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Action potential changes associated with a slowed inactivation of cardiac voltage-gated sodium channels by KB130015

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1 We have studied the acute cardiac electrophysiological effects of KB130015 (KB), a drug structurally related to amiodarone. Membrane currents and action potentials were measured at room temperature or at 37°C during whole-cell patch-clamp recording in ventricular myocytes. Action potentials were also measured at 37°C in multicellular ventricular preparations.

2 The effects of KB were compared with those of anemone toxin II (ATX-II). Both KB and ATX-II slowed the inactivation of the voltage-gated Na^+ current (I_{Na}). While KB shifted the steady-state voltage-dependent inactivation to more negative potentials, ATX-II shifted it to more positive potentials. In addition, while inactivation proceeded to completion with KB, a noninactivating current was induced by ATX-II.

3 KB had no effect on I_{K1} but decreased $I_{Ca\text{-}L}$ The drug also did not change I_{to} in mouse myocytes.

4 The action potential duration (APD) in pig myocytes or multicellular preparations was not prolonged but often shortened by KB, while marked APD prolongation was obtained with ATX-II. Short APDs in mouse were markedly prolonged by KB, which frequently induced early afterdepolarizations.

5 A computer simulation confirmed that long action potentials with high plateau are relatively less sensitive to a mere slowing of I_{Na} inactivation, not associated with a persisting, noninactivating current. In contrast, simulated short action potentials with marked phase-1 repolarization were markedly modified by slowing I_{Na} inactivation.

6 It is suggested that a prolongation of short action potentials by drugs or mutations that only slow I_{Na} inactivation does not necessarily imply identical changes in other species or in different myocardial regions.

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Abbreviations: APD, action potential duration; EAD, early afterdepolarization; $I_{Ca\text{-}L}$, L-type Ca^{2+} current; I_{K1}, inward-rectifying background K^+ current; I_{Na}, voltage-dependent Na^+ current; I_{to}, transient outward K^+ current; KB, KB130015 ([2-methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran])

Introduction

A dysfunction of voltage-dependent Na^+ channels is the basis for many congenital cardiac, skeletal muscle and neurological diseases (Balser, 2001; Goldin, 2001). Several genetic mutations in the cardiac Na^+ channel have been detected, which result in altered inactivation and may cause a specific type of the long QT syndrome, a condition associated with high propensity to arrhythmias (for recent reviews, see Bennett, 2000; Chiang & Roden, 2000; Li *et al*., 2000; Bezzina *et al*., 2001; Grant, 2001; Miyamoto *et al*., 2001). In addition to slowing inactivation, these mutations are also frequently associated with a persisting, noninactivating opening of a small fraction of Na^+ channels. The exact roles played by these two factors in causing the long QT syndrome are not fully clear.

Slowed inactivation of Na^+ channels may also be induced by pharmacological agents. Na^+-channel modifying agents (e.g. DPI-201-106, BDF-9148, BDF-9196, BDF-9198, etc.) have been proposed as possible new pharmacological tools in the treatment of congestive heart failure (Muller-Ehmsen *et al*., 1997; 1998; Flesch & Erdmann, 2001), since they are expected to increase the intracellular Na^+ concentration ($[Na^+]_i$), with a consequent positive inotropic action *via* an effect on Na^+ – Ca^{2+} exchange. However, the beneficial use of these agents is limited by a potential arrhythmogenic action (Stump *et al*., 2000), attributed to their ability to prolong the action potential duration (APD) and the QT interval on the electrocardiogram (Amos & Ravens, 1994; Stump *et al*., 2000; Flesch & Erdmann, 2001). Since many Na^+-channel modifying agents that slow inactivation also induce a noninactivating current, it would be of interest to identify or design drugs which display either one of these electrophysiological properties, in order to assess their relative importance in the prolongation of the APD and of the QT interval.

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KB130015 (KB) is a new drug, synthetically derived by chemical substitution from amiodarone (Carlsson *et al*., 2002). The drug has been designed with the purpose of retaining the antiarrhythmic effects of amiodarone but without the side effects. We have shown recently that KB causes the following effects on Na^+ channels: (1) a marked slowing of the inactivation, (2) a shift of the voltage-dependent inactivation to more negative potentials, (3) a slowing of the recovery from inactivation, and (4) a shift of the threshold for activation to more negative potentials (Macianskiene *et al*., 2003). The Na^+ channel inactivation, although slowed by KB, still preceded to completion during maintained depolarization. Hence the drug could be used to test the consequences of slowed Na^+ channel inactivation in the absence of persisting, noninactivating current. In the present study, we examined the extent to which the slowing of Na^+ channel inactivation by KB impacts on the APD.

Methods

The measurements were performed on ventricular isolated, single myocytes, and on ventricular multicellular preparations. The study has been carried out in accordance with the Declaration of Helsinki and with the institutional guidelines for the care and use of laboratory animals.

Single cells and multicellular preparations

In most experiments, we used pig or mouse ventricular myocytes. Cardiomyocytes from other species (rabbit, guinea-pig, man) were also obtained occasionally. The methods used for cell dissociation have been described before (Stankovicova *et al*., 2000; Hernandez-Benito *et al*., 2001; Macianskiene *et al*., 2002), and involved the arterial perfusion, at 37°C, of a piece of the (pig or human) left ventricular wall or of the whole (mouse, rabbit, or guinea-pig) heart with a Ca^{2+}-free Tyrode solution containing protease (0.1 mg ml^{-1}; type XIV, Sigma) and collagenase (1.4 mg ml^{-1}; type A, Boehringer, Manheim, Germany). Dissociated cells were stored at room temperature (21–22°C). Experiments on single myocytes were carried out at room temperature or, when explicitly mentioned, at 37°C.

Multicellular preparations consisted of pig left ventricular trabecular muscles or mouse thin papillary muscles. They were mounted in an experimental bath (see Mubagwa *et al*., 1997) and were superfused with standard Tyrode solution (see composition below) at 37°C.

Ion currents in myocytes

Measurements in single cells were carried out in the whole-cell patch-clamp recording mode (see Macianskiene *et al*., 2003), using heat-polished borosilicate glass electrodes (1–3 MΩ, when filled with internal solution) connected to an Axopatch 200A or 200B amplifier (Axon Instruments, Foster City, CA, U.S.A.). An analog-to-digital interface controlled by the pClamp 8.1 software (Axon Instruments) was used to generate command pulses and acquire data.

Various ion currents were measured using appropriate voltage protocols. To measure the voltage-dependent Na^+ current (I_{Na}), step depolarizations were given every 1 s from a holding potential (V_H) of −80 mV. To measure the L-type Ca^{2+} current ($I_{Ca\text{-}L}$), a 1-s prepulse to −40 mV was used (to inactivate Na^+ and eventual T-type Ca^{2+} channels) before activating the current and the protocol was repeated every 5 s. During measurements of I_{Na} or $I_{Ca\text{-}L}$, K^+ currents were blocked by substituting intracellular and extracellular K^+ with Cs^+. The transient outward K^+ current (I_{to}) was measured as described previously (Hernandez-Benito *et al*., 2001).

