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INHIBITION OF G PROTEIN COUPLED AND ATP SENSITIVE POTASSIUM CURRENTS BY KB130015, AN AMIODARONE DERIVATIVE

by

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DMSO = dimethylsulfoxide;

EGTA ethylene glycol-bis(2aminoethlether)-N,N,N`,N`-tetraacetic acid;

HEPES = N-(2-hydroxyethyl) piperazine-N`(2-ethanesulfonic acid);

T_3 = 3,3`,5-triodo-L-thyronine

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Abstract:

KB130015 (KB), a novel compound derived from amiodarone, has been proposed to have antiarrhythmic properties. Its effect on the G protein-coupled inward rectifying K^+ current ($I_{K(ACh)}$ or $I_{K(Ado)}$), ATP sensitive K^+ current ($I_{K(ATP)}$), and background inward rectifying current (I_{K1}) were studied in guinea-pig atrial and ventricular myocytes by the whole-cell voltage clamp technique. Receptor-activated $I_{K(ACh/Ado)}$, induced in atrial myocytes by the stimulation of either muscarinic or Ado receptors was concentration-dependently ($IC_{50} \approx 0.6$-0.8 μM) inhibited by KB. Receptor-independent, GTP-γ-S-induced and background $I_{K(ACh)}$, which contributes to the resting conductance of atrial myocytes, were equally sensitive to KB ($IC_{50} \approx 0.9$ μM). $I_{K(ATP)}$ induced in atrial myocytes during metabolic inhibition with 2,4-dinitrophenol (DNP) was also suppressed by KB, whereas I_{K1} measured in ventricular myocytes was insensitive to the drug (KB ≤ 50 μM). While being effective when applied from the outside, intracellular application of KB via the patch pipette affected neither $I_{K(ACh)}$ nor $I_{K(ATP)}$. T_3, which shares structural groups with KB, did not have an effect on the K^+ currents. Consistent with the effects on single myocytes, KB did not depolarize the resting potential but antagonized the shortening of action potential duration by CCh or by DNP in multicellular preparations and antagonized the shortening of action potential duration by ACh in single myocytes. It is concluded that KB inhibits $I_{K(ACh)}$ and $I_{K(ATP)}$ by direct drug-channel interaction at a site more easily accessible from extracellular side of the membrane.

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Class III antiarrhythmic agents are effective drugs and are commonly used in ischemic heart disease. Suppression and/or prevention of arrhythmias are due to a prolongation of the action potential duration and of refractoriness. Amiodarone is considered as a class III drug and is known to be among the most effective drugs in the prevention of ventricular and supraventricular arrhythmias. Its action mechanisms at the cellular level include chronic effects, obtained after prolonged drug application, such as the suppression of the delayed repolarizing current I_{Ks} and an interaction with the thyroid hormone signal transduction. The drug also has short-term or acute effects, including an inhibition of various ion currents (for review see Kodama et al., 1999).

The clinical benefits of amiodarone are complicated by many undesirable side effects. To develop drugs with an improved pharmacological profile, new molecules based on its structural modification have been synthesized. KB130015 ([2-methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran]; KB) is one such novel compound, that has been suggested to have antiarrhythmic properties and to inhibit the binding of T_3 to nuclear receptors (Carlsson et al., 2002). Its relative effectiveness compared to amiodarone is not yet known. Given the structural similarity, some KB effects can be expected a priori. However, no prediction can be made about which of the amiodarone effects are conserved, or about their magnitude. As an example is the unexpected effect of KB on Na^+ channel inactivation, which is inexistent with amiodarone (Macianskiene et al., 2003b). Other structural analogs of amiodarone such as dronaderone (SR33589) have been shown to exert chronic and acute effects on ion channels similar to those of the parent agent amiodarone. The effects of KB on other K^+ channels have not been investigated so far but could be important. Cardiac K^+ currents that are acutely inhibited by amiodarone and its analog dronaderone include the background inward rectifying current (I_{K1}), the G protein-coupled inwardly rectifying currents (GIRK or $I_{K(ACh)}$ or $I_{K(Ado)}$), and the ATP-sensitive current ($I_{K(ATP)}$ (see Kodama et al., 1997; Altomare et al., 2000; Guillemare et al., 2000). In addition, while initial reports suggested that the fast component of the delayed rectifier, I_{Kr}, is insensitive, a few recent studies indicate that it is inhibited by high concentrations of amiodarone (IC_{50} = 38 µM; Kamiya et al., 2001).

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In the present study we investigated the effects of KB on inward rectifying potassium currents in isolated guinea-pig atrial and ventricular myocytes. $I_{K(ACh)}$ and $I_{K(ATP)}$ were measured in atrial cells. We found both currents to be markedly inhibited by KB, possibly via a direct drug-ion channel interaction. I_{K1} was studied in ventricular cells but was found to be insensitive to KB. In multicellular preparations, KB did not depolarize the resting membrane but antagonized APD shortening by CCh in atrial appendages, or by DNP in papillary muscles.

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METHODS

The experiments were done in guinea-pig isolated atrial and ventricular myocytes or multicellular preparations. The study was performed in accordance with the Declaration of Helsinki and the institutional guidelines for the care and use of laboratory animals.

Isolation and culture of myocytes

Single atrial myocytes were isolated via a Langendorff perfusion method previously described in detail (Banach et al., 1993). Atrial myocytes were kept in culture for up to 48 hours at 37 °C. The culture medium was the bicarbonate-buffered M199 (Gibco, Dreiech, Germany) containing gentamycin and kanamycin (each at 25 pg ml^{-1}; Sigma, Deisenhofen, Germany). The medium was not supplemented with fetal calf serum in order to prevent a time-dependent loss of sensitivity to ACh or Ado (Bunemann and Pott, 1995). Cells were plated at a density of several hundred per culture dish. About 50-70 % of the cells attached within 16 to 24 hours. No differences were found between freshly isolated and cultured myocytes in the effects studied here.

Multicellular preparations

Left atrial appendages and left ventricular papillary muscles were dissected from guinea pig hearts. The preparations were pinned to the bottom of a tissue chamber and continuously superfused with the Tyrode solution equilibrated with 100 % O_2. They were electrically stimulated with bipolar electrodes at 1 Hz with pulses of 2 ms duration and twice the diastolic threshold.

