Slowing of the Inactivation of Cardiac Voltage-Dependent Sodium Channels by the Amiodarone Derivative 2-Methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran (KB130015)

R. MACIANSKIENE, S. VIAPPIANI, K. R. SIPIDO, and K. MUBAGWA

Centre for Experimental Surgery and Anaesthesiology (R.M., S.V., K.M.) and Laboratory of Experimental Cardiology (K.R.S.), University of Leuven, Leuven, Belgium

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ABSTRACT

2-Methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran (KB130015 or KB) is a new drug, structurally related to amiodarone and to thyroid hormones. Its effects on cardiac voltage-dependent Na⁺ current (I\textsubscript{Na}) were studied in pig single ventricular myocytes at 22°C using the whole-cell (with [Na⁺]\textsubscript{i} = [Na⁺]\textsubscript{o} = 10 mM) and cell-attached patch-clamp techniques. KB markedly slowed I\textsubscript{Na} inactivation, due to the development of a slow-inactivating component (t\textsubscript{slow} \approx 50 ms) at the expense of the normal, fast-inactivating component (t\textsubscript{fast} \approx 2–3 ms). The effect was concentration-dependent, with a half-maximally effective concentration (K\textsubscript{eff}) of 2.1 μM. KB also slowed the recovery from inactivation and shifted the voltage-dependent inactivation (∆V\textsubscript{50} \approx -15 mV; K\textsubscript{eff} \approx 6.9 μM) and activation to more negative potentials. Intracellular cell dialysis with 10 μM KB had marginal or no effect on inactivation and did not prevent the effect of extracellularly applied drug. In cell-attached patches, extracellular KB prolonged Na⁺ channel opening. Amiodarone (10 μM) and 10 μM 3,5-diiodo-L-thyropionic acid had no effect on inactivation and did not prevent KB effects. 3,3',5-Triiodo-L-thyronine (T\textsubscript{3}) also had no effect on inactivation, but at 10 μM it increased I\textsubscript{Na} amplitude and partially prevented the slowing of inactivation by KB. These data suggest the existence of a binding site for KB and T\textsubscript{3} on Na⁺ channels.

Voltage-dependent Na⁺ channels play an important role in the initiation and conduction of electrical signals in excitable cells. A dysfunction of Na⁺ channels, as may occur after genetic mutation or as a consequence of drug action, is the basis for a variety of cardiac arrhythmias (e.g., those related to the long QT and the Brugada syndromes), skeletal muscle diseases, and epileptic seizures (for reviews, see Fozzard and Hanck, 1996; Catterall, 2000; Balser, 2001; Goldin, 2001). On the other hand, Na⁺ channels are targets on which a variety of substances (hormones, neurotransmitters, and drugs) act to modify cellular function. Therefore, drugs acting on Na⁺ channels are widely used as antiarrhythmic agents, muscle relaxants, or antiepileptics.

Among antiarrhythmic agents, amiodarone has proven to be the most effective drug against ventricular arrhythmias (Kodama et al., 1999). However, given the many undesirable side effects of the drug, there has been a constant interest in synthesizing new molecules that can retain its beneficial effects but be devoid of the side actions. 2-Methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran (KB130015, hereafter called KB) is a new drug whose structure is related to that of amiodarone and thyroid hormones. KB is reported hereafter called KB to antagonize the action of 3,3',5-triiodo-L-thyronine (T\textsubscript{3}) by binding onto thyroid hormone nuclear receptors (Carlsson et al., 2002). Given the known actions of amiodarone on many ion channels, the major target channels being delayed rectifier K⁺ channels and voltage-dependent Na⁺ channels (Carmeliet and Mubagwa, 1998), KB may be expected to have similar electrophysiological effects. KB has been proposed as a potential antiarrhythmic agent, possibly devoid of some side effects of its congener (Carlsson et al., 2002). In the present study, we report marked acute effects of KB on the inactivation of Na⁺ channels. These effects were unexpected based on those known for amiodarone (Follmer et al., 1987; Kohlhardt and Fichtner, 1988; Kodama et al., 1999; Maltsev et al., 2001), but a few studies have reported similar effects with high concentrations of thyroid hormones (Craelius et al., 1990; Harris et al., 1991; Dudley and Baumgarten, 1993).

ABBREVIATIONS: T\textsubscript{3}, 3,3',5-triiodo-L-thyronine; [X]\textsubscript{o}, extracellular concentration of X; DITPA, 3,5-diiodo-L-thyropionic acid; DMSO, dimethyl sulfoxide; [X]\textsubscript{i}, intracellular concentration of X.
Materials and Methods

The measurements were performed on pig isolated single ventricular myocytes. 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.