Action potentials in myocytes and in multicellular preparations

Action potentials in isolated cardiomyocytes were measured under current-clamp in ruptured or perforated patch recording modes. The stimulus consisted of a 2-ms rectangular current pulse applied at 0.5–3 Hz *via* the patch electrode.

Action potentials in multicellular preparations were induced by electrically stimulating with the help of a punctate Ag–AgCl electrode placed on the surface of the tissue. Membrane potentials were measured with microelectrodes filled with 3 M KCl (resistance: 20–30 MΩ) connected to a GeneClamp500 (Axon Instruments) amplifier. The signals were digitized at 4 kHz with WindAq (DataQ Instruments; Akron, OH, U.S.A.). APDs were measured at 50% (APD_{50}) and 90% (APD_{90}) repolarization.

Solutions and drugs

The composition of the standard Tyrode solution used during cell isolation and during action potential measurements was (in mM): 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.9 $MgCl_2$, 0.33 NaH_2PO_4, 10 Na-HEPES (pH 7.4) and 10 glucose. The solution was bubbled with 100% O_2 during cell dissociation or during experiments on tissues. To measure I_{Na}, a low-$[Na^+]_o$ Tyrode (composition in mM: 10 NaCl, 135.4 CsCl, 2.6 $MgCl_2$, 0.1 $CaCl_2$, 0.33 NaH_2PO_4, 10 Cs-HEPES and 10 glucose; pH 7.4) was used to optimize voltage control. During measurements of L-type Ca^{2+} currents ($I_{Ca\text{-}L}$), the extracellular solution was identical to the normal Tyrode solution except that K^+ was replaced with Cs^+. To measure I_{to} without interference from drug-modified I_{Na}, Na^+-free solutions were used, in which Na^+ was completely replaced by NMDG, while $[Ca]_o$ was decreased to 0.18 mM. The patch pipette solution contained (in mM): 155 CsCl or KCl, 1 $MgCl_2$, 5 Na_2ATP or MgATP, 1 EGTA, 0.1 Na_2GTP, 5 Cs-HEPES or K-HEPES (pH 7.25). For perforated-patch recordings, 325 μM amphotericin B and 1.8 mM $CaCl_2$ (to cause cell death in case of patch rupture) were added to the pipette solution.

KB was obtained from Karo-Bio, Huddingen, Sweden. Anemone toxin II (ATX-II; from *Anemonia sulcata*) and all other drugs or products were from Sigma Chemie (Bornem, Belgium). Stock solutions (10–100 mM) of KB were prepared in DMSO, which had no effect of its own at the highest concentration used (0.1%, v v^{-1}).

Data analysis

Data were analyzed using Clampfit (Axon Instruments) or Origin (Microcal, Northampton, MA, U.S.A.). Average data are expressed as mean ± s.e.m. Statistical comparison was made using a two-tailed *t*-test or ANOVA followed by Tukey–Kramer multiple comparison test.

Modeling of I_{Na} and action potential

The Luo–Rudy computer model of ion channel currents and action potential (Clancy & Rudy, 1999; http://www.cwru. edu/med/CBRTC/LRdOnline/content.htm) was used to test whether the observed action potential changes can be qualitatively consistent with the changes of I_{Na}. To avoid division by zero in voltage clamp simulations with settings of the membrane potential to multiples of 10 mV, the original constants −30 and −50 in the expressions of the 'taud' and 'tauxs1' variables were replaced by, respectively, −30.1 and −50.1. Unlike in the simulation of action potentials, in voltage clamp mode fixed time steps (0.001 ms) were used. The program was implemented in the Delphi™, version 7.0 (Borland) environment and run on a personal computer. When indicated, the transient outward current I_{to} was incorporated (Dumaine *et al*., 1999), using a conductance G_{Ito} of 4 mS μF^{-1}. To simulate the effect of the drug, the default kinetic parameters of the fast Na^+ channel given in Clancy & Rudy (1999) were modified as indicated in the legend of Figure 9.

Results

Slowing of Na^+ current inactivation by KB and ATX-II

Typical effects of KB on Na^+ current (I_{Na}) are illustrated in Figure 1a and are compared with those of ATX-II, illustrated in Figure 1b. I_{Na} was induced from a holding potential (V_H) of −80 mV. Both drugs markedly slowed I_{Na} inactivation. However, they had opposite effects on I_{Na} peak amplitude: KB markedly decreased, whereas ATX-II increased I_{Na} amplitude. Similar results were obtained in five cells with KB (10 μM) and four cells with ATX (200 nM). To illustrate the relative change of I_{Na} by these agents, for each cell the current trace in the presence of either KB or ATX was normalized using the peak amplitude of the control current (in the absence of drug). Average traces of the normalized I_{Na} at −30 mV are displayed in Figure 1c and d for KB and ATX, respectively. The results confirm the opposite effects of KB and ATX on I_{Na} amplitude. The inset in Figure 1d shows that the increase of I_{Na} by ATX remained relatively larger than that by KB throughout the whole pulse duration. In addition, while I_{Na} inactivated completely after >200 ms in the presence of KB, a non-inactivating component persisted in the presence of ATX even after 1 s.

The changes of peak current amplitude are likely due to the drug effects on voltage-dependent inactivation. Figure 2a,b (upper panels) shows the effects on I_{Na} measured at −30 mV following 1-s prepulses to various potentials. Besides slowing the time course of inactivation, no drug had any marked effect on the peak amplitude of I_{Na} induced from the most negative potentials. However, the relative decrease of I_{Na} with depolarized prepulses was different between the two drugs. Inactivation curves obtained from these data (Figure 2a,b, lower panels) show that KB shifted the steady-state I_{Na} inactivation to more negative potentials (see Macianskiene *et al*., 2003), whereas ATX-II shifted it in the opposite direction. These results help explain the opposite effects of both drugs observed on the I_{Na} peak amplitude when using −80 mV as V_H (see Figure 1), with no effect on I_{Na} amplitude when using a V_H of −120 mV (Figure 2c, d).