Solutions

For measurements in isolated myocytes the culture medium was replaced by a solution containing (mM): NaCl 120, KCl 20, $CaCl_2$ 2, $MgCl_2$ 1, glucose 10, HEPES-NaOH 10, pH 7.4. Pipettes were filled with a solution containing (mM): K-aspartate 110, KCl 20, MgATP 5, $MgCl_2$ 1, EGTA 2, GTP 0.1, HEPES-NaOH 10, pH 7.4. For activation of $I_{K(ATP)}$ $MgCl_2$ was substituted for MgATP in an equimolar concentration. The chosen K^+ concentrations yield an equilibrium potential for K^+ of -48 mV. The Tyrode solution used for multicellular preparations contained (in mM): NaCl 144, KCl 4,

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$CaCl_2$ 1.8, $MgCl_2$ 1, HEPES-NaOH 10, glucose 5, pH 7.4. Experiments on isolated cells were carried out at room temperature (21-24 °C). For multicellular preparations, the bath temperature was kept at 36±1 ^oC, and pO_2 was 580-600 mmHg.

Materials

Standard salts were from Merck (Darmstadt, Germany). HEPES, EGTA, MgATP, GTP, ACh-chloride, carbamylcholine-chloride (CCh), adenosine (Ado) and DNP were from Sigma (Deisenhofen, Germany). KB (free acid) was from Karo-Bio AB, Huddinge, Sweden, and was dissolved as a 100-200 mM stock solution in DMSO. The highest DMSO concentration used (0.1 % v/v) had no direct effect on the membrane currents and potentials under study nor did it affect the action of ACh, Ado or DNP.

Ion current measurements and action potential measurements in single atrial myocytes

Membrane currents were measured under whole-cell patch clamp (Hamill et al., 1981). Pipettes were fabricated from borosilicate glass with filament (Clark Electromedical, Pangbourne, UK) on a horizontal puller (DMZ, Munich, Germany) and were filled with the solution listed below. The DC resistance of the filled pipettes ranged from 3 to 6 MΩ. Currents were measured by means of a patch clamp amplifier (List LM/EPC 7, Gießen, Germany). Signals were analog filtered (corner frequency 1-3 kHz) and were digitally stored on the hard disk of a computer equipped with a software package for voltage control, data acquisition, and data evaluation (ISO-2 by MFK, Frankfurt, Germany). Capacitance and series resistance were partially compensated. Unless stated otherwise the holding potential was -90 mV, chosen to cause large inward K^+ currents because of the strong inward rectifying properties of G protein-coupled currents and an extracellular K^+ concentration of 20 mM. Fast voltage ramps (-120 to +60 mV; 500 ms) were generated to provide current-voltage relations and to monitor the electrical access to the cell. Drug-induced changes in outward current were measured during the voltage ramps, and no qualitative difference was detected in the observed drug effects on inward and outward currents. Rapid exchange of superfusion solution was performed by means of solenoid-operated valves, which permitted switching between up to 6 different solutions. The halftime

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of solution exchange was about 0.5 s, as measured from the time course of $I_{K(ACh)}$ block upon switching from the standard (high-K^+) to a K^+-free, 5 mM Cs^+-containing solution.

Current-clamp experiments were also performed in the whole cell recording mode at 37±1.0°C. External and pipette solutions were the same as those used in the whole cell voltage-clamp experiments. However a physiological external K^+ concentration was chosen (5 mM). After the establishment of the whole cell clamp mode rectangular 2 ms currents were applied at a rate of 0.2 Hz. After a stabilization of action potential configuration experiments were performed.

Action potentials in multicellular preparations

For measurements in the multicellular preparation transmembrane potentials were recorded with glass microelectrodes filled with 3 M KCl (resistance: 7-10 MΩ). The microelectrodes were connected to the input stage of a high-impedance amplifier (Nihon Kohden MEZ-7101). The amplified signals were displayed on a dual-beam oscilloscope (C1-69) and sampled at 10 kHz using a 16-bit analogue-to-digital converter (PCL816; Advantech France, Levallois Perret, France). After an equilibration period of 50-60 min with stable impalement, control recordings were first made. Then the atrial preparations were exposed to carbachol (CCh; 10 µM, for 10-15 min) followed by a washout of the agonist (15-25 min). Afterwards, the same preparations were exposed to KB (50 or 200 µM; for 15-20 min), and CCh was reapplied and washed out in the presence of KB. In ventricular preparations, control measurements were followed by an application of 2,4-dinitrophenol (DNP; 50-100 µM) for 10 min, and of KB (200 µM; 25 min) added on top of DNP. Action potential durations were measured at 50 % (APD_{50}) and 90 % (APD_{90}) repolarization.

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RESULTS

KB inhibits agonist-induced and background muscarinic potassium current

In guinea pig atrial myocytes freshly isolated or kept in serum-free culture for several hours, saturating concentrations of ACh (10 μM) induced the G protein-coupled inward rectifying current $I_{K(ACh)}$, with a mean current density of -74±9 pA/pF at -90 mV (n=7). Figure 1A shows a representative current trace. Adding KB (50 μM) to the superfusion solution in the continued presence of ACh caused a complete inhibition of $I_{K(ACh)}$. The current-voltage (I-V) relationship of the ACh-induced current, obtained by subtracting currents in basal conditions (labeled in Figure 1A as "a") from the current in the presence of ACh (labeled in Figure 1A as "b"), is displayed in Figure 1B (filled squares) and shows the strong inward rectification typical for $I_{K(ACh)}$. The KB-inhibited current (Figure 1B, unfilled squares), obtained by subtracting the current in the presence of KB ("c" in Figure 1A) from that in the presence of ACh ("b" in Figure 1A), exhibited characteristics identical to those of $I_{K(ACh)}$, suggesting that the drug inhibited this current.

KB effectively inhibited both inward and outward components of $I_{K(ACh)}$. The KB-sensitive current was quantitatively larger than the ACh-induced current (see less inward current at holding potential in the presence of KB compared to pre-drug level in Fig. 1A). Since an agonist-independent opening of $K_{(ACh)}$ channels has been shown to contribute to the background current in atrial cells (Sakmann et al., 1983; Okabe et al., 1990; Kaibara et al., 1991), our results thus suggest that KB inhibited not only the ACh-induced $I_{K(ACh)}$ but also the basal G protein activated current. The I-V relationship of the KB-inhibited background current (not illustrated) also displayed the same strong inward rectification as $I_{K(ACh)}$.

