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The slow delayed rectifier K+ current is differently regulated under baseline conditions and following β-adrenergic stimulation in canine ventricular cardiomyocytes

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Sympathetic activation robustly increases the slow delayed rectifier K^+ current (I_{Ks}) in the mammalian ventricular myocardium, however, exact downstream pathways involved in the β-adrenergic regulation of the current are not fully elucidated yet. This study examined the Ca^{2+} sensitivity of I_{Ks} and the contribution of the protein kinase A (PKA) and the calcium/calmodulin kinase II (CaMKII) pathways in regulating I_{Ks} in isolated canine ventricular myocytes. Experiments were carried out under ß-adrenergic receptor activation (10 nM isoproterenol) and under baseline conditions (without isoproterenol). I_{Ks} was measured as an HMR-1556 sensitive current with the action potential voltage clamp technique under the physiological intracellular calcium homeostasis of the cells. Reducing intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) with 1 µM nisoldipine decreased the peak and mid-plateau densities of I_{Ks}, reduced the current integral, and increased the time-to-peak value. In contrast, ß-adrenergic receptor activation by isoproterenol resulted in larger I_{Ks} densities and integral, and shorter time-to-peak value. These effects of isoproterenol on I_{Ks} were significantly smaller when the CaMKII inhibitor 1 µM KN-93 was present in the cells, but the PKA inhibitor 3 µM H-89 did not exert such effect. Importantly, all effects of isoproterenol on I_{Ks} have fully developed even in the presence of 1 µM nisoldipine. Under baseline conditions the mid-plateau density of I_{Ks}, was significantly smaller in the presence of KN-93, H-89 or nisoldipine, while peak I_{Ks} density and the current integral were significantly smaller only in nisoldipine. In conclusion, many different signaling pathways are involved in regulating I_{Ks}. Under baseline conditions the regulation is strongly $[Ca^{2+}]_i$-dependent, with PKA and CaM-CaMKII involved, whereas during ß-adrenergic stimulation it is $[Ca^{2+}]_i$-independent and supposes a pivotal role of EPAC-mediated activation of CaMKII.

Keywords Slow delayed rectifier K^+ current, Sympathetic activation, Protein kinase A, Calcium/calmodulin-dependent protein kinase II, Action potential voltage clamp, Canine cardiomyocytes

Abbreviations

AC	Adenylate cyclase
AIP	Autocamtide-2 related inhibitory peptide
Akt	Ser/Thr kinase isolated from the AKR mouse spontaneous thymoma cells
AP	Action potential

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APVC	Action potential voltage clamp
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
CACNA1C	Voltage-gated calcium channel subunit alpha1C (generating $I_{Ca,L}$)
CaM	Calmodulin
CaMKII	Calcium/calmodulin-dependent protein kinase II
$Ca_v1.2$	Alias of CACNA1C
EPAC	Guanine nucleotide exchange protein activated by cAMP
ISO	Isoproterenol
$I_{Ca,L}$	L-type Ca^{2+} current
I_{Ks}	Slow delayed rectifier K^+ current
KCNQ1	Voltage-gated potassium channel subfamily Q member 1 (generating I_{Ks})
$K_v7.1$	Alias of KCNQ1
NISO	Nisoldipine
NOS	Nitrogen monoxide synthase
PKA	Protein kinase A
PCLε	Phospholipase C-epsilon
PP1	Protein phosphatase 1
Rad	Ras-related glycolysis inhibitor and calcium channel regulator protein
Rap	Ras-proximate GTP binding protein
RyR	Ryanodine receptor

Sympathetic activation of the heart is an important mechanism for fight-or-flight response, encompassing changes in cellular electrophysiology, calcium handling, and contractility. The action potential (AP) configuration is modified based on the changes of several inward and outward ion currents. One of these outward currents, the slow delayed rectifier K^+ current (I_{Ks}), is a crucial component of the repolarization reserve[1,2] and is also robustly increased by sympathetic stimulation in the canine heart[3,4]. However, the precise regulatory mechanisms of I_{Ks} are not fully elucidated yet. It is widely accepted that many effects of catecholamines on cardiac K^+ channels are mediated via the protein kinase A (PKA) pathway, while others are mediated through distinct, partially independent mechanisms such as the Ca^{2+}-sensitive calcium/calmodulin-dependent protein kinase II (CaMKII) pathway[5–7]. Notably, sympathetic activation leads to an increase in intracellular calcium concentration ($[Ca^{2+}]_i$), at least partly due to a PKA-, and Rad-mediated increase in L-type Ca^{2+} current ($I_{Ca,L}$). The reported findings on the ß-adrenergic regulation of cardiac I_{Ks} are highly dependent on the specific experimental conditions and species studied, further complicated by the extensive crosstalk between the downstream signaling pathways of ß-adrenergic receptor activation[8].

This study was designed to delineate the contributions of the PKA and CaMKII pathways in the acute ß-adrenergic receptor mediated modulation of I_{Ks} in canine ventricular cardiomyocytes under physiological conditions. These conditions included (1) unbuffered $[Ca^{2+}]_i$ for the physiological intracellular calcium homeostasis, (2) the use of action potential voltage clamp (APVC) technique to visualize the I_{Ks} current under an actual ventricular AP, and (3) activating ß-adrenergic receptors with low (not saturating) concentration of isoproterenol (10 nM). Canine myocytes were chosen for their electrophysiological properties, which closely resemble those of human cardiomyocytes[9–12].