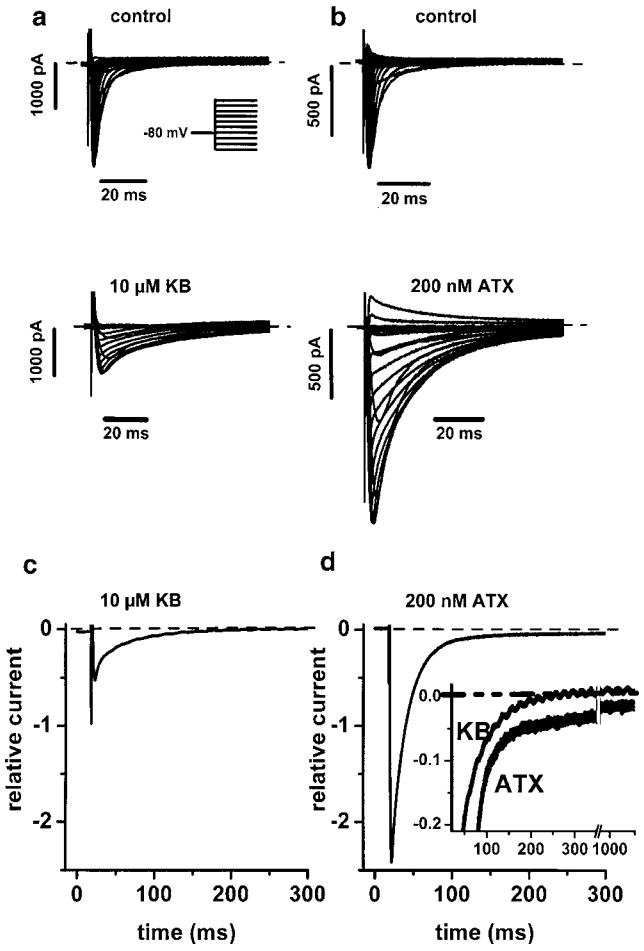
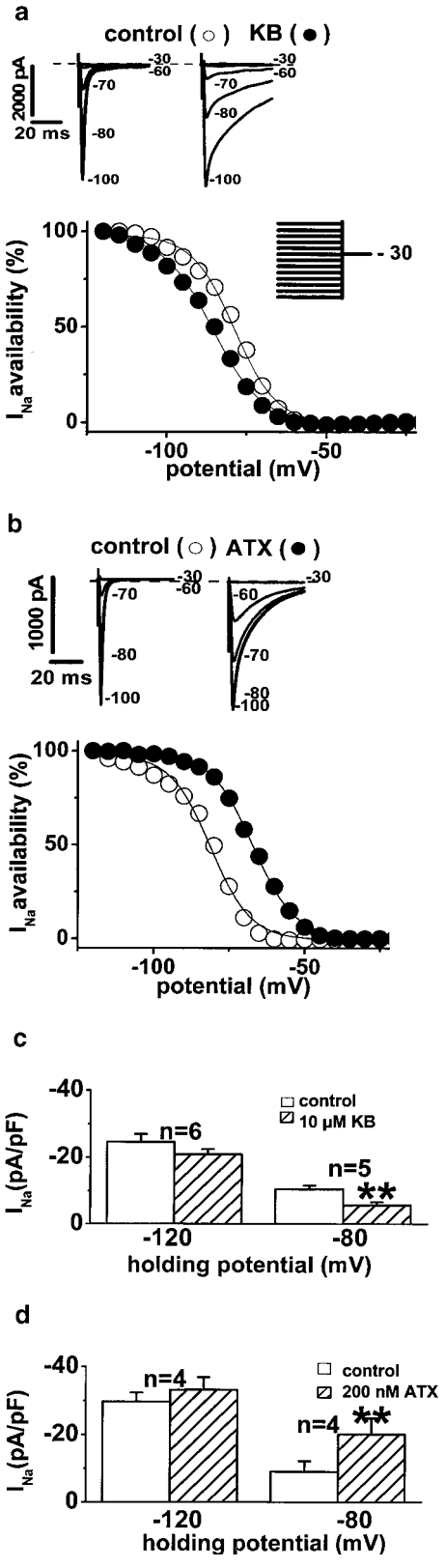


Figure 1 Comparison of the effects of KB and ATX-II on voltage-dependent Na^+ current (I_{Na}). (a,b) I_{Na} traces elicited by depolarizations to various potentials before (upper panels) and following 10-min exposure (lower panels) to 10 μM KB (a) or 200 nM ATX-II (b). Inset in (a): voltage pulse protocol. Holding potential (V_H): −80 mV. Test potentials between −120 and 0 mV (a) or +20 mV (b). (c,d) Average normalized I_{Na} traces at −30 mV in the presence of 10 μM KB (c; $n=5$) or 200 nM ATX (d; $n=4$). I_{Na} in the presence of drug in each cell was normalized relative to the I_{Na} peak amplitude before exposure to the drug in the same cell. Inset in (d) superimposed traces from (c) and (d) shown at different current gain. The horizontal dashed lines indicate zero current level. Pig ventricular myocytes. $[Na]_o = [Na]_i = 10$ mM; K^+-free, Cs^+-containing external and internal solutions; room temperature.

Different effects of KB and ATX-II on APD in pig preparations

In the above and in previous experiments, extracellular and intracellular Na^+ concentrations of 10 mM were used to decrease I_{Na} amplitude and improve voltage control. In a few experiments in the present study, we used the normal Tyrode ($[Na^+]_o = 150$ mM) and a K^+-based internal solution to allow recording of both I_{Na} and action potential in the same cell. I_{Na} and action potentials were elicited at 1 Hz, alternately during steps to −30 mV in the whole-cell voltage clamp mode ($V_H = -80$ mV) or during pacing in current clamp conditions. Figure 3a shows superimposed I_{Na} traces obtained during 1-s depolarizations before and after application of 10 μM KB. Despite technical limitations for accurately measuring fast and large currents, the KB effects were essentially similar to those



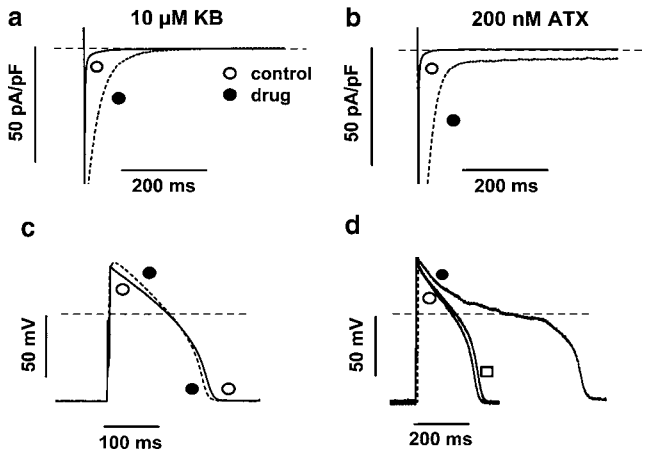


Figure 3 Comparison of KB and ATX-II effects on I_{Na} and action potentials in pig cardiac myocytes. (a,b) Superimposed I_{Na} traces induced at −30 mV in control (unfilled circles) and in the presence (filled circles) of either 10 μM KB (a) or 200 nM ATX-II (b). (c,d) Superimposed action potential recorded under current-clamp conditions in the same cells, in control (unfilled circles) and in the presence (filled circles) of either 10 μM KB (c) or 200 nM ATX-II (d). In (d) action potential labeled with unfilled square was obtained after 10-min washout of ATX-II. Horizontal dashed lines indicate 0 mV level. Stimulation *via* the patch pipette at 1 Hz. $[Na]_o = 150$ mM, $[Na]_i = 10$ mM; K^+-containing external and internal solutions; ruptured-patch recording; room temperature.

obtained with low $[Na]_o$ (see Figure 1). Identical results could be obtained when recording with the perforated-patch method, or when using myocytes from mouse ($n = 3$), rabbit ($n = 2$), guinea-pig ($n = 1$), and human heart ($n = 3$) (not illustrated). These results indicate that KB exerts its effects under physiological conditions and in different animal species. Despite the marked slowing of I_{Na} inactivation, there was practically no change of the APD in the same cell (Figure 3c). KB caused a small elevation of the plateau that was followed by a slight APD shortening. In contrast, superimposed traces obtained before and after application of 200 nM ATX-II show that the toxin-induced slowing of I_{Na} inactivation was associated with a persisting, noninactivating current (Figure 3b), and a marked APD prolongation in the same cell (Figure 3d).

