

# RESEARCH PAPER

## PKC inhibition results in a $K_v1.5 + K_v\beta1.3$ pharmacology closer to $K_v1.5$ channels

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### BACKGROUND AND PURPOSE

The  $K_v\beta1.3$  subunit modifies the gating and pharmacology of  $K_v1.5$  channels in a PKC-dependent manner, decreasing channel sensitivity to bupivacaine- and quinidine-mediated blockade. Cardiac  $K_v1.5$  channels associate with receptor for activated C kinase 1 (RACK1), the  $K_v\beta1.3$  subunit and different PKC isoforms, resulting in the formation of a functional channelosome. The aim of the present study was to investigate the effects of PKC inhibition on bupivacaine and quinidine block of  $K_v1.5 + K_v\beta1.3$  channels.

### EXPERIMENTAL APPROACH

HEK293 cells were transfected with  $K_v1.5 + K_v\beta1.3$  channels, and currents were recorded using the whole-cell configuration of the patch-clamp technique. PKC inhibition was achieved by incubating the cells with either calphostin C or bisindolylmaleimide II and the effects of bupivacaine and quinidine were analysed.

### KEY RESULTS

The voltage-dependent inactivation of  $K_v1.5 + K_v\beta1.3$  channels and their pharmacological behaviour after PKC inhibition with calphostin C were similar to those displayed by  $K_v1.5$  channels alone. Indeed, the  $IC_{50}$  values for bupivacaine were similar in cells whose PKC was inhibited with calphostin C or bisindolylmaleimide II. Similar results were also observed in the presence of quinidine.

### CONCLUSIONS AND IMPLICATIONS

The finding that the voltage-dependence of inactivation and the pharmacology of  $K_v1.5 + K_v\beta1.3$  channels after PKC inhibition resembled that observed in  $K_v1.5$  channels suggests that both processes are dependent on PKC-mediated phosphorylation. These results may have clinical relevance in diseases that are characterized by alterations in kinase activity.

### Abbreviation

PAH, pulmonary arterial hypertension

## Table of Links

TARGETS	LIGANDS
Kv1.5 channel	Bupivacaine
PKC	Quinidine
PKCβ	Calphostin C
α-adrenoceptor	
β-adrenoceptor	
PKA	

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a,b,c).

## Introduction

The activation of K<sub>v</sub>1.5 channels generates the ultra-rapid outward potassium current ( $I_{Kur}$ ), which plays an important role in human atrial repolarization and the maintenance of the resting membrane voltage in the pulmonary vascular system (Archer *et al.*, 1998; Ravens and Wettwer, 2011). Indeed, mutations in and down-regulation of, K<sub>v</sub>1.5 channels are involved in the development of atrial fibrillation and pulmonary arterial hypertension (PAH) (Yuan *et al.*, 1998; Olson *et al.*, 2006; Remillard *et al.*, 2007; González *et al.*, 2010). The slow and partial inactivation and the voltage-dependence of these channels underlie their important role in the regulation of the duration of atrial action potentials (Fedida *et al.*, 1993; Snyders *et al.*, 1993). This slow inactivation is modified by the assembly of K<sub>v</sub>1.5 subunits with β subunits (K<sub>v</sub>β1.2, K<sub>v</sub>β1.3 and K<sub>v</sub>β2.1) that are present in the human myocardium (Uebele *et al.*, 1996; 1998). The K<sub>v</sub>β1.3 subunit modifies the electrophysiological characteristics of K<sub>v</sub>1.5 channels, inducing a fast and partial inactivation, a greater degree of slow inactivation, a shift of the activation curve towards more negative potentials, a decrease in the sensitivity of the channel to blockade induced by antiarrhythmic drugs and local anaesthetics, and a decrease in the degree of stereoselective blockage (González *et al.*, 2002; Decher *et al.*, 2005; Arias *et al.*, 2007).