Results

We studied the effects of ß-adrenergic receptor activation on the parameters of I_{Ks} by using 10 nM isoproterenol (ISO). In this relatively low concentration of ISO, as shown in Fig. 1A, the peak density of I_{Ks} was larger (0.32 ± 0.06 A/F vs 1.36 ± 0.34 A/F; see also Fig. 2C) and the time-to-peak value was shorter (153.5 ± 4.7 ms vs 128.9 ± 6.5 ms, Fig. 1A inset) compared to control conditions. Opposite differences were observed when calcium entry, and therefore $[Ca^{2+}]_i$ was reduced by the application of 1 µM nisoldipine (NISO). As demonstrated in Fig. 1B, peak I_{Ks} density was lower (0.32 ± 0.06 A/F vs 0.19 ± 0.02 A/F; see also Fig. 3C) and the time to peak I_{Ks} density took longer time (153.5 ± 4.7 ms vs 170.9 ± 4.1 ms, Fig. 1B inset). The true current–voltage relationship of I_{Ks} under a ventricular action potential is shown in black in Fig. 1C, D, under control conditions, and in the presence of ISO (purple trace in Fig. 1C) or NISO (blue trace in Fig. 1D). These curves also indicate that I_{Ks} activates earlier (i.e. at more positive membrane potentials on the graph of Fig. 1C) under ß-adrenergic receptor activation and later (i.e. at more negative voltages in Fig. 1D) when $[Ca^{2+}]_i$ was decreased by nisoldipine. In addition, the mid-plateau current density (current density measured at the halving point between the AP peak and 90% repolarization level) was also larger in the ISO-treated cells (0.23 ± 0.03 A/F vs 1.04 ± 0.26 A/F; Fig. 2B) and smaller in cells treated with NISO (0.23 ± 0.03 A/F vs 0.06 ± 0.01 A/F; Fig. 3B), similarly to the current integral, which was more than fourfold larger in ISO (28.2 ± 5.3 mC/F vs 135.7 ± 32.1 mC/F; Fig. 2D) and almost 50% smaller in NISO (28.2 ± 5.3 mC/F vs 15.4 ± 1.3 mC/F; Fig. 3D) compared to control conditions. All these differences evoked by ISO or NISO in the parameters of I_{Ks} were statistically significant ($p < 0.05$).

To investigate the signal transduction pathways mediating the effect of ß-adrenergic receptor stimulation by ISO, cells were pretreated either with the CaMKII inhibitor 1 µM KN-93, or with the PKA inhibitor 3 µM H-89, or with both, before adding 10 nM ISO to the bath. The effect of ISO on the parameters of I_{Ks} was significantly smaller in cases when CaMKII was inhibited, either by 1 µM KN-93 alone or in combination with 1 µM KN-93 and 3 µM H-89 together, as represented in Fig. 2A. The peak current densities (Fig. 2C) were 1.36 ± 0.34 A/F in ISO, whereas 0.66 ± 0.16 A/F when KN-93 was applied before ISO, and 0.62 ± 0.12 A/F when both KN-93 and H-89 was applied before ISO. The mid-plateau densities (Fig. 2B) were 1.04 ± 0.26 A/F, 0.41 ± 0.11 A/F and 0.29 ± 0.07 A/F, whereas the current integrals (Fig. 2D) were 135.7 ± 32.1 mC/F, 65.2 ± 11.9 mC/F and 56.7 ± 12.8 mC/F, respectively.

