BJP British Journal of Pharmacology
BPS BRITISH PHARMACOLOGICAL SOCIETY

Effects of ß-adrenoceptor stimulation on delayed rectifier K+ currents in canine ventricular cardiomyocytes

Journal:	*British Journal of Pharmacology*
Manuscript ID:	2009-BJP-1701-RP.R2
Manuscript Type:	Research Paper
Date Submitted by the Author:	08-Sep-2010
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Publication category:	Cardiovascular pharmacology
Keywords:	Cardiovascular pharmacology < A. General fields, B. Mediators, Receptors, Transporters, Channels & Ligands, K-channels < B. Mediators, Receptors, Transporters, Channels & Ligands, Adrenaline/noradrenaline < B. Mediators, Receptors, Transporters, Channels & Ligands, Control of cardiac function < D. Functional systems, Molecular pharmacology < A. General fields, Antiarrhythmic drugs < C. Drug classes

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September 8, 2010

Dear Professor Ahluwalia,

Please, find enclosed the revised version of our original report entitled "Effects of ß-adrenoceptor stimulation on delayed rectifier K^+ currents in canine ventricular cardiomyocytes" (MS#: 2009BJP-1701-RP.R1). Your and the referees' suggestions were strictly followed and new experiments were performed according to these comments. Changes made in the manuscript are indicated with red colour, detailed responses to the reviewers are enclosed below. We hope that the revised version of our manuscript will meet your and the referee's satisfaction and the manuscript will be considered suitable for publication in British Journal of Pharmacology.

Best regards,

Peter P. Nanasi
Professor of Physiology

Response to the Editor

We have made the following major changes in the manuscript in accordance with the comments of the Editor and Reviewer 2:

1. The effect of ISO was tested in the presence of the selective beta-1 blocker CGP-20712A (300 nM), which has fully prevented the effect of ISO on I_{Kr} - similarly to results obtained with 100 nM metoprolol. This information has been included to the Results section and presented also in Fig. 2.C.
2. All PKC data (including text and figures) have been removed from the revised manuscript.
3. We followed the BJP's nomenclature for drugs, receptors and ion channels (according to BJP's Guide to Receptors and Channels). This statement, (referring to the relevant citation from Alexander et al.) has been appended to the end of the Methods section. According to this, "beta-adrenergic" was replaced with "beta-adrenoceptor" and "isoproterenol" with isoprenaline.

We thank the kind help of the Editor, since we believe that her/his suggestions resulted in a substantial improvement of the manuscript.

Response to Reviewer 2

1. The effect of ISO was tested in the presence of the selective beta-1 blocker CGP-20712A (300 nM), which has fully prevented the effect of ISO on I_{Kr} - similarly to results obtained with 100 nM metoprolol. This information has been included to the Results section and presented also in Fig. 2.C.
2. Reviewer 2 has convinced us that there were uncertainties regarding the interpretation of our data with the PKC blockers chelerythrine and GF-109203X. Indeed, - in absence of data obtained from expressed HERG channels - we cannot exclude direct effects of these agents on I_{Kr}. Therefore – in accordance with the Editor's suggestion – all PKC data have been removed from the manuscript.
3. We are indebted to Reviewer 2 for the idea that helped to explain the differences observed between the effects of ISO on I_{Kr} and I_{Ks}. We have accepted this argumentation and included it in the Discussion section.

We thank the kind help of the Reviewer, since we believe that his/her suggestions resulted in a substantial improvement of the manuscript.

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Effects of ß-adrenoceptor stimulation on delayed rectifier K^+ currents in canine ventricular cardiomyocytes

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Running title: ß-adrenoceptor stimulation and cardiac K^+ currents

Summary

Background and purpose. While the slow delayed rectifier K^+ current (I_{Ks}) is known to be enhanced by stimulation of ß-adrenoceptors in several mammalian species, phosphorylation-dependent regulation of the rapid delayed rectifier K^+ current (I_{Kr}) is controversial.

Experimental approach. In the present study, therefore, the effect of isoprenaline (ISO), activators and inhibitors of the PKA pathway on I_{Kr} and I_{Ks} were studied in canine ventricular myocytes using the whole cell patch clamp technique.

Key results. I_{Kr} was significantly increased (by 30-50 %) following superfusion with ISO, forskolin or intracellular application of PKA activator cAMP analogues (cAMP, 8-Br-cAMP, 6-Bnz-cAMP). Inhibition of PKA by Rp-8-Br-cAMP had no effect on baseline I_{Kr}. The stimulating effect of ISO on I_{Kr} was completely inhibited by selective $ß_1$-adrenoceptor antagonists (metoprolol and CGP-20712A), by the PKA inhibitor Rp-8-Br-cAMP, and by the PKA activator cAMP analogues, but not by the EPAC activator 8-pCPT-2'-O-Me-cAMP. In comparison, I_{Ks} was increased threefold by activation of PKA (by ISO or 8-Br-cAMP), and strongly reduced by the PKA inhibitor Rp-8-Br-cAMP. The ISO-induced enhancement of I_{Ks} was decreased by Rp-8-Br-cAMP and completely inhibited by 8-Br-cAMP.

Conclusions and implications. The results indicate that stimulation of $ß_1$-adrenoceptors increases I_{Kr}, similar to I_{Ks}, via activation of PKA in canine ventricular cells.

Keywords: ß-adrenoceptors, isoprenaline, cAMP, PKA, delayed rectifier K^+ current, I_{Kr}, I_{Ks}, dog myocytes.

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Abbreviations: I_{Kr}, rapid delayed rectifier K^+ current; I_{Ks}, slow delayed rectifier K^+ current; ISO, isoprenaline; PKA, protein kinse A; PKC, protein kinase C; EPAC, exchange protein directly activated by cAMP.