Figure 2 Comparison of the effects of KB and ATX-II on voltage-dependent inactivation of I_{Na}. (a,b) Superimposed I_{Na} traces, elicited by depolarizations to −30 mV following 1-s prepulses to various levels (upper panels), and inactivation curves (lower panels) in the absence (unfilled circles) and in the presence (filled circles) of either 10 μM KB (a) or 200 nM ATX-II (b). Inset in (a): voltage pulse protocol. Holding potential (V_H): −80 mV. Horizontal dashed lines indicate zero current level. Fitting of the inactivation curves was made using Boltzmann distribution functions. Average parameters of the Boltzmann functions for ATX: $V_{0.5} = -83 \pm 2.85$ mV, slope $= 5.7 \pm 0.3$, in control; $V_{0.5} = -72 \pm 3.7$ mV, slope $= 7.5 \pm 0.4$, in the presence of the toxin ($n = 4$; $P < 0.05$ vs control). For average KB data, see Macianskiene *et al.* (2003). (c,d) Pooled data on peak amplitude of I_{Na} at −30 mV using V_H of either −120 or −80 mV in control (unfilled columns) and in the presence (hatched columns) of 10 μM KB (c; $n = 5$–6) or 200 nM ATX-II (d; $n = 4$). Pig ventricular myocytes. $[Na]_o = [Na]_i = 10$ mM; K^+-free, Cs^+-containing external and internal solutions; room temperature. $^{**}P < 0.01$ for drug vs control (paired t-test).

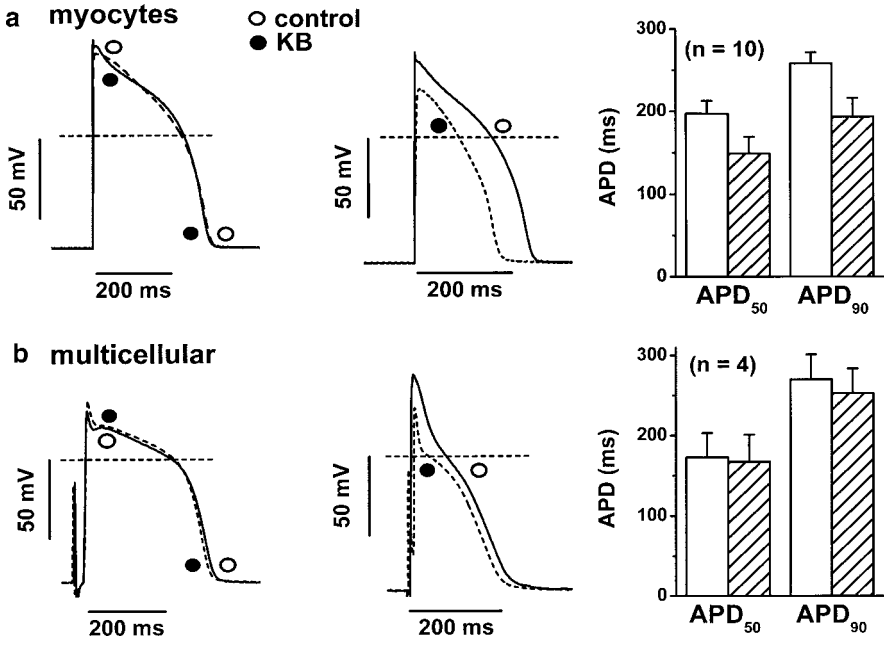


Figure 4 Variable effects of KB on action potentials in pig preparations. Superimposed action potential traces (leftmost and middle panels; different cells) recorded in control (unfilled circles) and in the presence of 10 μM KB (filled circles), and average durations (columns; rightmost panels) of the APD at 50% (APD_{50}) and 90% repolarization (APD_{90}). Horizontal dotted lines indicate 0 mV level. Pacing frequency: 1 Hz. (a) Pig ventricular myocytes; room temperature; (b) pig ventricular trabecula; 37°C.

Table 1 Effect of KB on the resting and action potentials of pig ventricular myocytes

	1 Hz (n = *10*)		*2.5 Hz* (n = *12*)		*3 Hz* (n = *8*)	
	Control	*KB*	*Control*	*KB*	*Control*	*KB*
V_{rest} (mV)	−74±1.5	−75±1.9	−74±1.8	−75±2.1	−75±1.4	−75±2.0
APA (mV)	131±1.4	113±3.0	117±4.3	105±3.8	123±2.1	99±5.1
APD_{50} (ms)	197±15.6	149±20.4**	139±7.0	125±13.1.4	139±6.2	111±15.3*
APD_{90} (ms)	258±13.4	194±22.5**	195±8.3.4	172±15.2	185±6.4.4	148±17.6*

Values are given as mean±s.e.m. Same cells used for various frequencies, in the absence and presence of 10 μM KB. Recordings made at room temperature. V_{rest}: resting potential; APA: amplitude of action potential; action potential duration (APD) measured at 50% (APD_{50}) or at 90% (APD_{90}) repolarization; *$P<0.05$; **$P<0.01$; compared to control.

The effects of both drugs were reversible. For KB, reversibility required washout with DMSO (0.1% v v^{-1}) for 40–60 min (see Figure 5a). For ATX-II, a 5–10-min washout in normal solution was sufficient (see Figure 3d).

Given that we had expected more prominent action potential changes due to the marked delay of I_{Na} inactivation by KB, we examined more extensively the effect of this drug. Figure 4 illustrates effects on action potentials recorded at room temperature in single myocytes (Figure 4a; two different cells), or under more physiological conditions at 37°C in multicellular preparations (Figure 4b; two different muscles). In single cells, some variability in the effect of KB was found between individual preparations. An increase (see Figure 3a) or a marked decrease of the overshoot and plateau, and either no change (Figure 4a, left) or a shortening (Figure 4a, right) of the global APD could be obtained in different cells. In no case was APD significantly prolonged. Pooled measurements of APD_{50} and APD_{90} at 1 Hz from 10 cells are given in Figure 4a (rightmost panel). Qualitatively identical results were obtained while pacing at different frequencies, and all measurements in pig myocytes are summarized in Table 1. On the average, a similar lack of APD prolongation was observed (not illustrated) in cells superfused at 37°C with no intracellular Ca^{2+} buffering by EGTA (APD_{90} at 1 Hz: 304±22.4 vs 293±30.4 ms, in control vs presence of 10 μM KB, respectively; $P>0.05$; $n=7$), although in two of these cells some prolongation was present. In multicellular preparations (Figure 4b), the results were qualitatively identical to those of single myocytes (APD_{90} at 1 Hz: 298±37.4 vs 265±34.1 ms, in control vs presence of 50 μM KB, respectively; $P>0.05$; $n=5$; 37°C). These data indicate that overall KB did not lengthen the APD in pig ventricular preparations.