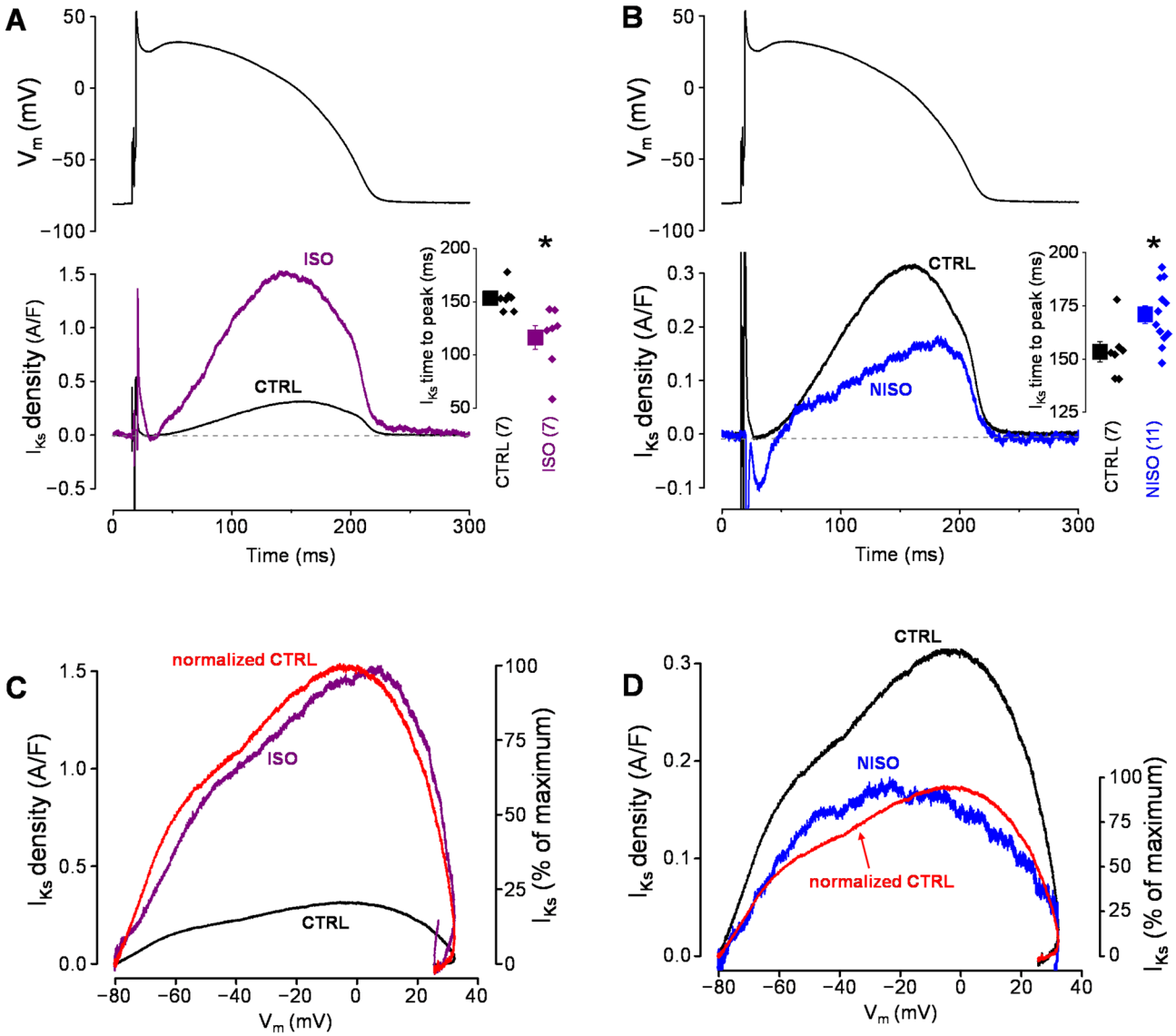

Fig. 1. Effect of 10 nM isoproterenol (ISO) and 1 µM nisoldipine (NISO) on I_{Ks} in canine ventricular cardiomyocytes. (**A**, **B**) Command action potentials (above), representative averaged I_{Ks} current traces (below), and I_{Ks} time to peak values (inserts below) obtained under APVC conditions in untreated control (black), in the presence of 10 nM ISO (violet), and 1 µM NISO (blue). (**C**, **D**) Current–voltage relationships obtained from the experiment shown on panel A and B (untreated control: black, ISO: violet, NISO: blue). Red curves were generated by scaling the control traces up or down to the amplitude of those obtained with ISO or NISO, respectively. On the relative scale, 100% is the maximal current density obtained either in CTRL (red) and in ISO (violet) (**C**), or in CTRL (red) and NISO (blue) (**D**).

While PKA blockade alone by 3 µM H-89 seemingly reduced the effect of ISO on I_{Ks} throughout the applied command AP pulse (as it appears on the representative traces of Fig. 2A), this effect did not reach statistical significance in any of the examined parameters of I_{Ks} peak density (0.80 ± 0.12 A/F), I_{Ks} mid-plateau density (0.56 ± 0.11 A/F), or the current integral (81.5 ± 15.5 mC/F).

Pretreating the cells with 1 µM NISO failed to modify the effects of ISO on I_{Ks} (Fig. 2) indicating that pathways independent of Ca^{2+}-entry dominate the ß-adrenergic receptor activated signal transduction in enhancing I_{Ks}.

We also tested the effects of 1 µM KN-93, 3 µM H-89 and 1 µM NISO on I_{Ks} without ß-adrenergic receptor activation. The mid-plateau I_{Ks} density was significantly lower either in the presence of KN-93 (0.10 ± 0.03 A/F) or H-89 (0.11 ± 0.03 A/F) than in the untreated control cells (Fig. 3A, B). Magnitudes of I_{Ks} peak current densities (Fig. 3C) and I_{Ks} current integrals Fig. 3D) were not significantly different in the presence of either KN-93 or H-89 compared to baseline conditions. In the presence of NISO, however, all these parameters were significantly smaller than in the untreated cell group.

While KN-93 is a potent and widely used inhibitor of CaMKII, it affects many other Ca/CaM-associated targets independently of CaMKII[13]. Therefore, we conducted some experiments with autocamtide-2 related

