhythmic drugs on ionic currents in human heart cells. We have shown that quinidine produces open-channel block of I_{to} in human atrial cells, whereas flecainide inhibits I_{to} in a fashion that suggests the highest affinity for the inactivated state (Wang *et al.*, 1995). Quinidine was found to inhibit I_{Kur} significantly at concentrations in the clinically relevant range, which suggests that I_{Kur} block may contribute to the drug's antiarrhythmic properties in the human, whereas flecainide had no detectable effect on I_{Kur} at concentrations as large as 10 μM (Wang *et al.*, 1995).

I_{to} and I_{Kur} appear to play important roles in human atrial repolarization (Shibata *et al.*, 1989; Escande *et al.*, 1987; Wang *et al.*, 1993b) and may therefore be important targets for antiarrhythmic drug action. The effects of class III drugs on I_{Kur} have not, to our knowledge, been studied. There is limited information about class III drug effects on I_{to}, and the results that have been obtained, largely relating to sotalol, are somewhat contradictory (Carmeliet, 1985; Berger *et al.*, 1989). We therefore designed the present study to evaluate the effects of three class III antiarrhythmic drugs, *d*-sotalol, E-4031 and ambasilide, on I_{to} and I_{Kur} in human atrial myocytes.

Methods

Isolation of human atrial cells. Specimens of human right atrial appendage were obtained from the hearts of 36 patients (58 ± 3 years old) undergoing aortocoronary bypass surgery. All patients were free of supraventricular tachyarrhythmias, and the atria were grossly normal at the time of surgery. The procedure for obtaining the tissue was approved by the Ethics Committee of the Montreal Heart Institute. Samples were quickly immersed in nominally Ca^{++}-free Tyrode's solution (100% O_2, 37°C) of the following composition (mM): NaCl, 126; KCl, 5.4; $MgCl_2$, 1.0; NaH_2PO_4, 0.33; dextrose, 10; HEPES (Sigma Chemicals, St. Louis, MO), 10; pH adjusted to 7.4 with NaOH. The myocardial specimens were chopped with scissors into cubic chunks and placed in a 25-ml flask containing 10 ml of the Ca^{2+}-free Tyrode's solution. The tissue was gently agitated by continuous bubbling with 100% O_2 and stirring with a magnetic bar. After an initial 5 min in this solution, the chunks were reincubated in a similar solution containing 390 U/ml collagenase (CLS II, Worthington Biochemical, Freehold, NJ) and 4 U/ml protease (Type XXIV, Sigma Chemicals). The first supernatant was removed after 45 min, and the chunks were reincubated in a fresh enzyme-containing solution. Microscopic examination was performed every 15 min to determine the number and quality of the isolated cells. When the yield appeared to be maximal, the chunks were suspended in a solution containing (mM): KCl, 20; KH_2PO_4, 10; glucose, 10; glutamic acid, 70; β-hydroxybutyric acid, 10; taurine, 10; EGTA, 10; albumin, 1%; pH adjusted to 7.4 with KOH, and gently pipetted.

Data acquisition. Only quiescent rod-shaped cells showing clear cross-striations were used. A small aliquot of the solution containing the isolated cells was placed in a 1-ml chamber mounted on the stage of an inverted microscope. Five minutes was allowed for cell adhesion to the bottom of the chamber, and then the cells were superfused at 3 ml/min with a solution containing (millimolar concentrations): NaCl, 126; KCl, 5.4; $MgCl_2$, 0.8; $CaCl_2$, 1.0; NaH_2PO_4, 0.33; HEPES, 10; glucose, 5.5; pH adjusted to 7.4 with NaOH. In order to minimize possible contamination from I_K, I_{K1}, $I_{K,Ach}$ and choline-activated K^+ current (Fermini and Nattel, 1994), the following chemicals were present during current recording: TEA (Sigma Chemicals, 10 mM, to inhibit I_K), $BaCl_2$ (Sigma Chemicals, 1 mM, to inhibit I_{K1}) and atropine (Sigma Chemicals, 1 μM, to inhibit $I_{K,Ach}$ and choline-activated K^+ current). In preliminary experiments and previously published studies (Wang *et al.*, 1993a; Wang *et al.*, 1993b), we found these interventions to be without effect on I_{to} and I_{Kur}. Sodium current was inhibited with the use of a holding potential of −50 mV and/or equimolar choline replacement of Na^+ in the superfusate. $CdCl_2$ (200 μM) was added to the superfusate to inhibit Ca^{++} current. 4AP was obtained from Sigma Chemicals, prepared as a 1 M stock solution with pH adjusted to 7.4 with the use of 1 N HCl and added at selected concentrations as specified below. Experiments were conducted at room temperature in order to resolve the rapid activation and deactivation kinetics of I_{Kur}; previous studies have shown that the amplitude of I_{Kur} at room temperature is similar to that at 37°C (Wang *et al.*, 1993b).

The whole-cell patch-clamp technique was employed to record ionic currents in the voltage-clamp mode. Borosilicate glass electrodes (O.D. 1.0 mm) were used, with tip resistances of 2.5 to 5 MΩ when filled with (millimolar concentrations): KCl, 130; $MgCl_2$, 1.0; HEPES, 10; EGTA, 5; Mg_2ATP, 5; Na_2-creatine phosphate, 5; pH adjusted to 7.4 with KOH, and were connected to a patch-clamp amplifier (Axopatch 1-D, Axon Instruments, Foster City, CA). Command pulses were generated by a 12-bit digital-to-analog converter controlled by pClamp software (Axon Instruments). Recordings were low-pass filtered at 1 kHz. Currents were digitized at a maximum frequency of 100 kHz (model TM 125, Scientific Solutions, Solon, OH) and stored on the hard disk of a personal computer.

Junction potentials were zeroed before formation of the membrane-pipette seal. Mean seal resistance averaged 11.6 ± 3.9 GΩ (n = 30). Several minutes after seal formation, the membrane was ruptured by gentle suction to establish the whole-cell configuration for voltage clamping. R_s was electrically compensated to minimize the duration of the capacitive surge on the current record and the voltage drop across the clamped cell membrane. R_s along the clamp circuit was estimated by dividing the time constant obtained by fitting the decay of the capacitive transient (τ_c) by the calculated cell membrane capacitance (the time-integral of the capacitive surge measured in response to 5-mV hyperpolarizing steps from a holding potential of −60 mV divided by the voltage drop).