Preparation of Pig Ventricular Myocytes. The methods used for the dissociation of pig cells have been described previously (Stankovicova et al., 2000; Macianskiene et al., 2002). In short, a piece of the left ventricular wall was excised with its supplying artery, and cannulated and perfused for 30 min at 37°C and at constant flow with an oxygenated Ca2+-free Tyrode’s solution, followed by a 20-min perfusion with a Ca2+-free Tyrode’s solution containing 0.1 mg ml⁻¹ protease (type XIV; Sigma-Aldrich, St. Louis, MO) and 1 mg ml⁻¹ collagenase (type A; Roche Diagnostics, Mannheim, Germany). After a 15-min washing perfusion with a 0.18 mM Ca2+-Tyrode’s solution, the tissue was removed from the perfusion and cut into small pieces. Cells were dispersed by gentle mechanical agitation and were stored at room temperature (21–22°C).

Electrophysiological Recordings in Myocytes. Membrane currents were measured using whole-cell or cell-attached configurations of the patch-clamp technique (Hamill et al., 1981). Heat-polished borosilicate glass electrodes (horizontal puller; Zeitz Instrumente, Munich, Germany), with tip resistances of 1 to 1.5 MΩ when filled with the internal solution, were connected to an Axopatch 200A or 200B amplifier (Axon Instruments, Union City, CA), and an analog-to-digital interface controlled by the pClamp software (Axon Instruments) was used to generate command pulses and acquire data. All experiments were carried out at room temperature.

Capacitance and series resistance were partially compensated. Whole-cell currents were generated by step depolarizations given from a holding potential (Vh) of −80 or −120 mV. Repetitive depolarizations to −30 mV were given every 1 s when changing from one solution to another. Upon reaching steady state in a given solution, depolarizations to various levels were given every 5 s to obtain current-voltage relationships or inactivation curves, or double pulses with varying intervals were given to −30 mV to obtain the recovery from inactivation. For steady-state inactivation, prepulses lasting 1 s were given to potentials between −120 and +40 mV (in 5-mV steps) before depolarizing to a test potential of −30 mV, and Na⁺ current (INa) after a given prepulse was normalized relative to the maximum current (induced by depolarizing directly from −120 mV). Single channel currents were recorded with cell-attached electrodes coated with Sylgard 184 (Dow Corning, Wiesbaden, Germany), whereas the intracellular potential was set close to zero using a 150 mM [K⁺]o solution. The pipette potential was held at +120 mV (relative to ground), and patch depolarization steps lasting 60 ms (for control measurements) or 400 ms (for measurements with KB) were given every 3 s. Current signals were filtered at either 2 kHz (for whole-cell currents) or at 5 to 10 kHz (for single channel currents) and were digitized at 10 or 20 kHz, respectively.

Using low [Na⁺]o and working at room temperature should reduce INa amplitude, making possible a study of this current under whole-cell patch clamp. Despite possibly persisting limitations in the quality of voltage control, the drug effects on the kinetics of whole-cell INa inactivation (see Results) were independent of the changes in peak INa amplitude or access resistance, indicating that they could not be attributed to a deteriorating voltage clamp. In addition, qualitatively similar results could be derived from experiments in the cell-attached patch-clamp mode, where voltage control is ideal.

Data were analyzed using Clampfit (Axon Instruments), Winased (Prof. G. Droogmans, University of Leuven, Leuven, Belgium) or Origin (Microcal, Northampton, MA). Normalized inactivation (or availability) curves were fitted using one single Boltzmann distribution function:

$$ availability = 1/[1 + \exp(\frac{V - V_{0.5}}{k})] $$

where $V_{0.5}$ is the potential of half-maximum inactivation and $k$ is the slope factor of the distribution curve. Concentration-effect relationships were fitted by the Hill equation:

$$ I_{Na} = A_{I} \cdot \exp(-n \cdot h \cdot D) + A_{2} \cdot \exp(-n \cdot h \cdot D) $$

where $A_{I}$ and $A_{2}$ are the amplitudes of the fast and slow decay components, respectively, and $h_{fast}$ and $h_{slow}$ are their respective time constants. Average data are given as mean ± S.E.M. Statistical comparison was made using a two-tailed t-test.