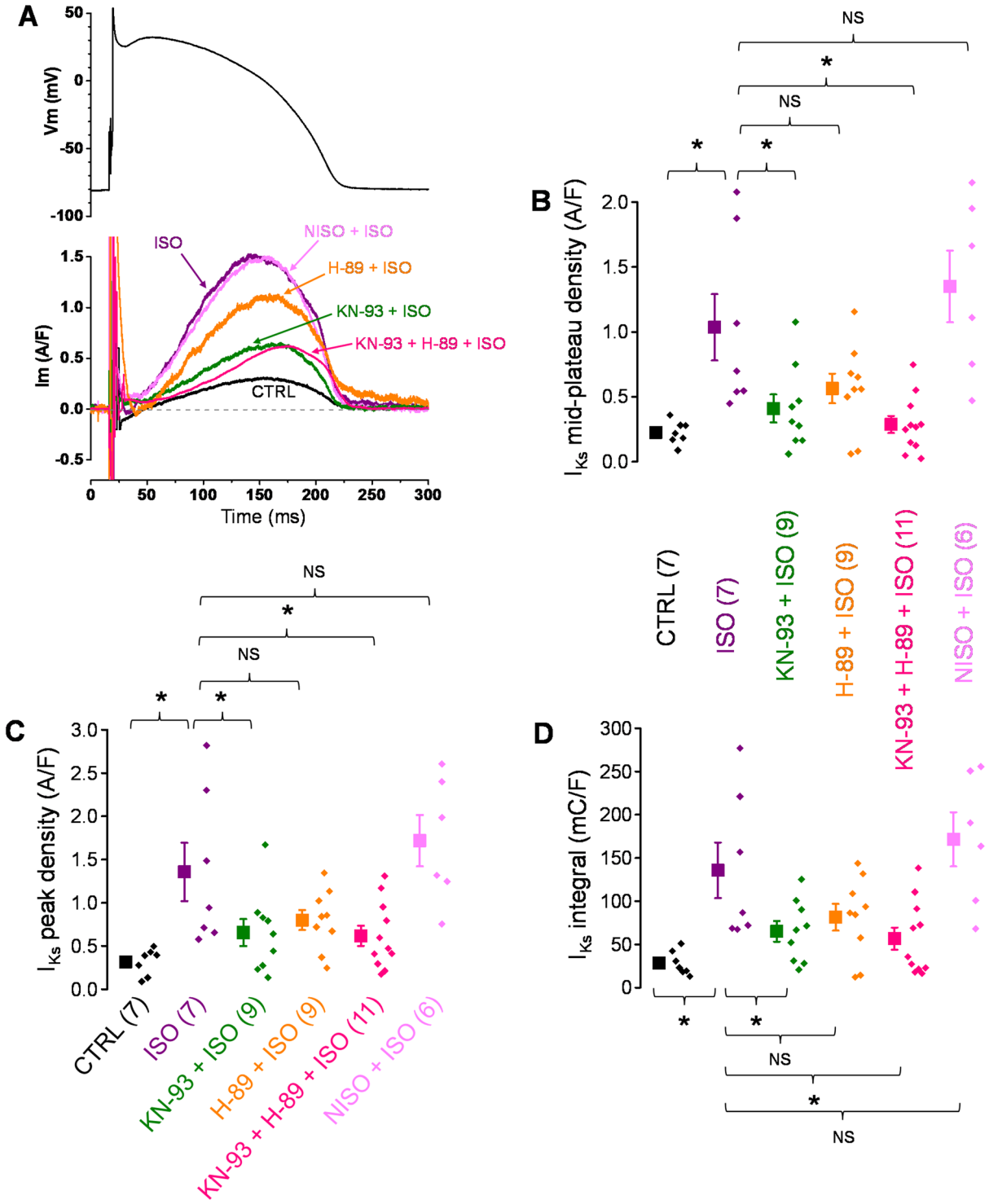

Fig. 2. Effect of pretreatment with 1 μM nisoldipine (NISO), 1 μM KN-93, 3 μM H-89 or KN-93 + H-89 on the isoproterenol (ISO) induced changes in I_{Ks}. (**A**) Command action potential (above) and representative averaged I_{Ks} current traces (below) obtained under APVC conditions in untreated controls, in the presence of 10 nM ISO, and in the further 4 groups when ISO was applied after pretreatment with 1 μM NISO, 1 μM KN-93, 3 μM H-89, or KN-93 + H-89. (**B**) Mid-plateau current densities, (**C**) peak current densities, (**D**) current integrals under the conditions on panel (**A**). Symbols and bars represent mean ± SEM, small dots represent individual data, numbers in parentheses indicate the number of cells studied. Asterisks indicate significant differences ($p < 0.05$) between groups determined using Dunnett's post-hoc test comparing data to the ISO group.

