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ION CHANNELS, RECEPTORS AND TRANSPORTERS

$[Ca^{2+}]_i$-induced augmentation of the inward rectifier potassium current (I_{K1}) in canine and human ventricular myocardium

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Abstract The inward rectifier K^+ current (I_{K1}) plays an important role in terminal repolarization and stabilization of the resting potential in cardiac cells. Although I_{K1} was shown to be sensitive to changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), the nature of this Ca^{2+} sensitivity—in spite of its deep influence on action potential morphology—is controversial. Therefore, we aimed to investigate the effects of a nonadrenergic rise in $[Ca^{2+}]_i$ on the amplitude of I_{K1} in canine and human ventricular myocardium and its consequences on cardiac repolarization. I_{K1}, defined as the current inhibited by 10 μM Ba^{2+}, was significantly increased in isolated canine myocytes following a steady rise in $[Ca^{2+}]_i$. Enhanced I_{K1} was also observed when $[Ca^{2+}]_i$ was not buffered by ethylene glycol tetraacetic acid, and $[Ca^{2+}]_I$ transients were generated. This $[Ca^{2+}]_i$-dependent augmentation of I_{K1} was largely attenuated after inhibition of CaMKII by 1 μM KN-93. Elevation of $[Ca^{2+}]_o$ in multicellular canine and human ventricular preparations resulted in shortening of action potentials and acceleration of terminal repolarization. High $[Ca^{2+}]_o$ enhanced the action potential lengthening effect of the Ba^{2+}-induced I_{K1} blockade and attenuated the prolongation of action potentials following a 0.3-μM dofetilide-induced I_{Kr} blockade. Blockade of I_{Ks} by 0.5 μM HMR-1556 had no significant effect on APD_{90} in either 2 mM or 4 mM $[Ca^{2+}]_o$. It is concluded that high $[Ca^{2+}]_i$ leads to augmentation of the Ba^{2+}-sensitive current in dogs and humans, regardless of the mechanism of the increase. This effect seems to be at least partially mediated by a CaMKII-dependent pathway and may provide an effective endogenous defense against cardiac arrhythmias induced by Ca^{2+} overload.

Keywords Canine/human myocardium · Inward rectifier K^+ current (I_{K1}) · Cytosolic Ca^{2+} · Action potential duration · Ventricular repolarization · Ba^{2+}

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Introduction

Cardiac repolarization, and consequently the duration of the ventricular action potential (APD), is tightly controlled by interactions and fine balance among various transmembrane ion currents [6, 43]. Plenty of these currents were shown to be sensitive to shifts in $[Ca^{2+}]_i$. A few of them (e.g., the L-type Ca^{2+} current, I_{CaL} and the Na^+/Ca^{2+} exchanger current, I_{NCX}) are predominantly inward, favoring depolarization [1, 2], while others (e.g., the Ca^{2+}-activated Cl^- current, I_{Cl}) are outward, facilitating repolarization [45, 47]. Even many "Ca^{2+}-independent" current, like the transient outward current (I_{to}) or the slow and fast components of the delayed rectifier K^+ current (I_{Kr} and I_{Ks}, respectively) were also found to be $[Ca^{2+}]_i$-sensitive to some extent, or at least being modulated by Ca^{2+}-dependent signaling pathways [16, 34, 40, 42].

The inward rectifier K^+ current (I_{K1}), an important repolarizing current, active in ventricular, atrial, and Purkinje

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cells, has long been known to play a crucial role in terminal repolarization. It is also a major contributor to the repolarization reserve of the heart [5]. Recent studies firmly support the idea that I_{K1} is subject to complex regulation by multiple interacting signaling pathways [13, 17, 20, 29, 36, 44]. Furthermore, all related studies agree that the current is $[Ca^{2+}]_i$-sensitive; however, experimental data on the direction and details of this Ca^{2+} dependence are highly controversial [7, 10, 13, 17, 19, 22, 28–31, 36, 44, 46]. For instance, I_{K1} was shown to be decreased by elevation of $[Ca^{2+}]_i$ in guinea pig [10, 28, 46] and rat [13] cardiac myocytes due to the $[Ca^{2+}]_i$-induced enhancement of the inward rectification of the channel. On the other hand, the increased activity of CaMKII kinase, which may also be a consequence of the elevated $[Ca^{2+}]_i$, reduced the amplitude of I_{K1} in mice, while I_{K1} was enhanced by CaMKII kinase in rabbit ventricular cells, but reduced in rats [44]. Since multiple controlling mechanisms converge on I_{K1}, the effect of a $[Ca^{2+}]_i$-induced shift on the current can hardly be predicted. Indeed, I_{K1} was shown to be subject to complex modulation by both inhibitory (via PKA/PKC) [13, 20] and activating (via CaMKII) Ca^{2+}-dependent pathways [44].

Considering the contradictory nature of the available experimental data, the primary aim of the present study was to elucidate the effect of $[Ca^{2+}]_i$ on the amplitude of I_{K1} and to evaluate the $[Ca^{2+}]_i$ dependence of the relative contribution of I_{K1} to ventricular repolarization in canine and human ventricular myocardium—as being the best known model for human cardiac tissues in terms of electrophysiology [38, 39].

I_{K1} is generally considered as a composite current of the Kir2.x and a few more, yet to be identified, background channels, all functioning between −80 and −30 mV and inhibited by Ba^{2+} when applied at low concentrations. Indeed, 10 μM $BaCl_2$ is considered as a relatively selective inhibitor of I_{K1} [5]. Therefore, in this study, I_{K1} was defined as the current blocked by 10 μM $BaCl_2$. $[Ca^{2+}]_i$ was increased via nonadrenergic ways to avoid the marked activation of PKA (and probably PKC) to minimize their modulatory effects on I_{K1}. In order to prevent any decline in the repolarization reserve, possibly caused by the enzymatic digestion process, all action potential measurements were performed in intact multicellular preparations excised from either a canine or an undiseased human heart. The results support a Ca^{2+}-induced and CaMKII-translated increase of the Ba^{2+}-sensitive current, which may contribute to adaptation of APD to conditions of elevated $[Ca^{2+}]_i$—independently of activation of the adrenergic pathway.

Methods

Experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals (USA NIH publication no 86–23, revised 1985). All experimental protocols were approved by the Ethical Committee for Protection of Animals in Research of the University of Szeged, Hungary (permit no. I-74-9/2009). Investigations performed in human cardiac samples conform to the principles outlined in the Helsinki Declaration. All experimental protocols were approved by the Regional and National Human Medical and Biological Research Ethics Committee, University of Szeged (permit no. 63/1997).

Canine preparations

Adult mongrel dogs of either sex, weighing 10–20 kg, were anesthetized with thiopental (30 mg/kg). The hearts were rapidly removed through right lateral thoracotomy and immediately rinsed with ice-cold Tyrode solution containing (in millimolar) the following: NaCl 144, NaH_2PO_4 0.33, KCl 4, $MgCl_2$ 1, glucose 5.5, HEPES 5, and $CaCl_2$ 2. The pH of the solution was adjusted to 7.4 with NaOH. A wedge-shaped section of the left ventricular wall, supplied by the left anterior descending coronary artery, was dissected and cannulated for isolation of single myocytes using the segment perfusion technique. Papillary muscles from the right ventricle were excised for simultaneous recording of action potentials and $[Ca^{2+}]_i$ transients.

Isolation of ventricular myocytes

The excised left ventricular segments were perfused through the anterior descending coronary artery using a gravity flow Langendorff apparatus. The perfusate was a modified MEM solution (Minimum Essential Medium Eagle, Joklik modification, Sigma, M-0518), supplemented with 1.2 mM $CaCl_2$, 10 mM HEPES, 2.5 g/l taurine, 0.175 g/l pyruvic acid, and 0.75 g/l ribose (pH=7.2). After removal of blood, the perfusate was switched for 10 min to nominally Ca^{2+}-free MEM. Dispersion of cells was achieved by an application of 0.5 g/l collagenase (Sigma type I) for 40 min in the presence of 50 μM $CaCl_2$. During the isolation procedure, the solutions were gassed with 100 % oxygen, and the temperature was maintained at 35 °C. Finally, the tissue was minced and gently agitated. The cells, freshly released from the tissue, were stored at room temperature before use. At least 60 % of the cells were rod-shaped and showed clear striation when the external Ca^{2+} was restored.

Human samples

Undiseased hearts (n=5) obtained from organ donors were explanted to obtain pulmonary and aortic valves for transplant surgery. Before cardiac explanation, the donors did not receive medication except furosemide, dobutamine, and

plasma expanders. Right ventricular papillary muscles were used in the experiments.

Measurement of I_{K1} in single cardiomyocytes

I_{K1} was recorded using the whole-cell version of the patch clamp technique. A drop of cell suspension was placed into a lucid chamber mounted on the stage of an inverted microscope. All measurements were performed at 37 °C. The cells were allowed to adhere for at least 10 min before starting superfusion with Tyrode solution. Micropipettes were fabricated from borosilicate glass capillaries (Clark Electromedical Instruments) using a microprocessor-controlled horizontal puller (Model P-97, Sutter Instruments). These electrodes had resistances of 1.5–2.5 MΩ, when filled with pipette solution, containing (in millimolar) the following: K-aspartate 100, KCl 40, K_2ATP 5, $MgCl_2$ 1, and HEPES 10, at pH=7.2. Pipette solutions were either unbuffered to allow $[Ca^{2+}]_i$ transients or were Ca^{2+} buffered by the addition of an appropriate mixture of ethylene glycol tetraacetic acid (EGTA) or BAPTA plus $CaCl_2$. The unbuffered solution was made using nominally Ca^{2+}-free distilled water, purchased from Sigma. Actual values of free Ca^{2+} in the pipette solutions ($[Ca^{2+}]_{pip}$) were calculated using WinMaxC [35]. Furthermore, the pipette solution containing high $[Ca^{2+}]$ was also verified by measuring the concentration of free $[Ca^{2+}]$ with a Ca^{2+}-sensitive electrode (World Precision Instruments Inc.). Ionic currents were recorded using an Axopatch 1D amplifier (Axon Instruments). Gigaseal has been established via gentle suction. The cell membrane was disrupted by either further suction or application of several short electrical pulses. Membrane currents were digitized under software control (pClamp 10.0, Axon Instruments) following low-pass filtering at 1 kHz with an analog-to-digital converter (Digidata 1440A, Axon Instruments). Sampling rate was set to 5 kHz. The applied voltage protocols are detailed in the Results section and shown in the figures as pertinent.