$I_{Kur}$  is highly modulated by α- and β-adrenoceptor stimulation (Li *et al.*, 1996) mediated by PKC and PKA, respectively (Murray *et al.*, 1994; Kwak *et al.*, 1999a,b). The expression levels of α- and β-adrenoceptors, as well as the release of catecholamines, can be modified in several cardiac pathologies (Schlaich *et al.*, 2003; 2005). In fact, cardiac hypertrophy is associated with an up-regulation of different PKC isoforms (Takeishi *et al.*, 1999; Braz *et al.*, 2002; Kerkela *et al.*, 2002; Hahn *et al.*, 2003). During PAH-associated right ventricular hypertrophy, the activity of GPCR kinases modulates the β<sub>1</sub>-adrenoceptor-mediated response (Piao *et al.*, 2012). Furthermore, one of the most effective treatments for atrial fibrillation is the administration of β-blockers (Kuhlkamp *et al.*, 2000); pharmacological and electrical remodelling are induced in patients chronically treated with these drugs (Valenzuela, 2003). In addition, a decrease in myocyte death

as a result of selective PKA inhibition has been proposed as one of the mechanisms responsible for the beneficial effects of β-blockers in heart failure patients (Sabbah, 2004; Zhang *et al.*, 2013). PKC and PKA activities are also required for K<sub>v</sub>1.5 channel modulation by the auxiliary subunits K<sub>v</sub>β1.2 and K<sub>v</sub>β1.3 (Kwak *et al.*, 1999a,b; Williams *et al.*, 2002). Indeed, calphostin C-mediated PKC inhibition removes many of the K<sub>v</sub>β1.3-dependent electrophysiological effects (Kwak *et al.*, 1999b). The presence of a K<sub>v</sub>1.5 channel complex (or channelosome) has recently been shown to be composed of several PKC isoforms (βI and βII), receptor for activated C kinase 1 (RACK1) and the K<sub>v</sub>β1.3 subunit in the rat ventricle, but not in the rat atria (David *et al.*, 2012).

K<sub>v</sub>1.5- or K<sub>v</sub>β1.3-phosphorylation is required for the K<sub>v</sub>β1.3-induced effects on K<sub>v</sub>1.5 channels (Kwak *et al.*, 1999b). Moreover, leucine at position 510 in the K<sub>v</sub>1.5 channel is involved in the binding of both the K<sub>v</sub>β1.3 subunit and bupivacaine. In addition, bupivacaine and quinidine share a common receptor site at K<sub>v</sub>1.5 channels, and this site is located at the S6 segment and involves both a polar and a hydrophobic interaction (through T507 and V514, respectively) (Yeola *et al.*, 1996; Franqueza *et al.*, 1997; González *et al.*, 2002; Decher *et al.*, 2005; Arias *et al.*, 2007). K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels expressed in cells treated with calphostin C exhibit an inactivation curve that is shifted towards more positive potentials compared with K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels expressed in control conditions (David *et al.*, 2012). Taken together, these results suggest that after PKC inhibition, both bupivacaine and quinidine should be more potent at inducing blockade.

In the present study, we analysed the electrophysiological and pharmacological consequences of PKC inhibition in K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels. The voltage-dependent activation of K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels after treatment with calphostin C or bisindolylmaleimide II was similar to that exhibited by K<sub>v</sub>1.5 channels alone. Also, the voltage-dependent inactivation and pharmacological activity of K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels after calphostin C- or bisindolylmaleimide II-mediated PKC inhibition was similar to that exhibited by K<sub>v</sub>1.5 channels alone. More importantly, the potency of bupivacaine and quinidine for blocking K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels expressed in cells treated with calphostin C or with bisindolylmaleimide II was more

similar to that displayed by  $K_v1.5$  channels than it was for that displayed by untreated  $K_v1.5 + K_v\beta1.3$  channels. In summary, PKC activity was found to regulate both the electrophysiological and pharmacological properties of  $K_v1.5 + K_v\beta1.3$  channels, indicating that PKC may be considered a new therapeutic target in some cardiac pathologies.

## Methods

### *Expression plasmids, cell culture and transient transfection*

Expression of human  $K_v1.5$  channels and the  $K_v\beta1.3$  subunit (Alexander *et al.*, 2013b) in pBK was performed as previously described (Uebele *et al.*, 1998; David *et al.*, 2012). Briefly, the human  $K_v1.5$  channel (–22 to 1894 nt) and the  $K_v\beta1.3$  subunit (–53 to 1500 nt) were inserted into the same pBK vector, with the  $K_v1.5$  channel placed 3' to the  $K_v\beta1.3$  subunit and preceded by an internal ribosome entry sequence, thus generating a bicistronic messenger RNA, as previously described (Kwak *et al.*, 1999b). Expression of human  $K_v1.5$  channels in pBK was performed as previously described (Arias *et al.*, 2007).