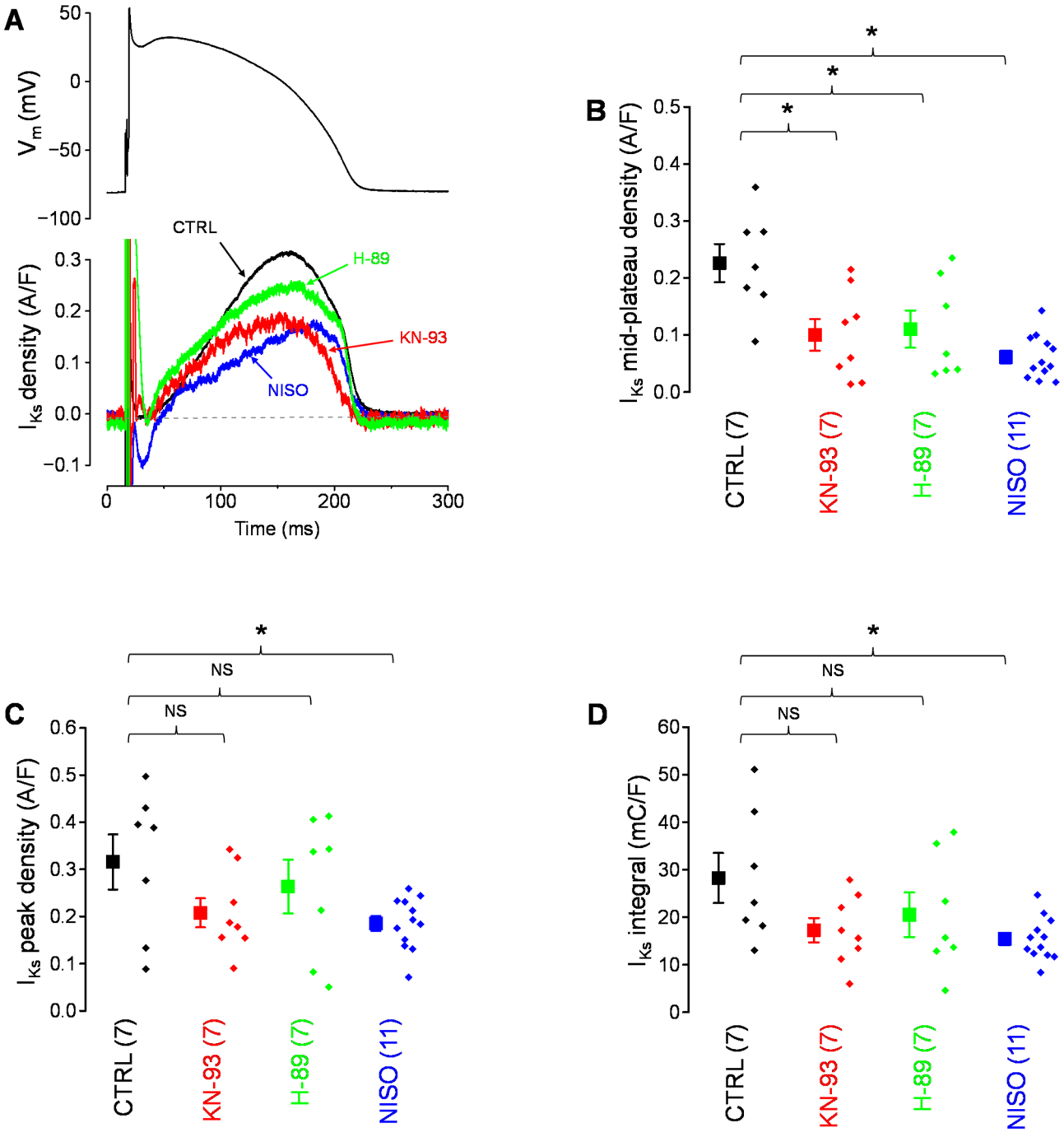

Fig. 3. Effect of 1 μM nisoldipine (NISO), 1 μM KN-93, and 3 μM H-89, on the parameters of I_{Ks} under baseline conditions. (**A**) Command action potential (above) and representative averaged I_{Ks} current traces (below) obtained under APVC conditions in untreated controls, in the presence of 1 μM NISO (blue), 1 μM KN-93, and 3 μM H-89. (**B**) Mid-plateau current densities, (**C**) peak current densities, and (**D**) current integrals under the conditions on panel (**A**). Symbols and bars are mean ± SEM, small dots represent individual data, numbers in parenthesis indicate the number of cells studied. Asterisks indicate significant differences ($p < 0.05$) between groups determined using Dunnett's post-hoc test comparing data to the control group.

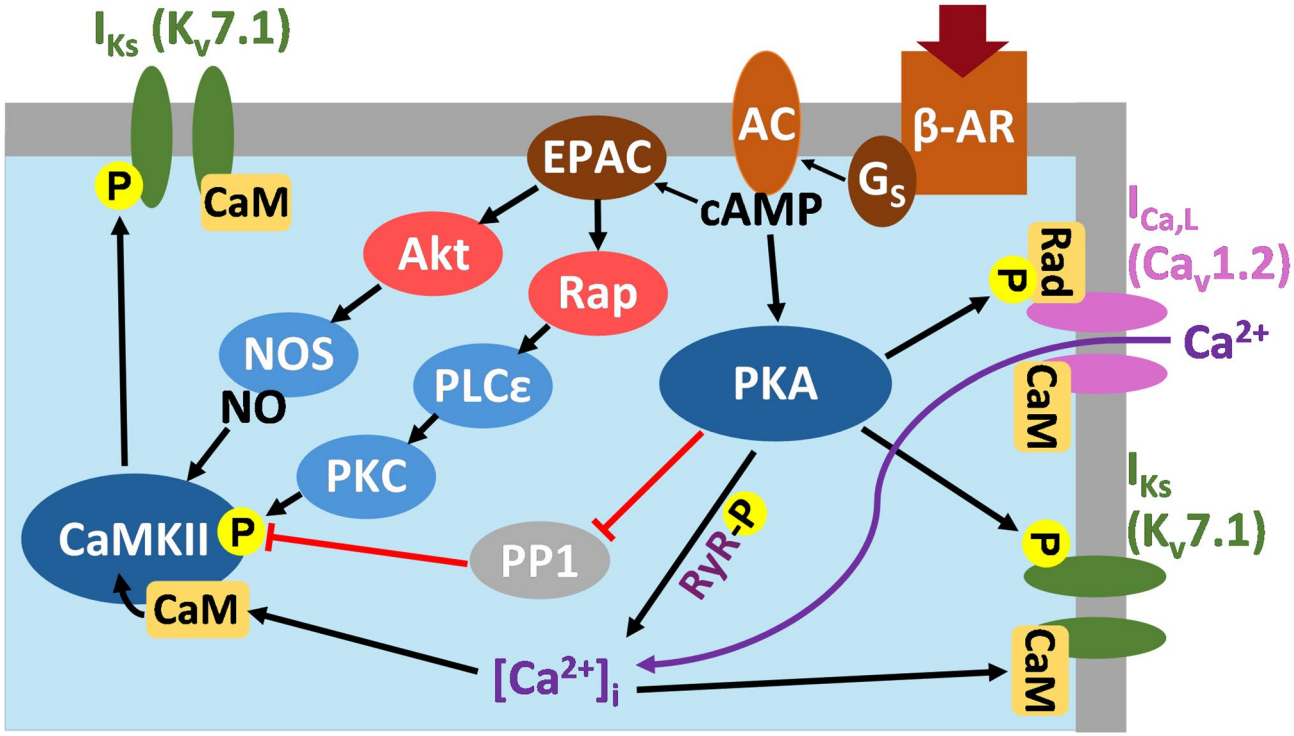

Fig. 4. Signal transduction pathways possibly involved in the regulation of I_{Ks} in canine ventricular cells. I_{Ks}: slow delayed rectifier K^+ current, K_v7.1: voltage-gated potassium channel subfamily Q member 1 (KCNQ1), ß-AR: beta-adrenergic receptor, G_s: stimulatory G protein, AC: adenylate cyclase, cAMP: cyclic adenosine monophosphate, EPAC: exchange protein activated by cAMP, Rap: Ras-proximate GTP binding protein, PCLε: phospholipase C epsilon, PKC: protein kinase C, Akt: serin/threonine kinase isolated from the AKR mouse spontaneous thymoma cells (also known as protein kinase B), NOS: nitrogen monoxide synthase, NO; nitrogen monoxide, PKA: protein kinase A, $I_{Ca,L}$: L-type Ca^{2+} current, Ca_v1.2: voltage-gated calcium channel subunit alpha1C (CACNA1C), Rad: Ras-related glycolysis inhibitor and calcium channel regulator protein, RyR: ryanodine receptor, $[Ca^{2+}]_i$: intracellular Ca^{2+} concentration, CaM: calmodulin, PP1: protein phosphatase 1. "P" in yellow circle symbolizes protein phosphorylation.

inhibitory peptide (AIP), a non-CaM-associated CaMKII inhibitor (Supplemetary Fig. 1) with and without β-adrenergic receptor activation. Data obtained with AIP reinforced our results obtained with KN-93 (see the Supplementary Results).