KB lengthens short action potentials in mouse myocytes

The above results suggest that the major difference in the KB and ATX-II effect on APD could be related to their different effects on I_{Na} magnitude, and to the absence and presence of a persisting I_{Na}, respectively. Since in the absence of a persisting I_{Na}, a slowed inactivation is likely to have more prominent effect on very short action potentials, we also examined the effect of KB in mouse ventricular myocytes. Because of a rapid run-down of $I_{Ca\text{-}L}$ in mouse myocytes during ruptured-patch recording under our experimental conditions (R. Macianskiene & K. Mubagwa, unpublished results), action potentials in this species were recorded under either ruptured patch ($n=8$;

Figure 5a) or perforated patch conditions ($n=7$; Figure 5b, c). Cells were obtained from the left ventricular free wall (Figure 5a,b) or from the septum (Figure 5c). Action potentials were usually longer in the perforated-patch recording mode. Figure 5a illustrates that in cells from the ventricular free wall, the action potential recorded under ruptured patch mode was very short and that KB caused a progressive prolongation of the action potential and induced early afterdepolarizations (EADs). In some cases, the EADs disappeared as the action potential grew longer and a clear plateau phase developed. Short action potentials recorded with the perforated patch method were also prolonged by KB (Figure 5b). In contrast, when the control APD was long, as during perforated patch recording, the early phase of repolarization could be delayed simultaneously with a shortening of the global APD (Figure 5c). All data from mouse myocytes are summarized in Table 2. Similar results were obtained in papillary muscle preparations ($n=2$; not illustrated). Altogether these results suggest that KB, which fails to increase the duration of long action potentials, markedly prolongs shorter action potentials.

Effects of KB on other membrane currents

The failure of KB to prolong the APD in pig preparations and its marked effect on short action potentials in mouse could also be due to changes in other membrane currents that, respectively, counterbalance or enhance the effect of a slowly inactivating I_{Na}. We therefore investigated the effect of KB (10 μM) on other membrane currents.

Steady-state currents Figure 6a shows current traces obtained using K^+-containing extra- and intracellular media, upon depolarizing for 1 s to various potentials ($V_H=-80$ mV), and Figure 6c shows the corresponding current–voltage relationships obtained by measuring the current at the end of the voltage step. KB had no effect at potentials where the time-dependent currents are absent (negative to -70 mV), but caused a marked slowing of the initial time-dependent inward currents following steps to potentials positive to -70 mV (i.e. , reflecting I_{Na}). At these potentials, there was no change of the steady-state current after the decay of the early inward currents. Steady-state current–voltage relationships obtained under control conditions and in the presence of KB were practically superimposable (Figure 6c), suggesting a lack of effect on I_{K1}.

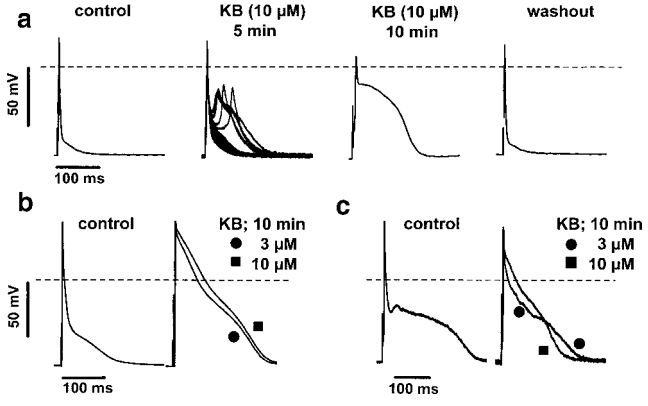


Figure 5 Effect of KB on action potentials in mouse myocytes. (a) Action potentials measured in control, at 5 and 10 min after application of 10 μM KB, and following 60-min washout of KB in 0.1% DMSO. Myocyte from the left ventricular free wall; ruptured patch recording. (b,c) Action potential recorded in control and in the presence of 3 or 10 μM KB; perforated patch recordings. Myocytes from the left ventricular free wall (b) and from the septum (c). Horizontal dashed lines indicate zero level; pacing at 1 Hz.

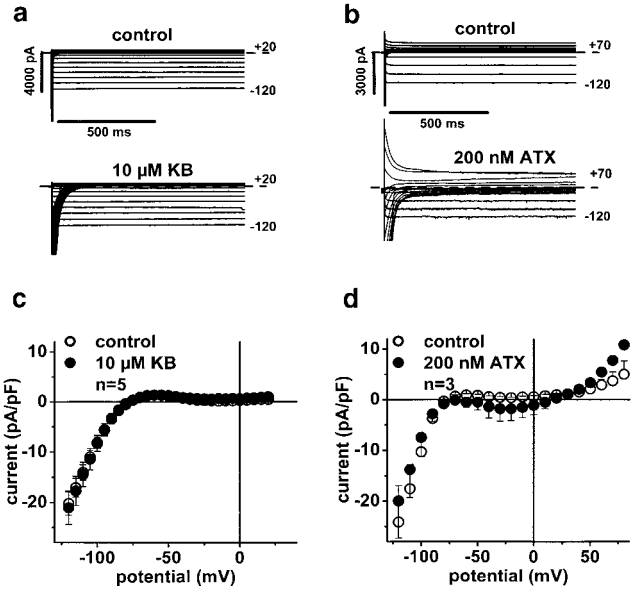


Figure 6 Effect of KB on steady-state currents. (a, b) Traces of currents elicited by 1-s steps to various potentials before (upper panels) and after application (lower panels) of either 10 μM KB (a) or 200 nM ATX-II (b). Horizontal dashed lines indicate zero current level. (c, d) Current–voltage relations in control (unfilled circles) and in the presence (filled circles) of either 10 μM KB (c) or 200 nM ATX-II (d). Currents measured at the end of the 1-s pulse. Notice the change of steady-state currents by ATX-II but not KB at potentials positive to -70 mV. Pig ventricular myocytes; K^+-containing external and internal solutions; $[Na]_o=150$ mM; $[Na]_i=10$ mM; room temperature.

Table 2 Effect of KB on the resting and action potentials of mouse ventricular myocytes

	Ruptured patch (n = 8)		*Perforated patch* (n = 7)	
	Control	*KB*	*Control*	*KB*
V_{rest} (mV)	−71±1.2	−72±1.0	−74±0.6	−76±1.5
APA (mV)	100±7.1	98±5.7	116±3.4	118±4.2
APD_{50} (ms)	6±0.6	48±18.2*	17±3.7	74±13.6*
APD_{90} (ms)	52±13.4	143±13.0*	155±16.5	198±10.4*

Values are given as mean±s.e.m. Same cells used in the absence and presence of 3 μM KB. Recordings made at room temperature, during pacing at 1 Hz. V_{rest}: resting potential; APA: amplitude of action potential; action potential duration (APD) measured at 50% (APD_{50}) or at 90% (APD_{90}) repolarization; *$P<0.05$; compared to control.