The effect of KB was concentration-dependent and reversible. Reversibility of the inhibition to the expected current level, taking into account the concurrent desensitization, was observed using low KB concentrations (Figure 2A), but the effects of high concentrations (≥ 10 μM) persisted for several minutes during drug washout. The concentration-effect curve obtained from experiments in 7 cells

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could be fitted by a Hill equation with IC_{50} of 0.82 µM and Hill coefficient of 1.12 (Figure 2B, continuous line).

To examine whether KB inhibition of $I_{K(ACh)}$ was due to an interaction at the muscarinic receptor level, or involved mechanisms downstream of the receptor, we tested its effect on G protein coupled K^+ current induced by other receptors. We therefore activated $I_{K(ACh)}$ by adenosine. Figure 3A illustrates an experiment that is representative for six different atrial myocytes, in which KB inhibited the adenosine-induced $I_{K(Ado)}$ (which is identical to $I_{K(ACh)}$). Again the effect was concentration-dependent (IC_{50}=0.57 µM; see Figure 2B, dashed line) and reversible within minutes. I-V relationships presented in Figure 3B show that the KB-sensitive current displayed the same strong inward rectifying properties as $I_{K(Ado)}$.

T_3, which shares structural groups with KB and whose nuclear receptors can bind KB (Carlsson et al., 2002), failed to mimic the inhibitory action of this drug on the adenosine-induced potassium current (concentrations tested ≤ 3 µM). Figure 3A shows an experiment being representative for 4 different myocytes. In this experiment 30 nM T_3, which is a physiological concentration of the hormone, had no effect on G-protein-dependent potassium current.

KB inhibits GTP-γ-S induced $I_{K(ACh)}$

The above experiments, in which KB inhibits $I_{K(ACh)}$ coupled to different receptors, make it unlikely that the drug acts at the receptor level. To further determine the level of KB action along the G protein-channel signal transduction cascade we tested the effect of KB on $I_{K(ACh)}$ activated by GTP-γ-S (500 µM). The non-hydrolysable nucleotide was added to the patch pipette solution and yielded an irreversible activation of $I_{K(ACh)}$ upon repeated exposure to ACh. Figure 4 illustrates representative results of similar experiments in 5 atrial myocytes. While dialyzing with GTP-γ-S, the application of ACh resulted in an activation of $I_{K(ACh)}$ to its maximal level (Figure 4A). The effect of ACh was only partially reversible after the first application and became irreversible after the third agonist application. Sustained $I_{K(ACh)}$ obtained after washout of ACh under these conditions was inhibited by KB

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(IC_{50}=0.89 µM; see Figure 2B, dotted line). The KB-sensitive current displayed strong inward rectifying properties (Figure 4B) and was larger than the current induced by GTP-γ-S and ACh (see less inward current at holding potential in the presence of KB compared to pre-drug level in Figure 4A), suggesting that KB inhibited both GTP-γ-S-induced and background $I_{K(ACh)}$. These experiments indicate that KB inhibits $I_{K(ACh)}$ either by interfering with the G protein (βγ-)subunit-channel interaction or by directly acting on $K_{(ACh)}$ ion channels.

KB inhibits the ATP-sensitive K^+ channel.

To investigate the sensitivity of $I_{K(ATP)}$ to KB we performed experiments in atrial myocytes and superfused the cells with DNP (100 µM). Figure 5 shows a representative experiment for 6 cells superfused with DNP. After application of the metabolic uncoupler, $I_{K(ATP)}$ was slowly activated and resulted in an increase of inward current at the holding potential of -90 mV. Superfusion with KB in the continued presence of DNP resulted in a suppression of the inward current increase. I-V relationship of the DNP-induced current showed weak inward rectification, which is typical for $I_{K(ATP)}$, and was superimposable to the KB-inhibited current, indicating that $I_{K(ATP)}$ was the target of both agents.

No effect on the background inward rectifier (I_{K1}) channel.

If KB is an inhibitor of G protein-coupled and ATP-sensitive channels other ion channels sharing structural similarities with these channels might be sensitive to the drug as well. For this reason we studied the effect of KB on inward rectifying potassium current I_{K1}. No effect of KB (10-50 µM) on that current was detected in 5 out of 5 ventricular myocytes (not illustrated). These results are consistent with data from pig ventricular myocytes (Macianskiene et al., 2003a).

No effect of KB applied via the patch pipette

KB is a lipophilic compound and may be expected to easily cross the cell membrane upon extracellular superfusion. So far the experiments did not differentiate between an intra- or an extracellular action site of the drug. To address this question we added KB (50 µM) to the patch

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pipette filling solution and cell dialysis was allowed to take place during at least 20 min. A representative experiment for 8 cells with this protocol is illustrated in Figure 6. After rupturing the membrane patch, the whole cell was exposed to short periods of superfusion with ACh in order to monitor $I_{K(ACh)}$. The first response to ACh was elicited 10 s after getting access to the cell interior. At this time diffusion of KB into the cell, if any, should be minimal since in experiments with intrapipette GTP-γ-S the first noticeable irreversible activation of $I_{K(ACh)}$ was observed only after a delay of about 40 s after patch rupture, with full activation being reached after a few minutes. Even after 30 min of cell dialysis with internal KB no inhibition of $I_{K(ACh)}$ was detected. However KB (50 µM) applied from the external side of the membrane completely inhibited $I_{K(ACh)}$. In line with the experiments described above, the background current was not affected by internal KB but was decreased by external KB. These results exclude that KB inhibits $I_{K(ACh)}$ by accessing its action site from the internal side of the membrane.

Influence of KB on action potentials in multicellular preparations and in single atrial myocytes

Given the above KB effects on membrane currents, the drug may be expected to antagonize the shortening of action potential induced by muscarinic receptor activation in atrial cells. Figure 7A illustrates the effect of carbachol (CCh) before or after pretreatment with KB in multicellular preparations. Under control conditions (in the absence of KB) action potentials were markedly shortened in the presence of 10 µM CCh (Figure 7A, left panel). After washout of the CCh effect (not illustrated; but see Table 1), treating the same preparation with 200 µM KB for 20 min, caused no major change of the action potential. Reapplication of CCh after stabilization with KB caused less marked APD shortening compared to control conditions (Figure 7A, right panel). Similar results were obtained in 5 preparations, as summarized in Figure 7B. APD_{90} in the presence of both CCh and 200 µM KB was not significantly different from the value in the presence of 200 µM KB alone, but was significantly ($P<0.05$) longer than the value in the presence of CCh before treatment with KB (Table 1). The inhibition of CCh effect by KB was concentration-dependent, and was less with 50 µM KB. In single atrial myocytes KB 50 µM almost completely reversed an ACh (10 µM) induced action

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potential shortening. An experiment being representative for 6 different cells is shown in Figure 8A. The summary of the data out of six different experiments (APD_{90}) is shown in Figure 8B.