Introduction

Delayed rectifier K^+ currents play pivotal role in repolarization of the cardiac action potential. In the ventricular myocardium of most mammalian species, including dog and human, the delayed rectifier K^+ current is composed of two independent components, a rapid delayed rectifier K^+ current (I_{Kr}) and a slow delayed rectifier K^+ current (I_{Ks}) (Gintant, 1996; Li et al., 1996). Both components are controlled by catecholamines, and are important targets for antiarrhythmic drug action. While I_{Ks} is known to be enhanced by stimulation of ß-adrenoceptors, phosphorylation-dependent regulation of I_{Kr} is controversial (Thomas et al., 2004). PKA-mediated phosphorylation of expressed HERG channels significantly decreased I_{Kr} tail currents (Thomas et al., 1999; Wei et al., 2002). Similarly, stimulation of ß-adrenoceptors by 10 µM isoprenaline (ISO) decreased I_{Kr} in voltage-clamped guinea-pig ventricular myocytes (Karle et al., 2002). In contrast to these results, Heath and Terrar (2000) found that I_{Kr} was enhanced by ISO in guinea-pig ventricular cells provided the conditions necessary to activate the PKC pathway were met, i.e. Ca^{2+} current was not blocked, cytosolic Ca^{2+} was not buffered, and the cell interior was not dialyzed. In the absence of relevant human data we decided to study the effects of ß-adrenoceptor stimulation on delayed rectifier K^+ currents in ventricular cardiomyocytes of the dog, a species that has action potential characteristics and properties of the underlying transmembrane ion currents most resembling those in

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humans (Szentandrássy et al., 2005; Szabó et al., 2005). It was found that ISO increased I_{Kr} significantly in canine ventricular myocytes by activation of PKA and this effect is mediated by stimulation of ß$_1$-adrenoceptors.

Methods

Single canine ventricular myocytes were obtained from hearts of adult mongrel dogs using the segment perfusion technique (Magyar et al., 2000). The animals (10-20 kg) were anaesthetized with ketamine hydrochloride (Calypsol) 5 mg kg^{-1} i.v. plus xylazine (CP-Xylazine) 0.04 mg kg^{-1}. After the chest had been opened, the heart was rapidly removed and the left anterior descending coronary artery was perfused using a Langendorff apparatus. Ca^{2+}-free JMM solution (Joklik modification of Eagle's Minimum Essential Medium, Sigma), supplemented with taurine (2.5 g l^{-1}), pyruvic acid (175 mg l^{-1}), ribose (750 mg l^{-1}), allopurinol (13.5 mg l^{-1}) and NaH_2PO_4 (200 mg l^{-1}), was used during the initial 5 min of perfusion to remove Ca^{2+} and blood from the tissue. After addition of $NaHCO_3$ (1.3 g l^{-1}) the pH of this perfusate was adjusted to 6.9 by equilibrating the solution with a mixture of 95% O_2 and 5% CO_2. Cell dispersion was performed for 30 min in the same solution containing, in addition, collagenase (660 mg l^{-1}, Worthington CLS-II), bovine serum albumin (2 g l^{-1}) and $CaCl_2$ (50 µM). During the isolation procedure the solutions were gassed with carbogen and the temperature was maintained at 37 ^{o}C. The cells were rod shaped and showed clear striation when the external calcium was restored.

I_{Kr} and I_{Ks} were recorded at 37^{o}C from Ca^{2+}-tolerant canine ventricular cells superfused with oxygenated Tyrode solution containing (in mM) NaCl 140, KCl 5.4,

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$CaCl_2$ 2.5, $MgCl_2$ 1.2, HEPES 5, glucose 10, at pH 7.4. This superfusate was supplemented with 5 µM nifedipine plus 1 µM E-4031 when measuring I_{Ks}, or 5 µM nifedipine plus 1 µM HMR-1556 when recording I_{Kr}, in order to eliminate L-type Ca^{2+} currents, I_{Kr}, or I_{Ks}, respectively. Suction pipettes, fabricated from borosilicate glass, had tip resistances of 1.5-2 MΩ after being filled with pipette solution composed of (in mM) K-aspartate 100, KCl 45, $MgCl_2$ 1, HEPES 5, EGTA 10, K-ATP 3. The pH of this solution was adjusted to 7.2 with KOH. Membrane currents were recorded with an Axopatch-200B amplifier (Axon Instruments) using the whole cell configuration of the patch clamp technique. After a high (1-10 GΩ) resistance seal had been established by gentle suction, the cell membrane beneath the tip of the electrode was disrupted by further suction or by applying 1.5 V electrical pulses for 1 ms. Ion currents were normalized to cell capacitance, determined in each cell using short hyperpolarizing pulses from -10 mV to -20 mV. The series resistance was typically 4-8 MΩ before compensation (usually 50-80%) prior to the measurement. Experiments were discarded when the amplitude of I_{Kr} or I_{Ks} was unstable within the initial 5 min of the experiment, or the series resistance was high or increased during the measurement. Outputs from the clamp amplifier were digitized at 20 kHz using an A/D converter (Digidata-1200, Axon Instruments) under software control (pClamp 6.0, Axon Instruments).

All values presented are arithmetic means ± SEM. Statistical significance of differences was evaluated by using one-way ANOVA followed by Student's *t* test for paired or unpaired data, as appropriate. Differences were considered significant when the *P* value was less than 0.05.

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The principles of laboratory animal care (NIH publication No. 85-23, revised 1985) and current version of the Hungarian Law on the Protection of Animals were strictly followed throughout the experiments. Drug and molecular target nomenclature conforms to Guide to Receptors and Channels (Alexander et al., 2009).