Recording of action potentials in multicellular preparations

Action potentials were recorded at 37 °C from the surface cell layer of ventricular papillary muscles using conventional microelectrode techniques. The preparations were mounted in a Plexiglas chamber, allowing continuous superfusion with O_2-saturated Tyrode solution. The muscles were stimulated by rectangular current pulses, having durations of 2 ms and amplitudes of twice the diastolic threshold, at a constant rate of 1 Hz. These pulses were delivered to the preparations through a pair of bipolar platinum electrodes coupled to an electrostimulator (Hugo Sachs Elektronik, model 215/II). Sharp microelectrodes, having tip resistance of 10–20 MΩ when filled with 3 M KCl, were connected to an amplifier (Biologic Amplifier, model VF 102). Voltage output from the amplifier was sampled using an A/D converter (NI 6025, Unisip Ltd). In order to optimize data processing during measurements, dual sampling rates were applied: in the initial 50 ms of the action potential, the sampling rate was set to 40 kHz, while the second, slow phase was digitized at 1 kHz. No further filtering was used.

Action potential duration, determined at 90 % level of repolarization (APD_{90}), was obtained using Evokewave v1.49 (Unisip Ltd). To ensure the physiological conditions of the preparations, ventricular muscle samples having action potential amplitudes less than 100 mV or showing a drift in APD during the control period were discarded. Efforts were made to maintain the same impalement throughout the whole experiment. When the impalement was dislodged, an adjustment was attempted. The measurement was continued if the action potential characteristics of the reestablished impalement deviated less than 5 % from the original values.

Monitoring $[Ca^{2+}]_i$ transients in multicellular samples and single cells

Multicellular samples were loaded with 25 μM Fluo 4-AM (Molecular Probes Inc.) for 50 min at room temperature. Isolated cells were loaded with 2 μM Fluo 4-AM for 15 min. Both were mounted in a low-volume imaging chamber (RC47FSLP, Warner Instruments) and field-stimulated at a rate of 1 Hz while continuously superfused with Tyrode solution at 37 °C. Fluorescence measurements were performed using an Olympus IX 71 inverted fluorescence microscope. Optical signals were recorded by a photon counting photomultiplier module (Hamamatsu, model H7828) sampled at 1 kHz. The dye was excited at 480 nm, and the emitted fluorescence was detected at 535 nm. Data acquisition and analysis were performed using the Isosys software (Experimetria, Hungary). Fluorescence traces recorded from multicellular samples were corrected for nonspecific background and bleaching. Alterations in $[Ca^{2+}]_i$ were expressed as changes in normalized fluorescence (F/F_0). Fluorescence traces recorded from single cells were only used to validate the absence/presence of $[Ca^{2+}]_i$ transients in the patch clamped cells. On these traces, no further processing, except smoothing, has been performed.

Drugs

All chemicals were purchased from Sigma, except for otherwise indicated. $BaCl_2$ (10 μM) was used to dissect I_{K1}. Dofetilide (gift from Gedeon Richter Ltd, Hungary) and HMR-1556 (Aventis Pharma) were dissolved in dimethyl sulfoxide. $BaCl_2$ was dissolved in distilled water performing stock solutions of 1–1 mM and 100 mM, respectively. KN-93 was purchased from Calbiochem and was also dissolved in dimethyl sulfoxide in a stock solution of 1 mM. Its final concentration was 1 μM, the same as that used in other

studies [9]. Following its application, time was allowed to reach at the maximal inhibition of CaMKII. For negative control, its inactive analog, KN-92, was used at the same concentration. All stock solutions were stored at 4 °C. Solutions were freshly made prior to the measurement.

Statistics

All values presented in this study are arithmetic means ± SEM. Statistical significance of the differences when making multiple comparisons was evaluated using repeated measures ANOVA + Bonferroni post hoc test. All other data were analyzed using Students *t* test for paired or unpaired data, as relevant. Differences were considered significant when p was less than 0.05.

Results

Ca^{2+}-dependent modulation of I_{K1} in isolated canine ventricular cells

To characterize its Ca^{2+} dependence, I_{K1} was determined in isolated canine left ventricular myocytes using the whole cell configuration of the patch clamp technique. In the first set of experiments, Ca^{2+} concentration in the pipette solution was adjusted to low (~160 nM) or high (~900 nM) levels by adding the appropriate mixture of Ca^{2+} and BAPTA to the pipette solution. Steady-state I_{K1} amplitudes were determined at the end of 300 ms of voltage pulses usually clamped to potentials ranging between −90 and −30 mV from the holding potential of −90 mV. I_{K1} was determined by repeating the protocol in the presence of 10 μM $BaCl_2$. As shown in Fig. 1a, b, within the voltage range of −70 to −40 mV, the use of high $[Ca^{2+}]_{pip}$ (and presumably elevated $[Ca^{2+}]_i$) significantly enhanced the magnitude of the Ba^{2+}-sensitive current compared to that recorded with low $[Ca^{2+}]_{pip}$ ($p<0.05$, $n=7$ for both groups, randomized from three dogs). Average values of membrane capacitance were also determined for the low and high $[Ca^{2+}]_{pip}$ groups: 145.11±8.3 and 138.77±10.21 pF were obtained, respectively. This difference was not significant statistically.

Ca^{2+}-dependent activation of I_{K1} during the action potential

Activation of I_{K1} during the AP was analyzed using the action potential clamp technique (i.e., by applying typical ventricular action potential waveforms as a command potential), as demonstrated in Fig. 2a. As previously, I_{K1} was determined as the current component dissected by 10 μM $BaCl_2$. In the presence of high $[Ca^{2+}]_{pip}$, the magnitude of peak I_{K1} was significantly higher than that in the presence of low $[Ca^{2+}]_{pip}$, without changing the time course of activation (Fig. 2b, c). As shown in Fig. 2d, the I–V relationship (phase-plane trajectory), obtained for I_{K1} by plotting the average current against the respective membrane voltage, reveals that the maximum of the current was shifted towards less negative membrane potentials (from −65±1.1 to −57±1.3 mV, $p<0.05$, $n=7$) in response to elevation of $[Ca^{2+}]_i$.

Effects of $[Ca^{2+}]_i$ transients on I_{K1}

To better characterize the beat-to-beat modulation of I_{K1} by changes in $[Ca^{2+}]_i$, we also tested the hypothesis that an unbuffered increase in $[Ca^{2+}]_i$ (e.g., during the normal $[Ca^{2+}]_i$ transient, generated by the cell in the absence of internal EGTA) may contribute to a higher amplitude of I_{K1}. In these experiments, I_{K1} was determined in voltage-clamped myocytes, both in the absence and presence of $[Ca^{2+}]_i$ transients, evoked using pipette solutions either containing 5 mM EGTA or without any calcium chelator, respectively. In order to trigger the onset of $[Ca^{2+}]_i$ transients prior to activation of I_{K1}, the voltage protocol started with a short prepulse stepping from the −80 mV holding potential to 0 mV for 50 ms. As expected, neither a $[Ca^{2+}]_i$ transient, nor the elevation of I_{K1} could be observed in the presence of 5 mM EGTA. In contrast, when EGTA was omitted from the pipette, the presence of $[Ca^{2+}]_i$ transients significantly increased the amplitude of I_{K1} (Fig. 3a, b). With EGTA in the pipette, the steady-state I_{K1} was relatively moderate (similar to the situation when normal $[Ca^{2+}]_o$ was applied; see Fig. 1); however, when EGTA was omitted from the pipette, the magnitude of the current was significantly increased in the −70 to −30-mV membrane potential range and also after hyperpolarizing the membrane to −90 mV ($p<0.05$, $n=5$, Fig. 3c). Small time-dependent changes of the current profile could be observed during these $[Ca^{2+}]_i$ transients; however, they remained within the experimental variance.

Mechanism of the Ca^{2+}-induced enhancement of I_{K1}

Since CaMKII was previously shown to increase the intensity of I_{K1} in rabbit ventricular myocytes [44], the relatively selective CaMKII inhibitor, KN-93 was used to test the involvement of this mechanism in canine cardiac cells. Three groups of myocytes were established: $[Ca^{2+}]_{pip}$ was set low (~160 nM) in the first (control) group and 1 μM of KN-92, the inactive analog of KN-93, was used; in the second and third groups, high $[Ca^{2+}]_{pip}$ (~900 nM) was applied in the presence of either the inactive KN-92 or the active KN-93 (1–1 μM each). Steady-state currents were determined at the end of 300 ms of voltage pulses ranging between −90 and −30 mV. I_{K1} was dissected using 10 μM $BaCl_2$. Without CaMKII inhibition (i.e., in the presence of KN-92), a significant difference could be observed between the low and high $[Ca^{2+}]_{pip}$ groups ($n=8$ in each group, randomized from four