Discussion

Our study was designed to delineate the contributions of the PKA and CaMKII pathways in the ß-adrenergic receptor mediated modulation of I_{Ks} in canine ventricular cardiomyocytes under physiological conditions. Based on our results, four conclusions can be made. (1) In canine ventricular cardiomyocytes, CaMKII is predominantly involved in mediating the effect of ß-adrenergic receptor activation on I_{Ks}, while the contribution of PKA may be limited. (2) The potential pathways mediating the effect of ß-adrenergic receptor activation on I_{Ks} are independent of $[Ca^{2+}]_i$ elevation, as 1 μM nisoldipine did not prevent the effects of isoproterenol on the parameters of I_{Ks}. (3) Under baseline conditions (without ß-adrenergic receptor activation), both PKA and CaMKII are somewhat involved in regulating I_{Ks}. (4) The baseline regulation of I_{Ks} is highly sensitive to $[Ca^{2+}]_i$, as all parameters of I_{Ks} were significantly smaller in the presence of 1 μM nisoldipine. The possible arrangement that meets all these four conditions is discussed in Fig. 4, summarizing the potential underlying signal transduction mechanisms involved in the regulation of I_{Ks}.

Under baseline conditions—theoretically—more than one mechanism depicted in Fig. 4 may be involved in regulating I_{Ks}. The $[Ca^{2+}]_i$ sensitivity of I_{Ks} observed in our experiments, which is similar to results reported in guinea pig[14], may be the consequence of contribution of the PKA → $I_{Ca,L}$/RyR activation → $[Ca^{2+}]_i$ increase → Ca^{2+}/CaM complex → CaMKII activation pathway. In addition to this, CaM increases I_{Ks} directly by binding to the K_v7.1 (KCNQ1) channel[5,15,16]. This CaM-dependent increase in I_{Ks} relies on PKA activation, but it is independent of CaMKII[17], and it may be responsible for the $[Ca^{2+}]_i$ sensitivity of I_{Ks} under baseline conditions, explaining why I_{Ks} was smaller in the presence of nisoldipine. Here, it must also be noted that $[Ca^{2+}]_i$ in the submembrane compartment (close to the ion channels located in the sarcolemma) reaches much higher levels and undergoes more rapid changes than the bulk cytosolic $[Ca^{2+}]_i$. According to this, Saucerman and Bers[18] suggested that local submembrane CaM and CaMKII are activated at each heartbeat, whereas cytosolic CaM and CaMKII are quite insensitive to cytosolic $[Ca^{2+}]_i$. In contrast, Bartos et al.[19] concluded that micromolar $[Ca^{2+}]_i$ in the

submembrane space is not required to maximize $[Ca^{2+}]_i$-dependent I_{Ks} activation during normal Ca^{2+} transients in rabbit ventricular cells. Their assumption is based on that I_{Ks} recorded during physiological Ca^{2+} transients was similar to I_{Ks} measured with $[Ca^{2+}]_i$ clamped at 500–600 nM. Our results also indicate that under baseline conditions in paced myocytes, I_{Ks} is partially activated in a Ca^{2+}-dependent fashion.

Additionally, PKA activation may reduce protein phosphatase 1 (PP1) activity leading to an overall increased phosphorylation level of CaMKII[20,21]. The contribution of this pathway to the regulation of baseline I_{Ks} may be limited due to its $[Ca^{2+}]_i$ insensitivity, even though PKA activation by various ways was able to significantly increase canine I_{Ks} in the presence of nifedipine[4].

In contrast to baseline conditions, the ISO-induced ß-adrenergic receptor activation mediated changes in I_{Ks} (1) were not sensitive to $[Ca^{2+}]_i$; (2) were minimally affected by PKA inhibition; but (3) were markedly suppressed by CaMKII blockade. This suggests two potential mechanisms responsible for mediating the effect of ISO, both based on the cAMP-induced activation of the guanine nucleotide exchange protein activated by cAMP (EPAC). One of these is the EPAC → Akt → NOS → NO → CaMKII pathway[22,23], while the other one is the EPAC → Rap → PLCε → PKC → CaMKII pathway[24,25]. In both pathways, CaMKII activation is independent of PKA activation and the magnitude of $[Ca^{2+}]_i$. Supporting this explanation, β-adrenergic stimulation activated CaMKII in a PKA-independent manner in cultured murine cardiac cells[26].

Another possibility for the seemingly minimal effect of PKA inhibition on the ISO-induced I_{Ks} enhancement is that 3 μM H-89 might *reduce* but not *completely eliminate* the ß-adrenergic receptor mediated PKA activation. The initial report on H-89[27] determined its dissociation constant for PKA to be 0.048 nM in a cell-free in vitro assay, and H-89 is usually used at concentrations of 1–10 μM for PKA blockade[17,28,29]. Using a fluorescence resonance energy transfer-based PKA activity assay, Bobin et al.[30] found that 10 μM H-89 was necessary to effectively reduce PKA activity induced by 1 nM ISO in adult rat ventricular myocytes, whereas 1 μM H-89 had only minor, insignificant effects.

We avoided using higher H-89 concentrations to prevent potential non-specific CaMKII blockade, which could occur due to H-89 interacting with the ATP binding site of CaMKII. Indeed, H-89 has been shown to inhibit CaMKII with a dissociation constant of approximately 30 μM in cell-free in vitro assays[27]. However, in pheochromocytoma cell line lysates, 30 μM H-89 did not significantly reduce CaMKII activity[27]. It is important to note that the effect of H-89 heavily depends on substrate and ATP concentrations[31], as well as the presence of potential activators[28]. Additionally, pheochromocytoma cells primarily express the neuronal CaMKIIα isoform, while CaMKIIδ is the major cytosolic isoform found in cardiac cells, which may exhibit different affinity for H-89.