Before R_s compensation, the decay of the capacitive surge had a time constant of 552 ± 36 μsec (cell capacitance of 87.7 ± 4.6 pF, n = 30). After compensation, the time constant was reduced to 166 ± 3 μsec. The initial R_s was calculated to be 6.3 ± 0.3 MΩ, and R_s was reduced to 2.2 ± 0.1 MΩ after compensation. Currents recorded during this study did not exceed 2 nA. The voltage drop across R_s therefore never exceeded 5 mV. Cells with significant leak currents, manifested as a conductance > 0.6 nS upon 10-mV hyperpolarization and depolarization from −60 mV, were rejected. If leak current changed over the course of an experiment, as indicated by a significant change (> 10 pA) in the holding current at −50 mV or by an increase in the membrane conductance at −60 mV, the experiment was terminated.

Data analysis. The amplitude of I_{to} was measured as the difference between the peak of the transient outward current and the sustained current at the end of the pulse, as previously described (Wang *et al.*, 1993b; Wang *et al.*, 1995). To record I_{Kur} in the absence of contamination by I_{to}, we used a 1-sec prepulse to +40 mV to inactivate I_{to} 10 msec before a depolarizing test pulse, a procedure that we have previously developed and validated (Wang *et al.*,

1993b). I_{Kur} was measured in two ways: 1) as described previously (Wang *et al.*, 1993b; Wang *et al.*, 1995), based on the maximum current upon depolarization in the presence of a depolarizing prepulse to inactivate I_{to}, and 2) in terms of the tail current upon repolarization from a depolarizing test potential to −20 mV.

Comparisons among groups were performed by ANOVA with Scheffé's contrasts. Single comparisons between base-line and drug data were performed with Student's *t* test, and a two-tailed probability of 5% was taken to indicate statistical significance. Group data are presented as mean ± S.E.M. Nonlinear curve fitting was performed using Clampfit in pClamp (Axon Instruments) or Sigmaplot software (Jandel Scientific, San Rafael, CA).

Results

Effects of *d*-sotalol and E-4031 on I_{to} and I_{Kur}. Concentrations of *d*-sotalol up to 500 μM, which fully inhibit I_{Kr} (Sanguinetti and Jurkiewicz, 1990) and are substantially higher than the maximum therapeutic concentration in the human (Wang *et al.*, 1986), failed to affect I_{to} recorded on 300-msec depolarizing pulses delivered at 0.1 Hz from −50 mV. Similarly, I_{Kur} elicited by 160-msec pulses from −50 mV (after a 1-sec prepulse to +40 mV to inactivate I_{to}) was not altered by the drug. Overall, concentrations of 5, 10, 50 and 500 μM *d*-sotalol (in five cells at each concentration), produced −1.2 ± 0.3, 0.9 ± 0.4, 1.5 ± 0.7 and −1.4 ± 0.9% changes in I_{to} and 0.8 ± 0.3, −2.1 ± 0.7, −1.3 ± 0.4 and 1.5 ± 0.9% changes in I_{Kur}, respectively, at +40 mV. When a train of 15 conditioning 90-msec pulses to +50 mV at a frequency of 1, 2 and 3.3 Hz was introduced before the test pulse to evaluate possible use-dependent actions, no effect of 100 μM *d*-sotalol on I_{to} or I_{Kur} was noted. I_{to} averaged 850 ± 65, 845 ± 59 and 831 ± 62 pA at +50 mV at 1, 2 and 3.3 Hz, respectively, before and 849 ± 62, 846 ± 61 and 829 ± 58 pA after 100 μM *d*-sotalol (P = NS for *d*-sotalol *vs.* control for each). Similarly, I_{Kur} averaged 499 ± 52, 488 ± 49 and 475 ± 46 pA at +50 mV at 1, 2 and 3.3 Hz, respectively, before and 490 ± 49, 490 ± 52 and 480 ± 50 pA after 100 μM *d*-sotalol (P = NS for *d*-sotalol *vs.* control).

E-4031 similarly failed to alter I_{to} or I_{Kur}—at concentrations of 1, 5 and 10 μM E-4031 (in five cells at each concentration), E-4031 produced 0.9 ± 0.2, −1.1 ± 0.4 and 0.8 ± 0.7% changes in I_{to} and −1.3 ± 0.8, 1.2 ± 0.7 and 0.9 ± 0.2% changes in I_{Kur} respectively at +40 mV. When a train of 15 conditioning 90-msec pulses to +50 mV at a frequency of 1, 2, and 3.3 Hz was introduced prior to the test pulse to evaluate possible use-dependent actions, no effect of 50 μM E-4031 on I_{to} or I_{Kur} was noted. I_{to} averaged 798 ± 60, 788 ± 69, and 770 ± 63 pA at +40 mV at 1, 2 and 3.3 Hz respectively before and 789 ± 58, 781 ± 56, and 768 ± 60 pA after 50 μM E-4031 (P = NS for E-4031 versus control for each). Similarly, I_{Kur} averaged 459 ± 49, 450 ± 45 and 442 ± 38 pA at +40 mV at 1, 2 and 3.3 Hz respectively before and 453 ± 47, 448 ± 46 and 449 ± 41 pA after 50 μM E-4031 (P = NS for E-4031 versus control for each).

Effects of ambasilide on I_{to}. The response of I_{to} to ambasilide is illustrated in figure 1. Figure 1A shows representative currents recorded in one cell under control conditions. Ambasilide produced a slight decrease in I_{to} at a concentration of 10 μM (fig. 1B). At a higher concentration (50 μM, fig. 1C), ambasilide decreased I_{to} substantially and caused apparent acceleration in the initial decay of I_{to} after peak values were attained. I_{to} inhibition by ambasilide was almost fully reversed after 20 min of drug washout (fig. 1D).