Solutions and Drugs. The composition of the standard Tyrode’s solution used during cell isolation was 150 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.9 mM MgCl₂, 0.33 mM NaH₂PO₄, 10 mM HEPES, and 10 mM glucose; pH was adjusted to 7.4 with NaOH. The solution was bubbled with 100% O₂. During measurements of whole-cell INa, the myocytes were superfused with a K⁺-free Tyrode’s solution containing 10 mM NaCl, 138 mM CsCl, 2.6 mM MgCl₂, 0.1 mM CaCl₂, 10 mM HEPES, and 10 mM glucose (pH adjusted to 7.4 with CsOH). Low [Na⁺]o was used to decrease INa to levels (<10 nA) that are compatible with maintained voltage control. Low [Ca²⁺]o was used to suppress ICa,L, whereas Mg²⁺ was increased to prevent opening of nonselective channels by the low (Ca²⁺) (Mubagwa et al., 1997; Macianskiene et al., 2001). The internal solution contained 10 mM NaCl, 120 mM Cs-glutamate, 20 mM tetraethylammonium-Cl, 5 mM MgATP, 0.1 mM Na₂GTP, 1 mM EGTA, and 5 mM HEPES (pH adjusted to 7.25 with CsOH). During measurements of unitary Na⁺ currents, the pipette was filled with a solution containing 150 mM Na-glutamate, 2.7 mM MgCl₂, and 10 mM HEPES (pH adjusted to 7.4 with KOH).

KB (free acid) was from Karo-Bio AB (Huddinge, Sweden). All other drugs and products were from Sigma Chemie (Bornem, Belgium). Stock solutions (10–100 mM) of KB, amiodarone, or 3,5-diido-t-thyrophosphonic acid (DITPA) were prepared in DMSO. Stock solutions of T₃ were prepared in either DMSO or NaOH. The highest DMSO concentration (0.1%, v/v) was without any effect of its own on either the INa amplitude (98 ± 3% of its basal level after 10 min) or the inactivation kinetics (2.0 ± 0.2 versus 1.9 ± 0.2 ms; basal versus vehicle; n_cells = 6; Ogura et al., 1995).

Results

KB Slows Na⁺ Channel Inactivation. Figure 1 illustrates typical effects of KB on INa recorded during step depolarizations from a Vh of −120 mV. Under control conditions (i.e., in the absence of drug; Fig. 1A, left), INa inactivated rapidly after reaching the peak level. After 5 min of exposure of the same cell to 10 μM KB, there was a marked slowing of inactivation (Fig. 1A, right). Superimposing INa recordings during long pulses at −30 mV in the absence or in the presence of KB (Fig. 1B) clearly shows that the INa decay in KB was remarkably slow but complete, with no maintained current. The inactivation of control INa in most cells could be fitted satisfactorily with one exponential, with a time constant ($\tau$) of 2.1 ± 0.7 ms (n_cells = 45) at −30 mV, but in 13% of the cells two exponentials ($\tau_{fast} = 2.1 ± 0.2 ms, \tau_{slow} = 20.8 ± 2.0 ms; n_cells = 7) were needed. In the latter cases, the slow component accounted for a minor part (8 ± 3.9%) of total INa. With 10 μM KB, the INa inactivation was domi-
nated by a slowly decaying component ($\tau_{\text{slow}} = 47.8 \pm 3.6$ ms, 77 ± 6% of total $I_{\text{Na}}$; $n_{\text{cells}} = 7$), but a fast component, with time constant ($\tau_{\text{fast}} = 3.4 \pm 0.5$ ms) similar to that of control was still present. Similar effects were obtained at other potentials, and the time constants in different cells are summarized in the inset of Fig. 1B. $I_{\text{Na}}$-voltage relationships from traces in Fig. 1A are shown in Fig. 1C: the threshold of $I_{\text{Na}}$ activation and the potential of maximum $I_{\text{Na}}$ were more negative in KB (filled symbols), suggesting a negative shift of the voltage-dependent activation. The $I_{\text{Na}}$ reversal potential was close to 0 mV (consistent with the experimental conditions used; $[\text{Na}^+]_i = [\text{Na}^+]_o = 10$ mM) and was unchanged by the drug.

KB was usually applied during repetitive depolarizations. Its effects developed progressively over time, and usually reached steady state within 5 to 10 min. In three experiments, applying KB for 10 min while continuously holding the potential at −120 mV (without depolarizing steps) caused typical effects on $I_{\text{Na}}$ induced by the first depolarizing pulse (data not shown). The current amplitude or time course of inactivation did not change significantly with additional depolarizing pulses (with 1-s interpulse interval), implying that there was no marked use dependence in the drug action at 1 Hz.

Concentration Dependence of KB Effects. Figure 2A shows traces obtained from another cell, successively exposed to control solution, and to 0.3, 3, and 100 μM KB. The slowing of $I_{\text{Na}}$ depended on the KB concentration. Fitting the $I_{\text{Na}}$ decay at −30 mV by a sum of two exponentials indicated that the major effect of KB consisted of inducing a slowly decaying $I_{\text{Na}}$ component and that changing the KB concentration mainly affected the relative amplitude on the fast versus slow components, with less marked effect on their time constants. The slowly decaying $I_{\text{Na}}$ component increased with drug concentration, at the expense of the fast-inactivating component. To quantitatively examine the concentration dependence of the KB effect, the relative magnitude of the slowly inactivating component ($A_s$; total $I_{\text{Na}} = A_f + A_s = 1$) was plotted as a function of the drug concentration (Fig. 2B). The relationship could be fitted by the Hill equation (see Materials and Methods), with a $K_{0.5}$ of 2.1 μM and a Hill coefficient of 1.1.