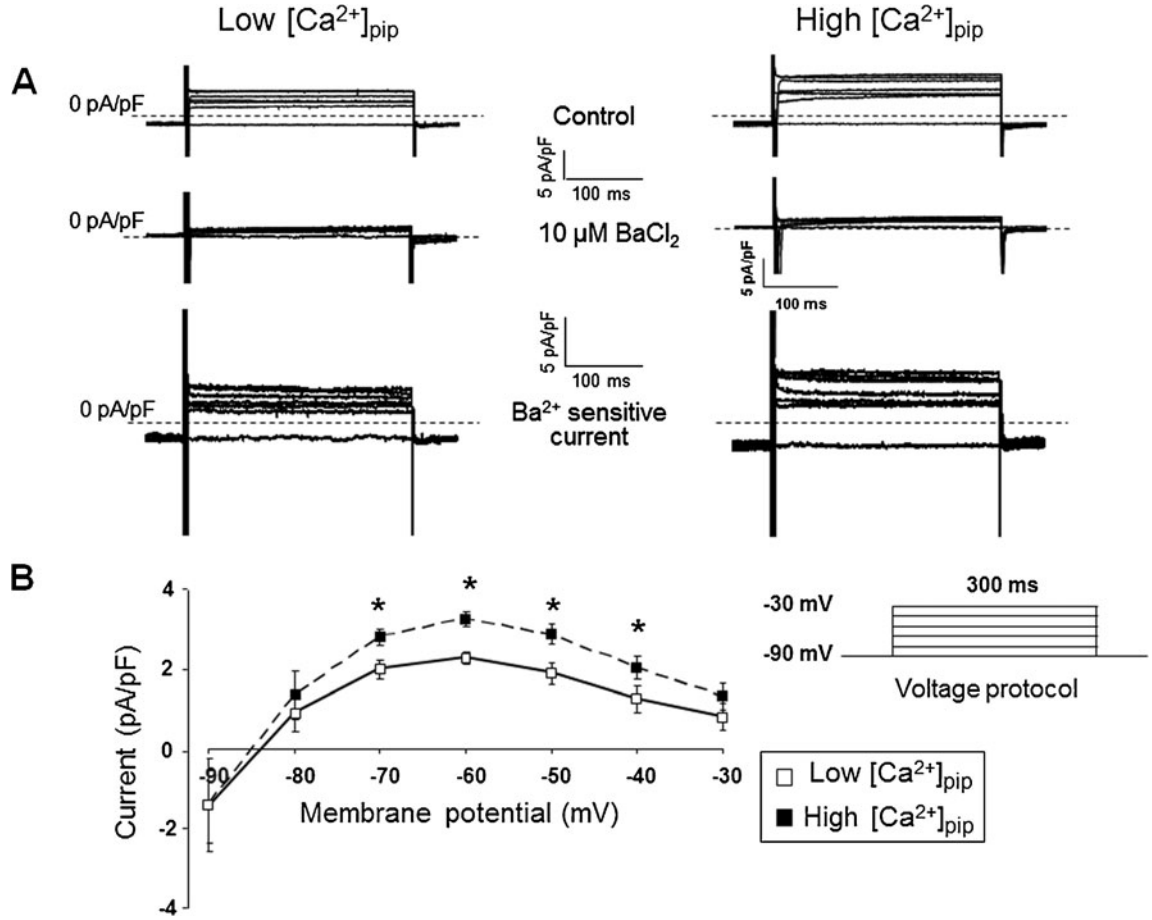

Fig. 1 $[Ca^{2+}]_{pip}$ dependence of the steady-state I_{K1} in isolated canine ventricular myocytes determined as the current blocked by 10 μM $BaCl_2$. **a** Representative sets of superimposed Ba^{2+}-sensitive current records and **b** the current–voltage relation curves obtained for I_{K1} with $[Ca^{2+}]_{pip}$ buffered to ~160 or to ~900 nM (*open* and *filled symbols*, respectively). Steady-state I_{K1} currents were determined at the end of 300 ms of test potentials ranging in amplitude from −90 to −30 mV. Statistical analysis (repeated measures ANOVA + Bonferroni ad hoc test) revealed that the difference between the two curves is significant ($p<0.05$). The *asterisks* indicate significant ($p<0.05$) differences between low and high $[Ca^{2+}]_{pip}$ results, obtained in seven cells isolated from three dog hearts

dogs). However, this difference was significantly decreased ($p<0.05$) following the inhibition of CaMKII by KN-93 (Fig. 4a, b).

To test the assumption that CaMKII activity changes during the Ca^{2+} transient, we performed a few additional experiments ($n=3$), in which the effect of Ca^{2+} influx via L-type Ca^{2+} channels on I_{K1} activation during the AP has been estimated using a Ca^{2+} channel blocker (Nifedipine, 10 μM). Data from these preliminary experiments (not shown) support some contribution of the Ca^{2+} influx to I_{K1} activation, but this contribution seems to be limited.

Effect of $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ transients and action potential configuration in multicellular canine ventricular preparations

Ca^{2+}-dependent alterations in action potential morphology were studied in canine right ventricular papillary muscles exposed to either 2 or 4 mM extracellular Ca^{2+} concentrations. This strategy was chosen in order to minimize the possible modulation of action potentials through the known intracellular signaling pathways, including adrenergic activation. On the other hand, multicellular preparations are known to display more stable action potentials than single cells; furthermore, the lack of enzymatic digestion may warrant a more "physiological" set of ion channels in the cell membrane of these cells. Elevation of $[Ca^{2+}]_o$ from 2 to 4 mM resulted in a significant (35±11 %) increase in the amplitude of $[Ca^{2+}]_i$ transients, without alterations in diastolic $[Ca^{2+}]_i$ (Fig. 5a, b). Elevation of $[Ca^{2+}]_i$ transients fully paralleled with the shortening of action potentials: APD_{90} decreased from 202.4±3.5 to 185.2±8.2 ms ($p<0.05$, $n=5$). This shortening of APD was associated with a markedly accelerated terminal repolarization (Fig. 5c, d). Indeed, the amplitude of $-dV/dt_{min}$ was increased by more than 40 % (from −1.13±0.06 to −1.59±0.05 V/s, $p<0.05$, $n=5$) after the elevation of $[Ca^{2+}]_o$. The action potential lengthening effect of the Ba^{2+}-induced suppression of I_{K1} was tested at both normal and elevated $[Ca^{2+}]_o$ (2 and 4 mM, respectively). As shown in

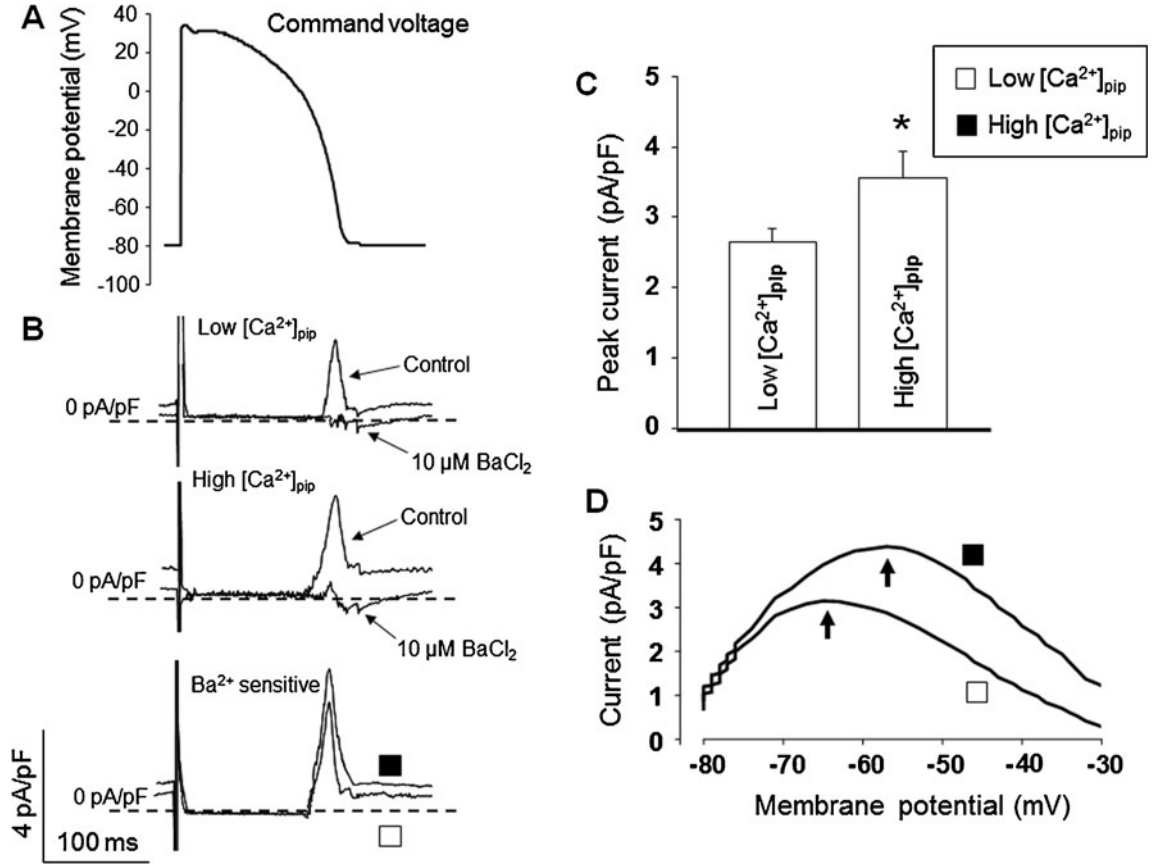

Fig. 2 Effects of increased $[Ca^{2+}]_{pip}$ on I_{K1} during a canine ventricular action potential. **a** A typical ventricular AP waveform has been applied as command voltage. I_{K1} current was dissected by the application of 10 μM $BaCl_2$. **b** A set of representative original recordings of I_{K1} current profiles and the calculated Ba^{2+}-sensitive current (*open* and *filled symbols*, respectively). **c** Peak I_{K1} currents evoked by the AP command in the presence of low (~160 nM) and high (~900 nM) $[Ca^{2+}]_{pip}$. The *asterisks* indicate significant ($p<0.05$) differences between low and high $[Ca^{2+}]_{pip}$ results, obtained in seven cells, (from three hearts)**. d** Current–voltage relationships, generated by plotting the magnitude of the (Ba^{2+}-sensitive) I_{K1} current against the corresponding membrane potential at high and low $[Ca^{2+}]_i$ (*open* and *filled symbols*, respectively). The *arrows* indicate I_{K1} current maximums

Fig. 6a, b, the $BaCl_2$-induced prolongation was significantly greater (nearly doubled) at 4 mM $[Ca^{2+}]_o$. APD_{90} was increased by 10 μM $BaCl_2$ from 185.2±8.2 to 231.2±13.1 ms at 4 mM $[Ca^{2+}]_o$ (Δ=19.6±1.9 %, $p<0.05$)—in contrast to the moderate, only 10.8±0.8 % lengthening effect of $BaCl_2$ obtained at 2 mM $[Ca^{2+}]_o$ (from 202.4±3.5 to 222.9±2.8 ms, $p<0.05$). This effect of $BaCl_2$ was fully reproducible, since following the application of $BaCl_2$ in the presence of 2 mM $[Ca^{2+}]_o$ $BaCl_2$ was washed out, and the Ba^{2+} challenge could be repeated in the presence of 4 mM $[Ca^{2+}]_o$ using the same preparation (Fig. 6c).