Corresponding data with ATX-II (200 nM) are shown in Figure 6b and d. ATX-II also did not change the time-independent currents at potentials negative to −70 mV and markedly slowed the initial time-dependent inward currents following steps to more positive potentials. However, steady-state currents were changed at potentials where I_{Na} is activated (Figure 6d). This result is also consistent with the existence of a persisting, noninactivating I_{Na} in the presence of ATX-II but not in the presence of KB (see also Figure 3b). The lack of ATX-II effect at the most negative potentials (where I_{Na} is not activated) indicates that the drug does not affect the inward rectifier I_{K1}.

Effect of KB on $I_{Ca\text{-}L}$

Figure 7a shows $I_{Ca\text{-}L}$ traces recorded in one cell before and after 10-min exposure to 10 μM KB. K^+ currents were blocked and I_{Na} inactivated by a 1-s prepulse to −40 mV (see Methods). KB caused a small decrease of the peak current amplitude but had no effect on the time course of inactivation (see inset of Figure 7a). Figure 7b and c presents pooled data of current–voltage relations and time constants, respectively. The magnitude of the decrease in peak current varied from cell to cell, but in a total of nine cells, the decrease in peak amplitude at +10 mV amounted to 35% of the control current. Similar effects (not illustrated) were obtained in three pig myocytes superfused at 37°C and not dialyzed with EGTA (decrease of the $I_{Ca\text{-}L}$ peak amplitude from -4.6 ± 0.7 to -3.3 ± 0.9 pA pF^{-1}; $P<0.05$) and in three human ventricular myocytes (decrease of $I_{Ca\text{-}L}$ from -2.1 ± 0.4 to -1.3 ± 0.5 pA pF^{-1}; $P<0.05$), with no effect on inactivation time course (τ_{fast}: 14 ± 4.5 and 12 ± 3.5 ms; τ_{slow}: 142 ± 24.9 and 140 ± 16.5 ms; in the human cells, under control conditions and in the presence of 10 μM KB, respectively).

Lack of effect of KB on the transient outward current (I_{to}) in mouse As the marked effect on APD in mouse ventricular preparations could be possibly attributed to a change of I_{to}, we also examined the effect of KB on this current. I_{to} was studied under conditions where external Na^+ was completely replaced with NMDG to avoid artefacts due to drug-induced change in I_{Na}. Figure 8 illustrates typical results, in which 10 μM KB failed to change both the time-independent currents at negative potentials (I_{K1}) and the time-dependent outward currents at potentials positive to −40 mV (I_{to}).

Discussion

The present study examined the consequences of the KB-induced slowing of I_{Na} inactivation on the APD. Under conditions where KB markedly slowed the Na^+ channel inactivation, the drug did not lengthen but frequently shortened the APD in pig ventricular myocytes. Since other Na^+ channel modifiers are found to be of little clinical usefulness for the treatment of heart failure because they induce an arrhythmogenic prolongation of the QT interval, it was of interest to examine the mechanisms responsible for the lack of APD prolongation by KB.

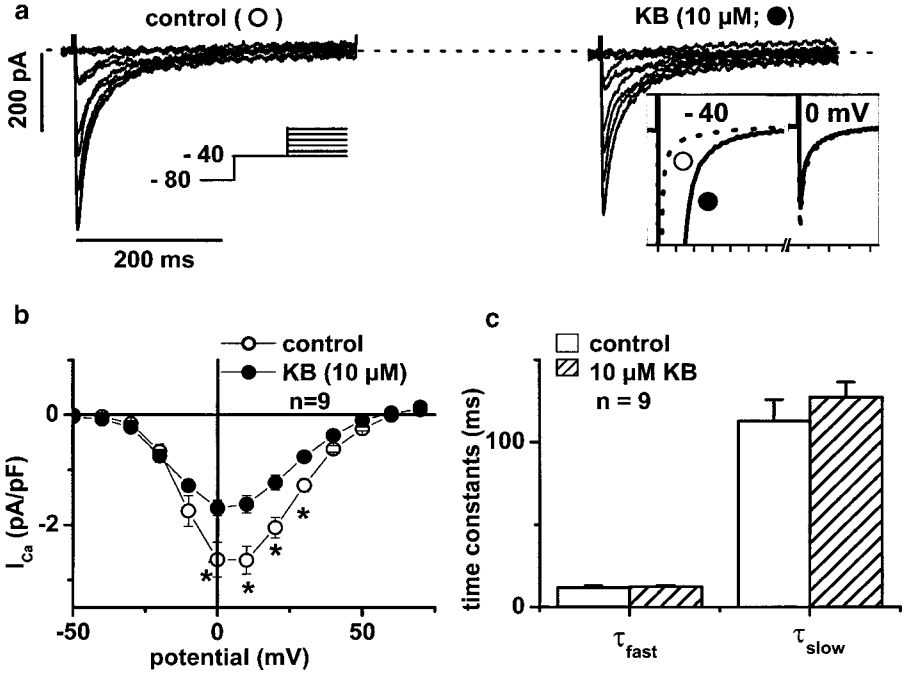


Figure 7 KB effect on the L-type Ca^{2+} current ($I_{Ca\text{-}L}$). (a) $I_{Ca\text{-}L}$ currents elicited by steps to various potentials in the absence (left) and in the presence (right) of 10 μM KB. The horizontal dotted line indicates zero current level. Inset in left panel shows the voltage protocol: holding potential (V_H) of −80 mV, prestep for 1 s to −40 mV to inactivate I_{Na} and any T-type Ca^{2+} current. Inset in right panel contrasts the failure of KB to change the time course $I_{Ca\text{-}L}$ (at 0 mV) at a time when it causes slowing of I_{Na} (during the prestep to −40 mV). (b) Current–voltage relations in control (unfilled circles) and in the presence (filled circles) of 10 μM KB. Pooled data from various cells ($n=9$). (c) Time constants (obtained using biexponential fitting) of $I_{Ca\text{-}L}$ inactivation measured at 0 mV, in control conditions (unfilled columns) and in 10 μM KB (filled columns). Same cells as in (b) pig myocytes; K^+-free, Cs^+-containing external and internal solutions; room temperature. *$P<0.05$ vs control.