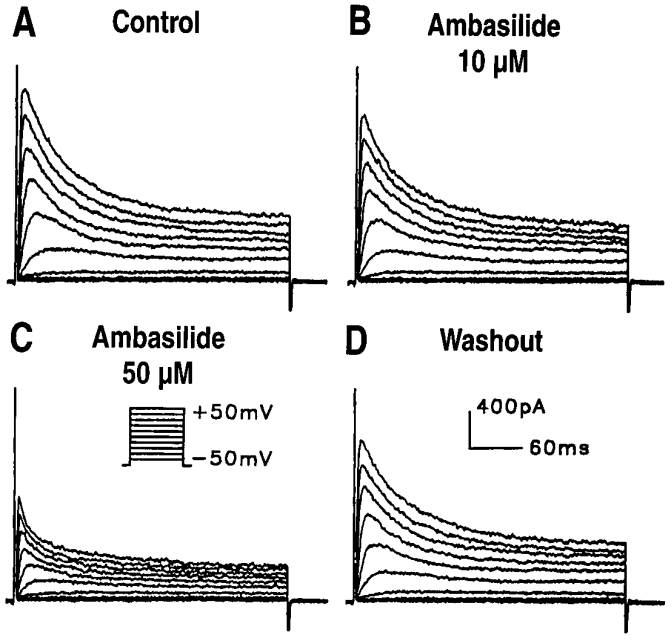

Fig. 1. Concentration-dependent effects of ambasilide on I_{to} in a representative cell. I_{to} was elicited by 300-msec depolarizing pulses from −50 mV to +50 mV at 0.1 Hz. Recordings are shown under control conditions (panel A) and then in the presence of 10 μM (panel B) and 50 μM (panel C) ambasilide, followed by washout (panel D).

Mean (± S.E.M.) I_{to} current amplitude in eight cells studied under control conditions, in the presence of 10, 50 and 100 μM ambasilide, and after 20 min of drug washout are shown in figure 2A. We were unable to study ambasilide concentrations higher than 100 μM because of limited drug solubility. The drug produced a concentration-related inhibition of I_{to}, which was fully reversed by washout. Drug effects were significant at $P < .01$ at all voltages for the two higher concentrations and at $P < .05$ at all voltages at the 10 μM concentration. Current amplitudes upon washout were not significantly different from those under control conditions at any voltage.

Figure 2B shows the mean percentage change in I_{to} at each TP for each drug concentration. Although significant changes from control were seen at all voltages (asterisks in figure), the effects of the drug were voltage-independent at all concentrations. Figure 2C shows the concentration dependence of drug inhibition of I_{to} on depolarization to +40 mV. The curve in figure 2C is the best-fit equation of the form

$$E = E_{max}\{1/[1 + (EC_{50}/C)^n]\}$$

where E is the effect at any concentration C, E_{max} is the maximal effect, EC_{50} is the concentration for half-maximal effect and n is the Hill coefficient. This equation was applied to data from each of the eight experiments in which I_{to} was recorded before and after ambasilide at each of the four concentrations shown. The mean EC_{50} was 22.6 ± 1.9 μM, with an average E_{max} of 62.4 ± 6.5% inhibition and a mean n of 1.6 ± 0.2.

The voltage-dependence of I_{to} activation and inactivation was evaluated in six myocytes each with the voltage protocols (applied at 0.1 Hz) shown in figure 2D. Inactivation was

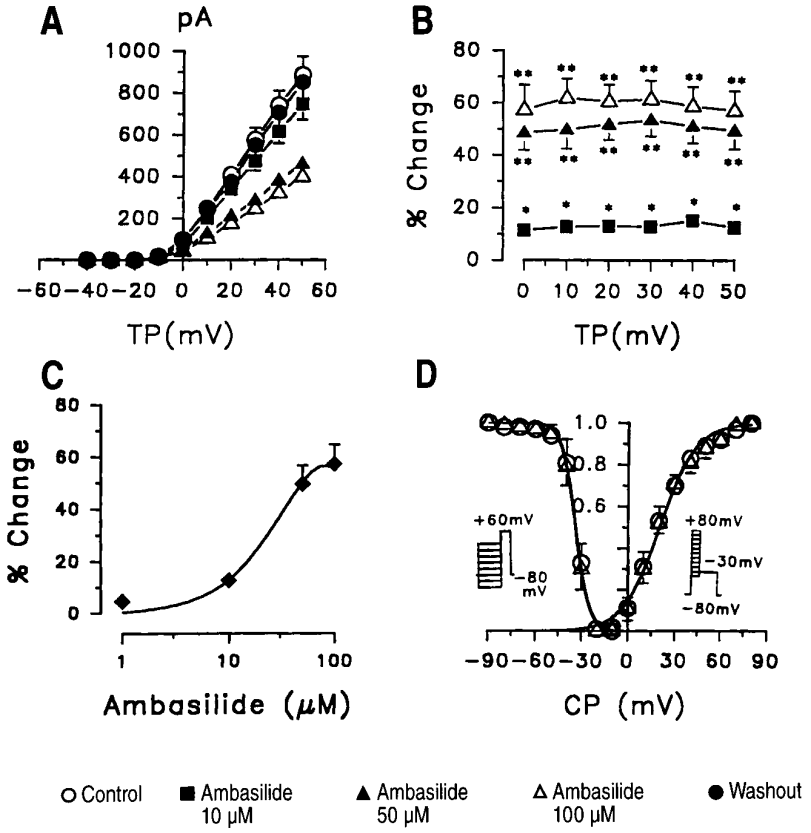

Fig. 2. A) Current-voltage relations under control, 10, 50 and 100 μM ambasilide and washout conditions (mean ± S.E.M., n = 8). B) Percent reduction in I_{to} (relative to control at the same voltage). *P < .05; **P < .01 *vs.* control (n = 8). C) Concentration-response relation for ambasilide effect on I_{to} at +50 mV. Symbols represent experimental data (mean ± S.E.M., n = 6 cells exposed to all concentrations); solid line is best-fit equation of the form $E = E_{max}(1 + [K/C]^n)$, where E is the effect at concentration C, E_{max} is the maximum effect, K is the concentration for half-maximal action, and n is the Hill coefficient. D) Mean data for voltage dependence of I_{to} inactivation and activation in the absence (circles) and presence (triangles) of 50 μM ambasilide (mean ± S.E., n = 6). Inactivation was assessed with a two-pulse protocol, with a 1-sec prepulse to voltages between −120 and +20 mV followed by a 1-sec test pulse to +60 mV. Activation was assessed by the tail current at −30 mV after 5-msec depolarizations to a variety of test potentials. The curves shown are fits of mean data by Boltzmann distribution equations (see text).

