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The amiodarone derivative, 2-Methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran (KB130015) induces a Na^+-dependent increase of $[Ca^{2+}]$ in ventricular myocytes

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Running title: **KB 130015 effects on Na^+ and Ca^{2+} handling in pig myocytes**

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ABSTRACT

2-Methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran (KB130015; KB) is a novel amiodarone derivative designed to retain the antiarrhythmic effects without the side-effects. Unlike amiodarone, KB slows Na^+ current inactivation and could, via an increase in $[Na^+]_i$, potentially lead to Ca^{2+} overload. We therefore studied the effects of KB on Na^+ and Ca^{2+} handling in single pig ventricular myocytes using the whole-cell ruptured patch-clamp technique and K_5Fluo-3 as $[Ca^{2+}]_i$ indicator. KB at 10 µM did not prolong action potential duration, but slightly increased the early plateau; spontaneous afterdepolarizations were not observed. The amplitude of the $[Ca^{2+}]_i$ transient was larger (434.9±37.2 nM *vs.* 326.8±39.8 nM at baseline, n=13, $P<0.05$) and the time to peak $[Ca^{2+}]_i$ was prolonged. During voltage clamp pulses, $[Ca^{2+}]_i$ transient peak was also larger (578.1±98.9 nM *vs.* 346.4±52.6 nM at baseline, $P<0.05$). Though L-type Ca^{2+} current was reduced (by 21.9% at +10 mV, n=9, $P<0.05$), sarcoplasmic reticulum Ca^{2+} content was significantly enhanced with KB. Forward Na/Ca exchange was significantly decreased after KB application, but reverse-mode of the Na/Ca exchanger was significantly larger, suggesting an increase in $[Na^+]_i$ with KB. This was confirmed by a two-fold increase of the $[Na^+]$-dependent current generated by the Na/K-ATPase (from 0.17±0.02 to 0.38±0.06 pA/pF, $P<0.05$). In conclusion, as predicted from the slowing of I_{Na} inactivation, KB130015 leads to an increase in $[Na^+]_i$ and consequently in cellular Ca^{2+} load. This effect is partially offset by a decrease in I_{CaL} resulting in a mild inotropic effect without the signs of Ca^{2+} overload and related arrhythmias usually associated with Na^+ channel openers.

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INTRODUCTION

Sudden cardiac death, mostly related to arrhythmias, is a major cause of mortality (Richter et al., 2005;Zipes and Wellens, 1998). Lethal arrhythmias occur in a many cardiac diseases, including congenital ion channel mutations without structural heart disease as well as genetic and acquired cardiomyopathies. Particularly in the setting of ischemic cardiomyopathy and heart failure, mortality due to ventricular arrhythmias is high and the search for efficient antiarrhythmic drugs has been frustrating. The recent introduction of device therapy with an implantable cardiac defibrillator, ICD, has been a major success in improving survival. It was superior to any medical therapy (The AVID Investigators, 1997;Bokhari et al., 2004). However, cost considerations and the impact on quality of life make it an option that is not always available (Epstein, 2004;Josephson and Wellens, 2004). Efficient and safe drugs for treatment of ventricular arrhythmias are still needed. In addition, association of pharmacotherapy with the ICD can substantially reduce the number of electroshocks thus improving efficiency of treatment and quality of life.

The major drawback of current antiarrhythmics are the earlier experiences of increased mortality in the CAST studies (The CAST Investigators, 1989). The newer class III agents with a pure K^+ channel blocking action have also been associated with pro-arrhythmia, notably a high incidence of “Torsade-de-Pointes” in the SWORD study (Waldo et al., 1996). They are not a first choice for ventricular arrhythmias occurring in the setting of ischemic cardiomyopathy where there is often already prolongation of the action potential at baseline. The multi-action drug amiodarone has not been reported to have the pro-arrhythmic effect of other class III agents (Amiodarone Trials Meta-

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Analysis Investigators, 1997). It has specific effects on multiple cardiac ion channels that differ in acute and in chronic treatment (Kodama et al., 1997). After myocardial infarction, treatment with amiodarone has been reported to prevent arrhythmic death (Cairns et al., 1997;Julian et al., 1997), but a recent trial in heart failure patients confirmed superiority of ICD (Bardy et al., 2005). Because of its low pro-arrhythmic potential, amiodarone remains a widely-used and efficient drug. However, its long-term clinical use is limited by its significant extracardiac toxicity. Because of close structural similarity with thyroid hormones, symptoms of hypothyroidism can occur. Corneal deposits and development of lung fibrosis can be major reasons for discontinuation of amiodarone treatment (Martin, 1990). Therefore several compounds have been developed based on the amiodarone structure, aiming to retain the efficiency and cardiac safety of amiodarone, with less of the extra-cardiac side-effects. 2-Methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran (KB130015; KB) is one of such new drugs (Carlsson et al., 2002). Preliminary data in guinea-pig suggest that KB has a toxicity profile more advantageous that amiodarone. In the same study in guinea-pig papillary muscle, KB has been shown to prolong action potential duration suggesting a potential antiarrythmic effect. Like amiodarone, it acts on many ion channels, including Ca^{2+}, Na^{+} and K^{+} channels (reviewed in (Mubagwa et al., 2003)). Unlike amiodarone, KB slows the inactivation of voltage-dependent Na^{+} channels (Macianskiene et al., 2003b). This effect is expected to increase intracellular [Na^{+}] and thus the cellular Ca^{2+} load through an increased influx via Na/Ca exchange, a potentially positive inotropic effect. However, KB also decreases L-type Ca^{2+} current, which would lower the cellular Ca^{2+} load (Macianskiene et al., 2003a). The net effect of these opposite actions is yet unknown and

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is of considerable interest given the earlier experience of increased mortality during chronic treatment in patients with heart failure with agents that increased cellular Ca^{2+} (Packer, 1993). Preliminary data indicated that KB increased cell shortening specially at high concentrations (Mubagwa et al., 2003). In the present study we therefore examined the effect of KB on cellular Ca^{2+} load and Na/Ca exchange function associated with alterations in increased Na^{+} influx. We used pig ventricular myocytes which have action potential duration and frequency behaviour close to that of humans.