Triangulation (defined as a difference between APD_{90} and APD_{25}) was also estimated to further quantify the Ca^{2+}-induced changes in action potential morphology. Statistical analysis revealed that triangulation by 10 μM $BaCl_2$ when tested at 2 mM $[Ca^{2+}]_o$ was significantly increased (from 84.4±4.3 to 102.4±4.4 ms, $p<0.05$). A significant and relatively even greater $BaCl_2$-induced increase in triangulation could be observed at 4 mM $[Ca^{2+}]_o$ (from 112.3±8.1 to 155.3±13.5 ms, $p<0.05$).

Ca^{2+} dependence of action potential morphology in undiseased human myocardium

Ca^{2+}-dependent effects of 10 μM $BaCl_2$ were different in human and canine right ventricular papillary muscles. In human preparations, APD_{90} was not lengthened by 10 μM $BaCl_2$ at 2 mM $[Ca^{2+}]_o$ (353.8±12.2 versus 362.4±14.3 ms, Δ=2.36±1 %, N.S., n=5 from two hearts). In contrast, the Ba^{2+}-induced lengthening of APD_{90} was significant when $[Ca^{2+}]_o$ was elevated to 4 mM (from 313.8±19.16 to 332.8±20.91 ms, Δ=6.02±0.5 %, $p<0.05$, Fig. 7a, b). Furthermore, elevation of $[Ca^{2+}]_o$ significantly accelerated the terminal phase of repolarization (from −0.44±0.02 to −0.49±0.03 V/s, $p<0.05$, Fig. 7c). Compared to canine papillary muscles, however, this effect was also weaker.

AP triangulation values were also calculated for human samples. Statistical analysis revealed that application of 10 μM $BaCl_2$ did not change significantly triangulation at 2 mM $[Ca^{2+}]_o$ (control: 188.8±11.6 ms; $BaCl_2$: 175.8±14.6 ms; n=5, N.S.). In contrast, at 4 mM $[Ca^{2+}]_o$, triangulation was

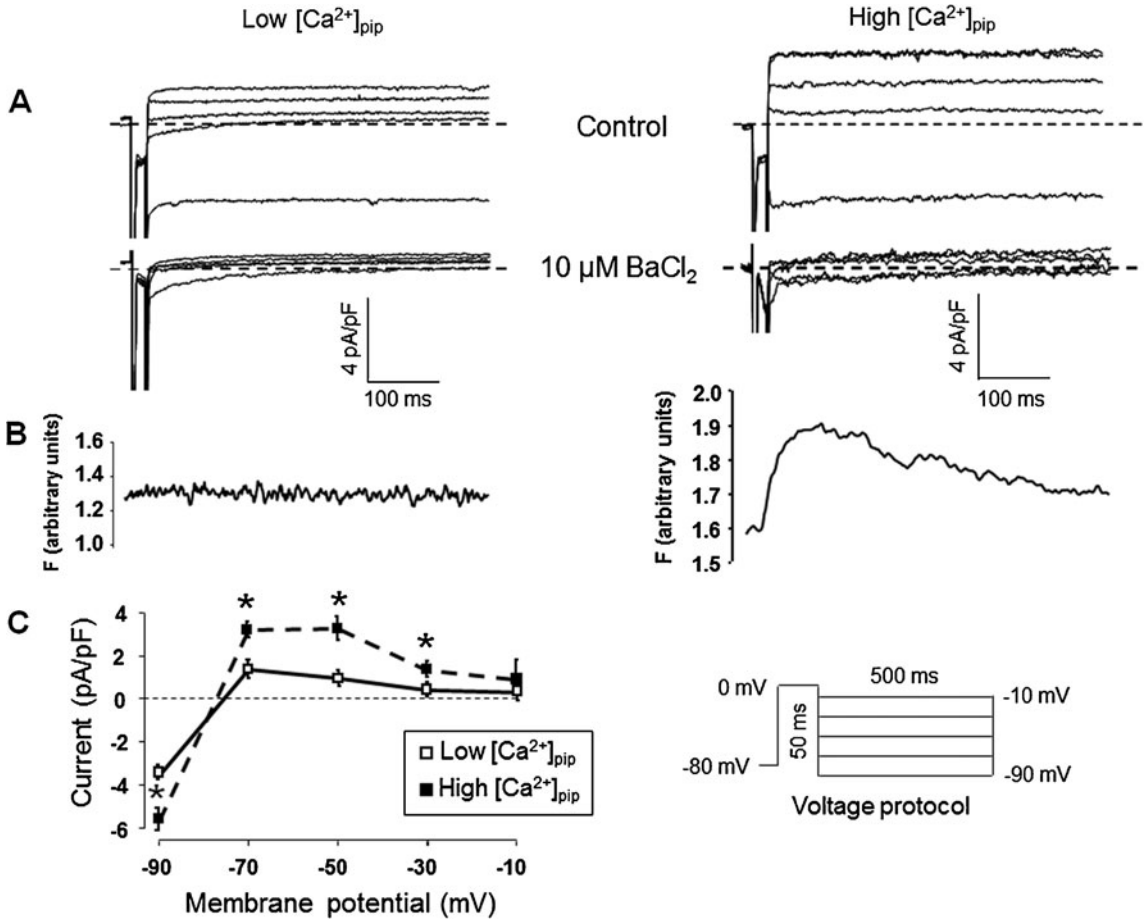

Fig. 3 Enhancement of I_{K1} in the presence of regular $[Ca^{2+}]_i$ transients in isolated canine ventricular myocytes. **a** Representative set of 10 μM Ba^{2+}-sensitive current records and **b** Fluo-4 fluorescence recordings (raw, uncorrected traces) obtained with 5 mM EGTA (low $[Ca^{2+}]_{pip}$) and without 5 mM EGTA (high $[Ca^{2+}]_{pip}$) in the pipette solution (*left* and *right panels*, respectively). $[Ca^{2+}]_i$ transients were evoked by 50 ms of prepulses clamped to 0 mV for 50 ms preceding the 500-ms test pulses—as shown in the insert. **c** Current–voltage relationships were obtained by plotting I_{K1} amplitudes, measured at the end of these test pulses, as a function of the respective test potential with (*open symbols*) and without (*filled symbols*) 5 mM EGTA in the pipette solution. Statistical analysis (ANOVA + Bonferroni post hoc) revealed significant difference between the low and high $[Ca^{2+}]_{pip}$ curves ($p<0.05$). The *asterisks* indicate significant ($p<0.05$) differences between low and high $[Ca^{2+}]_{pip}$ results, obtained in five myocytes isolated from three dog hearts

significantly increased (control: 194.8±11.7 ms; $BaCl_2$: 212.2±10.6 ms; $n=5$, $p<0.05$). Human and canine triangulation data were also compared, and a significant difference was revealed between all four corresponding pairs of experimental groups.

$[Ca^{2+}]$-dependent contribution of I_{K1} to the repolarization reserve in canine myocardium

If the magnitude of the Ba^{2+}-sensitive current was increased by elevation of $[Ca^{2+}]_o$, its relative contribution to the repolarization reserve should also be enhanced. In order to characterize the $[Ca^{2+}]$-dependent redistribution of repolarizing currents, the contribution of I_{Kr} and I_{Ks} to repolarization was estimated at normal and high $[Ca^{2+}]_o$ levels. As summarized in Fig. 8, prolongation of APD_{90} induced by 300 nM dofetilide was significantly less at 4 than 2 mM $[Ca^{2+}]_o$ (at 2 mM $[Ca^{2+}]_o$; control: 205.2±6.5 ms; Dofetilide: 253.2±9.3 ms, $p<0.05$; at 4 mM $[Ca^{2+}]_o$, control: 185.8±8.1 ms, Dofetilide 208.4±9.2 ms, N.S.; for the relative increase 23.5±1.8 versus 12.2±1.1 %, $p<0.05$, $n=5$). Selective inhibition of I_{Ks} by 0.5 μM HMR-1556 had no significant APD_{90} lengthening effect in either 2 or 4 mM $[Ca^{2+}]_o$ (at 2 mM $[Ca^{2+}]_o$: control: 206.3±5.9 ms, HMR-1556: 211.5±6.1 ms, N.S.; at 4 mM $[Ca^{2+}]_o$: control: 191.4±6.7 ms, HMR-1556: 193±4.8 ms, N.S.) (Fig. 8b, c) congruently with a very limited contribution of I_{Ks} to cardiac repolarization under control conditions. These findings demonstrate that the APD-shortening effect of high $[Ca^{2+}]_o$ cannot be attributed to accelerated activation of either I_{Kr} or I_{Ks} due to the concomitant elevation of the plateau potential. These results are just the opposite of those observed with inhibition of I_{K1} (Figs. 6 and 7). Since the elevation of $[Ca^{2+}]_i$ significantly alter the contribution of the Ba^{2+}-sensitive current to terminal repolarization, this effect may have