We also expected KB to antagonize the shortening of action potential induced upon K_{ATP} channel opening with DNP. This antagonism was tested in papillary muscles. It was rather difficult to stably record action potentials in the presence of DNP (100 µM), because of a frequent occurrence of contracture or of a complete loss of excitability in several experiments. Successful recordings in 4 preparations using 50 µM DNP (n=2) or 100 µM DNP (n=2) showed that KB could partly reverse the APD shortening caused by the metabolic poisoning. Figure 9A illustrates a typical result, and Figure 9B and Table 2 summarize the data from all preparations. The APD prolongation caused by KB was significant ($P<0.01$) but remained modest and did not restore the duration to its initial value.

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DISCUSSION

The objective of the present study was to examine the effects of the new amiodarone-like compound KB130015 (KB) on cardiac K^+ currents. The results show that KB inhibits the G-coupled inward-rectifying K^+ current $I_{K(ACh)}$ and the ATP-sensitive current $I_{K(ATP)}$ in atrial cells. KB suppressed $I_{K(ACh)}$ activated indirectly via various receptors or directly by GTP-γ-S. Consistent with the suppression of $I_{K(ACh)}$ measured in single cells, KB decreased the APD shortening caused by the muscarinic agonist CCh in multicellular preparations. Similarly the drug suppressed $I_{K(ATP)}$ activated by metabolic inhibition with DNP in myocytes, and partially reversed the APD shortening by DNP in papillary muscles. In contrast, KB had no effect on I_{K1} recorded in myocytes and on the resting potential in multicellular preparations.

KB shares structural similarity with amiodarone, from which it was derived by chemical substitution (Carlsson et al., 2002), with the aim to produce a drug with less side effects and improved bioavailability. Preliminary animal toxicity studies of KB suggest that side effects following its chronic administration occur at doses similar to or higher than those of amiodarone, used as reference compound (Carlsson et al., 2002; supplementary materials at http://pubs.acs.org). Amiodarone, which is one of the most effective antiarrhythmic drugs, exerts many effects at the cellular level after either acute or chronic administration (Kodama et al., 1997). While it is still unclear which of the complex effects of amiodarone are critical for its antiarrhythmic action, its effects on the function and expression of various ion channels are likely to play a predominant role. Amiodarone or its analogs, when acutely applied, decrease the availability or the open probability of many ion channels, including voltage-dependent Na^+ channels, L-type Ca^{2+} channels and various K^+ channels (see Kodama et al., 1997). Hence KB could be expected to exert similar effects. However, besides the recently described and unexpected effects of KB on voltage-dependent Na^+ and Ca^{2+} channels (Macianskiene et al., 2003b), its effects on other ion channels are largely unknown.

$I_{K(ACh)}$ represents the prototypical G protein gated K^+ channel, and can be activated via the M_2 muscarinic receptor (m_2AChR; for review see Kurachi, 1995; Dascal, 1997; Dascal, 2001) or via the

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A_1 adenosine receptor (A_1-AdoR; Kurachi et al., 1986). Our data show that $I_{K(ACh)}$ activated by agonists to either of these receptors (ACh and Ado, respectively) was suppressed by KB. The KB-sensitive current had the same reversal potential (-48 mV) and a similar (inward-rectifying) shape of the I-V relationship as the current induced by the agonists. Under our experimental conditions, $I_{K(ACh)}$ reached a peak shortly after the start of the agonist application, and then decayed to a steady level due to desensitization (Carmeliet and Mubagwa, 1986). KB applied on top of the agonists suppressed $I_{K(ACh)}$ with a time course markedly faster than that of desensitization. In addition, upon washout of low KB concentrations, $I_{K(ACh)}$ rapidly returned to its steady state level. The fast onset of and recovery from KB effect indicates that $I_{K(ACh)}$ suppression by KB involved a mechanism different from desensitization. The KB effect is likely to involve a mechanism downstream from the agonist-receptor interaction, at the G protein or $K_{(ACh)}$ channel level. This was suggested by the equal potency to suppress ACh and Ado effects (see Fig. 2B) and was confirmed by the ability of KB to suppress $I_{K(ACh)}$ induced by GTP-γ-S. These results are similar to those obtained with amiodarone and dronaderone, which also inhibit $I_{K(ACh)}$ induced via various receptors or by internal GTP-γ-S (Watanabe et al., 1996; Altomare et al., 2000; Guillemare et al., 2000). The potency of KB to suppress $I_{K(ACh)}$ (IC_{50} = 0.8 μM) is similar to that of amiodarone (IC50= 1-2.4 μM; Watanabe et al., 1996; Guillemare et al., 2000), but much lower compared to that reported for dronedarone (10-63 nM; Altomare et al., 2000; Guillemare et al., 2000). Our finding that KB suppresses activation of G protein-gated K+ channels by muscarinic and adenosine agonists does not exclude the possibility that it also acts at the receptor level. The parent compound, amiodarone, interferes with binding to the muscarinic receptor (Cohen-Armon et al., 1984; Colvin et al., 1989). As in the case of amiodarone, KB actions may be more potent at, but not restricted to the channel.