evaluated with 1-sec CPs from voltages between −120 and +20 mV, followed by a 1-sec test pulse to +60 mV. Activation was analyzed on the basis of tail currents on repolarization to −30 mV after a 5-msec conditioning pulse from −80 mV to potentials between −20 and +80 mV. Mean data for activation and inactivation, along with best-fit Boltzmann distribution curves, under control conditions and in the presence of 100 μM ambasilide are shown in figure 2D. The equation used for curve fitting was

$$I/I_{max} = 1/\{1 + \exp[(V_{1/2} - V)/k]\}$$

where I is I_{to} amplitude (for inactivation) or tail current (for activation) at a conditioning pulse voltage V, $V_{1/2}$ is the voltage for half-maximal activation and k is a slope constant (positive for activation, negative for inactivation). Mean values for $V_{1/2}$ and k under control conditions were −33.5 ± 3.4 mV and −4.5 ± 0.6 mV, respectively, for inactivation and were +20.4 ± 2.2 mV and +11.5 ± 0.9 mV for activation. In the presence of 50 μM ambasilide, corresponding values were −34.1 ± 3.3 mV and −4.5 ± 0.5 mV for inactivation and +20.8 ± 2.1 mV and 11.6 ± 0.9 mV for activation, respectively. Thus ambasilide did not alter the voltage dependence of I_{to}.

Effects of ambasilide on I_{Kur}. The effects of ambasilide on I_{Kur} in a representative myocyte are illustrated in figure 3A. I_{Kur} was recorded with the use of the protocol shown in the inset, including a 1-sec prepulse to +40 mV to inactivate I_{to}, followed by a 160-msec test pulse delivered at 0.1 Hz to a variety of potentials between −40 and +50 mV (results at +50 mV are shown in the figure). Current under control conditions (fig. 3A) shows the rapid activation with little or no inactivation typical of I_{Kur}. Ambasilide (100 μM) caused a substantial reduction in both step current elicited by depolarization and tail current at −20 mV. The effect of ambasilide was qualitatively similar to, although quantitatively somewhat less than, that of 4AP at a concentration (5 mM) we have previously shown to block I_{Kur} fully (Wang *et al.*, 1993b). The drug-sensitive difference currents shown in figure 3B indicate the similar morphology of the current inhibited by ambasilide and 4AP. Figure 3C shows the mean ± S.E.M. current-voltage relationships of step current sensitive to 100 μM ambasilide (▲) and 5 mM 4AP (△), which were quite similar in form.

Figure 3D shows an analysis of the concentration-dependence of ambasilide effects on I_{Kur} at different test potentials, based on step currents recorded with the protocol illustrated in figure 3A. Ambasilide produced a concentration-dependent inhibition of I_{Kur} that was reversible upon drug washout. The percent change in current produced by the drug is shown as a function of test potential in figure 3E. Although ambasilide produced significant changes relative to control at all concentrations and every voltage (indicated by the asterisks), there was no significant voltage-dependence of drug action. Figure 3F shows the best-fit concentration-response relation for inhibition of I_{Kur} step current at +50 mV (open diamonds) with the use of a relation of the form $E = E_{max}\{1/[1 + (EC_{50}/C)^n]\}$, as presented above. When fitted to data in each experiment, this relation provided mean values of 75 ± 7% for E_{max} and 34.2 ± 2.9 μM for EC_{50} in six cells.

One problem in analyzing I_{Kur} step current data is that in measuring relative to the zero current level, it is assumed that no other currents are present. Although a variety of currents, including I_K, I_{to}, I_{K1}, $I_{K,ACh}$, I_{Na} and I_{Ca}, are inhibited by the contents of the superfusate and/or the voltage