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METHODS

Cell isolation

Single left ventricular myocytes were enzymatically isolated from the hearts of domestic pigs of either sex (body weight 41±4 kg, N=10) as previously described (Stankovicova et al., 2000). We used only cells isolated from the midmyocardial layer, mostly from the LV anterior free wall, but in a number of experiments also from the LV posterior wall. Cells were stored at room temperature and used within 24 hours.

Solutions and drugs

All experiments were performed in normal Tyrode solution (in mM): NaCl 137, KCl 5.4, $MgCl_2$ 0.5, $CaCl_2$ 1.8, Na-Hepes 11.8, glucose 10; pH 7.40. The pipette solution for whole-cell patch clamp contained (in mM): K-aspartate 120, NaCl 10, KCl 20, K-Hepes 10, MgATP 5, K_5fluo-3 0.05; pH 7.2.

A stock solution of KB 130015 (a gift from Karo-Bio, Huddinge, Sweden) was prepared in DMSO at a concentration of 10 mM. The final concentration to be used was obtained by diluting 1:1000 (v/v) in normal Tyrode. A 1:1000 dilution of the solvent in normal Tyrode solution had no effects by itself (n_{cells}=9, data not shown).

$NiCl_2$ (Sigma-Aldrich, Bornem, Belgium) was dissolved directly in the Tyrode solution at 2.5 mM.

Dihydroouabain, DHO, (Sigma-Aldrich, Bornem, Belgium) was prepared as a 1 mM stock in distilled water and diluted 1:100 in the Tyrode solution before use.

Experimental setup

$[Ca^{2+}]_i$ transients were measured with fluo-3 as a $[Ca^{2+}]_i$ indicator. The setup for fluorescence and membrane currents recording was as described before (Antoons et al.,

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2002). The fluorescence signals were corrected for the background recorded after the seal formation. This signal was further calibrated to $[Ca^{2+}]_i$ values, using a F_{max} reading obtained at the end of the experiment (Trafford et al., 1999; Antoons et al., 2002).

Experimental protocols

We recorded action potentials and $[Ca^{2+}]_i$ transients in current clamp mode, applying 5 ms current injections to trigger action potentials. During voltage clamp experiments, the holding potential was –70 mV. For measuring L-type Ca^{2+} current, I_{CaL}, the Na^+ current was inactivated by a prepulse to –50 mV. I_{CaL} was taken as the difference between the peak inward current and the current at the end of the pulse during depolarizations to –40 up to +50 mV.

The Ca^{2+} content of the sarcoplasmic reticulum (SR) was measured during a fast application of 10 mM caffeine at a holding potential of -50 mV following a train of 10 conditioning pulses from –70 to +10 mV at 1 Hz.

The amplitude of the reverse-mode of Na/Ca exchange current was measured as the Ni-sensitive current during a depolarizing step to +40 mV. The current generated by the Na/K-ATPase was measured as DHO-sensitive current at a holding potential of -50 mV following a train of 10 conditioning pulses from –70 to +10 mV at 1 Hz.

All the experiments were done at 37°C.

Statistics

All data are shown as mean ± SEM. As statistical test a paired Student's t-test was used for single measurements and ANOVA was used for multiple measurements.

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RESULTS

KB effects on the $[Ca^{2+}]_i$ transient during action potentials

Fig. 1A illustrates the effect of KB on the action potential and the pooled data from 13 myocytes. On average KB did not prolong the action potential duration (APD_{90} 318.8±25.9 ms *vs.* 352.5±23 ms in control, CTRL). Resting membrane potential was unchanged, but the early repolarization tended to be less with an increase of V_m at 15 ms in 10/13 cells. Fig. 1B illustrates the corresponding effect on the $[Ca^{2+}]_i$ transient with mean data for $[Ca^{2+}]_i$ at rest and at peak. Resting $[Ca^{2+}]$ was significantly enhanced with KB (182.2±18.9 nM *vs.* 121.8±12.7 nM in CTRL, n_{cells}= 25, $P<0.01$) and the amplitude of the $[Ca^{2+}]_i$ transient was increased (peak $[Ca^{2+}]_i$= 434.9±37.2 nM *vs.* 326.8±39.8 nM in CTRL, $P<0.05$). The kinetics of the $[Ca^{2+}]_i$ transient are given in Fig. 1C. Time to peak was significantly prolonged and the time from onset of depolarization to half-relaxation was also prolonged, although the rate of relaxation itself was not altered.

KB effects on the $[Ca^{2+}]_i$ transient during depolarizing steps of fixed duration and amplitude

To exclude the secondary effects of changes in action potential profile on Ca^{2+} handling we examined the $[Ca^{2+}]_i$ transients during a single voltage step of 250 ms from -70 mV to +10 mV, illustrated in Fig. 2. As during action potentials, peak $[Ca^{2+}]_i$ was significantly larger with KB (578.1±98.9 nM *vs.* 346.4±52.6 nM in CTRL, $P<0.05$) and the rise of the $[Ca^{2+}]_i$ transient was slower (time to peak 195.9±11.8 ms *vs.* 130.3±9.2 ms in CTRL, $P<0.01$). Rate of relaxation was unchanged.

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KB reduces I_{CaL} but increases Ca^{2+} release

In order to examine the role of I_{CaL} in the increased $[Ca^{2+}]_i$ transient, we subsequently used a double step protocol to separate the Na^+ current, I_{Na}, and the L-type Ca^{2+} current, I_{CaL}. This approach is illustrated in Fig. 3A: a step from -70 to -50 mV activated I_{Na} and was followed by a step to +10 mV activating I_{CaL}. As expected, KB slowed I_{Na} inactivation as manifested in the current trace during the step to –50 mV. This step, which in control induced a small $[Ca^{2+}]_i$ transient most likely related to spurious activation of Ca^{2+} channels (Sipido et al., 1995), now had a large $[Ca^{2+}]_i$ transient in the presence of KB. During the step to +10 mV we saw a decrease of I_{CaL}, but the amplitude of the accompanying $[Ca^{2+}]_i$ transient was significantly increased. Fig. 3B summarizes the data on the $[Ca^{2+}]_i$ transients during the step to +10 mV and shows that time to peak is now unchanged.