Results

Effect of ISO on I_{Kr}

I_{Kr} was activated by 250 ms depolarizing pulses to +10 mV applied at a rate of 0.05 Hz. I_{Kr} was characterized as tail current amplitudes determined as the difference between the peak current and the pedestal value observed following repolarization to the holding potential of -40 mV in the presence of 5 µM nifedipine plus 1 µM HMR-1556. Exposure of myocytes to ISO for 3-4 min increased I_{Kr} tail amplitude in a readily reversible manner (Figure 1a-c). The effect of ISO developed rapidly, the maximal effect was typically achieved within 2-3 min. These current tails (including the baseline amplitude as well as the ISO-induced component) were fully eliminated by 1 µM E-4031 (the I_{Kr} tail amplitude decreased to 0.01±0.002 pA/pF in the presence of E-4031, n=5), therefore the current can be considered to be purely I_{Kr} mediated by HERG channels (Figure 1d). The ISO-induced enhancement of I_{Kr} was caused by a leftward shift in the voltage-dependence of activation of I_{Kr} (the midpoint potential was shifted from +5.6±0.9 mV to -4.6±1.2 mV), while no significant change was found in the slope factor (6.7±0.5 versus 7.4±0.5 mV^{-1}) in the 4 myocytes studied (Figure 1.e).

The stimulating effect of ISO on I_{Kr} was concentration-dependent, 1 µM ISO increased I_{Kr} by 37±3 % (Figure 2a). By fitting these results to the Hill equation an EC_{50} value of 13.6±2.5 µM and a Hill coefficient of close to unity were obtained. Since 100

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nM ISO resulted in a nearly maximal activation of I_{Kr}, this concentration was used in the following experiments. The effect of ISO was fully prevented by pretreatment with either 100 nM metoprolol or 300 nM CGP-20712A - both are known to be selective inhibitors of ß$_1$-adrenoceptors at these concentrations (Figure 2b and c).

The signal transduction pathway mediating the ISO-induced stimulation of I_{Kr} was investigated using specific PKA activators and inhibitors. The effect of ISO was mimicked (i.e. I_{Kr} was increased in a similar extent) by various types of PKA activators, including 3 µM forskolin, 250 µM intracellular cAMP or 8-Br-cAMP (Figure 3). Similar results were obtained with 100 µM 6-bnz-cAMP (selective PKA activator with no effect on EPAC), while the same concentration of 8-pCPT-2'-O-Me-cAMP, a cAMP analogue known to activate EPAC without altering the activity of PKA (Holz et al., 2008), failed to enhance I_{Kr}. Intracellular application of 100 µM Rp-8-Br-cAMP, a cAMP analogue that is a selective PKA inhibitor, had no effect on baseline I_{Kr}.

The effect of ISO on I_{Kr} was fully prevented by pretreatment with some PKA activators (forskolin, 8-Br-cAMP, and 6-bnz-cAMP), and by the PKA inhibitor (Rp-8-Br-cAMP). It was only partially eliminated in the presence of cAMP, while the EPAC activator 8-pCPT-2'-O-Me-cAMP had no effect (Figure 3). These results indicate that the ISO-induced enhancement of I_{Kr} is critically dependent on activation of PKA.

Effect of ISO on I_{Ks}

I_{Ks} was activated by 3 s long depolarizing pulses to +30 mV delivered at a rate of 0.1 Hz from the holding potential of -40 mV. Tail currents, obtained after repolarization in the presence of 5 µM nifedipine plus 1 µM E-4031, were used to characterize I_{Ks}. Exposure

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of myocytes to 10 and 100 nM ISO increased I_{Ks} tail amplitude to 194±28 and 293±41 %, of the control, respectively, in a largely reversible manner (Figure 4). The current was fully eliminated by 1 µM HMR-1556, indicating that it was purely I_{Ks}.

Baseline I_{Ks} was significantly reduced by selective inhibition of PKA using 100 µM intracellular Rp-8-Br-cAMP (1.63±0.22 pA/pF in control, n=13 versus 0.47±0.06 pA/pF in the presence of Rp-8-Br-cAMP, n=5). On the other hand, full activation of PKA by loading the pipette with 250 µM of the non-hydrolyzable cAMP derivative 8-Br-cAMP, increased I_{Ks} to 4.53±0.63 pA/pF (n=5), which was three times higher than its control value (Figure 5). The effect of ISO on I_{Ks} was reduced in the presence of Rp-8-Br-cAMP, and ISO failed to enhance I_{Ks} any more when PKA was fully activated by 8-Br-cAMP.

Discussion and conclusions

In the present study the effects of the ß-adrenoceptor agonist ISO on the two components of the delayed rectifier K^+ current, I_{Kr} and I_{Ks}, were studied and compared in canine myocytes. This is the first time an enhancement of I_{Kr} by ISO has been demonstrated in canine ventricular cells, which may be an important mechanism of defense against lengthening of action potentials in the case of ß-adrenoceptor stimulation. This ISO-induced enhancement of I_{Kr} seems to be mediated by activation of PKA, since the effect of ISO was eliminated after either inhibition or full activation of PKA. It must be noted, however, that cAMP – in contrast to 8-Br-cAMP and 6-bnz-cAMP – failed to fully prevent the action of ISO. This may be explained by the proper compartmentalization of the PKA-channel complex, suggesting that the submembrane phosphodiesterase barrier

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may limit the accessibility of PKA from the intracellular side (Jurevicius and Fischmeister, 1996; Fischmeister et al., 2006). Thus cAMP – but not 8-Br-cAMP – might partially be degraded locally by phosphodiesterase.