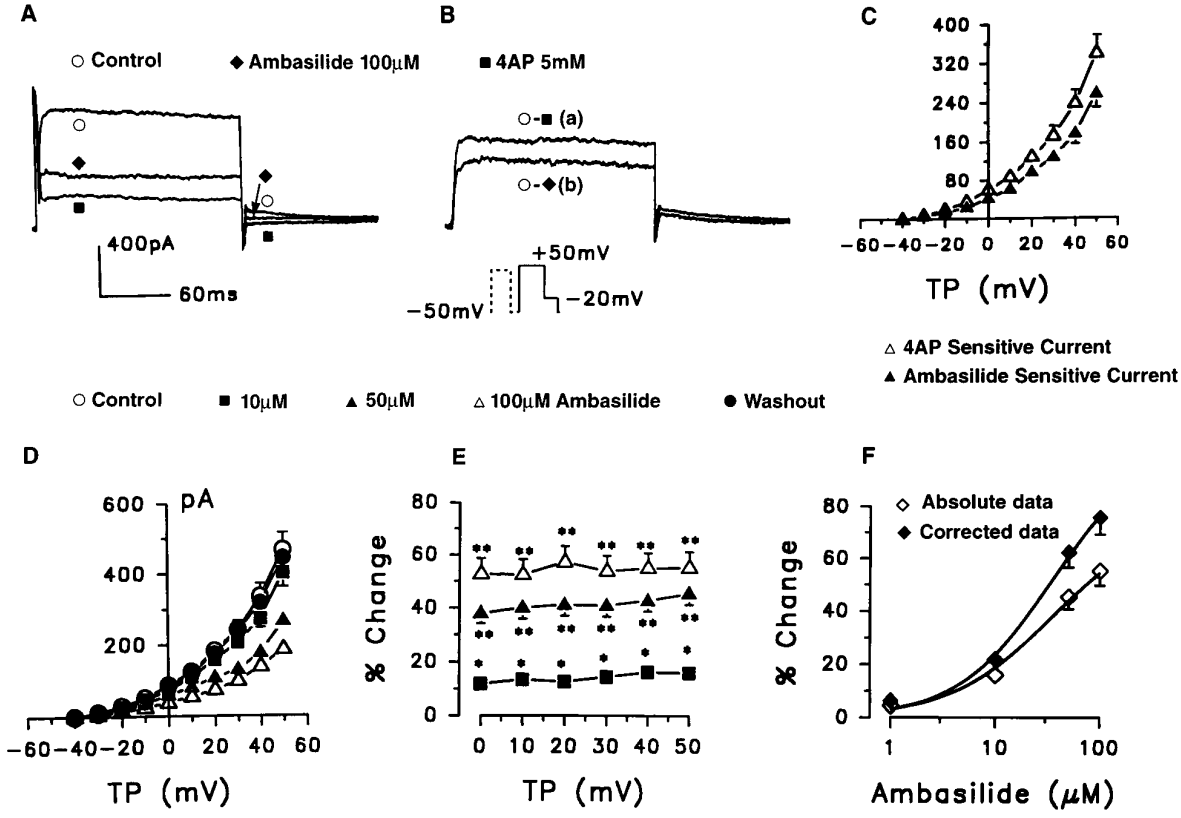

Fig. 3. A) I_{Kur} recorded from one cell with the protocol shown in the inset of panel B, under control conditions and then after exposure to 100 μM ambasilide and 5 mM 4AP. B) Currents sensitive to 5 mM 4AP (a) and to 100 μM ambasilide (b), based on digital subtraction of currents in the presence of drug from those in the absence of drug in panel A. C) Current-voltage relation of 4AP- and ambasilide-sensitive current obtained as illustrated in panels A and B. D) Current-voltage relations for step current under control, 10 (■), 50 (▲) and 100 (△) μM ambasilide and washout conditions (mean ± S.E.M., $n = 8$). E) Percent reduction in I_{Kur} (relative to control at the same voltage). *P < .05; **P < .01 *vs.* control ($n = 8$). F) Concentration-response relation for ambasilide inhibition of I_{Kur} at +50 mV. Open symbols represent experimental data (mean ± S.E.M., $n = 6$ cells exposed to all concentrations); solid lines are best-fit concentration-response equations. Filled symbols show data corrected for nonspecific component by calculating ambasilide's effects as (b/a) × 100%, where b is current inhibited by the drug at a given concentration and a is current inhibited by 5 mM 4AP, obtained as shown in figure 3B.

protocol, other conductances, such as that of the time-independent nonselective cation channel (Crumb *et al.*, 1995), can carry current upon depolarization. To obtain a more precise indication of the amplitude of I_{Kur} step current, and of the effect of ambasilide on it, we analyzed the effect of 100 μM ambasilide on 4AP-sensitive current obtained as illustrated in figure 3A to C. Because a depolarizing prepulse was used, I_{to} was suppressed and the only 4AP-sensitive component was I_{Kur}. Figure 3B shows 4AP-sensitive current (a), obtained by digital subtraction of current in the presence of 4AP from control, and ambasilide-sensitive current (b), obtained by subtraction of current in the presence of ambasilide from control. For each cell, ambasilide's effect on I_{Kur} can be calculated specifically by relating ambasilide-sensitive current (b in fig. 3B) to 4AP-sensitive current (a in fig. 3B) and expressing the ambasilide effect (in terms of percent change in I_{Kur}) as (b/a) × 100. When this is done, the results shown by the filled diamonds in figure 3F are obtained. When the corrected concentration-response data were fitted by the E_{max} equation given above, the EC_{50} averaged 34.7 ± 3.1 μM, and E_{max} for I_{Kur} inhibition averaged 103% ± 9%.

In order to obtain an independent estimate of the magnitude of the specific ambasilide effect on I_{Kur}, we analyzed time-dependent tail currents as shown in figure 4. I_{Kur} tail currents were recorded with 180-msec conditioning depolarizations to potentials between −40 and +50 mV, followed by repolarization to −20 mV for 120 msec. Because I_K is negligible as a result of the short pulse duration, the presence of TEA in the superfusate, and study at room temperature, and because I_{to} is fully inactivated by the end of the conditioning pulse, I_{Kur} is the only component that gives rise to the tail current. Figure 4A shows typical currents recorded with this protocol and indicates a concentration-dependent inhibitory effect of the drug on tail currents. Figure 4B shows the relation between tail current and CP potential in five cells under control conditions, in the presence of various ambasilide concentrations and after drug washout. The drug produced a concentration-dependent inhibition of tail current at all voltages, an effect that was completely reversible upon washout. Drug effects were significant at all voltages and concentrations and were similar at all voltages (fig. 4C). Figure 4D shows the concentration-dependence for inhibition of I_{Kur} tail current elicited by a depolarization to +50 mV, as determined in five cells. The best-fit E_{max} equation is shown by the solid line, which agrees very closely with the concentration-response relation obtained from corrected step currents (filled diamonds in fig. 3F), whose best-fit concentration-response curve is reproduced as the dashed line in figure 4D. Overall, the tail current analysis provides mean values of 104 ± 10% for E_{max} and 34 ± 3 μM for EC_{50}.

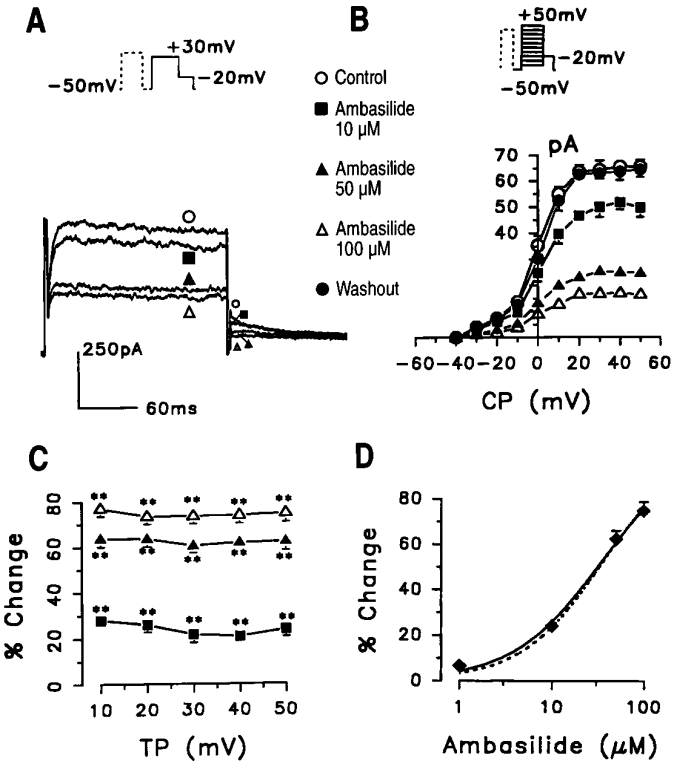

Fig. 4. Effects of ambasilide on I_{Kur} tail currents. A) Recordings of I_{Kur} obtained in one cell with the voltage protocol shown in inset, under control, ambasilide (10, 50 and 100 μM) and washout conditions. B) Current-voltage relations (mean ± S.E.M. for five cells exposed to each condition) for I_{Kur} tail currents recorded as shown in the inset. C) Percent change in I_{Kur} tail current produced by various concentrations of ambasilide. **P < .01 *vs.* control at same voltage. D) Concentration-response relation for ambasilide inhibition of tail currents. Symbols are mean data at +50 mV; solid line is best-fit concentration-response curve. Dashed line is concentration-response curve for drug effects on step current, corrected for 4AP-sensitive component as shown by filled symbols and corresponding curve fit in figure 3F.