In a similar protocol as in Fig. 3A, we studied further the effect of KB on the amplitude and the voltage-dependence of I_{CaL} by varying the amplitude of the second depolarizing step. KB decreased significantly the amplitude of I_{CaL} (by 21 % at +10 mV, n_{cells}=9, $P<0.05$) without affecting its voltage-dependence (Fig.4A). Despite the reduction in I_{CaL}, KB significantly increased the amplitude of the associated $[Ca^{2+}]_i$ transient (at +10 mV, peak $[Ca^{2+}]_i$ transient 419.9±46.4 nM *vs.* 302.6±58.6 nM in CTRL, $P<0.05$) (Fig. 4B). Note that this effect is smaller than what is seen during a single step from –70 to +10 mV (Fig. 3B).

A decrease in I_{CaL} associated with an enhanced Ca^{2+} release suggests an increase in Ca^{2+} availability in the SR. Therefore we estimated SR Ca^{2+} content by emptying the SR with

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a fast application of 10 mM caffeine (Fig. 5A). The amplitude of the $[Ca^{2+}]_i$ transient, measured as the difference between the peak $[Ca^{2+}]_i$ transient and diastolic $[Ca^{2+}]_i$, was significantly larger with KB (670.9±163.6 nM *vs.* 345.1±56.7 nM in CTRL, $P<0.05$) consistent with a larger SR Ca^{2+} content (Fig. 5B). However, the integrated Na/Ca exchange current, which is normally a precise indicator for the amount of Ca^{2+} released from the SR (Varro et al., 1993) was not significantly increased (Fig. 5C). This can only be explained by an altered Na/Ca exchanger function and decreased removal of Ca^{2+} by the (forward mode) of the Na/Ca exchanger. Consistent with this hypothesis, the rate of relaxation of the caffeine-induced $[Ca^{2+}]_i$ transient was much slower in the presence of KB (time constant of $[Ca^{2+}]_i$ decay 1624.5±133.1 ms *vs.* 1186.8±60.2 ms in CTRL, $n_{cells}=10$,$P<0.01$) (Fig. 5D). Likewise peak Na/Ca exchange current was reduced (-1.34±0.13 pA/pF *vs.* -1.71±0.12 pA/pF in CTRL, $P<0.05$) (Fig. 5E) and declined with a longer time constant (448.6±38.3 ms *vs.* 346.4±31.1 ms in CTRL, $P=0.057$) (Fig. 5F). Such behavior of the forward mode Na/Ca exchanger could be due to increased intracellular $[Na^+]$ and would lead to incomplete removal of the Ca^{2+} released from the SR out of the cell. We indeed observed that $[Ca^{2+}]_i$ at the end of the caffeine pulse remained significantly above baseline in the presence of KB (26.8±5.3 nM, $n_{cells}=10$) and returned to baseline only after removal of caffeine, whereas this was not the case in control. With incomplete removal the integral of the Na/Ca exchange current is no longer reliably reflecting SR content, explaining why we failed to observe a significant increase in the integrated NCX current.

Increased $[Na^+]_i$ and incomplete removal of Ca^{2+} are also expected to lead to fast reloading of the SR after removal of caffeine. This was tested by applying a second pulse

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of caffeine 10 s after washout of the first application, with the membrane potential held constant at –50 mV. As illustrated Fig. 6A, a 2nd caffeine application in CTRL conditions did not evoke a second Ca^{2+} release (no transient increase of $[Ca^{2+}]_i$, only an increase of baseline $[Ca^{2+}]_i$ by less than 100 nM). However, with KB, a second release of Ca^{2+} from the SR was observed as a $[Ca^{2+}]_i$ transient during the 2nd caffeine pulse, consistent with incomplete removal during the first application and fast reloading of the SR (peak of the $[Ca^{2+}]_i$ transient during the 2nd pulse of caffeine was 316.6±49.7 nM *vs.* 156.15±25.7 nM for the maximal value of $[Ca^{2+}]_i$ during caffeine application in CTRL, n_{cells}=10, P<0.01, Fig. 6B).

The effect of KB on the Na/Ca exchanger is mediated by an increase in $[Na^+]_i$

If the reduction in forward mode Na/Ca exchange is due to an increase in $[Na^+]_i$, we expect to see an increase in reverse mode Na/Ca exchange. Therefore, we measured the outward current during a step to +40 mV and blocked by 2.5 mM $NiCl_2$ before and after KB application. This Ni-sensitive current was indeed significantly increased after KB application (0.65±0.14 pA/pF *vs.* 0.37±0.09 pA/pF in CTRL, n_{cells}=7, P<0.01, Fig. 7A). To further elucidate whether there is indeed an increase in $[Na]_i$, we measured the Na/K-ATPase current which is directly related to changes in $[Na]_i$ if membrane potential is constant (reviewed in (Glitsch, 2001;Verdonck et al., 2003)). Therefore, we measured the current suppressed by 10 μM dihydroouabain, at -50 mV, following a train of pulses from -70 to+10 mV. At this potential, $I_{Na/K}$ is small (0.17±0.02 pA/pF) but it was more than two-fold increased with KB (0.38±0.06 pA/pF, n_{cells}=5, P<0.05, Fig. 7B).

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DISCUSSION

KB 130015, at a dose of 10 μM which significantly slows I_{Na} inactivation, increases the amplitude of the Ca^{2+} transient during action potentials but without inducing spontaneous Ca^{2+} release; an increase of $[Ca^{2+}]_i$ is also seen during square voltage steps. The SR Ca^{2+} content is increased, but the L-type Ca^{2+} current is reduced. The enhanced cellular Ca^{2+} load may result from a reduced forward mode Na/Ca exchange and increased reverse mode of Na/Ca exchange. These changes in Na/Ca exchange activity can be related to an increase in $[Na^+]_i$.