**KB Effects on Na+ Current Amplitude Depend on Holding Potential.** The above-mentioned data (Fig. 1C) indicate that the KB effect on the $I_{\text{Na}}$ amplitude was marked at potentials close to the activation threshold. The effect also depended on $V_H$. Figure 3A shows superimposed $I_{\text{Na}}$ recorded under control conditions and in the presence of 10 μM KB, during steps to −30 mV from a $V_H$ of −80 mV, either without prepulse (Fig. 3A, left) or with a 1-s prepulse to −120 mV (Fig. 3A, right). Although the most prominent drug effect was a slowing of inactivation, there was in addition a marked decrease of peak $I_{\text{Na}}$ amplitude, the decrease being relatively more marked when depolarizing directly from $V_H$ of −80 mV. To avoid any drug effect, due to holding at $V_H$ of −80 mV, that may not be removed by the 1-s hyperpolarization to $−120$ mV, in further experiments a continuous $V_H$ of either −80 or −120 mV was used. Figure 3, B and C, show pooled current-voltage relationships from various cells in which $I_{\text{Na}}$ was recorded at various potentials, in control conditions (unfilled symbols) and in the presence of 10 μM KB (filled symbols). Control $I_{\text{Na}}$ was smaller with a $V_H$ of −80 mV than with a $V_H$ of −120 mV (compare unfilled circles in Fig. 3, B and C), as expected from less channel availability at −80 mV. KB decreased $I_{\text{Na}}$ at all potentials when using a $V_H$ of −80 mV (at −30 mV, decrease from $−10.4 \pm 1.1$ to $−5.6 \pm 0.9$ pA/pF; $P < 0.01$, $n_{\text{cells}} = 5$), whereas it increased $I_{\text{Na}}$ at threshold potentials and had marginal or no significant effect at potentials more than −40 mV when using a $V_H$ of −120 mV (at −30 mV, $−24.6 \pm 2.4$ versus $−20.9 \pm 1.5$ pA/pF, in control and in KB, respectively; $P > 0.05$, $n_{\text{cells}} = 6$).
Shift of Voltage-Dependent Inactivation. The dependence of KB effect on the prestep or $V_H$ suggests a change of the voltage-dependent inactivation. Figure 4A shows $I_{Na}$ induced at −30 mV after 1-s prepulses to different levels ($V_H = -120$ mV), in the absence (Fig. 4A, left) and in the presence (Fig. 4A, right) of 10 μM KB. The steady-state availability (or inactivation) curve obtained from such tracings in 14 cells was concentration dependently shifted to more negative potentials in the presence of KB (Fig. 4B). The change in potential of half-maximum inactivation ($\Delta V_{0.5}$) is given as a function of the KB concentration in Fig. 4C. Assuming that 100 μM KB caused maximal effect ($\Delta V_{0.5}$ of −15 ± 3.5 mV), the average relationship ($n_{cells} = 3–7$ for each concentration) could be fitted by the Hill equation with a $K_{0.5}$ of 6.9 μM KB ($n_{Hill} = 1.2$).

Slowing of Recovery from Inactivation. We also examined the effect of KB on the recovery from inactivation. Figure 5A shows $I_{Na}$ traces from a typical experiment in which double pulses were given at −30 mV, with increasing interpulse interval at $V_H$ of −120 mV, before and during treatment with 10 μM KB. The first pulse was 1 s in duration (of which only the initial 40 ms are displayed in traces of Fig. 5A) and allowed complete inactivation of $I_{Na}$, even in the presence of KB. The second pulse allowed a recovery of $I_{Na}$ as a function of the interpulse interval.

Fig. 2. Concentration dependence of the slowing of $I_{Na}$ inactivation by KB. A, $I_{Na}$ traces elicited by depolarizations to various potentials in the absence (control) and in the presence of 0.3, 3, or 100 μM KB. Horizontal dotted line indicates zero current level. $V_H$ = −120 mV. B, relationship between relative amplitude of the slow component induced by KB and drug concentration. Data were fitted to the Hill equation (see Materials and Methods). $A_s$ and $A_f$ are the magnitudes of the slow and fast components of $I_{Na}$, respectively, $K_{0.5}$ is the half-maximum effective concentration, and $n_{Hill}$ is the Hill coefficient.