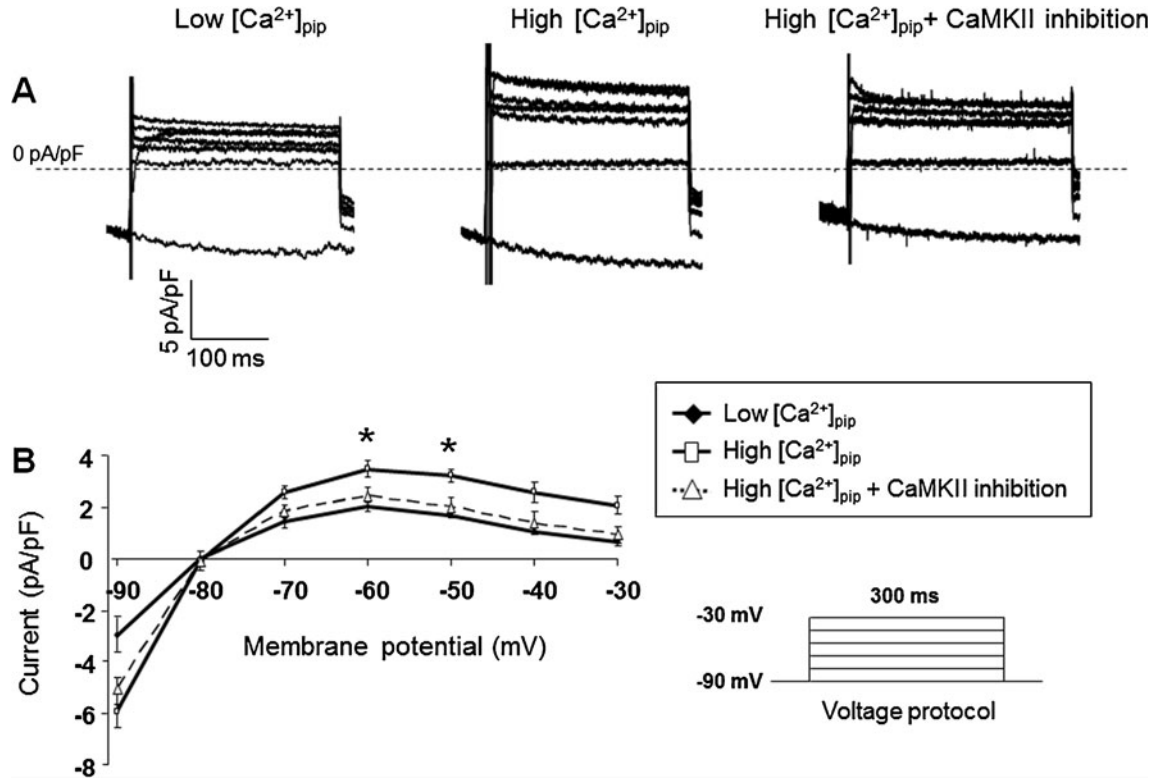

Fig. 4 Sensitivity of the $[Ca^{2+}]_{pip}$-induced augmentation of I_{K1} to inhibition of CaMKII. **a** Representative I_{K1} current sets evoked by 300 ms of pulses to test potentials ranging between −90 and −30 mV. Ba^{2+}-sensitive (10 μM) current was determined at low $[Ca^{2+}]_{pip}$ plus KN-92 (*left panel*), high $[Ca^{2+}]_{pip}$ plus KN-92 (*middle panel*), and high $[Ca^{2+}]_{pip}$ + KN-93 (*right panel*, CaMKII inhibition). **b** Steady-state current–voltage relationships obtained for the Ba^{2+}-sensitive currents under various experimental conditions (eight cells isolated from four dog hearts in each group). Statistical analysis (ANOVA + Bonferroni) revealed significant difference between the low and high $[Ca^{2+}]_{pip}$, as well as the low $[Ca^{2+}]_{pip}$ and high $[Ca^{2+}]_{pip}$ + CaMKII inhibition curves ($p<0.05$). The *asterisks* indicate significant ($p<0.05$) differences between the high $[Ca^{2+}]_{pip}$ and $[Ca^{2+}]_{pip}$ + CaMKII inhibition groups

also a considerable influence on the magnitude of repolarization reserve.

Discussion

Ca^{2+} dependence of I_{K1} and AP morphology

I_{K1} is an essential repolarizing current playing important role in terminal repolarization and in stabilizing the resting membrane potential [5, 25]. As such, it is an important component of cardiac repolarization reserve [5]. The regulation of I_{K1} is complex because of the multiple interacting signaling pathways converging on the current [13, 17, 20, 29, 36, 44]—many of those are known to be $[Ca^{2+}]_i$-sensitive. Nevertheless, experimental data on the nature and details of the Ca^{2+} dependence of I_{K1} itself are quite controversial. In some studies, the effect of a $[Ca^{2+}]_i$ rise on I_{K1} (or on Kir2 channels) was an inhibition [28–30], while in other studies, I_{K1} was found to increase with rising $[Ca^{2+}]_i$ [7, 17, 19, 36]. Possible reason for this scattering of data may be the marked differences in experimental conditions and the wide variety of the animal models used. However, even under very similar experimental conditions, the results may often be divergent. Due to the special importance of I_{K1} in shaping action potential morphology (especially in case of Ca^{2+} overload), the aim of the present study was to elucidate the effects of $[Ca^{2+}]_i$ rise on I_{K1} under conditions when the majority of the other Ca^{2+}-sensitive regulatory mechanisms are relatively inactive. For this reason, we selected nonadrenergic ways to increase $[Ca^{2+}]_i$, namely elevation of $[Ca^{2+}]_o$ in multicellular preparations and direct modulation of $[Ca^{2+}]_i$ when using buffered or unbuffered pipette solutions in single cell experiments. In these cases, although a number of Ca^{2+}-dependent signaling pathways may simultaneously be activated, the elevation of $[Ca^{2+}]_i$ is most probably not related to activation of PKA/PKC pathways, allowing thus a relatively consistent interpretation of the results.

Convincing data support the observation that cardiac AP morphology is modulated by a large number of $[Ca^{2+}]_i$-dependent and $[Ca^{2+}]_i$-independent signaling pathways. Early clinical studies demonstrated that changes in serum Ca^{2+} level induced alterations in QT interval [8, 14, 33]. $[Ca^{2+}]_i$-dependent shifts in AP configuration are involved in heart

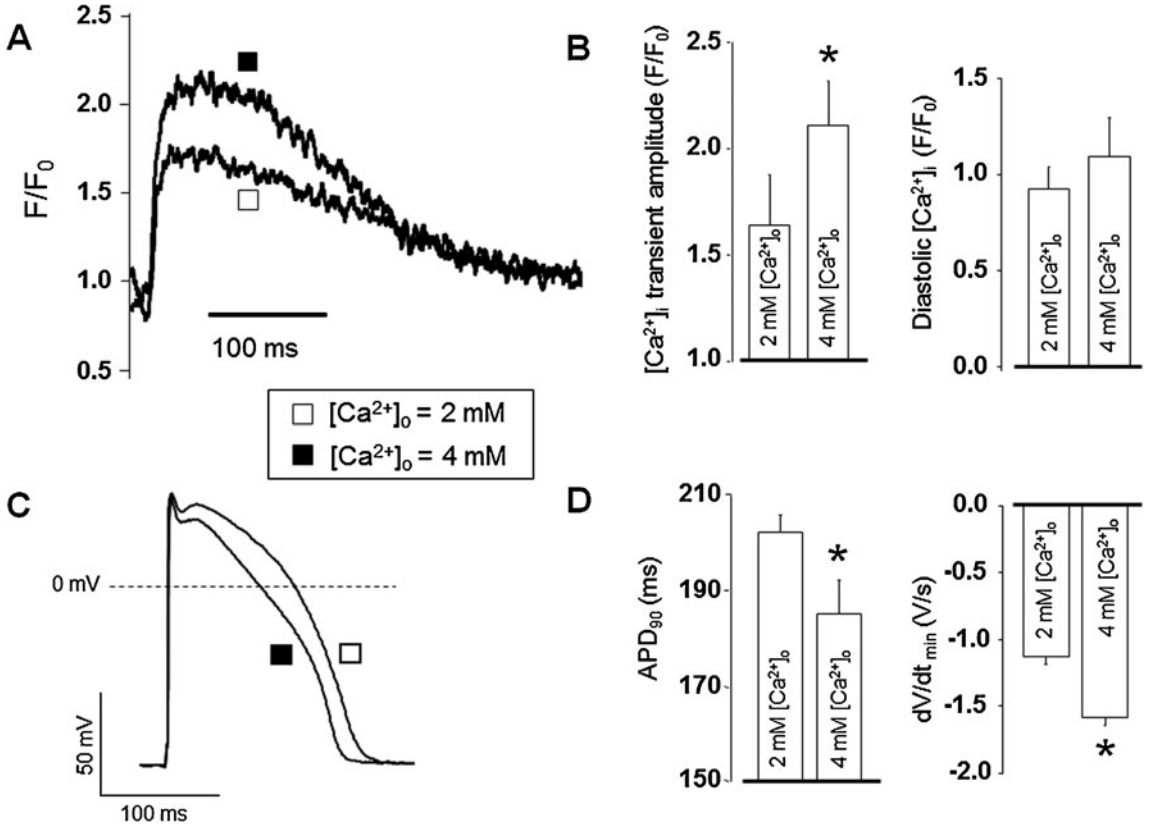

Fig. 5 $[Ca^{2+}]_o$ dependence of $[Ca^{2+}]_i$ transients (**a**, **b**) and action potential morphology (**c**, **d**) in multicellular canine ventricular preparations (papillary muscles). **a** Representative $[Ca^{2+}]_i$ transients and **b** corresponding, superimposed action potentials in the presence of 2 or 4 mM $[Ca^{2+}]_o$ (*open* and *filled symbols*, respectively). $[Ca^{2+}]_i$ transients were recorded using the fluorescent $[Ca^{2+}]_i$ indicator, Fluo-4. Fluorescence traces were corrected for nonspecific background and bleaching prior to quantitative analysis. **c** Average values obtained for the amplitude of $[Ca^{2+}]_i$ transients and diastolic $[Ca^{2+}]_i$ levels. **d** Average action potential duration (APD_{90}) and minimal rate of terminal repolarization (dV/dt_{min}) values obtained from five papillary muscles (isolated from five dogs) in the presence of 2 or 4 mM $[Ca^{2+}]_o$. The *asterisks* indicate significant ($p<0.05$) differences observed between the low $[Ca^{2+}]_o$ and high $[Ca^{2+}]_o$ results

failure and ischemia–reperfusion injury and may increase the incidence of cardiac arrhythmias. In spite of its crucial role, the impact of perturbations in $[Ca^{2+}]_i$ homeostasis on AP morphology is still poorly understood. Even novel, sophisticated mathematical models are unable to explain the inverse relationship between the length of the QT interval and $[Ca^{2+}]_i$ [15].