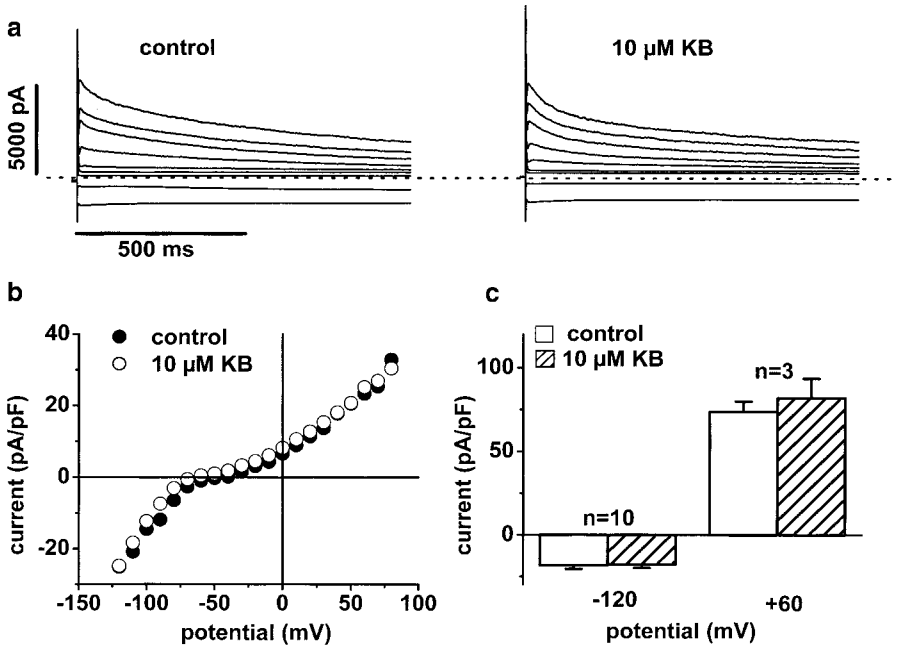


Figure 8 Lack of effect of KB on I_{to} and I_{K1} in mouse myocytes. (a) Traces of currents elicited by steps to various potentials before (left panel) and after application (right panel) of 10 μM KB. Horizontal dotted lines indicate zero current level. (b) Current–voltage relations in control (unfilled circles) and in the presence (filled circles) of 10 μM KB. Currents measured at peak level. (c) Pooled data on amplitude of currents at -120 mV (largely I_{K1}; $n=10$) and $+60$ mV (largely I_{to}; $n=3$). Notice lack of KB effect. $[Na]_o=[Na]_i=0$ mM; K^+-containing external and internal solutions; room temperature.

In our previous study (Macianskiene *et al*., 2003), slowing of I_{Na} inactivation by KB was obtained while using low $[Na^+]_o$ (to decrease the driving force for Na^+) and low $[Ca^{2+}]_o$ (to avoid contamination of inward currents by $I_{Ca\text{-}L}$). The present study confirms and extends these results by showing that KB causes a qualitatively similar slowing of I_{Na} inactivation when physiological $[Na^+]_o$ and $[Ca^{2+}]_o$ are used. In addition, we now show that KB effects are slowly reversible when DMSO is included in the solution during washout. In the early study, we had noticed that washout with normal Tyrode for 20–30 min was not associated with a removal of the drug effect. The slow time course of reversibility, the need for DMSO to remove the drug effect, and our previous observation that intracellular KB was ineffective, all suggest that KB accumulates within the lipid phase of the membrane, from where it is able to interact with its action site on the Na^+ channel.

We compared the effects of KB with those of ATX-II, a toxin known to change the inactivation of cardiac Na^+ channels (Isenberg & Ravens, 1984). Whereas both drugs slowed I_{Na} inactivation, they differed in the ability to induce persisting, noninactivating current (present with ATX-II but not with KB; see Figures 1, 3 and 6) and in their effects on the voltage-dependent steady-state inactivation (shift to more positive potentials with ATX-II, but to more negative potentials with KB; see Figure 2). The different shifts in steady-state inactivation resulted in opposite effects (decrease with KB, increase with ATX-II) on the amplitude of I_{Na} induced from -80 mV.

Because of the prolonged opening of Na^+ channels by KB and ATX-II, we had expected that either drug would prolong the APD, albeit to different extents. However, we found that in pig ventricular myocytes only ATX-II was able to prolong APD. In most cases, no prolongation or a marked APD shortening was obtained with KB. The difference between KB and ATX-II effects on APD (see Figure 3) is likely due, at least in part, to the differences in their effects on I_{Na}. In other studies, differences have been also noticed between the channel modifiers DPI201-2065, BDF9648 and BDF9698 in their ability to prolong the APD and were attributed to different effects on other ion channels (Ravens *et al*., 1991; Amos *et al*., 1994; Yuill *et al*., 2000). Such a situation could also apply in the case of KB and ATX. To account for the absence of an APD prolongation by KB, the following possibilities could be invoked: (1) concomitant direct effects of KB on other ion channels, whose currents counterbalance the effect of slow-inactivating I_{Na}; (2) indirect effects on other currents, due to changes in the early part of the action potential, induced by drug-modified I_{Na}; (3) relative insensitivity of long action potentials to an effect of slowed I_{Na} inactivation in the absence of a persisting current. These possibilities are now examined.

In pig cells, we did not find any effect on steady-state currents. However, there was a significant decrease of $I_{Ca\text{-}L}$ (Figure 7). The mechanism underlying this decrease and the reason for the observed variability between cells were not elucidated in the present study. A similar decrease of $I_{Ca\text{-}L}$ has been observed with amiodarone and was attributed to a negative shift of the steady-state voltage-dependent inactivation (Nishimura *et al*., 1989). Since a marked decrease was not obtained in all cells (see example of Figure 7), it is unlikely to be the sole explanation of the general absence of APD prolongation by KB. In pig cells, the transient outward K^+ current (I_{to}) is not significantly expressed (Macianskiene *et al*., 2002), and its eventual increase cannot therefore be invoked for a counterbalancing effect relative to I_{Na}.

Changes of the early part (overshoot level or initial repolarization) of the action potential due to modified I_{Na}

may indirectly modify the ion current balance and the repolarization rate during the subsequent part of the plateau. In some pig cells, an elevation of the early part of the plateau was obtained in the presence of KB. Such an effect may lead to more inactivation of I_{Na} and $I_{Ca\text{-}L}$, and more activation of K^+ currents, effects that will tend to shorten the APD. However, in other cells a decrease of the overshoot and a lowering of the plateau level were obtained. These apparently contradictory results are probably accounted for by the opposing consequences of slowed inactivation and of decreased initial I_{Na} availability. On the one hand, the slowing of I_{Na} inactivation will favor an elevation of the plateau. On the other hand, due to the KB-induced shift of voltage-dependent steady-state inactivation to more negative potentials, fewer Na^+ channels are available at the resting potential, and the initial I_{Na} may be decreased, favoring both a lower overshoot and a lower plateau. The relative contribution of both effects probably determines the net change in overshoot and plateau.

Finally, it is possible that long action potentials with high plateau are relatively insensitive to a slowly inactivating I_{Na} if inactivation can still proceed to completion (i.e., if there is no maintained, noninactivating current). The APD is more likely to increase if I_{Na} is increased and contributes a persisting, noninactivating current during the plateau of the action potential, as is the case with ATX-II (see Figure 2; and Boutjdir *et al*., 1994), while less APD prolongation will occur if I_{Na} amplitude is decreased and the current fully inactivates shortly after the beginning of a long plateau. Since plateau levels in pig cells are at potentials above 0 mV, the effect of KB may be minimized by a faster I_{Na} inactivation and a decrease of the electrochemical driving force for Na^+ at more positive potentials.