Fig. 3. Dependence on the holding potential of KB effects on $I_{Na}$ amplitude. A, superimposed $I_{Na}$ traces, elicited by depolarizations to $V_H$ of −80 mV. Insets, voltage-pulse protocol. Horizontal dotted line indicates zero current level. Left, depolarization without prestep. Right, depolarization after 1-s prestep to −120 mV. B and C, $I_{Na}$-voltage relations in control (○) and in the presence (●) of 10 μM KB. $V_H$ of −80 mV (B) or −120 mV (C).
function of the interpulse interval. The time course of the relative recovery measured in five cells (I_{Na} in the second pulse as percentage of I_{Na} in the first pulse) is shown in Fig. 5B and illustrates that the removal of inactivation was slowed in the presence of KB. Two exponential components were sufficient to account for the recovery process. In control conditions, a fast component with a time constant (\( \tau_{\text{fast}} \)) of \( 25 \pm 2.8 \) ms accounted for \( 50 \pm 2.2\% \) of the total recovery, whereas a slow component (\( 50 \pm 2.3\% \)) had a time constant (\( \tau_{\text{slow}} \)) of \( 117 \pm 20.2 \) ms. In the presence of 10 \( \mu M \) KB, the two components were slowed (\( \tau_{\text{fast}} = 55 \pm 13.2 \) ms, \( \tau_{\text{slow}} = 318 \pm 123.8 \) ms; Fig. 5C, left). To assess the change in the relative contributions of both components, the recovery in KB was fitted using the same time constants as in control. The slow recovery component was predominant in the presence of KB (Fig. 5C, right). In addition, it was also noticed that a small \( I_{Na} \) recovered after brief interpulse intervals (e.g., traces labeled “a” in Fig. 5A, right, and inset) inactivated faster than a larger \( I_{Na} \) recovered after longer intervals (e.g., traces labeled “b” in Fig. 5A, right, and inset). This result is also consistent with a slower recovery of slowly inactivating Na channels.

**Effect of Tetrodotoxin and Interaction with Other Drugs Related to Thyroid Hormones.** \( I_{Na} \) in control conditions and in the presence of KB could be largely suppressed by 10 \( \mu M \) tetrodotoxin (n_{cell} = 3; data not shown), suggesting that sensitivity to this toxin was not suppressed by KB.

Due to their structural resemblance, we also examined a possible interaction between KB and amiodarone. Amiodarone (10 \( \mu M \)) itself had no effect on \( I_{Na} \) induced from a V_{0.5} of 30 mV after 1-s conditioning steps to various potentials. The fitting lines are Boltzmann distribution functions (see Materials and Methods) drawn using the mean of parameters obtained in each experiment (control: \( V_{0.5} = -79 \pm 1.5 \) mV, slope = \( 4.8 \pm 0.2 \); 10 \( \mu M \) KB: \( V_{0.5} = -84 \pm 2.7 \) mV, slope = \( 4.5 \pm 0.3 \); 100 \( \mu M \) KB: \( V_{0.5} = -94 \pm 2.4 \) mV, slope = \( 6.0 \pm 0.4 \); \( P < 0.05 \) for \( V_{0.5} \) in KB versus control; \( P > 0.05 \) for slopes in KB versus control). C, relationship between shift in potential of half-maximum inactivation (\( V_{0.5} \)) and drug concentration.

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**Fig. 4.** Effect of KB on steady-state inactivation of \( I_{Na} \). A, current traces obtained at \(-30 \) mV after 1-s prepulses to various levels in control conditions (left) and in the presence of 10 \( \mu M \) KB (right). Horizontal dotted line indicates zero current level. \( V_{1/2} \) of -120 mV. Prepulses in the traces shown are to \(-105, -95 \) (identical with trace following \(-105 \)), \(-85, -75, -65 \), and \(-55 \) mV. B, availability (or inactivation) curves in the absence (○) and in the presence of 10 \( \mu M \) KB. Left inset, voltage pulse protocol. Right inset, superimposed currents, obtained by a double-pulse protocol, with varying interval between the two pulses. Data obtained by normalizing currents measured at \(-30 \) mV after 1-s conditioning steps to various potentials. The fitting lines are Boltzmann distribution functions (see Materials and Methods) drawn using the mean of parameters obtained in each experiment (control: \( V_{0.5} = -79 \pm 1.5 \) mV, slope = \( 4.8 \pm 0.2 \); 10 \( \mu M \) KB: \( V_{0.5} = -84 \pm 2.7 \) mV, slope = \( 4.5 \pm 0.3 \); 100 \( \mu M \) KB: \( V_{0.5} = -94 \pm 2.4 \) mV, slope = \( 6.0 \pm 0.4 \); \( P < 0.05 \) for \( V_{0.5} \) in KB versus control; \( P > 0.05 \) for slopes in KB versus control). C, relationship between magnitude of shift in potential of half-maximum inactivation (\( V_{0.5} \)) and drug concentration.