Mechanisms underlying the increase in $[Ca^{2+}]_i$ with KB

In the presence of a reduction of I_{CaL}, the first explanation for the increase in the amplitude of the $[Ca^{2+}]_i$ transient with KB is the increase in SR Ca^{2+} content resulting from the higher intracellullar $[Na^+]$. We have to also consider however, that with the increase in $[Na^+]$, enhanced influx of Ca^{2+} via reverse mode Na/Ca exchange during depolarization can contribute to this increase. We have previously found that reverse mode per se is a very inefficient trigger for Ca^{2+} release and that the Ca^{2+} release seen with the Na^+ current most likely represents spurious activation of Ca^{2+} channels rather than activation of release channels via reverse mode (Sipido et al., 1995;Sipido et al., 1997). This view has been supported by others (reviewed in (Bers, 2002)), though there have been reports that Ca^{2+} release triggered by reverse mode Na/Ca exchange following Na^+ influx via the channel, had a specific profile(Lipp and Niggli, 1994). Others have argued that Ca^{2+} influx via Na/Ca exchange can modulate and re-inforce the trigger function of I_{CaL} (Litwin et al., 1998). In Fig. 3B we saw that the increase in amplitude of

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the $[Ca^{2+}]_i$ transient activated by I_{CaL} alone during the step from –50 to +10 mV is less than the increase in the $[Ca^{2+}]_i$ transient during the action potential or the voltage clamp step from –70 to +10mV that activated both I_{Na} and I_{CaL}. This can be taken to support the idea that Ca^{2+} influx associated with the Na^+ current contributes to the enhanced $[Ca^{2+}]_i$ transient with KB. It is not absolute proof however, as the release with the step from –70 to –50 has partially depleted the sarcoplasmic reticulum. Another argument in favor of a role for reverse mode, is the prolongation of the time to peak $[Ca^{2+}]_i$ during action potentials and single square voltage steps from –70 to +10mV, but not in the steps from -50 to +10 mV. This is compatible with the presence of a slower process triggering Ca^{2+} release or contributing direct Ca^{2+} influx in the presence of Na^+ current only. This could be reverse mode Na/Ca exchange, in particular in the presence of a reduced I_{CaL}. Another argument in favor of increased Ca^{2+} influx via reverse mode Na/Ca exchange during depolarization comes from examining the Ca^{2+} flux balance. With a depolarizing step, Ca^{2+} extrusion via Na/Ca exchange on repolarization should match the Ca^{2+} influx during the step (Bers, 2002;Bridge et al., 1990;Trafford et al., 1997). We calculated the integral of the Na/Ca exchange forward mode on repolarization after the single depolarizing pulse and found that it was unchanged with KB (data not shown). However, as Ca^{2+} influx via I_{CaL} is reduced we expect to find a reduced value, and thus this observation can support the concept that there has been additional Ca^{2+} influx via reverse mode Na/Ca exchange.

Is KB a useful inotropic and/or antiarrhythmic agent?

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The finding of a moderate increase in Ca^{2+} release without spontaneous Ca^{2+} oscillations can be taken as favorable in comparison to other Na^+ channel openers such as DPI 201-106, BDF 9148 and BDF 9198 (reviewed in (Flesch and Erdmann, 2001)). To some extent this must be seen as a dose effect. Indeed, in a few experiments we used KB at 50 μM and saw a larger increase in $[Ca^{2+}]_i$ and $[Na^+]_i$, with arrhythmogenic effects (data not shown). On the other hand, there is a genuine difference between KB and these substances namely the presence of Ca^{2+} channel blockade that limits total Ca^{2+} influx and protects against excessive Ca^{2+} loading. Caution remains however in the failing heart as $[Na^+]_i$ is already elevated, and a further increase in $[Na^+]_i$ might more rapidly lead to Ca^{2+} overload.

Whereas the effects on systolic Ca^{2+} can thus overall be considered rather favorable, the effects on diastolic Ca^{2+} are less favorable. Indeed, the total duration of the $[Ca^{2+}]_i$ transient is somewhat prolonged and diastolic $[Ca^{2+}]_i$ levels are slightly increased, factors that both will likely reduce diastolic filling in the intact heart. This is particularly relevant for patients with a compromised diastolic function as often observed in heart failure (Kass et al., 2004).

In contrast to 'classical' Na^+ channel openers, KB does not markedly prolong the action potential and its potential anti-arrhythmic activity can thus not be related to a typical class III effect. However there are some unique properties that may help to explain the observed protection (Mubagwa et al., 2003). Triggered early and delayed afterdepolarizations are important mechanisms underlying ventricular arrhythmias in heart failure; KB may partially reduce the likelihood of both these mechanisms.

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Early afterdepolarizations have been ascribed to re-activation of I_{CaL} (January and Riddle, 1989;Zeng and Rudy, 1995) and thus a partial block of I_{CaL} may be a favorable property of KB. It could be argued that Na^+ window currents could provide EADs with KB, as seen with anemone toxin II (Boutjdir et al., 1994) or veratridine (Verdonck et al., 1991). A difference with these substances is however that they induce a large persistent current that is again not seen with KB. Inward Na/Ca exchange current in the setting of enhanced Ca^{2+} loading can provide the conditioning current to allow the EAD (Volders et al., 1997;Wehrens et al., 2000;Zeng and Rudy, 1995). As we have seen, the inward Na/Ca exchange current is not increased but rather reduced with KB, probably because of the higher intracellullar Na^+ and leftward shift of the reversal potential. This same mechanism, i.e. a reduced forward Na/Ca exchange current, can also help to reduce the likelihood and amplitude of delayed afterdepolarizations if spontaneous Ca^{2+} release would occur. Lastly, the higher amplitude of the outward Na/K pump current may exert a stabilizing influence on the resting membrane potential. As elegantly demonstrated by Pogwizd et al., a reduction in outward I_{K1} at the resting membrane potential as seen in the rabbit with heart failure, is an important factor in facilitating delayed afterdepolarizations (Pogwizd et al., 2001). KB would counteract enhanced excitability by providing additional outward current at the resting membrane potential.

Despite its delaying of Na^+ channel inactivation, KB does not prolong the action potential. The reduction of I_{CaL} is one element that contributes, as discussed before (Mubagwa et al., 2003). In the present study we identify another, namely the larger Na/K pump current. The lack of an increase in action potential duration is favourable to reduce EADs and may also be favourable for the diastolic function.