It was demonstrated that to abolish the ISO-induced positive inotropic effect in the intact rat heart, H-89 was required in 30 μM concentration[17], which is rather high. This suggests that in addition to PKA, high concentrations of H-89 might interfere with other kinases, such as CaMKII, involved in transducing β-adrenergic receptor activation to target proteins. This interference could affect processes like the phosphorylation of the ryanodine receptor and phospholamban, as seen in the experiments by Ferrero et al.[17], or activation of I_{Ks} in the present study.

It might seem to be surprising that none of the ISO-mediated effects could be prevented by pretreating the cells with 1 μM NISO. One might suppose that ISO could activate $I_{Ca,L}$ to such a great extent that it overcomes the $I_{Ca,L}$ blocking effect of NISO. This possibility can be excluded by previous results showing that even a higher ISO concentration (100 nM) was not able to increase $I_{Ca,L}$ substantially in the presence of 1 μM NISO[32] and no mechanical activity of the cardiomyocytes was observed under these experimental conditions, suggesting very low $[Ca^{2+}]_i$ levels.

In conclusion, many different signaling pathways are involved in regulating I_{Ks}. Under baseline conditions the regulation is strongly $[Ca^{2+}]_i$-dependent, with PKA and CaM-CaMKII involved, whereas during ß-adrenergic stimulation it is $[Ca^{2+}]_i$-independent and supposes a pivotal role of EPAC-mediated activation of CaMKII.

Study limitations

Our study was designed to describe the contributions of the PKA and CaMKII pathways in the acute (short-term) ß-adrenergic receptor mediated modulation of I_{Ks} in canine ventricular cardiomyocytes under physiological conditions. The limitations of the study originate from its design: (1) the highly complex nature of adrenergic signaling in the heart, (2) possible species-specific differences (3) time course of ß-adrenergic receptor activation, and (4) changes under pathological conditions.

β-adrenergic signaling is complex, involving not only PKA and CaMKII, but also many other pathways. β-adrenergic agonists can activate both β_1- and β_2-adrenergic receptors. While β_1-receptors regulate cardiac function solely through the "classical" cAMP-PKA-CaMKII pathway, β_2-receptors can be coupled to both the "classical" and the "non-classical" pathway[33]. This latter involves switching the β_2-receptor coupling from G_s to G_i and consequently activating two main signaling pathways: the phosphatidylinositol 3-kinase—Akt, and the mitogen-activated protein kinase cascade[33,34]. Exploring these pathways in their entire complexity was well beyond the aim and focus of the current study. It should also be mentioned that several other pathways beyond β-adrenergic signaling (e.g. ATP, phosphatidylinositol 4,5-bisphosphate, protein kinase G) can also regulate I_{Ks}.

While canine models are commonly used in cardiac research, and channel proteins responsible for generating I_{Ks} (both the pore-forming subunit and the regulatory subunit) are similarly distributed in canine and human myocardium[12], there can be species-specific differences in channel proteins and in signaling pathways between human and canine hearts. Therefore, results from canine myocytes may not fully translate to human physiology. Ideally, similar studies in human-derived cells (or in humanized models) shall be conducted to confirm whether the observed mechanisms of the present study also exist in human cardiomyocytes.

Chronic (long-term) effects of sustained β-adrenergic receptor activation (such as in chronic stress, heart failure or under other chronic cardiovascular conditions) may differ. Chronic β-adrenergic stimulation can lead

to desensitization of receptors and substantial changes in intracellular signaling pathways. Further studies are needed to explore the effects of chronic β-adrenergic activation, and to investigate different cardiac disease conditions (e.g. heart failure), as the adaptations observed under these conditions could reveal additional mechanisms in I_{Ks} regulation or potential changes in the role of PKA and CaMKII. Here are a few examples of how long-term β-receptor activation may affect I_{Ks}. In cultured adult guinea pig cardiomyocytes, Zhang et al.[35] found that sustained β-adrenergic activation by isoproterenol for 48 h reduced I_{Ks}, contrary to acute isoproterenol exposure. This can be explained either by the desensitization of the adrenergic receptors, or by the downregulation of I_{Ks} channel protein expression. Shrugg et al.[36] found that in cultured HEK-293B cells 12–24 h of sustained β-adrenergic receptor stimulation also reduced I_{Ks}, which was prevented by CaMKII inhibition. Similarly, CaMKIIδ overexpression reduced I_{Ks}. This effect did not occur in S484A KCNQ1 mutant channels, which cannot be phosphorylated by CaMKII. In a canine model of cardiac hypertrophy, Stengl et al.[37] found a reduction of I_{Ks}, together with a blunted β-adrenergic response in left ventricular cells 30 days after the induction of hypertrophy by ablation of the AV node.

Methods

The present study is reported in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Animals

Adult mongrel dogs of either sex, bred for research purposes were purchased from a local breeder. The animals were 12–18 months old, weighing 8–14 kg at the time of sacrifice. Experimental dogs were anesthetized with intramuscular injections of 10 mg/kg ketamine hydrochloride (Calypsol, Richter Gedeon, Hungary) + 1 mg/kg xylazine hydrochloride (Sedaxylan, Eurovet Animal Health BV, The Netherlands) according to the protocol approved by the Institutional Animal Care Committee of the University of Debrecen (license No: 2/2020/DEMÁB, 9/2015/DEMÁB). All animal procedures conformed to the guidelines from Directive 2010/63/EU of the European Parliament.