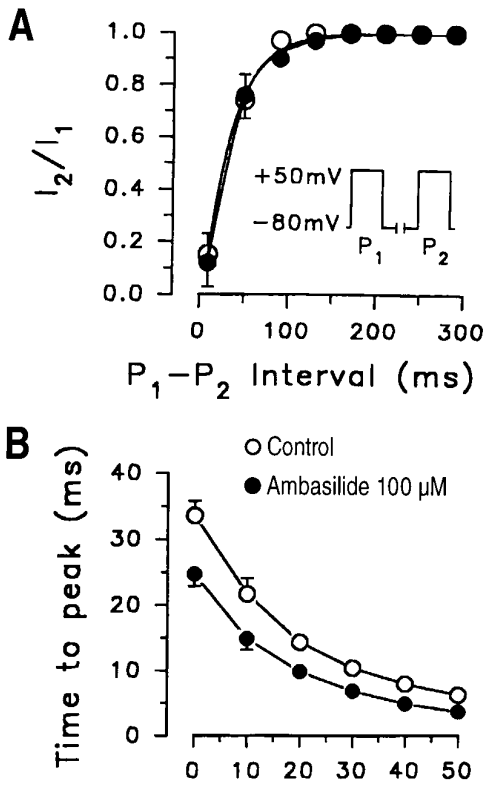

Fig. 5. A) Time course of recovery from inactivation of I_{to} in absence (open circles) and presence (closed circles) of 100 μM ambasilide obtained with protocol in inset. Results are mean ± S.E.M. of five cells studied under both conditions. Best-fit exponential recovery curves are provided. B) Time to peak of I_{to} as a function of test potential under control conditions and in the presence of 100 μM ambasilide (mean ± S.E.M., $n = 6$, reduction by drug significant at P < .01 for all voltages).

Possible effects of ambasilide on I_{Kur} activation were evaluated by fitting tail current data in each experiment with the Boltzmann distribution equation provided above. Under control conditions, values for $V_{1/2}$ and k averaged −4.0 ± 0.4 mV and 7.5 ± 0.8 mV, respectively, whereas in the presence of 100 μM ambasilide, the corresponding values were −3.9 ± 0.4 mV and 7.2 ± 0.7 mV, respectively. Thus ambasilide did not alter the voltage dependence of I_{Kur} activation.

State-dependence of ambasilide actions. The above results show that ambasilide inhibits I_{to} and I_{Kur} in a concentration-dependent and voltage-independent fashion. To evaluate further the possibility of state-dependent blocking actions, we assessed the time-dependence of block. If ambasilide interacted preferentially with open or inactivated I_{to} channels with recovery from the rested state slower than spontaneous recovery from inactivation, then slowed recovery after a depolarizing pulse would result. Figure 5A presents an analysis of the time-dependent recovery of I_{to} as determined with the two-pulse protocol shown in the inset. Mean values for I_{to} of the test pulse (P_2) normalized to current during a basic pulse (P_1, at 0.1 Hz) are shown as a function of the P_1 to P_2 interval in six cells. The best-fit exponential curves to each set of data are shown; they are very similar. Exponential curve fitting to recovery data in each cell provided recovery time constants of 31.8 ± 2.9 and 33.2 ± 3.0 msec under control and 100 μM ambasilide conditions, respectively, which indicated no change in I_{to} recovery kinetics.

The results shown in figure 5A do not exclude state-dependent actions of ambasilide; they simply indicate that if such actions exist, then unblocking upon repolarization cannot be substantially slower than spontaneous recovery from inactivation. Visual inspection of I_{to} recordings in figure 3 suggests that I_{to} attains a peak and inactivates more rapidly in the presence of higher concentrations of ambasilide than in the absence of the drug. Figure 5B shows an analysis of the time from the onset of depolarization to peak I_{to} before and after exposure to 100 μM ambasilide in six cells. Ambasilide significantly decreased the time to peak current at all voltages, a result consistent with an open-channel blocking action (Wang *et al.*, 1995).

To evaluate further the possibility that ambasilide causes open-channel block of I_{to}, we evaluated the inactivation of I_{to} in the absence and presence of ambasilide. Figure 6A shows a representative recording of I_{to} upon depolarization to 50 mV for 300 msec under control conditions. Current inactivation was well fitted by a monoexponential relation as shown. In the presence of 50 μM ambasilide, a monoexponential relation no longer fitted I_{to} well, as illustrated by the best-fit monoexponential relation in figure 6B. A biexponential relation, on the other hand, fitted the data well (fig. 6C). Similar findings were obtained in all experiments in the presence of

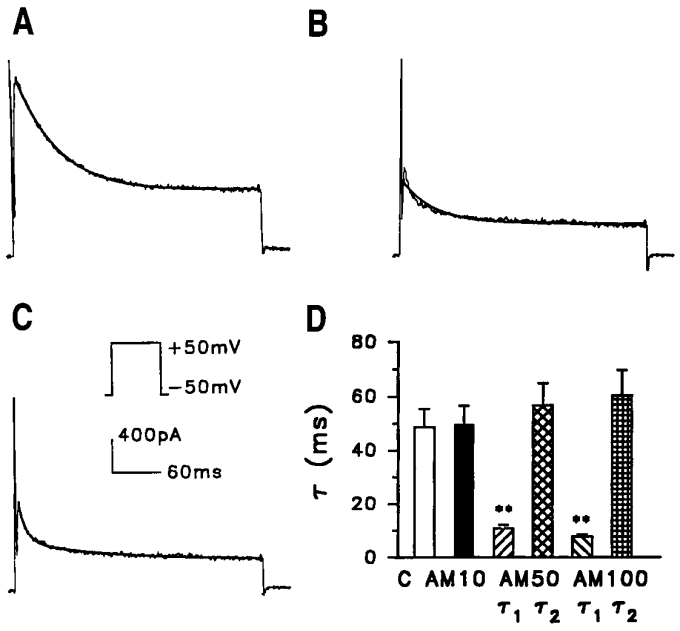

Fig. 6. Effects of ambasilide on the time course of I_{to} inactivation. Representative recordings from one cell upon depolarization to +50 mV under control conditions (panel A) and in the presence of 50 μM ambasilide (panels B and C). Best-fit monoexponential relations to raw data are shown in panels A and B, whereas panel C shows a biexponential fit to results in the presence of ambasilide. D) Time constants of I_{to} inactivation under control and drug conditions. C = control; AM10, AM50 and AM100 = 10, 50 and 100 μM ambasilide, respectively. **P < .01 *vs.* control.