In strong support of the PKA-dependent enhancement of I_{Kr} is the finding that when the current was observed in the presence of cAMP analogues it was markedly elevated resulting in permanent activation of the enzyme. However, in contrast to our results, I_{Kr} was shown to be reduced following activation of PKA in oocytes expressing HERG channels (Thomas et al., 1999; Wei et al., 2002). The reason for this discrepancy is not clear; it may be due to the lack of other important members of the underlying signal transduction pathway in the oocytes, but it may reflect interspecies differences as well.

Similar to our results, Heath and Terrar (2000) found that I_{Kr} was enhanced by 10 µM ISO in guinea-pig ventricular cells if the conditions required to activate the conventional PKC isoenzymes were met, i.e. Ca^{2+} current was not blocked, cytosolic Ca^{2+} was not buffered, and the cell interior was not dialyzed. They concluded that this stimulating effect was mediated via activation of the PKC pathway, involving crosstalk between PKA and PKC. However, activation of the conventional PKC isoforms with thymelatoxin was shown to decrease I_{Kr} in oocytes (Thomas et al., 2003). Furthermore, our experimental conditions did not favour the activation of conventional PKC isoenzymes, since Ca^{2+} current was blocked by 5 µM nifedipine, the cytosolic Ca^{2+} was strongly buffered by 10 mM EGTA, and the cell interior was dialyzed.

Similar to I_{Kr}, I_{Ks} was also equally enhanced by exposure to ISO and intracellular application of 8-Br-cAMP; however, marked differences were observed between I_{Kr} and I_{Ks} in response to ISO after inhibition of PKA. Rp-8-Br-cAMP strongly compromised

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baseline I_{Ks}, but failed to modify baseline I_{Kr} at all. This may indicate a more marked contribution of the cAMP/PKA pathway to the basal activity of I_{Ks} compared to that of I_{Kr}. On the other hand, pretreatment with Rp-8-Br-cAMP fully prevented the effect of ISO on I_{Kr} but only decreased it on I_{Ks}. Thus it appears that a moderate suppression of the cAMP/PKA pathway is sufficient to blunt the effect of ISO on I_{Kr}, which may be the consequence of a less effective stimulus transduction targeting the HERG channel. However, it is also possible that different PKA isoenzymes with different sensitivities to inhibitors are involved in mediating the effects of ß-adrenoceptor stimulation to I_{Kr} and I_{Ks}. Hence, further studies are required to elucidate the differences between the fine tuning of ß-adrenoceptor stimulation of I_{Kr} and I_{Ks}.

In summary, I_{Kr}, similar to I_{Ks}, is enhanced by ISO in canine ventricular myocytes via activation of the cAMP/PKA system. Due to the particular importance of ß-adrenoceptor stimulation in controlling cardiac repolarization and the susceptibility to arrhythmias, the detailed mechanism of regulation may be a promising subject of further studies.

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Financial support for the studies was provided by grants from the Hungarian Research Fund (OTKA-K68457, OTKA-K73160, CNK-77855), the Hungarian Ministry of Health (ETT-060/2006), and the Medical and Health Science Center of University of Debrecen, (MEC-14/2008). The authors thank Mrs Vighné Katalin Horváth for excellent technical assistance.

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Figure legends

Figure 1. Effects of isoprenaline (ISO) on I_{Kr} current. (a-c) I_{Kr} current traces showing tail currents as relaxation of current during repolarization to -40 mV under control conditions (a), following exposure to 100 nM ISO (b), and after washing the cells in ISO-free solution (c). (d) Representative experiment demonstrating the effect of 100 nM ISO on I_{Kr} and the full suppression of the ISO-induced current by 1 µM E-4041. (e)Voltage-dependence of activation of I_{Kr} in control and in the presence of 100 nM ISO. Tail current amplitudes measured at each test potential was normalized to the respective tail current obtained at +20 mV. Symbols and bars are means ± SEM, n=4.

Figure 2. (a) Cumulative concentration-dependent effect of isoprenaline (ISO) on I_{Kr} tail current amplitude studied in 8 myocytes. (b, c) Effects of 100 nM ISO in the presence of 100 nM metoprolol (n=6) and 300 nM CGP-20712A (n=4). I_{Kr} amplitudes were normalized to their respective control values. Columns and bars indicate means ± SEM. *Denote significant (P<0.05) differences from control (100 %) determined using paired t-test.

Figure 3. Role of the cAMP/PKA pathway in regulation of I_{Kr}. Lefthand column of each pair shows I_{Kr} tail current amplitudes measured in control (no drug, n=13), following superfusion with forskolin (3 µM, n=6), and after internal application of cAMP (250 µM, n=9), 8-Br-cAMP (250 µM, n=8), 6-bnz-cAMP (100 µM, n=8), 8-pCPT-2'-O-Me-cAMP (100 µM, n=6) and Rp-8-Br-cAMP (100 µM, n=6). Righthand column of each pair

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indicates I_{Kr} tails obtained following superfusion with 100 nM ISO. Data are means ± SEM, *Indicate significant (P<0.05) differences from the control (no drug) I_{Kr} amplitude, determined using unpaired t-test, $^{+}$Denote ISO-induced differences from the respective pre-ISO values, determined using paired t-test.

Figure 4 Effects of isoprenaline (ISO) on I_{Ks} current. (a-c) I_{Ks} tail current traces obtained at repolarization to -40 mV under control conditions (a), following exposure to 100 nM ISO (b), and after washing the cells in ISO-free solution (c). (d)Representative experiment demonstrating the effect of 100 nM ISO on I_{Ks} and the full suppression of the ISO-induced current by 1 µM HMR-1556. (e) Average data obtained in 11 myocytes showing the cumulative effect of 10 nM and 100 nM ISO on I_{Ks} tail current amplitude. Columns and bars are means ± SEM values, *Indicate significant (P<0.05) differences from control determined using paired t-test.