**Fig. 5.** Effect of KB on the recovery from inactivation. A, superimposed currents, obtained by a double-pulse protocol, with varying interval between the two pulses. \( V_{1/2} \) of -120 mV. Test potential, -30 mV. Pulses given every 5 s. Horizontal dotted line indicates zero current level. Left, in the absence of drug. Right, in the presence of 10 \( \mu M \) KB. Left inset, voltage pulse protocol. Right inset, traces after 20-ms (a) and 500-ms (b) interval. Note fast inactivation of the trace after shorter interval. B, amplitude of \( I_{Na} \) during the second pulse expressed relative to \( I_{Na} \) induced from a prepulse to various levels in control conditions. Hatched columns, 10 \( \mu M \) KB. *P < 0.05 for KB versus control. C, time constants and amplitudes of the biexponential fitting of inactivation. Unfilled columns, control conditions. Hatched columns, 10 \( \mu M \) KB. *P < 0.05 for KB versus control.
−120 mV and did not slow the inactivation process (Fig. 6A). In five cells, $\dot{I}_{Na}$ at −30 mV inactivated with $\tau$ of 2.1 ± 0.2 and 1.8 ± 0.2 ms before and after 10 min in the presence of 10 µM amiodarone. However, the drug shifted the inactivation curve to more negative potentials and slowed the recovery from inactivation of $I_{Na}$ ($n_{cells} = 4$; data not shown). Application of KB on top of amiodarone still induced its usual slowing of inactivation (Fig. 6A, right), enhanced the negative shift of the inactivation curve, and further slowed the recovery from inactivation. Because, on the one hand, KB like amiodarone may share some receptors with thyroid hormones, and on the other hand these hormones have been reported to slow $I_{Na}$ inactivation (Craelius et al., 1990; Harris et al., 1991; Dudley and Baumgarten, 1993), we also examined the effect of T3 and of its analog DITPA and their interaction with KB. T3 at physiological concentrations (10 pM–1 nM; $n_{cells} = 3$) did not affect $I_{Na}$. Even at higher concentrations (10 nM, $n_{cells} = 2$; or 10 µM, $n_{cells} = 6$), T3 failed to slow $I_{Na}$ inactivation under our experimental conditions (Fig. 6B). However, at 10 µM T3 increased $I_{Na}$ peak amplitude (Fig. 6B, middle; Huang et al., 1999). In addition, we noticed that in the presence of 10 µM T3 the effect of 10 µM KB (applied on top of T3) on inactivation was markedly reduced (Fig. 6B, right). DITPA at 10 µM ($n_{cells} = 2$) did not affect $I_{Na}$ or prevent the KB effect (data not shown). The above-mentioned results with amiodarone and T3 are summarized in Fig. 6C, which presents the relative magnitude of the slowly inactivating $I_{Na}$ component in the different experimental conditions.

**Preferential Binding of KB from Extracellular Side.** The removal of KB for up to 30 min was not associated with a washout of its effect ($n_{cells} = 3$). Because the drug is lipophilic and could reach an intracellular site of action even when given extracellularly, we tested the effect of adding KB to the cell-dialyzing pipette solution. In 11 cells, intracellular dialysis with 10 µM KB had no apparent effect on $I_{Na}$, which inactivated as fast as in untreated cells ($\tau = 2.5 ± 0.6$ ms, after 12–15 min of dialysis with KB; $V_{0.5}$ of inactivation of $-79 ± 1.4$ mV; $P_{0.05}$ versus $-80 ± 1.2$ mV in 26 control cells). In addition, these cells still responded to extracellularly applied KB (data not shown). In four other cells there was a modest slowing of $I_{Na}$ inactivation while dialyzing with 10 µM KB ($\tau_{fast} = 1.7 ± 0.1$ ms, $\tau_{slow} = 32.1 ± 2.3$ ms; and $A_{c} = 13 ± 3.4$% of total $I_{Na}$). However, even in these cells the slowing of inactivation became most marked when the same drug concentration was further applied externally. Taken together, these results do not allow excluding access of KB to its binding site from the intracellular medium, but indicate that the drug is more effective when applied externally.

To further examine the membrane side from which the drug reached its binding site, we measured single Na+$^+$ channel activity in cell-attached patches. Figure 7 compares recordings obtained without and with KB added to the pipette solution. When KB was not included in the pipette solution (Fig. 7A), multiple Na+$^+$ channel openings were clustered in the first 10 ms of the voltage step and hardly seen at potentials negative to −70 mV. In contrast, when 10 µM KB was present in the pipette (i.e., on the extracellular side of the patch; Fig. 7B), the channel openings were present for a longer time (during the first 100 ms of the voltage step; notice difference in time scale between A and B) but also decayed slowly with time. In addition, channel openings were frequently observed at potentials more negative than −70 mV. Typical ensemble-average currents obtained from such recordings in another experiment with 10 µM KB in the pipette are illustrated in Fig. 7C and confirmed that the channel inactivation was markedly slowed by the application of KB on the external side of the patch. Under these conditions, the inactivation time constant was 28 ± 6.6 ms ($n_{cells} = 3$) at −30 mV, a value that is of the same order of magnitude as the dominant slow time inactivation of whole-cell $I_{Na}$ (Fig. 1B). The current-voltage relationship obtained using ensemble averages has the shape expected for $I_{Na}$, and reversed at an extrapolated potential of $>+50$ mV (consistent with $[Na^+]_{pipette} = 150$ mM). When KB was applied in the bath, no or less marked effect on the patch channels was obtained (data not shown). These results are also consistent with an easier access of the drug from the extracellular side.