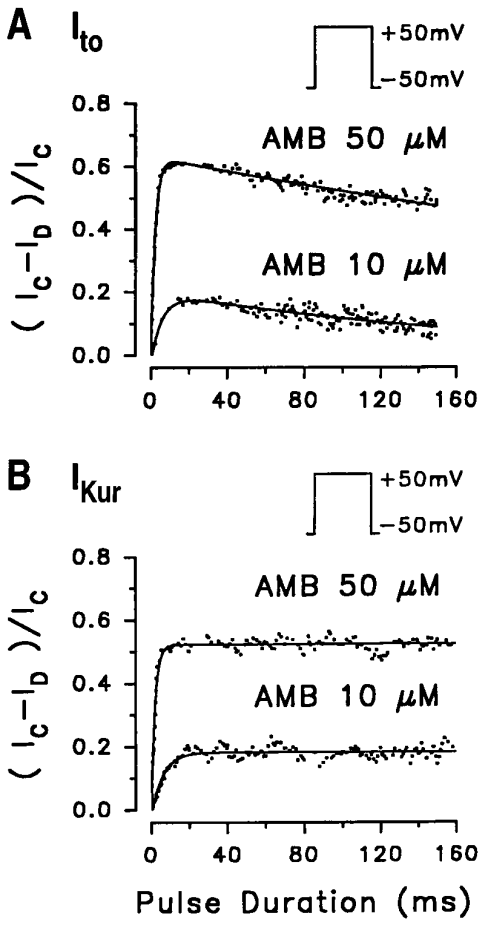

Fig. 7. The time course of development of inhibition of I_{to} (panel A) and I_{Kur} (panel B) by 10 and 50 μM ambasilide upon depolarization to +50 mV from a holding potential of −50 mV. Reductions in current over time are expressed as changes from control values (I_C) to values in the presence of ambasilide (I_D). The curves are least-squares fits to the equations $f = a + b \times [1 - \exp(-t/\tau_1)] \times \exp(-t/\tau_2)$ in panel A and $f = a - b \times \exp(-t/\tau)$ in panel B. The average time constants are as follows: for I_{to}, τ_1 6.4 ± 0.5 msec and 2.8 ± 0.3 msec, τ_2 525 ± 60 msec and 588 ± 62 msec at 10 and 50 μM ambasilide, respectively; for I_{Kur}, 6.5 ± 0.8 msec and 2.7 ± 0.3 msec at 10 and 50 μM ambasilide.

50 and 100 μM ambasilide. Mean rate constants for six cells studied under control conditions and at each drug concentration are shown in figure 6D. In the presence of 10 μM ambasilide, the time constant of inactivation was not altered. At higher concentrations, there is a slower component with a time constant similar to that under control conditions and a faster component whose time constant decreases with increasing drug concentration. These results are consistent with high-affinity open-channel block causing rapid current decay in a concentration-dependent fashion.

The possibility of open-channel block was further pursued with the analysis shown in figure 7. The pulse protocols shown were used to record I_{to} (fig. 7A) and I_{Kur} (fig. 7B) under control conditions and in the presence of 10 and 50 μM ambasilide. Drug-induced block was then plotted as a function of time after the onset of the pulse. For I_{Kur}, the results shown were obtained in a cell lacking I_{to}, which we have shown to have the same I_{Kur} properties as cells possessing I_{to} (Wang *et al.*, 1993b), in order to avoid potential complicating effects of the prepulse. Block was found to develop in a time-dependent fashion, with an exponential onset as shown by the curve fits in the figure. The rate of block development increased with increasing concentration; time constants at 10 and 50 μM averaged 5.8 ± 0.8 and 2.5 ± 0.4 msec for I_{to} and 6.1 ± 0.8 and 2.1 ± 0.3 msec for I_{Kur}, respectively. In the case of I_{to}, there was also a slower time-dependent unblocking phase during sustained depolarization, similar to previous observations with 4AP (Wang *et al.*, 1995).

The time-dependent onset of block is consistent with an open-channel blocking mechanism. The rate constant for block onset should equal $kD + l$, where k is the rate constant for drug binding to open channels, D is drug concentration and l is the unbinding rate constant. With the use of the experimentally determined mean values indicated above, k and l for ambasilide block of I_{to} and I_{Kur} can be estimated, and doing so yields values of 0.0057 μM^{-1} $msec^{-1}$ and 0.166 $msec^{-1}$, respectively, for I_{to} and 0.0078 μM^{-1} $msec^{-1}$ and 0.156 $msec^{-1}$ for I_{Kur}. The rate constants for drug binding and unbinding can be used to estimate the dissociation constant for the drug-channel interaction, and doing so yields values of 20 μM for I_{Kur} and 29 μM for I_{to}. These values are of the same order of magnitude as the directly (and independently) determined EC_{50} values of 34 and 23 μM for I_{Kur} and I_{to}.

Discussion

We have shown that the class III antiarrhythmic agents E-4031 and *d*-sotalol do not alter I_{to} or I_{Kur} in human atrial myocytes. On the other hand, the experimental class III drug ambasilide inhibited both currents. These results point to the

possibility of developing class III antiarrhythmic agents with a different profile of channel blocking actions from previous class III drugs, whose use has been limited by proarrhythmic properties (Hondeghem and Snyders, 1990).

Comparison with previous studies of ionic mechanisms of the drugs studied. Several studies have indicated that E-4031 is a highly selective blocker of I_{Kr} in animal cells (Sanguinetti and Jurkiewicz, 1990; Colatsky *et al.*, 1990). The present study extends the specificity of E-4031 action by excluding a blocking effect on I_{Kur} and I_{to} in human atrium. The specificity of sotalol's blocking action has been less clear. Carmeliet (1985) showed a high degree of selectivity for I_K but significant effects on other currents at higher concentrations. Sanguinetti and Jurkiewicz (1990) showed a high degree of sotalol selectivity for the I_{Kr} component, but other studies have suggested relatively strong effects on I_{to} (Berger *et al.*, 1989). The present study indicates that E-4031 and *d*-sotalol, even at very high concentrations, have no effect on human atrial I_{to} or I_{Kur}.