Figure 5 Role of the cAMP/PKA pathway in regulation of I_{Ks}. In (c), lefthand column of each pair shows I_{Ks} tail current amplitude measured in control (no drug, n=11), and after internal application of Rp-8-Br-cAMP (100 µM, n=5) and 8-Br-cAMP (250 µM, n=5). Righthand column of each pair indicates I_{Ks} tails obtained following superfusion with 100 nM ISO. Data are means ± SEM, *Indicate significant (P<0.05) differences from the control (no drug) I_{Ks} amplitude determined using unpaired t-test, $^{+}$Denote ISO-induced differences from the respective pre-ISO values determined using paired t-test.

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Statements of conflicts of interests

None

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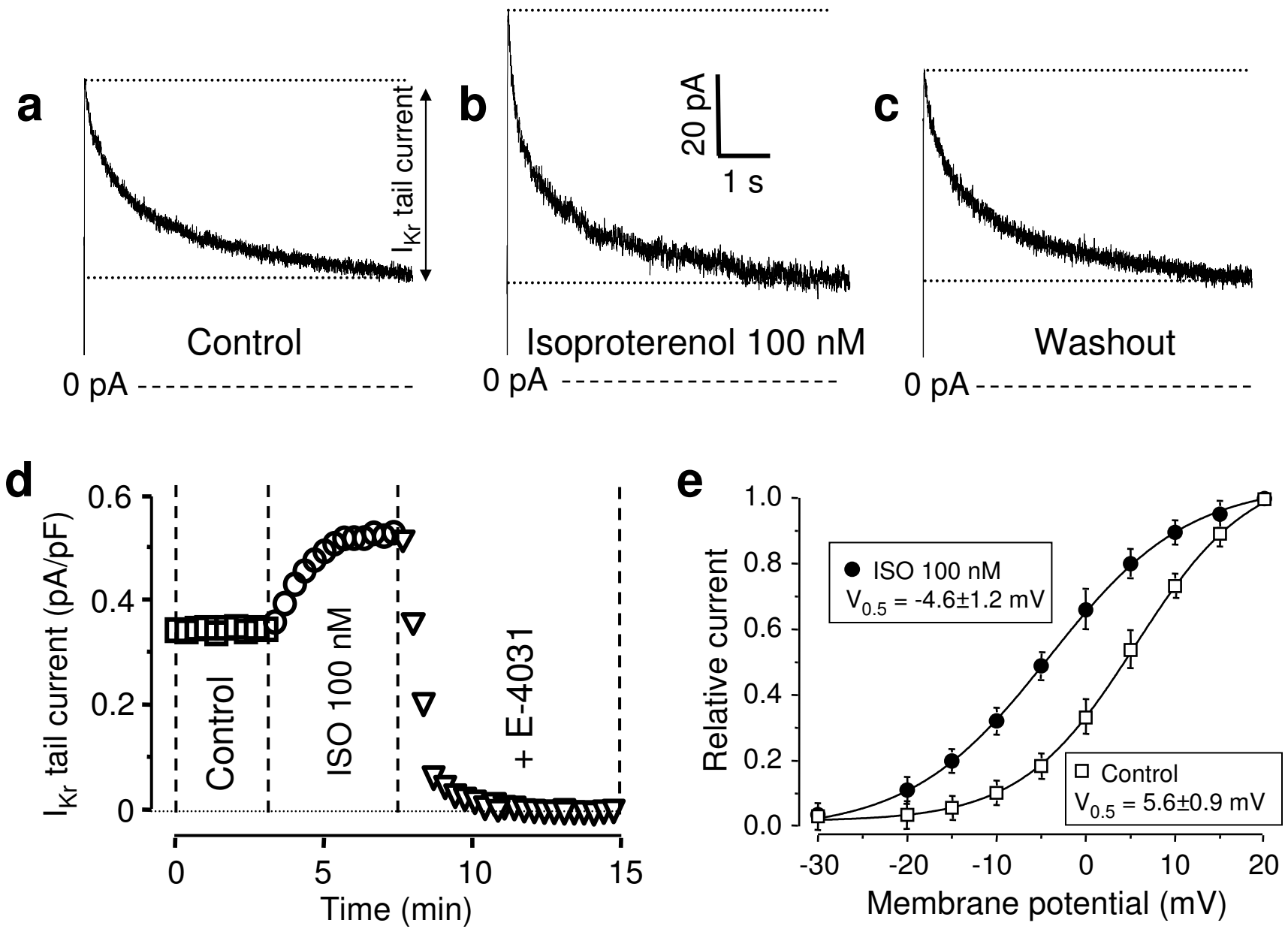
a
b
c
I_{Kr} tail current
20 pA
1 s
Control
Isoproterenol 100 nM
Washout
0 pA
0 pA
0 pA
d
e
I_{Kr} tail current (pA/pF)
Control
ISO 100 nM
+ E-4031
Time (min)
Relative current
ISO 100 nM
$V_{0.5}$ = -4.6±1.2 mV
Control
$V_{0.5}$ = 5.6±0.9 mV
Membrane potential (mV)