During KB application the current at holding potential usually became smaller than the basal level. I-V relationships also showed quantitatively larger KB-sensitive currents compared to agonist- or GTP-γ-S-induced currents. This indicates that KB had another effect in addition to suppressing the effect of agonists and GTP-γ-S. The extra-current suppressed by KB showed inward rectification and reversal potential similar to those of $I_{K(ACh)}$. Agonist-independent opening of $K_{(ACh)}$ channels has been

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shown to contribute to the background current in atrial cells (Sakmann et al., 1983; Okabe et al., 1990) and reflects basal activation of $G_{i(K)}$ molecules by non-liganded receptors (Kaibara et al., 1991). Our results thus suggest that in the presence of KB not only the ACh-induced $I_{K(ACh)}$ but also basal G protein activated current was inhibited. Since agonist-independent opening of $I_{K(ACh)}$ may contribute to basal current in atrial cells, it has been proposed that APD prolongation by amiodarone and its analogs could contribute to antiarrhythmic action. Our experiments on atrial preparations, KB was applied alone after washout of CCh. At 50 µM there was no effect, but at 200 µM the drug caused a slight prolongation of the APD (see Table 1). It is not possible to interpret this effect solely in terms of an effect on $I_{K(ACh)}$ because effects on other ion channels could contribute to the net change. KB has effects on Na^+ and Ca^{2+} channels (Macianskiene et al., 2003a). Other ion currents (e.g. the Na^+-K^+ pump, the Na^+-Ca^{2+} exchange, or the Na^+-activated K^+ currents, etc., that are sensitive to intracellular Na^+ concentrations) might also be indirectly affected by the expected increase in Na_i due to prolonged Na^+ channel opening (Macianskiene et al., 2003b). In addition, the contribution of $I_{K(ACh)}$ might be overwhelmed by a larger contribution of I_{K1}, especially at the resting potential.

In the present study, $I_{K(ATP)}$ in the whole cell configuration was activated by uncoupling mitochondrial respiration from oxidative phosphorylation with 2,4-dinitrophenol (DNP). Both the DNP-induced current and the current suppressed by KB in the presence of DNP showed similar inward-going rectification. Although outward rectifying currents have been induced by metabolic inhibition in other studies (Isenberg et al., 1983), inward rectification may be induced by intracellular Mg^{2+} and Na^+ (Horie et al., 1987; Ciani and Ribalet, 1988). Inward rectifying K(ATP) currents have been obtained under experimental conditions similar to those used in the present study (Brandts et al., 2000). Hence we can conclude that $I_{K(ATP)}$ induced by DNP was reversibly suppressed by KB. A similar effect (IC_{50}=2.3 µM) has been previously noted using amiodarone on $I_{K(ATP)}$ induced by ATP depletion or by bimakalim in inside-out membrane patches of rat ventricular cells (Holmes et al., 2000). However, other studies showed a lower potency (IC_{50}=19 µM) of amiodarone on the K^+ conductance induced by ATP depletion in rat cardiocytes or a lack of effect on $I_{K(ATP)}$ induced by the K^+ channel opener

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KRN2391 in Xenopus oocytes (Sakuta et al., 1992). The reason for this discrepancy remains unclear, but the difference in results might have been due to the cell type or the experimental conditions.

Of the K^+ currents tested, I_{K1}, which represents the strong inward rectifying background current responsible for setting the resting potential in atrial and ventricular myocytes, was the least sensitive to KB effect. Our results with guinea-pig ventricular cells are similar to those obtained in pig cells, where KB was found to have no effect on I_{K1} or on the resting potential (Macianskiene et al., 2003a).

Our data also show that KB could antagonize the APD shortening induced by m_2AChR activation in intact atrial muscle and isolated atrial myocytes. Similarly the drug partially reversed the APD shortening induced by DNP in the multicellular preparation of ventricular muscle. In the multicellular preparation, KB concentrations of 50 µM were little or not effective and a concentration as high as 200 µM had to be given to obtain an effect. The reasons for the low potency of KB in multicellular preparations are unknown but could involve diffusion limitations imposed by superfusing instead of perfusing the preparations. This may result in reduced drug availability in the cell from which recordings are made. The same reason can be invoked to explain only partly the low efficacy of KB to oppose DNP-induced APD shortening. Additional factors (e.g. effects on other ion channels) implicated in the DNP effects or a change of sensitivity of $K_{(ATP)}$ channels by DNP are likely to be involved.

Although KB is highly lipophilic and its effect at high concentrations could not be easily washed out, the onset and washout of the effect of low concentrations was fast (see Figure 2). The latter finding makes it likely that KB was acting from a site easily accessible from the extracellular medium. This is supported by experiments in which intracellularly dialyzed KB failed to inhibit $I_{K(ACh)}$ (see Figure 6). A similar failure of intracellular KB (in contrast to exracellular KB) to modify Na^+ channels has been observed (Macianskiene et al., 2003b). For amiodarone, based on 1) the high lipophilicity, 2) the slow time course of its action, and 3) the ability of bath applied molecules to block $I_{K(ACh)}$ in cell-attached patches, it has been proposed that its effect on $I_{K(ACh)}$ is caused by drug diffused into the intracellular

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medium (Altomare et al., 2000). However, the same criteria would apply if the drug can diffuse within the membrane to reach its action site. Given the above-mentioned failure of intracellular KB to cause any effect, we propose that KB inhibits $I_{K(ACh)}$ and $I_{K(ATP)}$ by binding to the channels or their subunits from within the cell membrane, at a sites more easily accessible from the external side of the bilayer.

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FOOTNOTES

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LEGENDS TO FIGURES

Figure 1.

Inhibition by KB of $I_{K(ACh)}$ activated via muscarinic receptors.

A. Current continuously recorded at the holding potential (-90 mV) and during regular short voltage ramps (appearing as vertical current deflections) under basal conditions, during exposure to ACh (10 μM) and during exposure to KB (50 μM) added on top of ACh. Periods of exposure to ACh and KB are indicated by horizontal bars. Upper dotted line: zero current level. Lower dotted line: current level at holding potential under basal conditions. Notice 1) the increase of inward current by ACh, 2) the suppression of the ACh effect in the presence of KB, and 3) a decrease of the inward current to a lower than basal level in the presence of KB.

B. Current-voltage (I-V) relationships of the ACh-induced current (unfilled squares) and of the KB-sensitive current (filled squares). The ACh-induced current was obtained by subtracting the trace labeled as "a" from the trace labeled as "b" in A. The KB-sensitive current was obtained by subtracting the trace labeled as "c" from the trace labeled as "b" in A.

Figure 2.

Reversibility and concentration-dependence of the KB effect on $I_{K(ACh)}$.

A. Current continuously recorded at the holding potential (-90 mV) and during regular short voltage ramps (appearing as vertical current deflections) under basal conditions, during exposure to ACh (10 μM) and during exposure to KB (1 μM) added on top of ACh. Periods of exposure to ACh and KB are indicated by horizontal bars. Dashed line: zero current level. Notice the rapid removal of 1 μM KB effect upon washout of this concentration.