Isolation of cardiomyocytes

Single canine myocytes were obtained by enzymatic dispersion using the segment perfusion technique, as described previously[38]. A wedge-shaped section of the ventricular wall, supplied by the LAD coronary artery, was cannulated and perfused with a nominally Ca^{2+}-free Joklik solution (Minimum Essential Medium Eagle, Joklik Modification). After a 5 min period required for washing out the blood, this solution was supplemented with collagenase (Type II, 1 mg/ml, final activity of 224 U/ml, Worthington Biochemical Co., Lakewood, NJ, USA) and 0.2% bovine serum albumin (Fraction V., Sigma) containing 50 μM Ca^{2+}; this perfusion lasted for 30 min typically. Finally, the tissue was minced to small chunks, the cells were released by gentle agitation and the normal extracellular Ca^{2+} concentration was restored. Before use the cells were stored in refrigerator at 15 °C in Minimum Essential Medium Eagle. This procedure yielded dominantly midmyocardial myocytes showing clear cross-striations.

Electrophysiology

Cells were perfused in a lucid chamber under an inverted microscope with a modified Tyrode solution at a rate of 1–2 ml/min. This solution contained (in mM): NaCl 121, KCl 4, $CaCl_2$ 1.3, $MgCl_2$ 1, HEPES 10, $NaHCO_3$ 25, and glucose 10; at pH = 7.35, with an osmolarity of 300 ± 3 mOsm. The bath temperature was set to 37 °C using a temperature controller (Cell MicroControls, Norfolk, VA, USA). Electrical signals were amplified and recorded (MultiClamp 700B or 700A, Molecular Devices, Sunnyvale, CA, USA) under the control of a pClamp 10 software (Molecular Devices) following analogue–digital conversion (Digidata 1440A or 1332, Molecular Devices). Electrodes having tip resistances of 2–3 MΩ when filled with pipette solution were fabricated from borosilicate glass. The pipette solution contained (in mM) K-aspartate 120, KCl 30, KOH 10, MgATP 3, HEPES 10, Na_2-phosphocreatine 3, EGTA 0.01, and cAMP 0.002; at pH = 7.3, osmolality = 285 ± 2 mOsm. The series resistance ranged typically between 4 and 8 MΩ. The experiment was discarded in all cases when the series resistance above 12 MΩ or increased by more than 50% during the experiment. For further details see references[12,39].

Experiments were performed using the APVC technique as previously described[40,41]. Cells were paced at a constant cycle length of 0.7 s, corresponding to the normal heart rate of dogs. To diminish the consequences of cell-to-cell variations in AP duration, "canonic" APs were applied as command signals, instead of using the own AP of the cell. The canonic AP was chosen to represent a midmyocardial canine AP with average parameters. This approach allowed the comparison of the individual current traces. I_{Ks} was defined as a 0.5 μM HMR-1556 sensitive current. In order to reduce the noise and trace-to-trace fluctuations, 20 consecutive current traces were recorded and averaged before and after the 5 min application of HMR-1556. I_{Ks} was obtained by subtracting the average post-drug current trace from the average pre-drug one. The trace subtraction and averaging were carried out by a different person than the experiment, therefore investigators were blinded to the applied chemicals during the analysis of raw data.

I_{Ks} was characterized by maximal current density (peak current), its density measured at the half-duration of the command AP (mid-plateau current), and the total charge carried by the current (integral). All parameters were normalized to cell capacitance, determined using hyperpolarizing pulses from + 10 to − 10 mV lasting for 15 ms.

Chemicals

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except for HMR-1556, KN-93 and H-89 which were purchased from Tocris Bioscience (Bristol, UK). HMR-1556 was used in the bath solution, whereas both kinase inhibitors (1 µM KN-93 and 3 µM H-89) were applied in the pipette solution.

ISO stock solution of 10 µM was freshly created right before each experiment, and this was diluted to 10 nM final concentration in the bath solution. This relatively low concentration of isoproterenol causes a significant but not saturating increase in I_{Ks} [4]. Similarly, Prabhakar et al.[42] found that in mouse hearts, 10 nM ISO caused a significant, but not saturating increase in both + dP/dt and – dP/dt. Recently, Saha et al.[43] found that in HEK-293B cells, the EC50 value of ISO on cAMP production increase was 7.7 nM.

Statistics

The sample size calculation was done with GPower 3.1 and was based on previous studies on the effect of ISO on I_{Ks} [4]. The used parameters resulted in a calculated sample size of 7 for each group (one-way ANOVA; 6 groups; effect size = 0.64; $\alpha = 0.05$; desired power = 0.8). Actual sample sizes for the different groups varied between 7 and 11. For the NISO + ISO group, one cell was excluded from data analysis after the subtraction (therefore n = 6 in this group), because it showed a partially inward I_{Ks} current trace, which is impossibl

Results are expressed as mean ± SEM values, n denotes the number of cells studied. Experimental groups were statistically compared with one-way ANOVA, followed by Dunnett's post-hoc test. In the experiments discussed in Figs. 1 and 3, the untreated control group was used as a reference, while in the "ISO-activated" comparison (Fig. 2), each group was compared to the 10 nM ISO treated group in the Dunnett's post-hoc test. Differences were considered statistically significant when $p < 0.05$.

Data availability

Data is available upon reasonable request. Inquiries regarding research data should be directed to the corresponding author.

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Conceptualization: T.B., P.P.N., B.H.; Cell isolation: C.D., J.O.; Recording of action potentials: Z.K., C.D., J.O.; Voltage clamp recordings: Z.K., C.D., J.O.; Analysis of data: B.H., J.M., N.S.; Writing-original draft preparation: P.P.N, B.H., Z.K; Writing-review and editing, B.H., T.B., P.P.N.; Visualization: B.H., Z.K.; Funding acquisition, P.P.N., N.S. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Institutional review board statement
The study was conducted according to the Directive 2010/63/EU of the European Parliament and approved by the Institutional Animal Care Committee of University of Debrecen (license No: 2/2020/DEMÁB and 9/2015/DEMÁB).

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