Fig.1

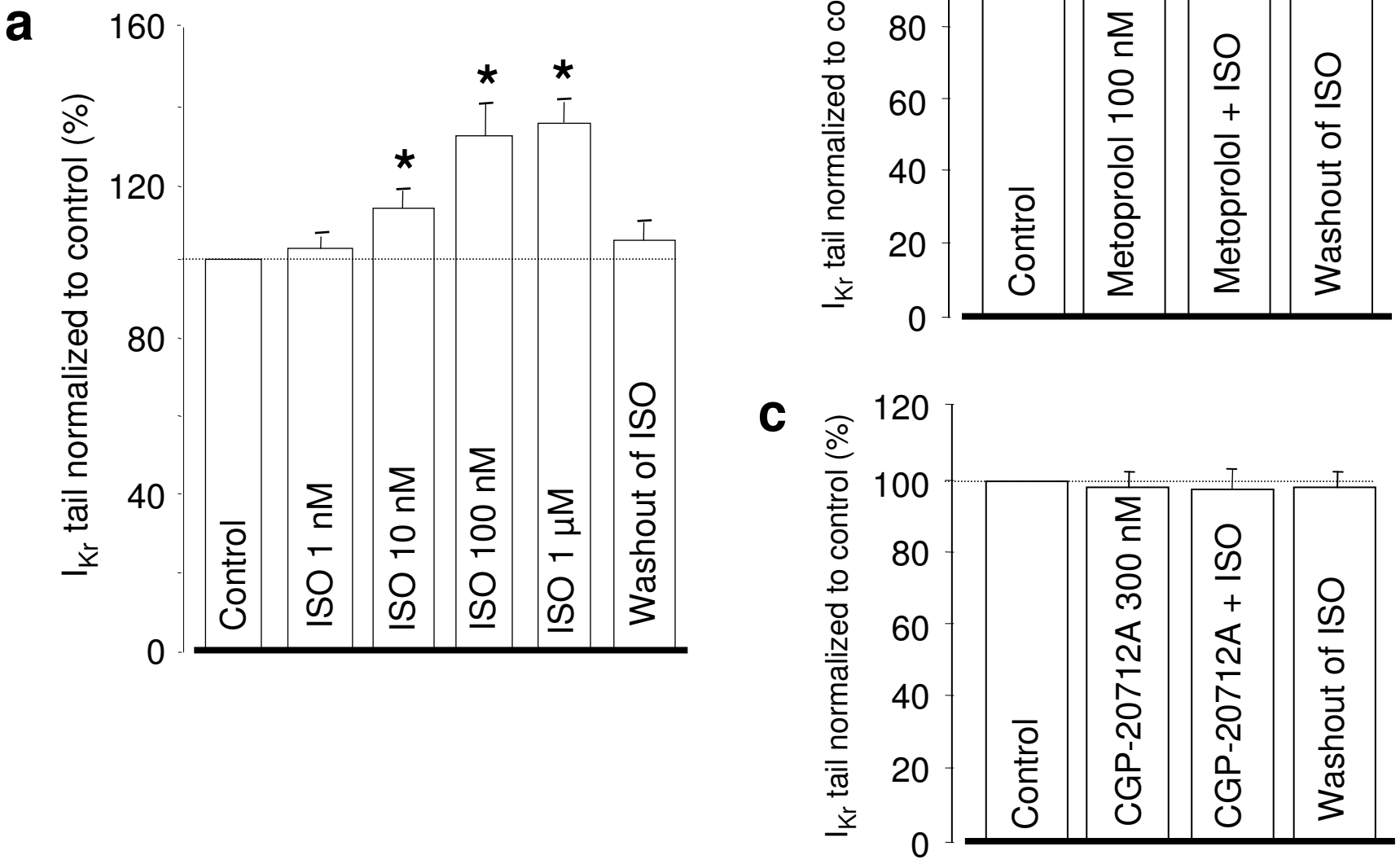
a
I_{Kr} tail normalized to control (%)
160
120
80
40
0
Control
ISO 1 nM
ISO 10 nM
ISO 100 nM
ISO 1 µM
Washout of ISO
*
*
*
b
I_{Kr} tail normalized to control (%)
120
100
80
60
40
20
0
Control
Metoprolol 100 nM
Metoprolol + ISO
Washout of ISO
c
I_{Kr} tail normalized to control (%)
120
100
80
60
40
20
0
Control
CGP-20712A 300 nM
CGP-20712A + ISO
Washout of ISO