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CONCLUSIONS

Slowing of the Na^+ channel inactivation by KB130015 leads to an increase in $[Na^+]_i$ and consequently in cellular Ca^{2+} load. This effect is partially offset by a decrease in I_{CaL} resulting in a mild inotropic effect without signs of Ca^{2+} overload and related arrhythmias. Activation of the Na/K pump current by the increased $[Na^+]_i$ may contribute to an anti-arrhythmic effect.

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FOOTNOTE

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LEGENDS FOR FIGURES

Figure 1: KB increases $[Ca^{2+}]_i$ transients evoked by action potentials

A, Typical example of an action potential recorded before and after 10 μM KB application with a plot of individual and averaged values of the action potential duration at 90 % of repolarization (APD_{90}) and membrane potential (V_m) at 15 ms after depolarization. B, The $[Ca^{2+}]_i$ transient recorded before and after 10 μM KB application with a plot of averaged values for $[Ca^{2+}]$ at rest and peak values of $[Ca^{2+}]_i$ (n_{cells}=25; **, P<0.01). C, Kinetics of the $[Ca^{2+}]_i$ transient with time to peak (TTP), time to 50% decline of $[Ca^{2+}]_i$, measured from the onset of depolarization (Time to Ca_{50}) and time constant of the rate of decline of $[Ca^{2+}]_i$. For the mean data, n_{cells}=13; *, P<0.05; **, P<0.01.

Figure 2: Effect of KB on the $[Ca^{2+}]_i$ transient during square voltage clamp steps

Example of the effect of KB application during single steps of 250 ms from -70 to +10 mV on the $[Ca^{2+}]_i$ transient (left panel) with pooled data for the peak values of the $[Ca^{2+}]_i$ transient, the time to peak (TTP) and the time constant of the rate of decline of the $[Ca^{2+}]_i$ transient. n_{cells}= 10; *, P<0.05; **, P<0.01.

Figure 3: Effects of KB on $[Ca^{2+}]_i$ transients with sequential activation of I_{Na} and I_{CaL}

A, example of the effect of KB washing on the membrane current and the associated $[Ca^{2+}]_i$ transient during a pulse from -70 to -50 mV followed by a step to +10 mV. B, Pooled data for the $[Ca^{2+}]_i$ transients activated by I_{CaL} during the second step: peak, TTP

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and the time constant for the rate of decline of $[Ca^{2+}]_i$ before and after KB. n_{cells}=11; *, P<0.05.

Figure 4: Voltage-dependence of I_{CaL} and the associated $[Ca^{2+}]_i$ transient

A, Voltage-dependence of L-type Ca^{2+} current measured as the peak-minus-end of pulse current before and after adding KB. B, Voltage-dependence of the peak $[Ca^{2+}]_i$ transient before and after KB. n_{cells}=9; *, P<0.05.

Figure 5: SR Ca^{2+} content and Ca^{2+} removal by the Na/Ca exchanger

A, Membrane currents and $[Ca^{2+}]_i$ transients evoked by 10 mM caffeine application for 12 s at a holding potential of -50 mV, following a train of pulses to +10 mV. The traces are the average of 10 cells. B, Pooled data of the amplitude of the evoked $[Ca^{2+}]_i$ transient, before and after KB. C, Integral of the inward Na/Ca exchange current. D, Time constant of the rate of decline of the caffeine-induced $[Ca^{2+}]_i$ transient. E, Peak inward Na/Ca exchange current induced by caffeine. F, Time constant of the rate of decline of the Na/Ca exchange current. n_{cells}=10; *, P<0.05; **, P<0.01.

Figure 6: Faster SR Ca^{2+} reloading in unstimulated cells.

A, Example illustrating the probing SR reloading in unstimulated cells at – 50 mV: 10 mM caffeine was applied for 12 sec and a 2nd caffeine application was given 8 to 10 s later (each application indicated by a horizontal line). B, Pooled data of the peak $[Ca^{2+}]_i$ values during the 2nd caffeine application. n_{cells}=10; *, P<0.05; **, P<0.01.

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Figure 7: KB increases reverse-mode Na/Ca exchange through increased $[Na^+]_i$

A, Individual and averaged data of the amplitude of Ni-sensitive outward current during a step to +40 mV before and after KB superfusion. n_{cells}=7; **, P<0.01. B, Pooled data of the amplitude of the outward DHO-sensitive current at -50 mV, recorded after a series of depolarizing pulses from -70 to +10 mV. n_{cells}=5; *, P<0.05.