B. Relationship between relative inhibition of $I_{K(ACh)}$ and drug concentration. $I_{K(ACh)}$ induced by either ACh (n=7), Ado (n=6) or GTP-γ-S (n=5). The amplitude of inhibition was measured as the difference between currents just before and at the end of the KB application. Data were fitted to the Hill equation (relative inhibition $=1/\{1+(IC_{50}/[KB])^p\}$; where [KB] is the drug concentration, IC_{50} is the half-maximum effective concentration, and p is the Hill coefficient).

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Figure 3.

Inhibition by KB of $I_{K(ACh)}$ activated via A_1 adenosine receptors.

A. Current continuously recorded at the holding potential (-90 mV) and during regular short voltage ramps (appearing as vertical current deflections) under basal conditions, during exposure to adenosine (Ado; 100 μM) and during exposure to KB (50 μM) or T_3 (30 nM) added on top of Ado. Periods of exposure to Ado, KB or T_3 are indicated by horizontal bars. Dotted line: zero current level. Notice the suppression of the Ado effect in the presence of KB but not in the presence of T_3.

B. Current-voltage (I-V) relationships of the Ado-induced current (unfilled squares) and of the KB-sensitive current (filled squares). The Ado-induced current was obtained by subtracting the trace labeled as "a" from the trace labeled as "b" in A. The KB-sensitive current was obtained by subtracting the trace labeled as "c" from the trace labeled as "b" in A.

Figure 4.

Inhibition by KB of $I_{K(ACh)}$ activated via the non-hydrolysable GTP analog GTP-γ-S.

A. Current continuously recorded at the holding potential (-90 mV) and during regular short voltage ramps (appearing as vertical current deflections) under basal conditions, during repeated exposures to ACh (10 μM) and during exposure to KB (50 μM). Cell dialyzed with pipette solution containing 500 μM GTP-γ-S. Periods of exposure to ACh or KB are indicated by horizontal bars. Dotted line: zero current level. Notice sustained $I_{K(ACh)}$ after last exposure to ACh and its suppression following exposure to KB.

B. Current-voltage (I-V) relationships of the KB-sensitive current, obtained by subtracting the trace labeled as "b" from the trace labeled as "a" in A.

Figure 5.

Inhibition by KB of $I_{K(ATP)}$ activated during metabolic inhibition with dinitrophenol (DNP).

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A. Current continuously recorded at the holding potential (-90 mV) and during regular short voltage ramps (appearing as vertical current deflections) under basal conditions, during exposure to DNP (100 µM) and during exposure to KB (50 µM) applied in the presence of DNP. Cell dialyzed with ATP-free pipette solution. Periods of exposure to DNP or KB are indicated by horizontal bars. Dotted line: zero current level. Notice the slow activation of $I_{K(ATP)}$ during the exposure to DNP and its suppression following exposure to KB. ACh induced $I_{K(ACh)}$ is inhibited as well.

B. I-V relationships of the DNP-induced (unfilled squares) and the KB-sensitive (filled squares) currents. The DNP-induced current was obtained by subtracting the trace labeled as "a" from the trace labeled as "b" in A. The KB-sensitive current was obtained by subtracting the trace labeled as "c" from the trace labeled as "b" in A. Weak inward rectification indicates $I_{K(ATP)}$ to be the activated and inhibited current respectively.

Figure 6.

Lack of effect of intracellularly applied KB of $I_{K(ACh)}$.

Current recorded at the holding potential (-90 mV) and during regular short voltage ramps (appearing as vertical current deflections) under basal conditions and during two exposures to ACh (10 µM). Dashed line: zero current level. Cell dialyzed with pipette solution containing 50 µM KB. Periods of exposure to ACh or to extracellular KB are indicated by horizontal bars. First exposure to ACh: 10 s after patch rupture. Second exposure to ACh: 30 min after patch rupture. Dotted line: zero current level. Notice similar effects of ACh during both applications.

Figure 7.

Reversal by KB of the effect of CCh in multicellular atrial preparations.

A. Typical action potentials measured in one single preparation in various conditions. Left panel: under basal control conditions, and after application of 10 µM CCh. Right panel: during application of 200 µM KB (after washout from the fist CCh exposure) and following re-exposure to CCh in the presence of KB. Dashed lines: zero potential level.

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B. Pooled data of action potential duration measured at 90 % repolarization (APD_{90}), in control and in the presence of CCh (10 µM) applied in 10 preparations before exposure or after 20-min pre-exposure to 50 µM (n=5) or 200 µM KB (n=5).

*: $P<0.05$ when comparing APD_{90} in the presence of CCh before exposure to KB vs. after pre-exposure to 50 µM KB.

Figure 8.

Effect of KB 50µM on action potential of atrial myocytes in the presence of ACh.

A. Representative experiment where ACh 10 µM induces a marked shortening of the action potential. Superfusion of the myocyte with KB completely reverses the ACh effect.

B. Summarized data of changes of action potential duration at 90% repolarization (APD_{90}).

*: $P<0.05$ when comparing APD_{90} in the presence of ACh before exposure to KB vs. after pre-exposure to 50 µM KB.

Figure 9.

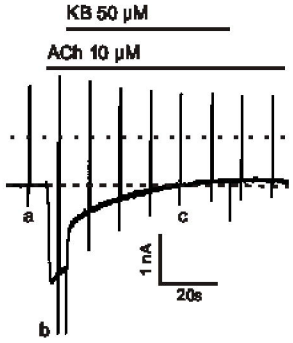
Reversal by KB of the effect of 2,4-dinitrophenol (DNP) in multicellular atrial preparations.

A. Typical action potentials measured in one single preparation under basal control conditions, after 10-min application of 100 µM DNP and after 25-min application of 200 µM KB on top of DNP.

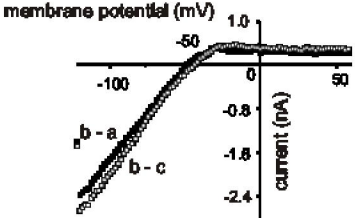
B. Pooled data (n=4) of action potential duration measured at 90 % repolarization (APD_{90}), in control, in the presence of DNP (50 µM, n=2; or 200 µM, n=2), and in the presence of KB (200 µM) applied on top of DNP.