Fig.2

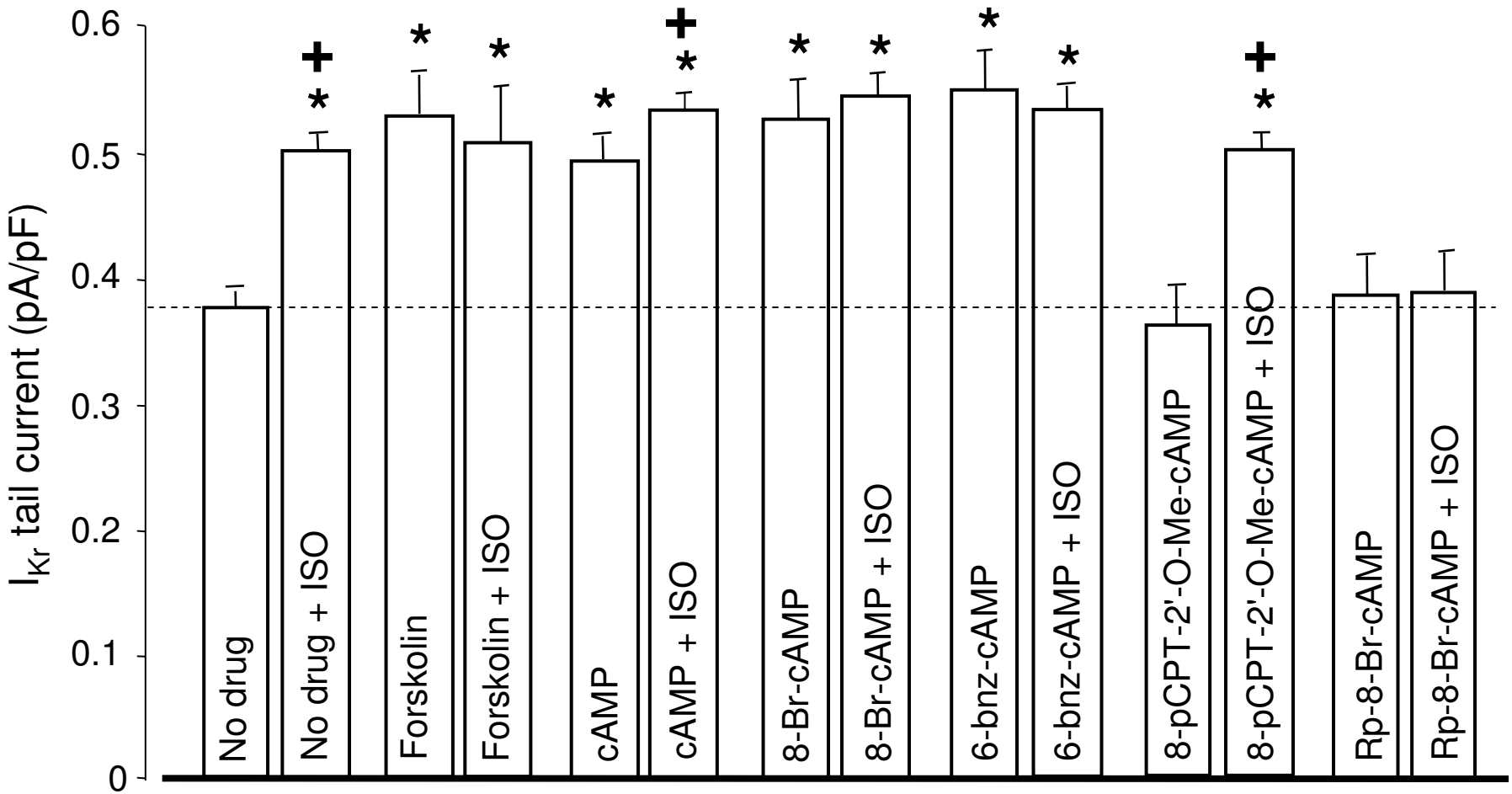
0.6
0.5
0.4
0.3
0.2
0.1
0
I_{Kr} tail current (pA/pF)
No drug
No drug + ISO
Forskolin
Forskolin + ISO
cAMP
cAMP + ISO
8-Br-cAMP
8-Br-cAMP + ISO
6-bnz-cAMP
6-bnz-cAMP + ISO
8-pCPT-2'-O-Me-cAMP
8-pCPT-2'-O-Me-cAMP + ISO
Rp-8-Br-cAMP
Rp-8-Br-cAMP + ISO

Fig.3

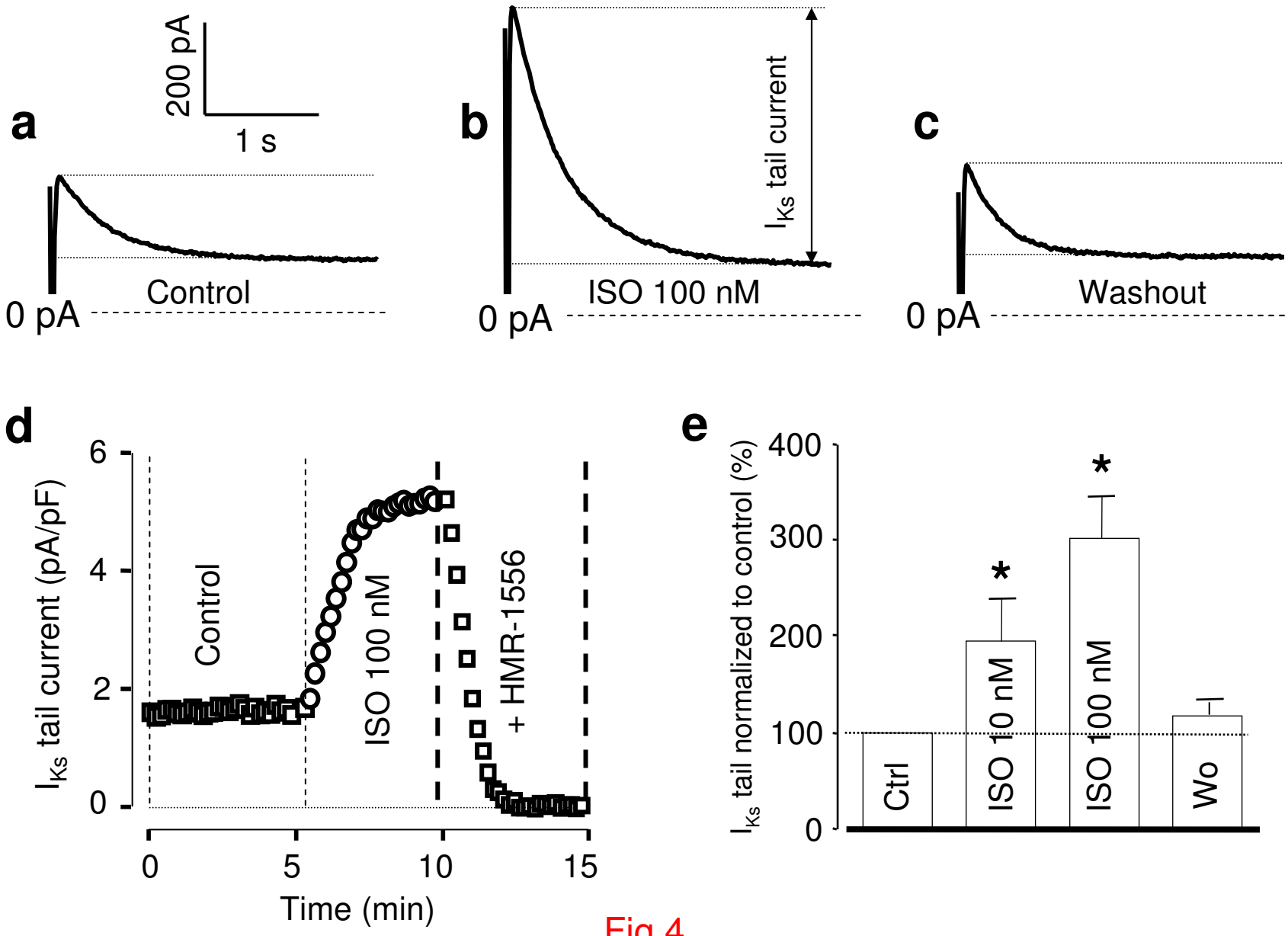
a
200 pA
1 s
Control
0 pA
b
I_{Ks} tail current
ISO 100 nM
0 pA
c
Washout
0 pA
d
I_{Ks} tail current (pA/pF)
Control
ISO 100 nM
+ HMR-1556
Time (min)
e
I_{Ks} tail normalized to control (%)
Ctrl
ISO 10 nM
ISO 100 nM
Wo