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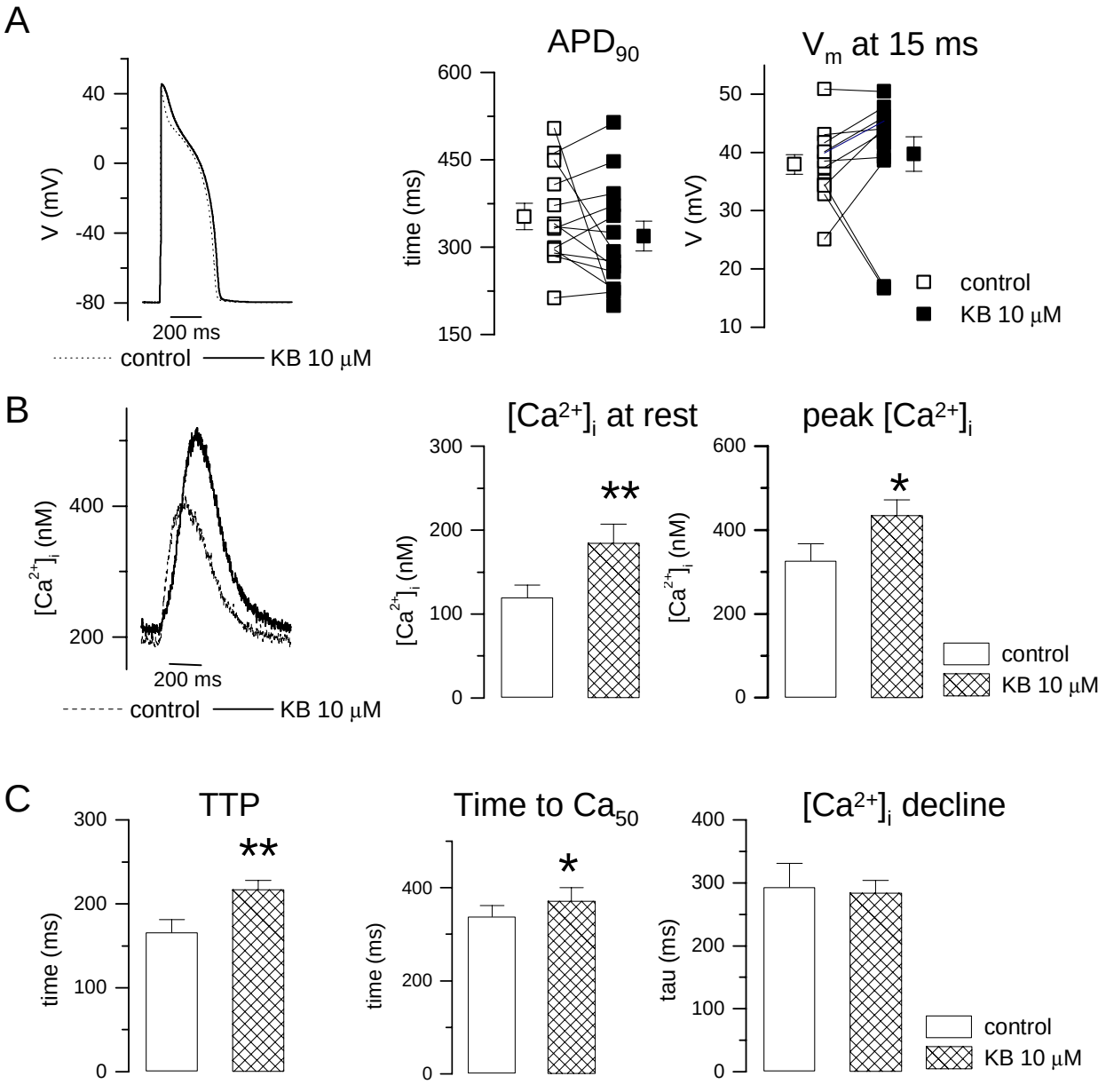

Figure 1

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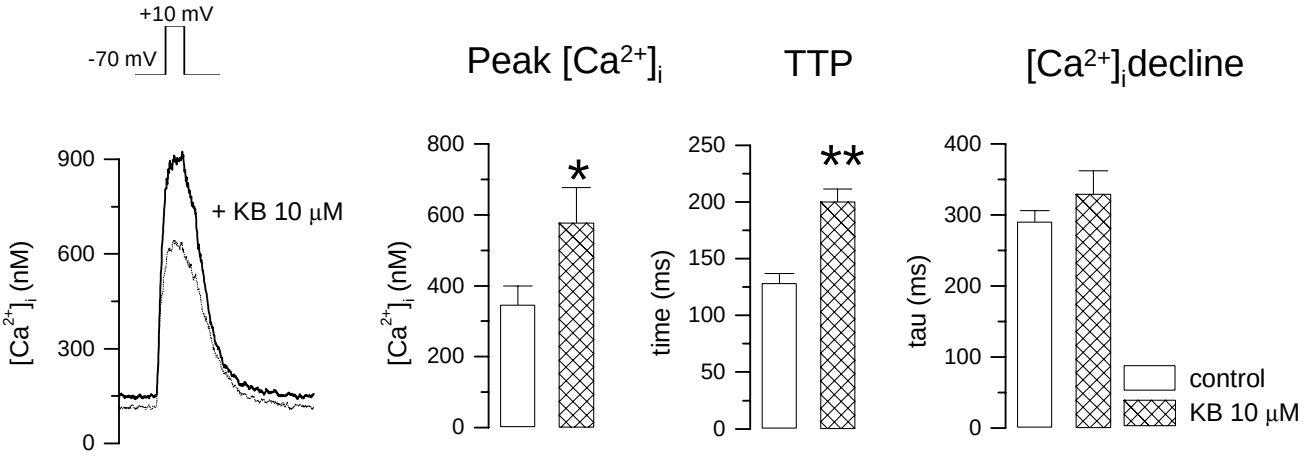