I_{CaL} and I_{NCX} are the primary factors believed to translate $[Ca^{2+}]_i$ shifts to membrane potential changes. $[Ca^{2+}]_i$-dependent acceleration of I_{CaL} inactivation, resulting in reduction of inward current during the action potential plateau, could explain the concomitant AP shortening [15]. Small conductance Ca^{2+}-activated (SK_{Ca}) channels also could provide a direct link between cellular Ca^{2+} handling and repolarization [41, 45]. However, our recent study demonstrated that a significant contribution of the apamin-sensitive SK current to ventricular repolarization is quite unlikely in canine and human hearts [12]. I_{Ks} could be another candidate to transfer $[Ca^{2+}]_i$ changes to APD. Lowering $[Ca^{2+}]_o$ increased I_{Ks} [21], while increasing the level of PKA also augmented I_{Ks} and enhanced the rate-dependent shortening of action potentials [24, 27]. Finally, Ca^{2+}-activated Cl^- current might also contribute to Ca^{2+}-induced adaptation of APD; however, investigation of its potential role is hampered by the lack of selective inhibitors. Since all these currents are quasi-simultaneously activated, the Ca^{2+}-induced APD adaptation is a highly complex process making the reliable dissection of the individual currents extremely difficult. In the present study, $[Ca^{2+}]_i$-dependent contribution of I_{K1} to repolarization was directly evaluated in isolated canine ventricular myocytes and papillary muscle preparations isolated from canine and undiseased human hearts.

$[Ca^{2+}]_i$ dependence of I_{K1} in isolated cardiomyocytes

In isolated cardiomyocytes, four sets of experiments were performed using the whole cell patch clamp technique. In the first set, steady-state I_{K1} current was determined at the end of depolarizing voltage steps as the Ba^{2+} sensitive current (Fig. 1). Secondly, I_{K1} current profiles were recorded during actual action potentials (Fig. 2). Effects of an unbuffered increase in $[Ca^{2+}]_{pip}$ on steady-state I_{K1} was investigated in the third set of experiments (Fig. 3), and the final set of

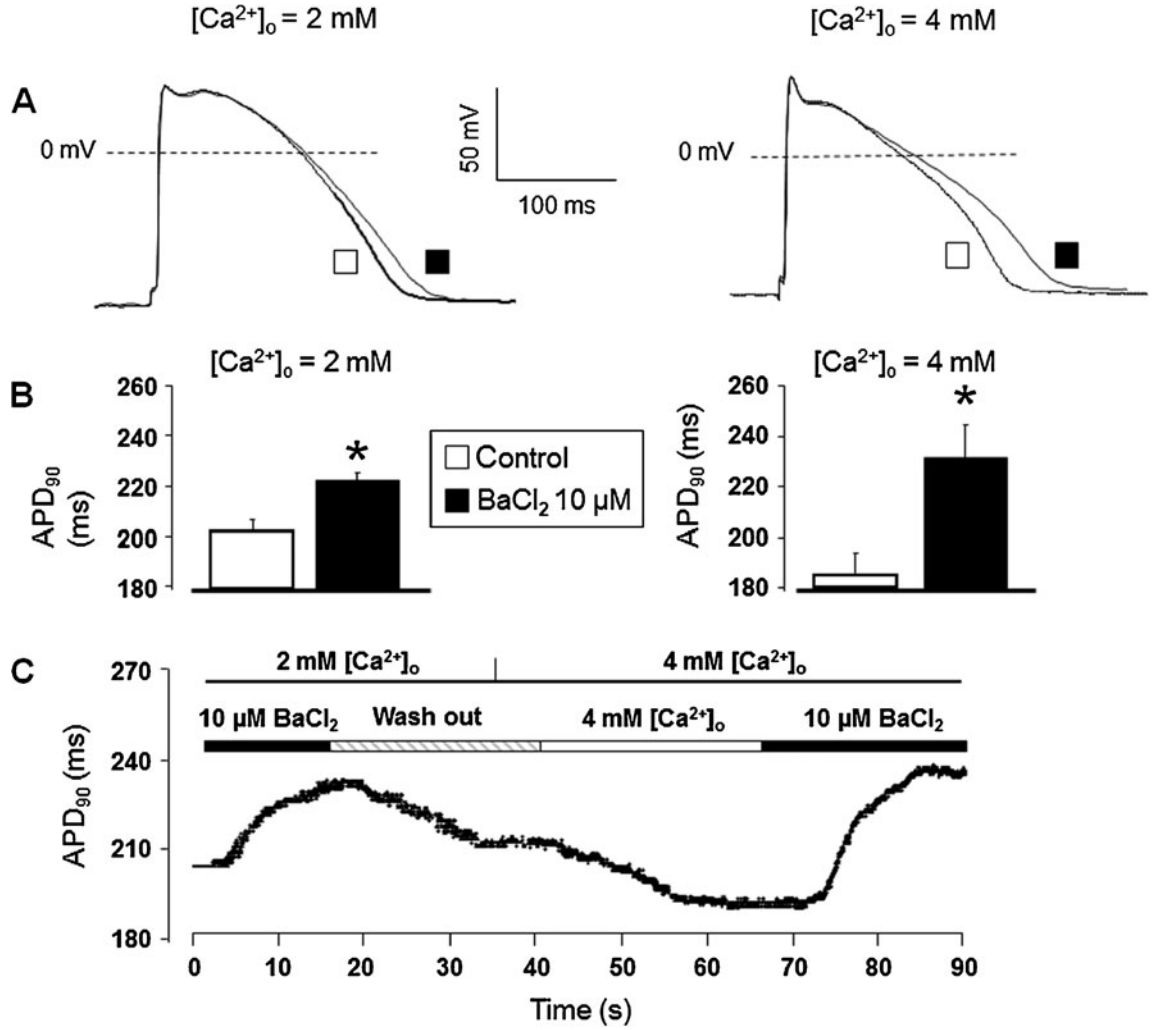

Fig. 6 $[Ca^{2+}]_o$ dependence of the action potential lengthening effect of I_{K1} blockade induced by 10 μM $BaCl_2$ in canine papillary muscles. **a** Representative superimposed action potentials recorded in the presence of 2 or 4 mM $[Ca^{2+}]_o$ prior to and after the application of 10 μM $BaCl_2$ (*open* and *filled symbols*, respectively). **b** Mean APD_{90} values measured under conditions specified above in five papillary muscles isolated from five dogs. The *asterisks* denote significant ($p<0.05$) changes induced by exposure to 10 μM $BaCl_2$. **c** Time course of development of the Ba^{2+}-induced APD lengthening in the presence of 2 and 4 mM $[Ca^{2+}]_o$, in a representative experiment from four performed

experiments revealed the mechanism involved in the $[Ca^{2+}]_i$-dependent activation of I_{K1} (Fig. 4). Independent of the experimental protocol applied, our data reflect a highly consistent upward shift in the I–V relationship obtained for I_{K1} in the physiologically relevant outward current range following the elevation of $[Ca^{2+}]_i$, of which response seems to be mediated to a large extent by the enhanced CaMKII activity.

Our results obtained in single cardiomyocytes are in full agreement with those of earlier studies in Purkinje fibers reporting increased I_{K1} and a higher contribution of I_{K1} to AP shortening following an increase in $[Ca^{2+}]_o$ [17]. These data also support previous studies dealing with the mechanism of hypoxia-induced APD shortening, where substantial contribution of $[Ca^{2+}]_i$-dependent elevation of I_{K1} to the early phase of shortening was demonstrated [31, 36, 37], in addition to the proposed changes in a few more $[Ca^{2+}]_i$-modulated currents, like I_{CaL}, I_{NCX}, or I_{Cl}. On the other hand, the present data, demonstrating an augmentation of I_{K1} in response to elevation of $[Ca^{2+}]_i$, seem to contradict to findings of some other studies [10, 13, 28, 29, 46] reporting a reduced steady-state I_{K1} following a rise of $[Ca^{2+}]_i$. In the case of the first three reports, the major reason for the discrepancy may be the highly unphysiological experimental conditions applied. In the study by Zaza et al. [46], the increased I_{K1} following the reduction of $[Ca^{2+}]_i$ could only be observed at membrane potentials corresponding to the plateau phase of the AP, leaving the terminal repolarization unaltered. Finally, while Fauconnier et al. [13] attributed the low density of I_{K1} in cardiomyocytes isolated from failing hearts to elevated diastolic $[Ca^{2+}]_i$ levels caused by an increased diastolic leak from the SR, their results could also be interpreted in a different way. In myocytes from failing a heart, an increased NCX activity together with decreased