The finding that in mouse ventricular cells KB markedly prolonged the APD and could induce EADs is in favor of the hypothesis that slowly inactivating I_{Na} is more likely to remodel short action potentials with fast early (phase 1) repolarization. During ruptured patch recording in mouse myocytes, the APD in control conditions is very brief, without clear plateau. Repolarization is terminated through a slow phase 3, that begins at potentials more negative than −50 mV. The most pronounced prolongation of the APD by KB was found in these cells, and was often associated with a decreased overshoot and the appearance of a clear plateau (see Figure 5a). The APD lengthening can be explained by the slowed inactivation of I_{Na}, and the decreased overshoot by a decreased I_{Na} peak amplitude due to enhanced steady-state inactivation. Our voltage-clamp results indicate that the repolarizing current I_{to} (which is prominent in mouse cells) is not modified in the presence of KB. I_{to} is expected to activate maximally at overshoot potentials. During the rapid repolarization due to I_{to}, I_{Na} has not had time to inactivate fully and may actually increase due to an increase in its driving force. This will induce EADs or a clear-cut plateau. The fact that EADs could take off from potentials as negative as −50 mV (see Figure 5a) is consistent with the possibility that they may result from an increased driving force of I_{Na}. Less APD remodeling by KB was induced on longer APDs recorded during perforated patch recording in mouse myocytes, in

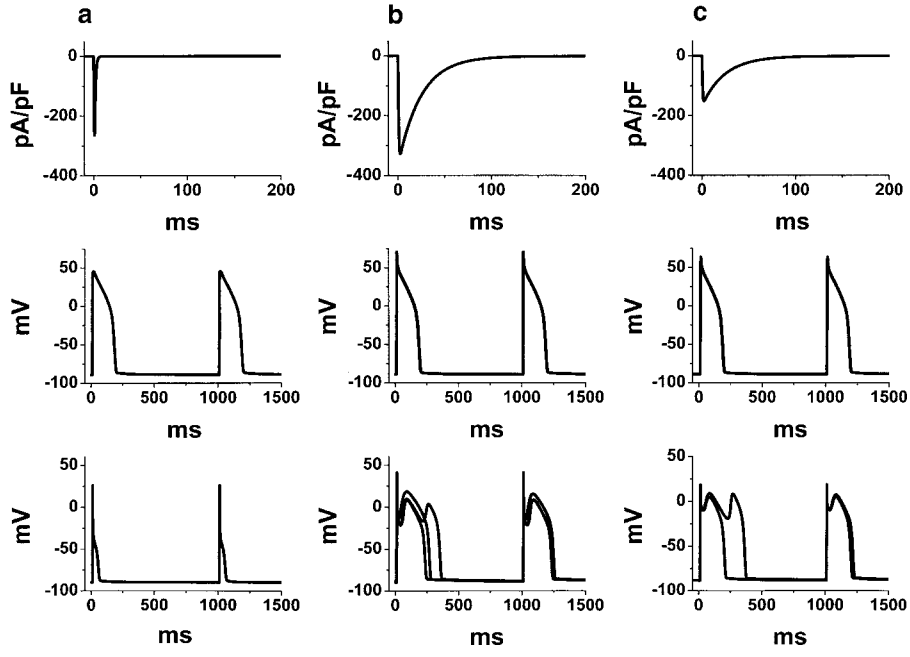


Figure 9 Simulation of the effect of slowed I_{Na} inactivation on the action potential. Na^+ currents (I_{Na}) and action potentials generated using different channel kinetic parameters. Upper panels: I_{Na} induced by simulated voltage-clamp from −80 to −30 mV. Middle panels: action potentials generated during simulated pacing at 1 Hz in the absence of the transient outward current (I_{to}). Lower panels: action potentials in the presence of a large I_{to} (maximal conductance 4 μS pF^{-1}). (a) Control conditions. Na^+ channel with standard kinetics and conductance, as given in the Luo–Rudy model formulated from single channel kinetics (Clancy & Rudy, 1999). (b) Na^+ channel with slowed inactivation kinetics but normal maximal conductance. The default rate constants of transitions from the open to the inactivated state (α_2) and from the closed to the open state (α_{13}) were decreased by a factor of 20 and 3.8, respectively, whereas the rate constant for the recovery from the inactivated to the closed state (α_3) was changed by a factor of 10. $\alpha_2 = 0.459 \times e^{V/29.68}$ m s^{-1}; $\alpha_{13} = 1/(0.1027 \times e^{-V/12} + 0.25 \times e^{-V/150})$ m s^{-1}; $\alpha_3 = 3.79 \times 10^{-8} \times e^{-V/5.2}$ m s^{-1}. (c) Na^+ channel with slowed inactivation kinetics and decreased maximal conductance. The rate constant α_2 and the maximal conductance (G_{Na}) were decreased. $\alpha_2 = 0.2 \times e^{V/29.68}$ m s^{-1}; $G_{Na} = 3$ instead of 16 μS pF^{-1}.

which the most consistent effect was a delay of the phase-1 repolarization. In some preparations, the latter effect was associated with a marked shortening of the APD, probably as a result of increased activation of repolarizing outward currents and inactivation of $I_{Ca\text{-}L}$ due to the prolonged phase 1 and the elevated plateau.

Our experimental results are also consistent with those of a computer simulation of I_{Na} and action potentials, used to test the hypothesis formulated above, that the relative effect of slowed I_{Na} inactivation on APD depends on the initial duration and on the plateau level. The results of the simulation are presented in Figure 9. The model has been shown to reproduce electrical changes due to mutations of the voltage-dependent Na^+ and HERG K^+ channels that cause a prolongation of APD and of the QT interval on the ECG (Clancy & Rudy, 1999; 2002). A slowly inactivating I_{Na} could be qualitatively reproduced by slowing the rate constants of the transitions between different states. However, the changes were associated with a marked increase in the peak amplitude of I_{Na} (Figure 9b), in contrast to our finding when measuring I_{Na} under voltage-clamp conditions with a V_H of -80 mV. To reduce I_{Na} to amplitudes equal to or smaller than control, we either changed the maximal conductance of I_{Na} (Figure 9c) or further reduced the rate constants of activation. The parameters used to change I_{Na} were then applied in a model of the action potential not incorporating I_{to}, to simulate the situation in pig cells (Figure 9, middle panels), or in a model incorporating a large I_{to}, to simulate the situation in mouse cells (Figure 9, lower panels). Action potentials generated by assuming a stimulation at 1 Hz showed no or little effect of slowing I_{Na} inactivation on the long, high-plateau action potentials in the absence of I_{to} (Figure 9, middle panels). In contrast, there was marked APD lengthening and induction of EADs in case of control short, action potentials in the presence of I_{to} (Figure 9, lower panels). Although the changes in channel kinetics used in the model may not represent a quantitative simulation of those induced by the drug, the results demonstrate the contrasting sensitivity of long or short action potentials to the effect of a slowly inactivating I_{Na}.

Conclusions

In contrast to ATX-II and to many other Na^+-channel modifying agents that increase APD, KB did not prolong but either did not change or did shorten APD in pig myocytes. It is proposed that APD lengthening is mainly obtained by agents that induce a maintained, noninactivating I_{Na}. Following an isolated slowing of inactivation, as may result from drugs such as KB or from mutations on the Na^+ channel, cells with short APDs will present more marked APD lengthening even under conditions where insignificant changes are produced in cells with long APDs. These results have practical implications, since they suggest that the consequences of drug- or mutation-induced change in Na^+ channel inactivation may differ between animal species with very short (e.g., mouse) or with long (e.g., pig, human) action potentials, and between different regions of the myocardium (e.g., subepicardial vs midmyocardial or subendothelial layers).

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