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Figure 2

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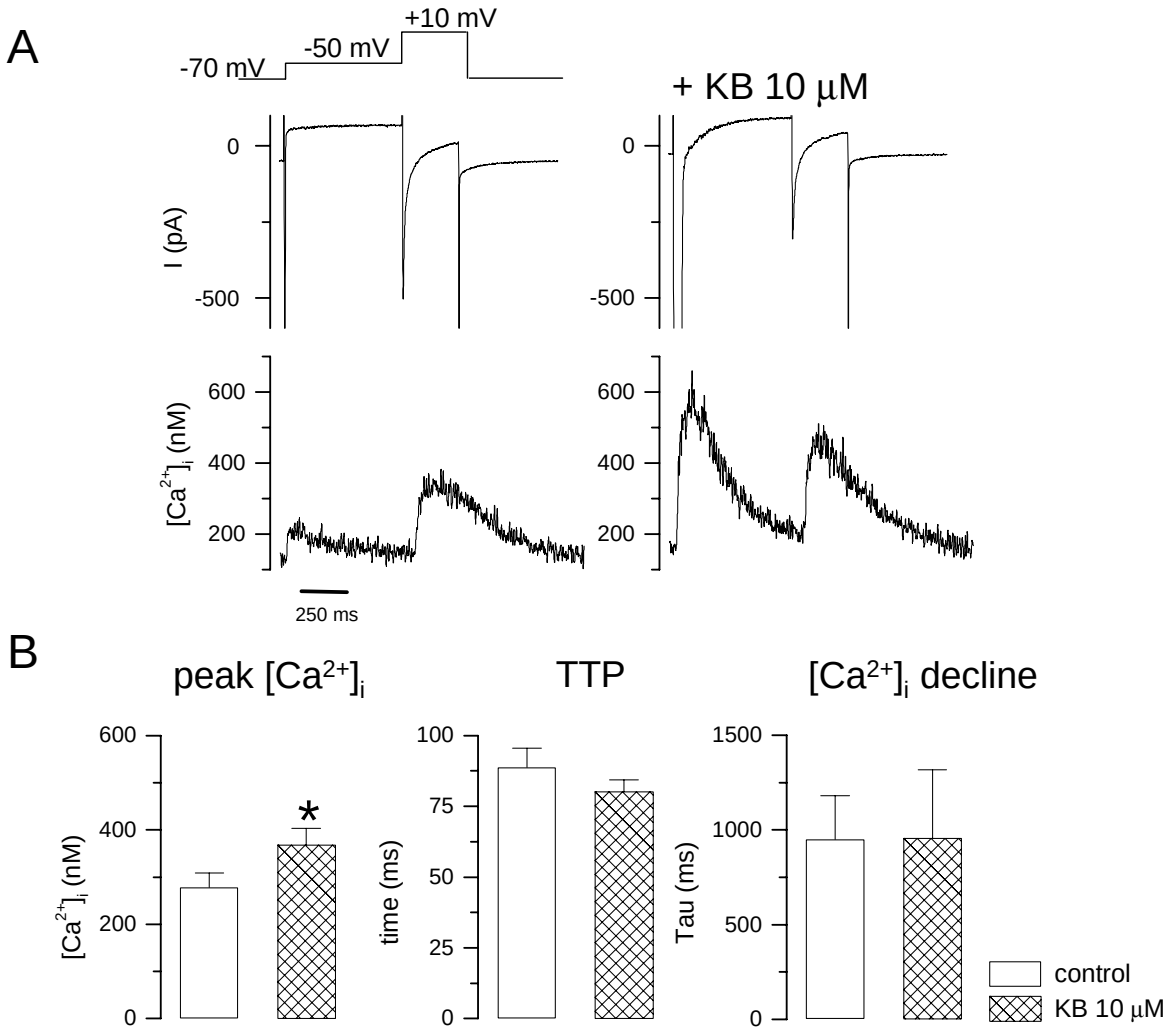

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Figure 3

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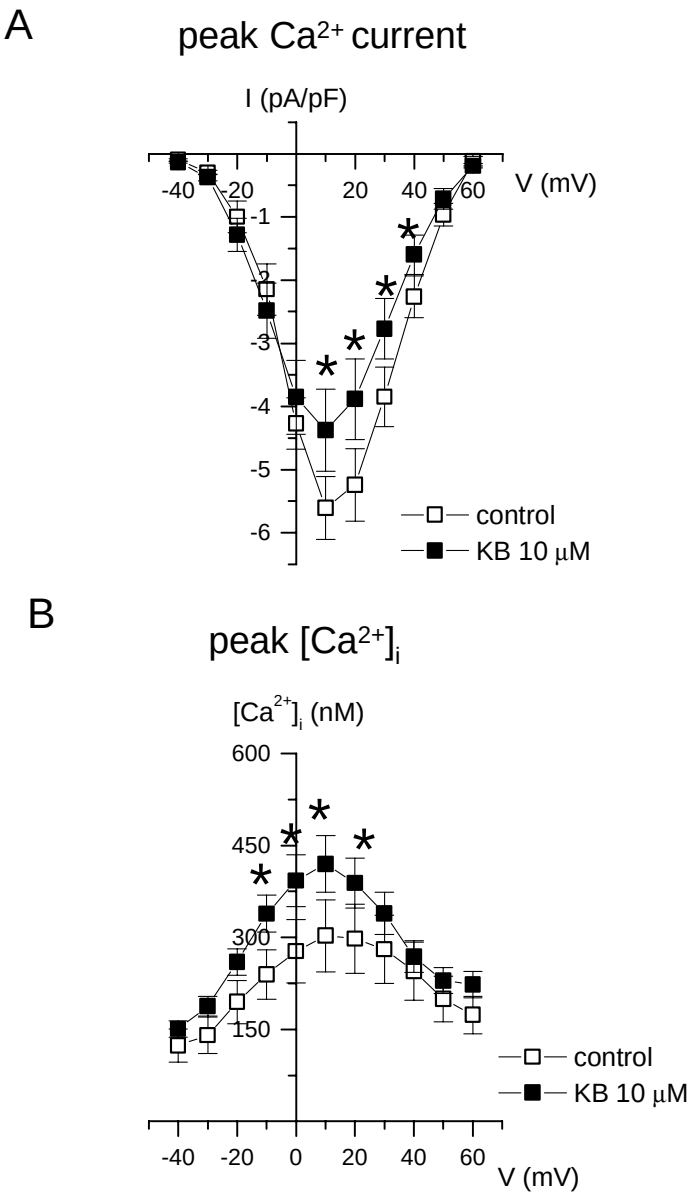