**Discussion**

**Effect on Inactivation.** The present study shows that KB, a drug related to amiodarone and to thyroid hormones, has marked effect on $I_{Na}$. The most prominent KB effect was a slowing of $I_{Na}$ inactivation, but the drug also had an effect on voltage-dependent activation and caused a change of $I_{Na}$ peak amplitude, depending on the voltage protocol.

The effect on the kinetics of $I_{Na}$ inactivation was unex-
pected given the known actions of amiodarone, which either does not change the kinetics of fast inactivation (Follmer et al., 1987; Kohlhardt and Fichtner, 1988) or accelerates the decay of late currents (Maltsev et al., 2001). It resembles that of many drugs, including animal or plant toxins, that interact with specific sites on the voltage-dependent Na⁺ channel and modify inactivation (Narahashi, 1996; Catterall, 2000; Goldin, 2001). At the molecular level, inactivation of Na⁺ channels and its coupling to activation involve many parts of the channel protein, comprising intracellular loops (e.g., between domain DIII and DIV of the α-subunit) but also intracellular, mid-, or even extracellular portions of certain transmembrane segments (Catterall, 2000; Goldin, 2001). Thus, inactivation can be modified by agents that act from either side of the membrane. For example, the binding site of insecticides such as DDT or pyrethroids can be accessed from the extracellular side of the membrane to change inactivation (Tatebayashi and Narahashi, 1994; Narahashi, 1996; Spencer et al., 2001). In the present study, the lack of a pronounced effect of intracellularly dialyzed KB in whole-cell experiments and the marked increase of channel activity by external KB in cell-attached patches are suggestive of a preferential access of KB from the external side.

The effect of KB on $I_{Na}$ peak amplitude depended on both $V_H$ and test potential. The marked decrease of $I_{Na}$ induced from $-80$ mV (Fig. 3, A and B), with less pronounced, no, or opposite effect when inducing $I_{Na}$ from $-120$ mV (Fig. 3C) is consistent with a shift of the $I_{Na}$ availability curve (Fig. 4B). Similar decreases of $I_{Na}$ amplitude and shifts of its inactivation curve are usually obtained by local anesthetic or class I antiarrhythmic drugs (Carmeliet and Mubagwa, 1998), but also by many other drugs that slow inactivation. Amiodarone also causes such an effect, which may contribute to its beneficial action against cardiac arrhythmias. The shift in inactivation curve by KB occurred at higher concentrations ($K_{0.5} \geq 6.9 \mu M$) compared with the effect on kinetics ($K_{0.5} = 2.1 \mu M$), making it plausible that different sites could be involved in the two effects. For example, binding at a local anesthetic site, accessible from the inside, could be invoked to account for the effect on voltage-dependent inactivation. However, the absence of a difference in $V_{0.5}$ of inactivation curve between cells internally dialyzed with KB and control cells, suggests that the shift of inactivation curve was not due to an internally accessible binding site.

KB applied while channels were rested at the holding potential, without any depolarizing pulse, produced its maximum effect on the first pulse. This indicates that the drug interacted either with the rested state and/or that there is very fast binding to open channels. At high drug concentrations, the largest component of the total $I_{Na}$ inactivated with a slow time constant, suggesting that under these conditions nearly all channels interacted with the drug. If such a KB binding were to occur only in the closed state, a significant increase in $I_{Na}$ amplitude should be obtained at all potentials in the presence of the drug due to less inactivation at the time of peak current. However, $I_{Na}$ was increased only at potentials less than $-50$ mV while using $V_H$ of $-120$ mV. This increase can be accounted for by a change of voltage-dependent activation. The lack of an $I_{Na}$ increase at potentials of maximum activation (more than $-40$ mV) therefore suggests that to a certain extent binding also involves the open state.