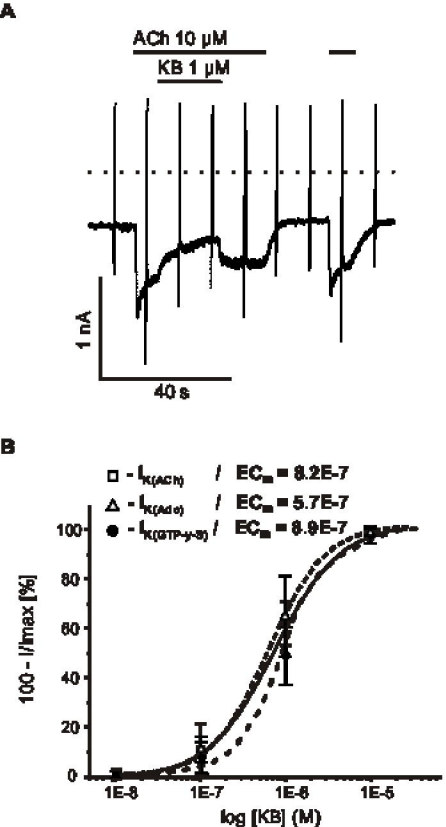
*: $P<0.05$ when comparing APD_{90} in the presence of DNP + KB vs DNP alone.

A

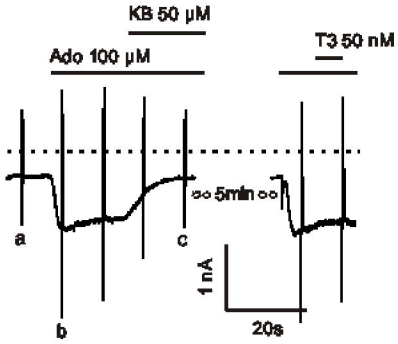

KB 50 µM
ACh 10 µM
a
b
c
1 nA
20s

B

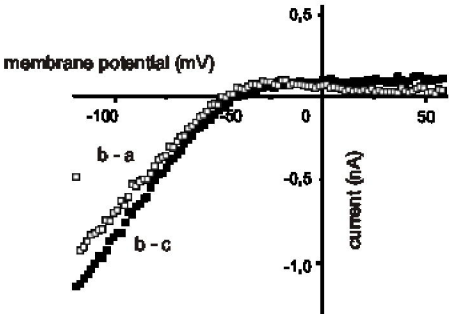

membrane potential (mV)
1.0
-50
-100
0
50
-0.8
-1.6
-2.4
current (nA)
b - a
b - c

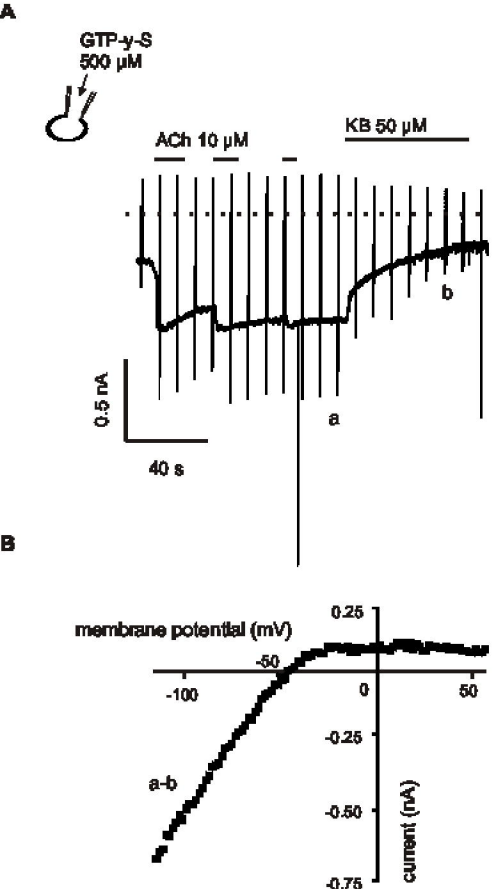

A
ACh 10 µM
KB 1 µM
1 nA
40 s
B
□ - I K(ACh) / EC50 = 8.2E-7
△ - I K(Ado) / EC50 = 5.7E-7
● - I K(GTP-γ-S) / EC50 = 8.9E-7
100 - I/Imax [%]
100
80
60
40
20
0
1E-8
1E-7
1E-6
1E-5
log [KB] (M)