Figure 4

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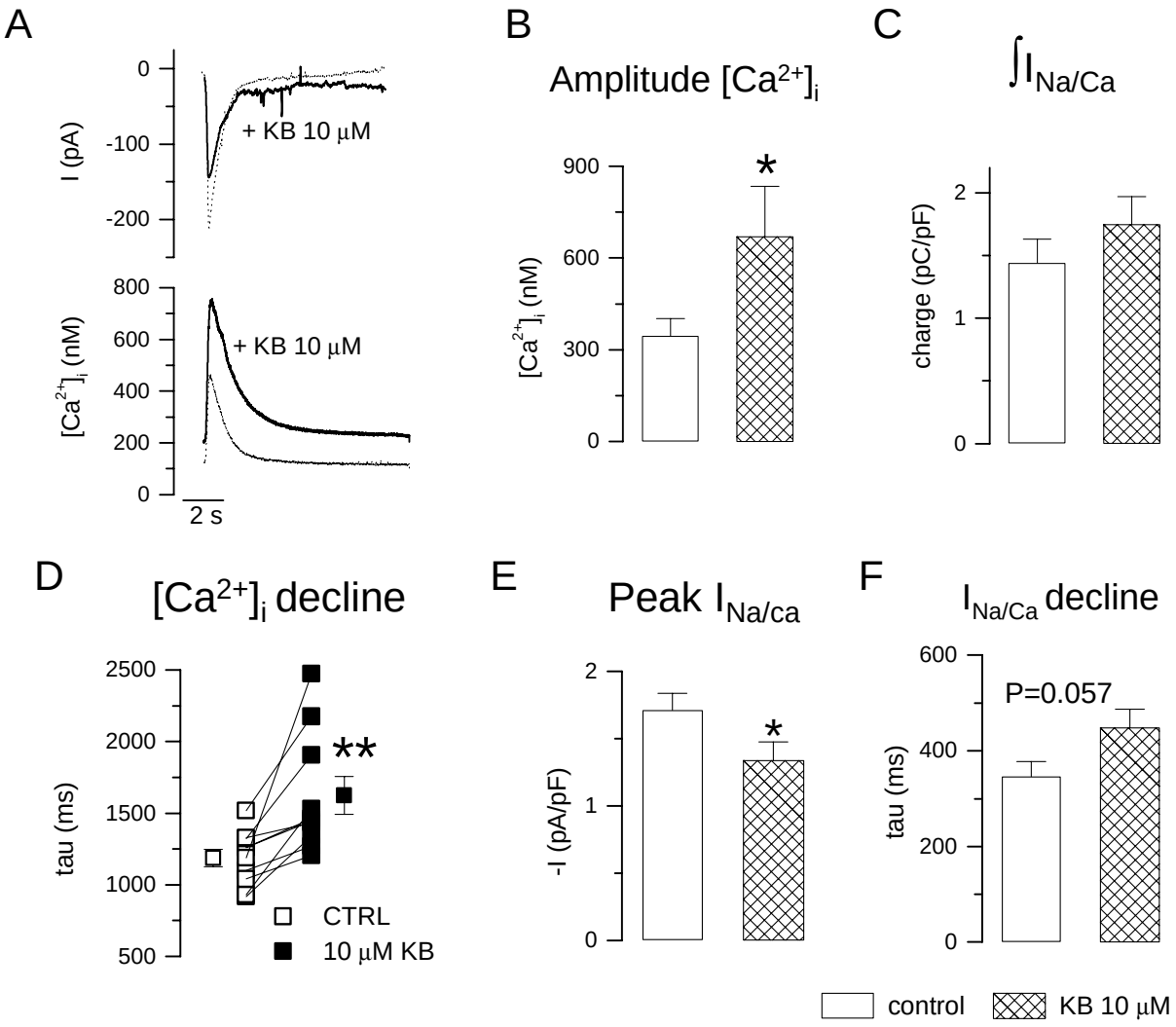

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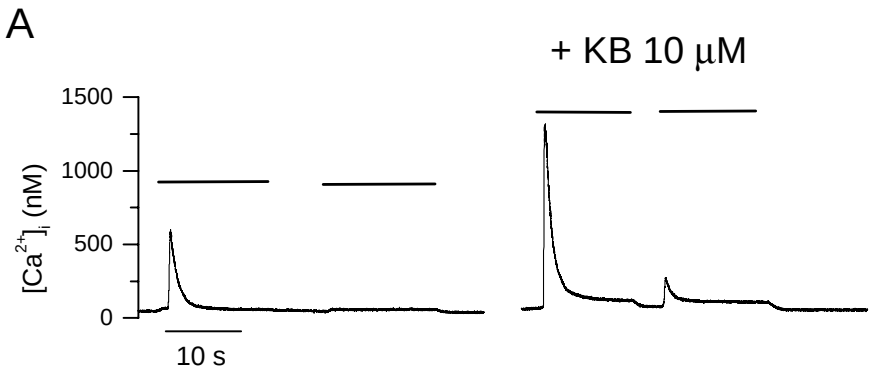

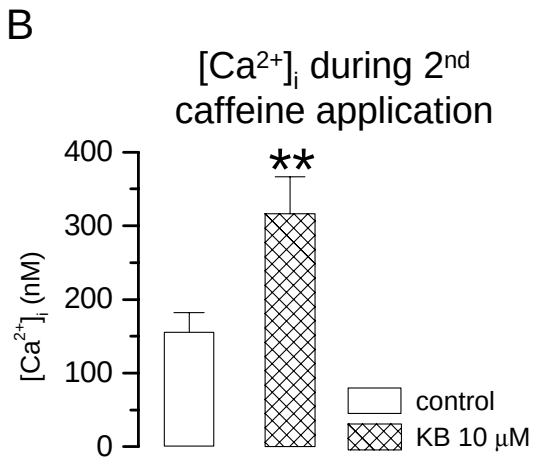

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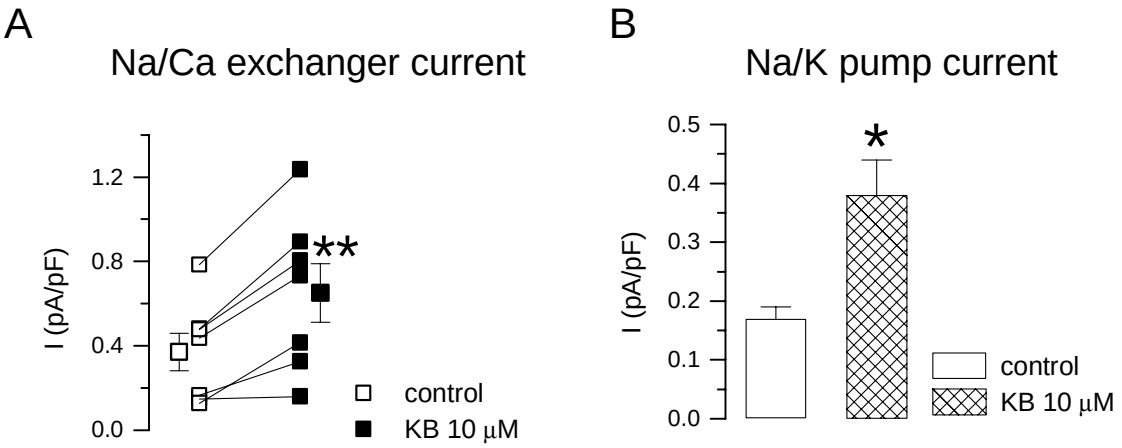

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Figure 7