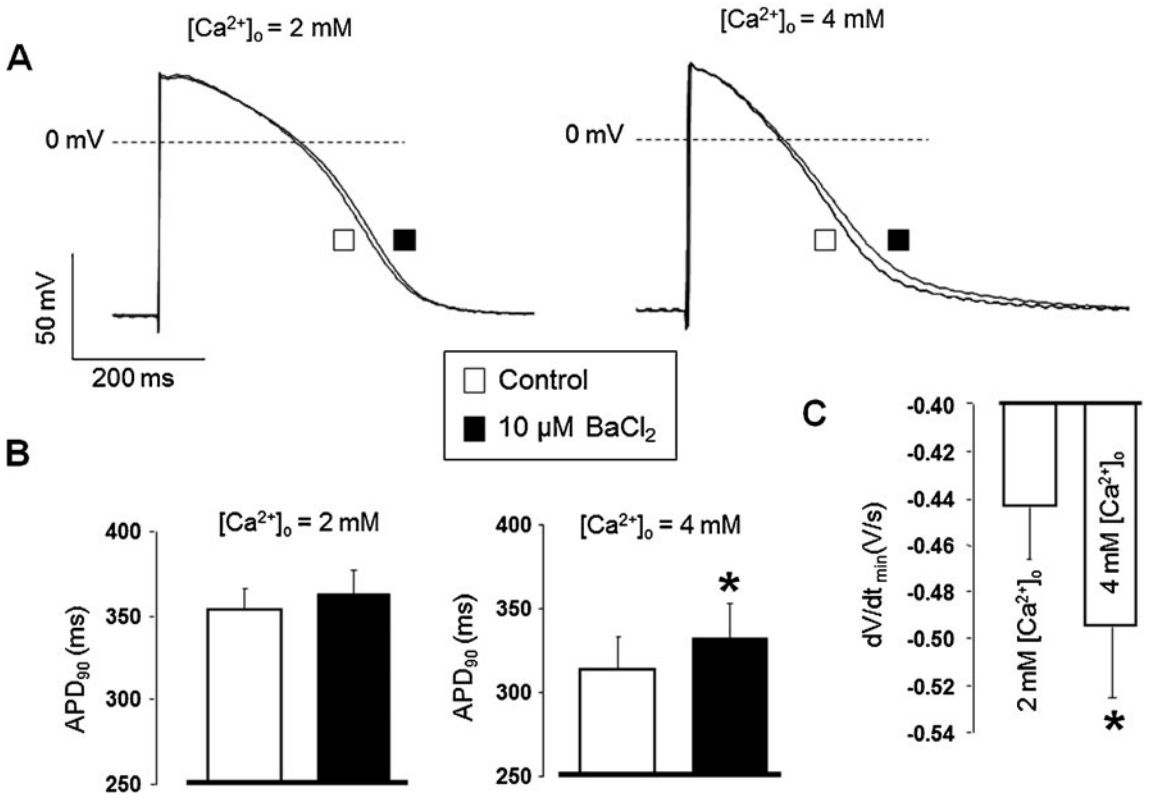

Fig. 7 $[Ca^{2+}]_o$ dependence of the Ba^{2+}-induced prolongation of action potentials in human papillary muscles isolated from undiseased hearts. **a** Representative superimposed pairs of action potentials recorded before and after the exposure to 10 μM $BaCl_2$ (*open* and *filled symbols*, respectively) in the presence of either 2 or 4 mM $[Ca^{2+}]_o$. **b** Average APD_{90} values showing the effect of 10 μM $BaCl_2$ in five human preparations isolated from two undiseased hearts in the presence of 2 or 4 mM $[Ca^{2+}]_o$. **c** Effect of $[Ca^{2+}]_o$ on the minimum rate of repolarization (dV/dt_{min}) in human papillary muscles ($n=5$). The *asterisk* in **b** indicates that in the presence of 4 mM $[Ca^{2+}]_o$ the application of 10 μM $BaCl_2$ induced a significant ($p<0.05$) change in APD_{90}. The *asterisk* in **c** indicates significant ($p<0.05$) differences in (dV/dt_{min}) between low and high $[Ca^{2+}]_o$ conditions

pumping rate of SERCA2 is known to result in a substantial loss of cellular Ca^{2+} content [4] leading subsequently to large decay in systolic $[Ca^{2+}]_i$ levels. Therefore, it may be quite probable that the average $[Ca^{2+}]_i$ seen by the Kir2.x channels was, indeed, decreased in these failing cells.

Ca^{2+}-induced changes of AP morphology in multicellular preparations

In papillary muscles isolated from canine and undiseased human hearts, $[Ca^{2+}]_i$ was increased by the elevation of $[Ca^{2+}]_o$. Increased $[Ca^{2+}]_o$ resulted in enhanced Ca^{2+} influx, and due to the autoregulative nature of cardiac Ca^{2+} cycling, a new equilibrium developed with elevated $[Ca^{2+}]_i$ and higher amplitude of $[Ca^{2+}]_i$ transients [11]. Indeed, as shown in Fig. 5a, b, switching to 4 mM $[Ca^{2+}]_o$ the amplitude of the $[Ca^{2+}]_i$ transient significantly increased with only a minor shift in diastolic $[Ca^{2+}]_i$. As expected [22], increased $[Ca^{2+}]_i$ was paralleled with a significant shortening of APD in both canine (Fig. 5c, d) and human (Fig. 7a, b) papillary muscles, indicating a $[Ca^{2+}]_i$-induced imbalance of the inward/outward current ratio. More importantly, the rate of terminal repolarization—considered to be proportional with the density of I_{K1}—was also significantly increased at high $[Ca^{2+}]_o$ (Figs. 5d and 7c).

In experiments shown in Figs. 5 and 6c, the recording of APs in 2 mM $[Ca^{2+}]_o$ always preceded the exposure to 4 mM $[Ca^{2+}]_o$. In principle, this sequence may carry the risk of a systematic error if the samples are unstable. In a set of additional time control experiments, however, no significant shifts in either AP morphology or APD_{90} during a 60-min control time period could be observed ($n=3$; data not shown).

Triangulation was increased by elevation of $[Ca^{2+}]_o$ to 4 mM, which represents a stronger shortening of APD_{25} than APD_{90}. Since the AP is a highly complex phenomenon, in principle, both increased and decreased triangulation may be feasible, since the momentary level of AP triangulation may also be modulated by concomitant changes in several further ion currents (e.g., I_{Kr}, I_{Ks}, I_{K1}, I_{Ca}, and I_{NCX}). Furthermore, the overall effect of the interdependence may also be enhanced if Ca^{2+}_i is substantially elevated since a few of these currents are also known to be modulated by Ca^{2+}_i. Consequently, one may speculate that the final level of triangulation can be, indeed, increased if I_{K1} is augmented by Ca^{2+}_i, since in the presence of

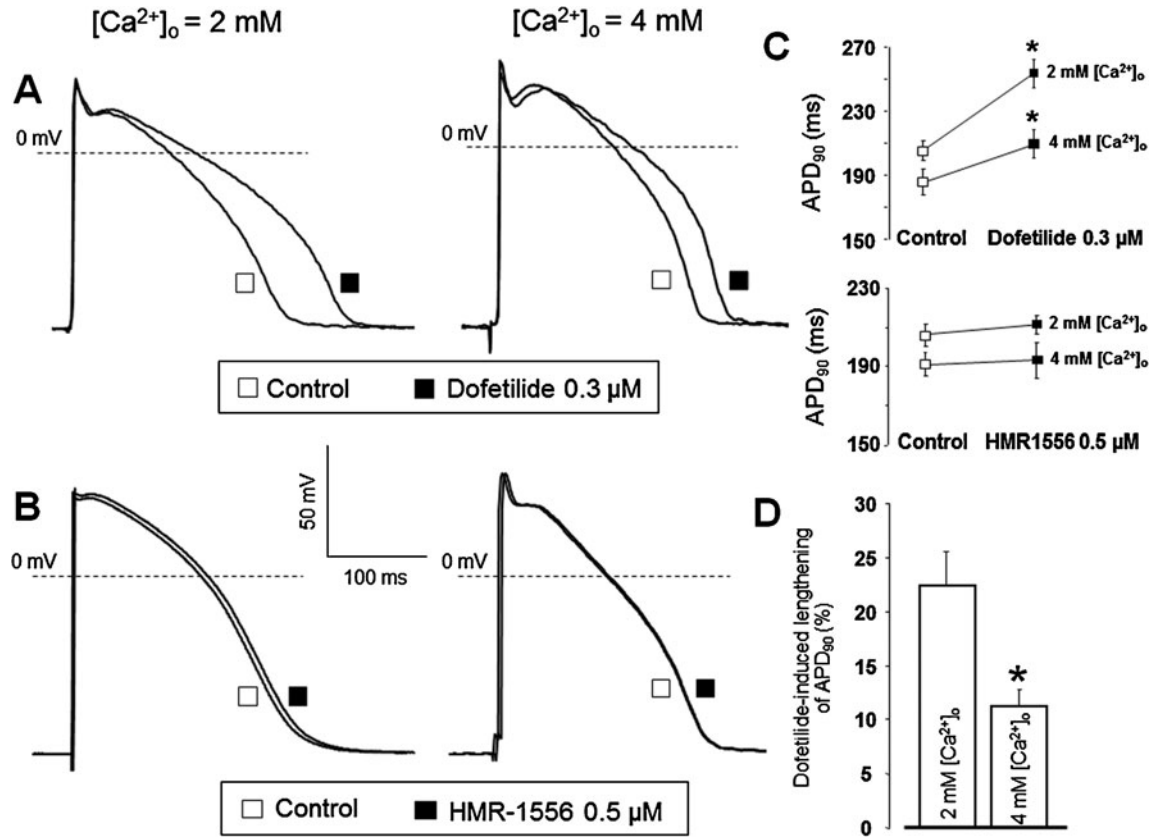

Fig. 8 In canine papillary muscles elevated $[Ca^{2+}]_o$ significantly attenuated the contribution of I_{Kr} to repolarization (i.e., its action potential lengthening effect) with no apparent effect on I_{Ks}. **a** Representative superimposed action potential pairs recorded in the presence of 2 or 4 mM $[Ca^{2+}]_o$, before and after the application of 0.3 μM dofetilide to block I_{Kr} (*open* and *filled symbols*, respectively). **b** Representative superimposed action potential pairs recorded in the presence of 2 or 4 mM $[Ca^{2+}]_o$, before and after the exposure to 0.5 μM HMR-1556 to block I_{Ks} (*open* and *filled symbols*, respectively). **c** Summary of action potential durations determined in the presence of 2 and 4 mM $[Ca^{2+}]_o$ in these experiments (control, *open symbols*; blockade, *filled symbols*); the *asterisks* indicate significant ($p<0.05$) changes from control. **d** Comparison of the effect of 0.3 μM dofetilide on action potential duration in the presence of 2 and 4 mM $[Ca^{2+}]_o$. Data are average values obtained from five preparations, each isolated from different dogs. The *asterisks* indicate significant ($p<0.05$) differences between the effect of dofetilide in low and high $[Ca^{2+}]_o$

high Ca^{2+}_i, I_{CaL} is substantially reduced (consequently APD_{25} is also reduced), while at the same time, forward I_{NCX} is probably enhanced, providing larger depolarizing currents in the range of APD_{90}. Our results are in line with the proposed primary role of I_{CaL} in the Ca^{2+}-induced shortening of action potentials [15]; however, the increased rate of terminal repolarization, and the more pronounced triangulation obtained with $BaCl_2$ in the presence of 4 mM $[Ca^{2+}]_o$ may rather be consistent with an additional mechanism, namely the contribution of enhanced I_{K1}.