Zhang *et al.* (1992) showed that ambasilide affects I_K in guinea pig ventricular myocytes in a fashion that differs from that of E-4031 and indicates significant block of I_{Ks}. The present observations indicate that ambasilide also causes significant inhibition of human atrial I_{to} and I_{Kur} at concentrations comparable to those that affect I_K in guinea pig (Zhang *et al.*, 1992).

Potential significance of our findings. Several newer class III agents appear to block the rapid component of I_K with relatively high selectivity (Colatsky *et al.*, 1990). These agents tend to have strong reverse use-dependent effects on repolarization (Wang *et al.*, 1994b; Hondeghem and Snyders, 1990; Jurkiewicz and Sanguinetti, 1993; Colatsky and Argentieri, 1994), which are associated with important risks of proarrhythmic reactions because of excessive delays in repolarization at slow HR (Hondeghem and Snyders, 1990; Nattel and Zeng, 1984). Balser *et al.* (1991) and Zhang *et al.* (1992) have suggested that class III agents without selectivity for I_{Kr} may have a more favorable profile of rate-dependent actions. *In vivo* studies suggest that amiodarone and ambasilide do, indeed, have less reverse use-dependent actions on repolarization than highly selective I_{Kr}-blocking compounds (Wang *et al.*, 1994b; Sager *et al.*, 1993). The present work points to another potentially interesting action of class III drugs: blockade of currents particularly important in repolarizing human atrial cells. Both I_{to} and I_{Kur} have been shown to play important roles in human atrial repolarization (Shibata *et al.*, 1989; Escande *et al.*, 1987; Wang *et al.*, 1993b). Furthermore, I_{Kur} appears to be absent in human ventricle (Li *et al.*, 1996). Therefore, blockade of I_{Kur} and/or I_{to} may be an advantageous property for class III compounds.

Mechanisms of channel blocking action. We found that ambasilide produced time-dependent block of I_{to} and I_{Kur}, a result that suggests an open-channel blocking mechanism. These properties are similar to blocking mechanisms we noted previously for quinidine on I_{to} and I_{Kur} (Wang *et al.*, 1995). Koidl *et al.* (1996) recently reported effects of ambasilide on rapidly and slowly inactivating components of I_{to} in human atrial cells. They observed inhibiting effects of the drug on each component, which they interpreted as representing blocking actions on I_{to} and I_{Kur}, respectively. They noted that ambasilide accelerated I_{to} inactivation, decreasing the time constants of both phases. The acceleration of I_{to} inactivation that they noted is compatible with an open-channel blocking action, as demonstrated in the present study. Although Koidl *et al.* (1996) suggested that the effect of ambasilide on the slowly inactivating component of I_{to} may be due to an effect on I_{Kur}, they did not study the latter directly. Our findings indicate that the effects of ambasilide they hypothesized on I_{Kur} do, in fact, occur and that the acceleration of inactivation they noted may be due to open-channel blockade. Unlike Koidl *et al.* (1996), we did not observe a slowly inactivating component of I_{to} in human atrial myocytes. The difference is probably due to differences in bath temperature. We studied currents at room temperature in order to observe accurately the rapid activation of I_{Kur} (Wang *et al.*, 1993b), whereas Koidl *et al.* (1996) worked at 37°C, at which temperature I_{Kur} inactivation might accelerate enough to be measurable during a 300-msec depolarizing pulse.

Potential limitations. Studies in native myocytes always present difficulties in terms of isolating the currents of interest. I_{to} is relatively distinct in terms of its rapid inactivation. I_{Kur} is more difficult to isolate, and we have used two previously described approaches (Wang *et al.*, 1993b): a depolarizing pulse to inactivate I_{to} and sensitivity to 4AP to identify the highly 4AP-sensitive I_{Kur} component. In addition, we analyzed effects on I_{Kur} tail currents and obtained results that were qualitatively consistent with the different methods used. The use of cloned channels in expression systems allows for clearer study of single currents but is limited by uncertainties regarding the relationship between cloned channels and their native counterparts, as well as by potential distortions due to differences between model expression systems and native tissues in the properties of membranes and in important intracellular regulatory processes.

I_{to} inhibition by ambasilide was incomplete even at maximally effective drug concentrations. This finding may have been due in part to limited solubility of the drug, which prevented us from using higher concentrations. On the other hand, like previous drugs that we have studied (Wang *et al.*, 1995), ambasilide required channel opening in order to produce block. Activation of I_{to} was faster than the rate of block development, and recovery from block was rapid. Therefore, at the time of peak I_{to}, ambasilide block was significantly less than when steady state was reached later in the pulse, so changes in peak current underestimate steady-state drug effects on the current at any given concentration.

The EC_{50} values for ambasilide block of I_{to} and I_{Kur} were in the range of 20 to 30 μM, and statistically significant effects on both were observed at a concentration of 10 μM. We were unable to find published reports of the drug's therapeutic concentrations in the human, but in previous studies in a dog model of atrial fibrillation (Wang *et al.*, 1994b), we found that the drug's therapeutic concentration was approximately 15 μM. Thus effective ambasilide concentrations would be expected to inhibit I_{to} and I_{Kur} significantly. Ambasilide also inhibits I_K, with 50% inhibition of I_{Kr} at about 5 μM and about 20% reduction in I_{Ks} at 10 μM (Zhang *et al.*, 1992). The drug's effect at therapeutic concentrations is therefore likely to result from actions on a variety of K^+ currents, including both components of I_K as well as I_{Kur} and I_{to}. It remains to be determined whether block of I_{Kur} and/or I_{to} gives clinical advantages to I_K-blocking class III drugs, such as ambasilide, over class III drugs (such as E-4031 and *d*-sotalol) that block

I_K without inhibiting I_{Kur} or I_{to}. Furthermore, it remains to be established whether class III drugs can be developed that act exclusively on IKur and/or I_{to} without affecting I_K.

Acknowledgments

The authors thank Johanne Doucet for technical assistance and Luce Bégin for typing the manuscript.

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Send reprint requests to: Dr. Stanley Nattel, Montreal Heart Institute, 5000 Belanger St. East, Montreal, Quebec, Canada H1T 1C8.