Fig.4

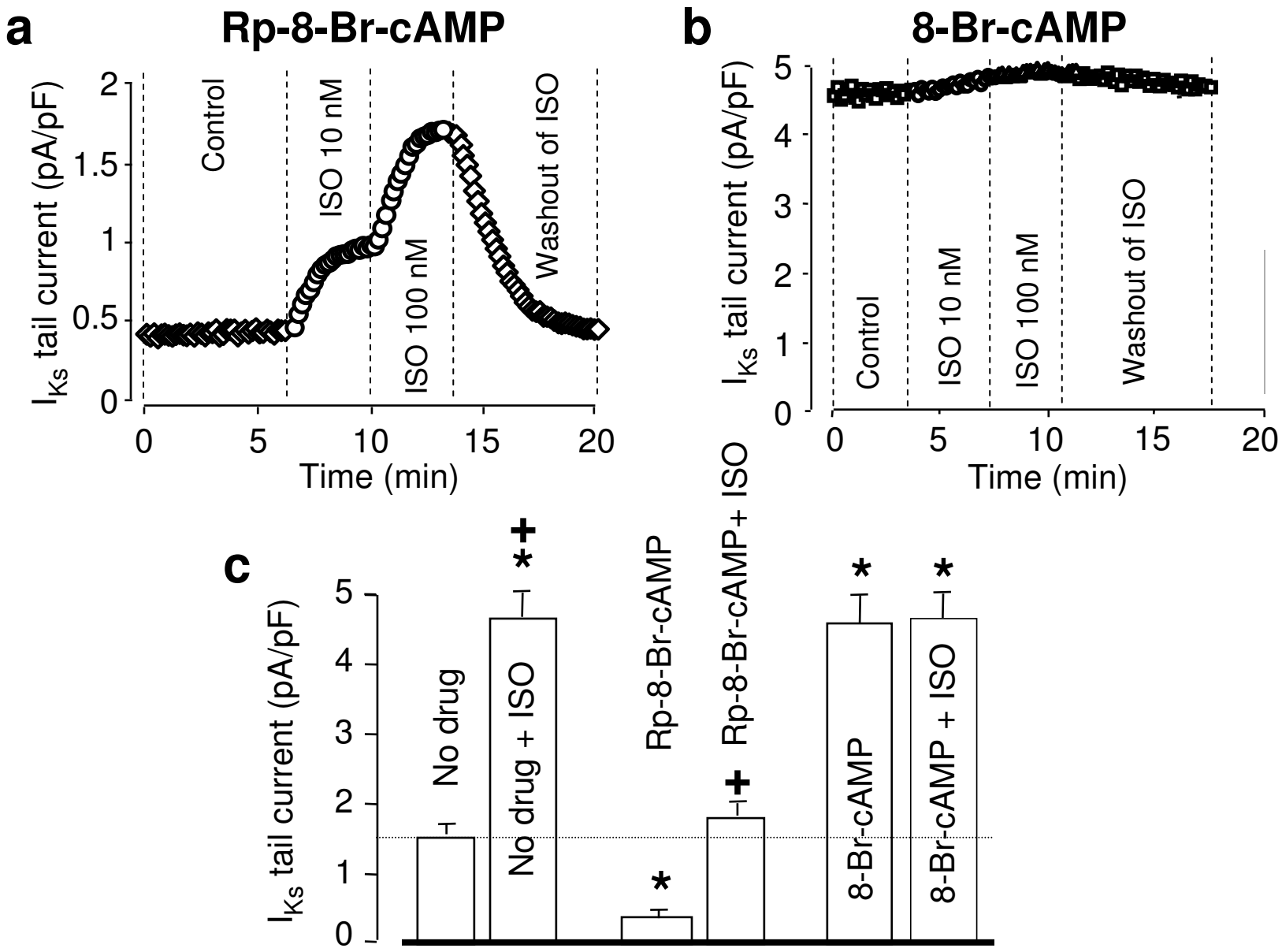
a
Rp-8-Br-cAMP
I_{Ks} tail current (pA/pF)
Control
ISO 10 nM
ISO 100 nM
Washout of ISO
Time (min)
b
8-Br-cAMP
I_{Ks} tail current (pA/pF)
Control
ISO 10 nM
ISO 100 nM
Washout of ISO
Time (min)
c
I_{Ks} tail current (pA/pF)
No drug
No drug + ISO
Rp-8-Br-cAMP
Rp-8-Br-cAMP+ ISO
8-Br-cAMP
8-Br-cAMP + ISO

Fig.5