Increased density of the repolarizing K^+ currents have also been proposed to modulate APD under conditions of elevated $[Ca^{2+}]_i$, especially when the shortening of APD was paralleled by elevated plateau potentials. Since all K^+ currents (I_{Kr}, I_{Ks}, I_{K1}) are known to contribute to some extent to terminal repolarization, their putative role in Ca^{2+}-induced APD shortening was further studied. The dofetilide-induced selective blockade of I_{Kr} resulted in a smaller prolongation of APD in high than in low $[Ca^{2+}]_o$ (Fig. 8a, d), while no significant changes could be observed following the application of the selective I_{Ks} inhibitor HMR-1556 (Figs. 8b, c). The finding that the APD lengthening effect of dofetilide was reduced at high $[Ca^{2+}]_o$ levels and no changes could be observed in the effect of HMR-1556 excludes the possibility of a Ca^{2+}-dependent augmentation of I_{Kr} or I_{Ks}. Although the compromised effects of these K^+ channel blockers could partially be ascribed to the $[Ca^{2+}]_o$-induced shortening of APD [3], allowing shorter time for activation of both delayed rectifiers, this argumentation fails to account for the marked enhancement of the APD lengthening effect of $BaCl_2$. Thus the reduced effect of the I_{Kr} and I_{Ks} blockade on APD is more likely a consequence of the increased contribution of another outward current, I_{K1}, providing this way an enhanced repolarization reserve capacity in response to elevation of $[Ca^{2+}]_i$. The present results provide direct experimental support for the complex mathematical model of Grandi et al. [15], who correctly predicted limited capability of the delayed rectifiers to accelerate repolarization when $[Ca^{2+}]_i$ is high.

Ca^{2+}-induced changes—canine vs. human

By comparing Figs. 6 and 7, it is evident that the effect of $BaCl_2$ on both the rate of terminal repolarization and APD was considerably weaker in human than in canine multicellular preparations (e.g., in human papillary muscles, 10 μM $BaCl_2$ failed to lengthen APD at 2 mM $[Ca^{2+}]_o$). This difference is likely related to a significantly smaller contribution of I_{K1} to ventricular repolarization in humans. This assumption is supported by our results in a parallel study (under publication) demonstrating that the contribution of I_{Kr} to the repolarization reserve is significantly larger in human than in canine ventricle. Furthermore, the full repolarization reserve in humans is substantially weaker than that in dogs, presumably also for the much smaller contribution of I_{K1}. These data may explain the moderate effect of Ba^{2+}, the relatively small extent of the Ca^{2+}-induced augmentation of the Ba^{2+}-sensitive current, and also the reduced speed of repolarization in human samples.

The substantially weaker I_{K1} (and other repolarizing K^+ currents) in human hearts may also well explain the large differences found between the triangulation data determined in canine and human samples. The significantly weaker repolarization force may be the primary cause of the largely elevated control levels in human samples at both low and high $[Ca^{2+}]_o$. Furthermore, it may also be the reason for the significantly reduced effect of 10 μM $BaCl_2$ on AP triangulation in human compared to dog samples.

Multifactor regulation of I_{K1}?

Beyond its direct $[Ca^{2+}]_i$ dependence, I_{K1} is also known to be sensitive to several factors modulating the phosphorylation/dephosphorylation state of channel proteins. I_{K1} was shown to be simultaneously targeted by PKA, PKC, and CaMKII with distinct roles. PKA and PKC reduced I_{K1} during adrenergic stimulation [20], while the current was enhanced following acute CaMKII activation [44]. In line with these results, our present data underline the proposed significant role of CaMKII activation in the Ca^{2+}-induced enhancement of the Ba^{2+}-sensitive current. It seems quite feasible that these distinct modulatory pathways (i.e., PKA, PKC, and CaMKII), by concomitantly targeting I_{K1}, may also jointly fine-tune it and subsequently improve the adaptation of APD to changing conditions. While sympathetic activation of PKA and PKC exert its crucial role in defining action potential waveform by modulating other ion currents, like I_{CaL}, I_{Ks}, and I_{Cl} [18], their proposed inhibiting effect on I_{K1} might be highly arrhythmogenic, due to subsequent reduction of the repolarization reserve. In this context, the rise in $[Ca^{2+}]_i$ and subsequent activation of CaMKII leading to augmented I_{K1} may counteract and limit this PKA/PKC-induced suppression, thus may largely contribute to the prevention of cardiac arrhythmias by normalizing APD. Substantially differing experimental conditions or pathologic states (e.g., heart failure) may result in "loss of balance" between these opposing pathways, resulting in significantly compromised I_{K1} and a consequently increased incidence of cardiac arrhythmias. The above-discussed enhancement of I_{K1} may be helpful when trying to interpret the contradictory results on Ca^{2+} dependence of the current.

Limitations of the study

I_{K1} versus Ba^{2+}-sensitive current

Since I_{K1} is a composite current flowing through a number of channel types not yet clarified, it should be emphasized that the magnitude of the actually measured I_{K1} may substantially depend on the experimental conditions applied. Similarly to most previous studies, we defined I_{K1} as the current sensitive to 10 μM Ba^{2+}. This dose of Ba^{2+} was shown to have no effect on other major repolarizing currents, like I_{to}, I_{Kr}, and I_{Ks}. [5]. However, we have very limited information about its possible effect on background leak channels [26], or on cardiac Ca^{2+} activated K^+ channels. Therefore, we should not assign the significant Ca^{2+} sensitivity of I_{K1} to a single channel type. In addition, we cannot rule out the possibility that putative apamin-insensitive SK channels [32] are, at least partially, responsible for the observed Ca^{2+} dependence found in the present study. Considering the complexity and nonlinear feature of the action potential, the relative contribution of the Ba^{2+}-sensitive and other currents to high Ca^{2+}-induced APD changes are not fully predictable and require further studies.

Low versus high concentrations of $[Ca^{2+}]_i$

In patch clamp studies, it is widely accepted that ion concentrations in the pipette shortly equilibrate with those in the cytosol. This assumption is rather feasible, especially for monovalent ions, but not necessarily true for $[Ca^{2+}]$. Some of the possible reasons are as follows: (1) the inherently high spatial and temporal heterogeneity of $[Ca^{2+}]$, (2) subsequent lack of a true average cytosolic $[Ca^{2+}]$, (3) slower intracellular diffusion of bivalent ions, and (4) uneven spatial/temporal activity of Ca^{2+} transporters etc. Consequently, a much simplified "average" $[Ca^{2+}]_i$, although should be rather close, is probably not identical to actual $[Ca^{2+}]_{pip}$. We have to mention, however, that in a few cases of high $[Ca^{2+}]_{pip}$, a minor contracture of the cell could also be observed as a sign of substantially enhanced intracellular Ca^{2+} content.

Steady-state $[Ca^{2+}]_i$ versus $[Ca^{2+}]_i$ transient?

Data presented in this study clearly demonstrate that, in addition to membrane potential changes, I_{K1} is, at least partially,

activated by increased $[Ca^{2+}]_i$ levels. In principle, this activation may either be direct via increased local $[Ca^{2+}]_i$ or indirect via $[Ca^{2+}]_i$-activated signaling pathways. In this $[Ca^{2+}]_i$-induced enhancement of I_{K1}, not only the steady elevation of $[Ca^{2+}]_i$, but also the AP-induced $[Ca^{2+}]_i$ transients may carry a role. Although the substantially different kinetics of the $[Ca^{2+}]_i$ transient and the I_{K1} current during the cardiac cycle seem to contradict this assumption, it should be emphasized that the activation is not necessarily direct and immediate. It may be mainly indirect showing some delay relative to the peak of the transient. Indeed, our present results support an important role for indirect activation of I_{K1} via CaMKII; however, the contribution of other signaling mechanisms cannot be ruled out either. Nonetheless, in the moment, we have no evidence for the direct effect of the $[Ca^{2+}]_i$ transient, only a few supporting arguments: (1) Based on the high spatial/temporal heterogeneity of $[Ca^{2+}]_i$, the existence of a true "average" value of $[Ca^{2+}]_i$ in cardiomyocytes is doubtful. The effect of local (spatial and/or temporal) Ca^{2+} movements on this functional heterogeneity is probably much larger than that reflected by the widely used fluorescence techniques based on Ca^{2+}-sensitive dyes and may significantly modulate the local activity of any Ca^{2+}-dependent process or enzyme. (2) During a cardiac cycle of large mammals including humans, the "time averaged" local $[Ca^{2+}]_i$ should be enhanced in active (when AP and $[Ca^{2+}]_i$ transient are present) compared to inactive cardiomyocytes (i.e., when $[Ca^{2+}]_i$ is close to its end-diastolic level). This is especially true for the submembrane regions surrounding the ion channels. (3) With increasing heart rate, both the magnitude of the $[Ca^{2+}]_i$ transient and the end-diastolic $[Ca^{2+}]_i$ level are enhanced. (4) Our present data support a significant role for CaMKII activation in the $[Ca^{2+}]_i$-induced elevation of I_{K1}. An activation delay of CaMKII or any other Ca^{2+}-dependent signaling pathway may well explain the time delay seen at the current increase. In fact, one may recognize very similar mechanism in Ca^{2+}-induced activation of several mitochondrial enzymes.

Concluding remarks

In summary, our results directly support the hypothesis [17, 23, 36, 44] that following a nonadrenergic $[Ca^{2+}]_i$ rise, the augmented I_{K1} may significantly contribute to shortening of APD in dogs and humans. In this case, the effect of increased $[Ca^{2+}]_i$, most probably mediated by CaMKII activation, may be an important physiological adaptation mechanism of the heart. Since substantially increased $[Ca^{2+}]_i$ is known to facilitate afterdepolarization-induced arrhythmogenesis, a $[Ca^{2+}]_i$-induced increase in I_{K1} may lead to the shortening of ventricular repolarization and enhancement of the repolarization reserve capacity. These factors could also be considered as an endogenous negative feedback limiting the proarrhythmic consequences of increased $[Ca^{2+}]_i$.

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Conflict of interest The authors declare that they have no conflict of interest.

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