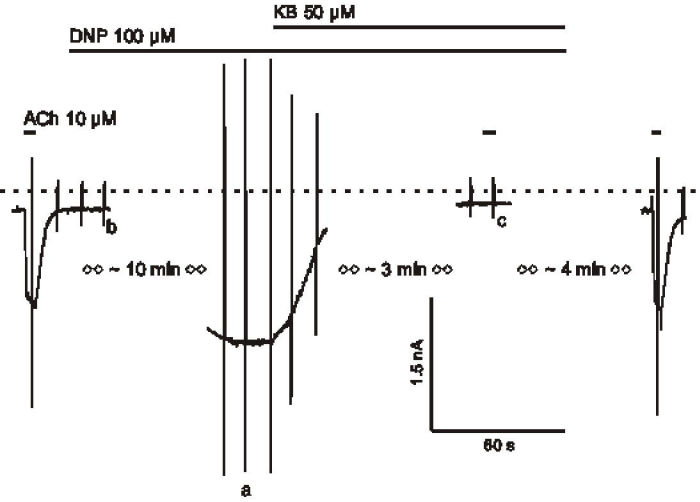
A

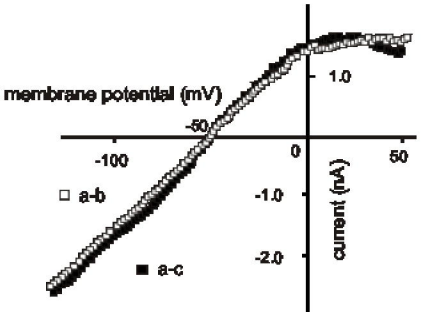

KB 50 µM
Ado 100 µM
T3 50 nM
5min
a
b
c
1 nA
20s

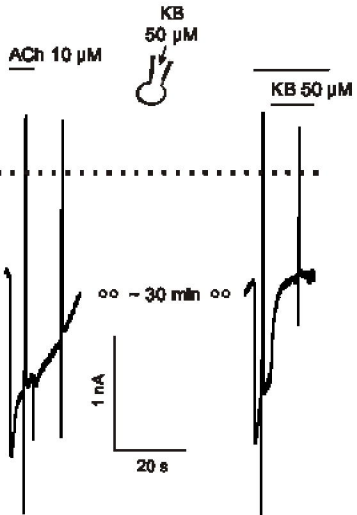
B

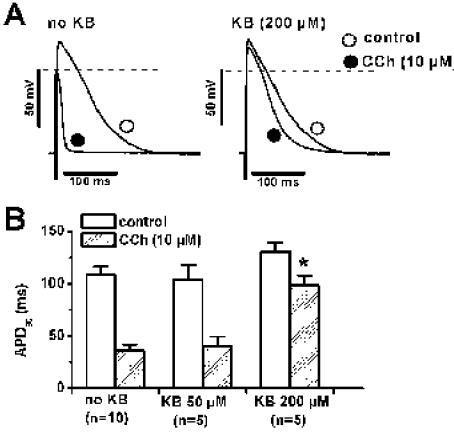

membrane potential (mV)
0,5
-100
-50
0
50
b - a
b - c
-0,5
current (nA)
-1,0

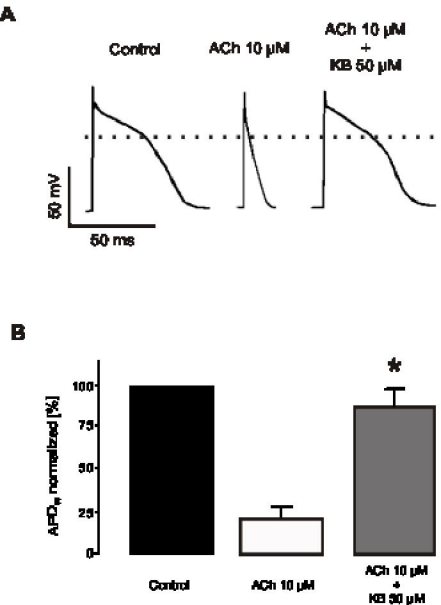

A
GTP-γ-S
500 µM
ACh 10 µM
KB 50 µM
b
0.5 nA
a
40 s
B
membrane potential (mV)
0.25
-50
-100
0
50
-0.25
a-b
-0.50
current (nA)
-0.75

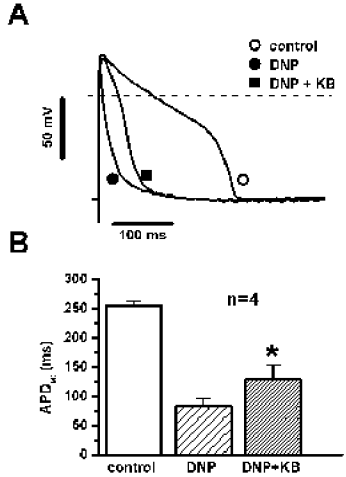

KB 50 µM
DNP 100 µM
ACh 10 µM
b
c
a
∞ ~ 10 min ∞
∞ ~ 3 min ∞
∞ ~ 4 min ∞
1.5 nA
60 s


membrane potential (mV)
-50
-100
0
50
1.0
-1.0
-2.0
current (nA)
□ a-b
■ a-c


KB
50 µM
ACh 10 µM
KB 50 µM
~ 30 min
1 nA
20 s


A
no KB
KB (200 µM)
control
CCh (10 µM)
50 mV
50 mV
100 ms
100 ms
B
control
CCh (10 µM)
150
100
50
0
APD90 (ms)
*
no KB
(n=10)
KB 50 µM
(n=5)
KB 200 µM
(n=5)


A
Control
ACh 10 µM
ACh 10 µM
+
KB 50 µM
50 mV
50 ms
B
APD90 normalized [%]
100
75
50
25
0
*
Control
ACh 10 µM
ACh 10 µM
+
KB 50 µM


A
control
DNP
DNP + KB
50 mV
100 ms
B
n=4
*
APD90 (ms)
300
250
200
150
100
50
0
control
DNP
DNP+KB

Influence of KB on the effect of CCh on membrane potentials of guinea pig atrial multicellular preparations

	no KB (n=10)			50 µM KB (n=5)			200 µM KB (n=5)	
	control	CCh (10 µM)	washCCh	control	CCh (10 µM)	wash CCh	control	CCh (10 µM)
V_{rest} (mV)	-75±2.1	-73±2.6	-74±2.5	-75±2.2	-73±2.5	-74±2.4	-79±2.3	-79±2.4
APA (mV)	114±4.7	89±5.3*	116±3.6	129±4.4	114±8.8	114±17.1	107±6.0	93±9.1
APD_{50} (ms)	53±5.5	14±1.9***	49±5.0	47±7.7	22.±5.6	53±4.4	55±7.0	42±4.4
APD_{90} (ms)	109±7.7	36±5.7***	109±8.4	104±14.3	40±9.7**	108±12.3	130±9.1	98±9.7

Values are given as mean SEM. Same preparations used for control and either 50 µM KB or 200 µM KB

Recordings made at 37 °C.

*: P<0.05; **: P<0.01; ***: P<0.001 compared to control (ANOVA and Tukey-Kramer multiple comparison test).

V_{rest}: resting potential

APA: amplitude of action potential

APD measured at 50 % (APD_{50}) or at 90 % (APD_{90}) repolarization

Influence of KB on the effect of 2,4 dinitrophenol (DNP) on membrane potentials of guinea pig papillary musc

	control	DNP	DNP + KB (200 µM)
V_{rest} (mV)	-83±0.4	-81±0.3	-81±0.2
APA (mV)	112±1.4	101±7.0	100±4.7
APD_{60} (ms)	205±12.3	33±12.1***	76±18.8*** ##
APD_{90} (ms)	255±7.6	83±13.3***	129±24.3*** #

Values are given as mean SEM (n=4).

Recordings made at 37 °C.

***: P<0.001 compared to control (ANOVA and Tukey-Kramer multiple comparison test).

#: P<0.05; ##: P<0.01, compared to DNP alone.

V_{rest}: resting potential

APA: amplitude of action potential

APD measured at 50 % (APD_{50}) or at 90 % (APD_{90}) repolarization