**Relation to Thyroid Hormones.** Given the reported slowing of $I_{Na}$ inactivation by T₃ (Craelius et al., 1990; Harris et al., 1991; Dudley and Baumgarten, 1993) and the structural similarity between this hormone and KB, we examined the effect of T₃, with the hypothesis that any T₃-induced slowing of inactivation was likely to involve the same membrane receptor as for KB. We were surprised to find that we could not reproduce the actions of T₃ on $I_{Na}$ inactivation reported by others, even at very high concentrations. The reason for this discrepancy with previous studies is not clear. But the same authors later obtained much less marked effect on the kinetics of inactivation in another study (Huang et al., 1999) and proposed that the T₃ effects could be influenced by...
the cell conditions. (Nevertheless, our study confirms that $T_3$ increases $I_{Na}$ peak amplitude; Huang et al., 1999.) We then tested whether $T_3$ was occupying the receptor involved in the KB action, but unable to induce the pharmacological effect. The decrease of KB effect when added on top of $T_3$ (Fig. 6, B and C) is in favor of this possibility, although a definitive proof would require more extensive and specific experimental techniques.

KB has been shown to interact with nuclear thyroid hormone receptors (Carlsson et al., 2002). Nuclear receptors mediate genomic effects that develop over hours or days. It is unlikely that the acute effects observed in the present study are mediated by such receptors. Extraneuronal receptors, which are also known to exist also for acute, nongenomic effects of thyroid hormones, are likely to be involved. The identity of the extraneuronal receptors is still unknown. The lack of response in most cells when including KB in the cytosol-dialyzing pipette solution and the marked effect of KB when the drug was applied to the external side of the patch are suggestive of an action site more easily accessible from the extracellular side. However, access to this site from an intracellular site cannot be completely excluded by the present experiments because in a few cases a small effect of KB applied via the pipette was obtained, and the ability of KB to be exchanged between pipette solution and cytosol is not known. At present we also cannot exclude the possibility of intermediate structures coupling the receptor for KB to the effector, i.e., the Na$^+$ channel. However, given the similarity with other inactivation-modifying drugs, it is possible that the Na$^+$ channel $\alpha$-subunit constitutes itself a membrane receptor for KB. Surprisingly, amiodarone, which is structurally closer to KB, did not antagonize the slowing of inactivation. Because only one concentration (10 $\mu$M) was tested, we cannot exclude the possibility that this negative result is due to a low-affinity interaction of amiodarone with the site responsible for slowing inactivation or the possibility that amiodarone is unable to induce the necessary conformation change.

**Practical Importance.** The effect of KB on $I_{Na}$ was not specific to the pig cells or to the special extracellular and cell-dialyzing solutions used to optimize $I_{Na}$ measurements. A similar effect could be obtained in ventricular myocytes of other species (guinea pig, rabbit, rat, and human) during superfusion with extracellular solutions containing 150 mM Na$^+$, or while recording with the perforated patch method (R. Macianskiene, S. Viappiani, K. Mubgwa, unpublished data).

KB action may be of importance for pathophysiological conditions such as arrhythmias and heart failure. KB has been proposed to have a protective effect during reperfusion after ischemia (Carlsson et al., 2002). However, because increased [Na$^+$]$_i$ is known to play a role in the deleterious effect of ischemia and reperfusion by promoting [Ca$^{2+}$]$_i$ overload, the slowing of $I_{Na}$ inactivation by KB, as described in the present study, will tend by itself to overload the cells with Na$^+$ and to enhance cell damage. In addition, slowed inactivation is generally associated with the long QT syndrome, with increased propensity to “tortades de pointes” arrhythmias and to fibrillation. Thus, a protective action based on this effect on Na$^+$ channel inactivation is unlikely. However, the “local anesthetic” action (shift in the inactivation curve), which implies that fewer channels are available to open from the resting potential, could partly offset the deleterious effect of slowed inactivation.

The slowing of Na$^+$ channel inactivation may be of interest for inotropy. Moderate increases in [Na$^+$], are proposed to be the basis of the beneficial effect of cardiac glycosides in supporting cardiac contraction in heart failure. Whereas the cardiac glycosides increase [Na$^+$], by inhibiting the Na$^+$–K$^+$-ATPase, such an increase can also be achieved by increasing Na$^+$ influx. There is increasing interest in a new class of positive inotropic agents (e.g., DPI-201–106, BDF-9148, BDF-9196, and LY-366634) that act by altering Na$^+$ channel function, because their action is preserved in failing myocardium (whereas the effect of cAMP-increasing agents tend to be limited; Muller-Ehmsen et al., 1997, 1998). For all positive inotropic agents, the beneficial use in heart failure is limited by their potential arrhythmogenic action. In this respect, it has been shown that many of the Na$^+$ channel-modifying agents increase the action potential duration (Amos and Ravens, 1994) and the QT interval on the electrocardiogram. It will be of interest to carefully examine whether KB lacks this side effect on action potential duration (Carlsson et al., 2002) and QT while probably retaining the positive inotropic action.

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**References**


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Address correspondence to: K. Mubagwa, Centre for Experimental Surgery and Anaesthesiology, University of Leuven, Campus Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium. E-mail: kanigula.mubagwa@med.kuleuven.ac.be