HEK293 cells were cultured in DMEM that had been supplemented with 10% FBS, 10 U·mL<sup>-1</sup> of penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 1% nonessential amino acids. For the electrophysiological experiments, cells plated in 35 mm dishes were transfected with  $K_v1.5 + K_v\beta1.3$  channels (0.8 µg) and a reporter plasmid expressing CD8 (1.8 µg) using Fugene 6 (at a 1:3 ratio of µg of DNA : µL of Fugene 6) (Promega, Madison, WI, USA). Prior to experimental use, the cells were incubated with polystyrene microbeads pre-coated with an anti-CD8 antibody (Dyna-beads M450, Life Technologies, Grand Island, NY, USA), as previously described (González *et al.*, 2001; 2002; Arias *et al.*, 2007).

### *Electrophysiological recordings and data acquisition*

The intracellular pipette filling solution contained the following (in mM): K-aspartate 80, KCl 42, phosphocreatine 3, KH<sub>2</sub>PO<sub>4</sub> 10, MgATP 3, HEPES-K 5 and EGTA-K 5 (adjusted to pH 7.25 with KOH). The bath solution contained the following (in mM): NaCl 140, KCl 4, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 1, HEPES-Na 10 and glucose 10 (adjusted to pH 7.40 with NaOH). Currents were recorded using the whole-cell configuration of the patch-clamp technique with a patch-clamp amplifier (Axopatch-200B Patch-clamp Amplifier; Molecular Devices Corp., Menlo Park, CA, USA) and were stored on a personal computer (TD Systems, Camarillo, CA, USA) with a Digidata 1440A analogue-to-digital converter (Molecular Devices Corp.). PClamp version 10 software was used for both data acquisition and analyses (Molecular Devices Corp.). Currents were recorded at room temperature (21–23°C) at a stimulation frequency of 0.1 Hz and were sampled at 4 kHz after antialias filtering at 2 kHz. The average pipette resistance ranged from 1 to 3 MΩ ( $n = 25$ ). Gigaohm seal formation was achieved by suction (2–5 GΩ,  $n = 25$ ). After seal formation, the cells were lifted from the bottom of the bath, and the membrane patch was ruptured with a brief additional suction. Microcal Origin 8.5 (OriginLab Co., Northampton,

MA, USA) and the CLAMPFIT utility of pCLAMP 10 were used to perform least-squares fitting and data presentation. Experiments were excluded from analysis if the voltage error estimate exceeded 5 mV after series resistance compensation. Deactivation was fitted to a mono- or bi-exponential process using the following equations:  $\gamma = A\exp(-t/\tau) + C$  or  $\gamma = A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2) + C$ , respectively, where  $\tau_x$  is the system time constant;  $A_x$  is the amplitude of the component of the exponential; and  $C$  is the baseline value. The voltage-dependence of the activation and inactivation curves was fitted using a Boltzmann equation:  $\gamma = 1/[1 + \exp[-(V - V_h)/s]]$ , where  $s$  represents the slope factor;  $V$  represents the membrane potential; and  $V_h$  represents the voltage at which 50% of the channels are open. In both cases, the maximum peak tail current or the maximum current generated during the application of the test pulse were plotted versus the previous membrane potential in order to generate the activation or inactivation curves respectively. Drug-induced block was measured at the end of 50 and 250 ms depolarizing pulses from –80 to +60 mV. The degree of inhibition obtained for each drug concentration was used to calculate the IC<sub>50</sub> and  $n_H$  values based on fitting these values to the Hill equation:  $1/[1 + [IC_{50}/(D)]^{n_H}]$ . Concentration–response curves with two components were fitted to a biphasic Hill equation.

### *Statistical analysis*

The data are presented as the means ± SEM. One-way ANOVA was used to compare more than two groups. Statistical significance was set at  $P < 0.05$ . The curve-fitting procedure used a nonlinear least-squares (Gauss–Newton) algorithm, and the results are displayed in linear and semilogarithmic formats with the difference plots. Goodness of fit was determined using the  $\chi^2$  criterion and by inspecting systematic non-random trends in the difference plot.

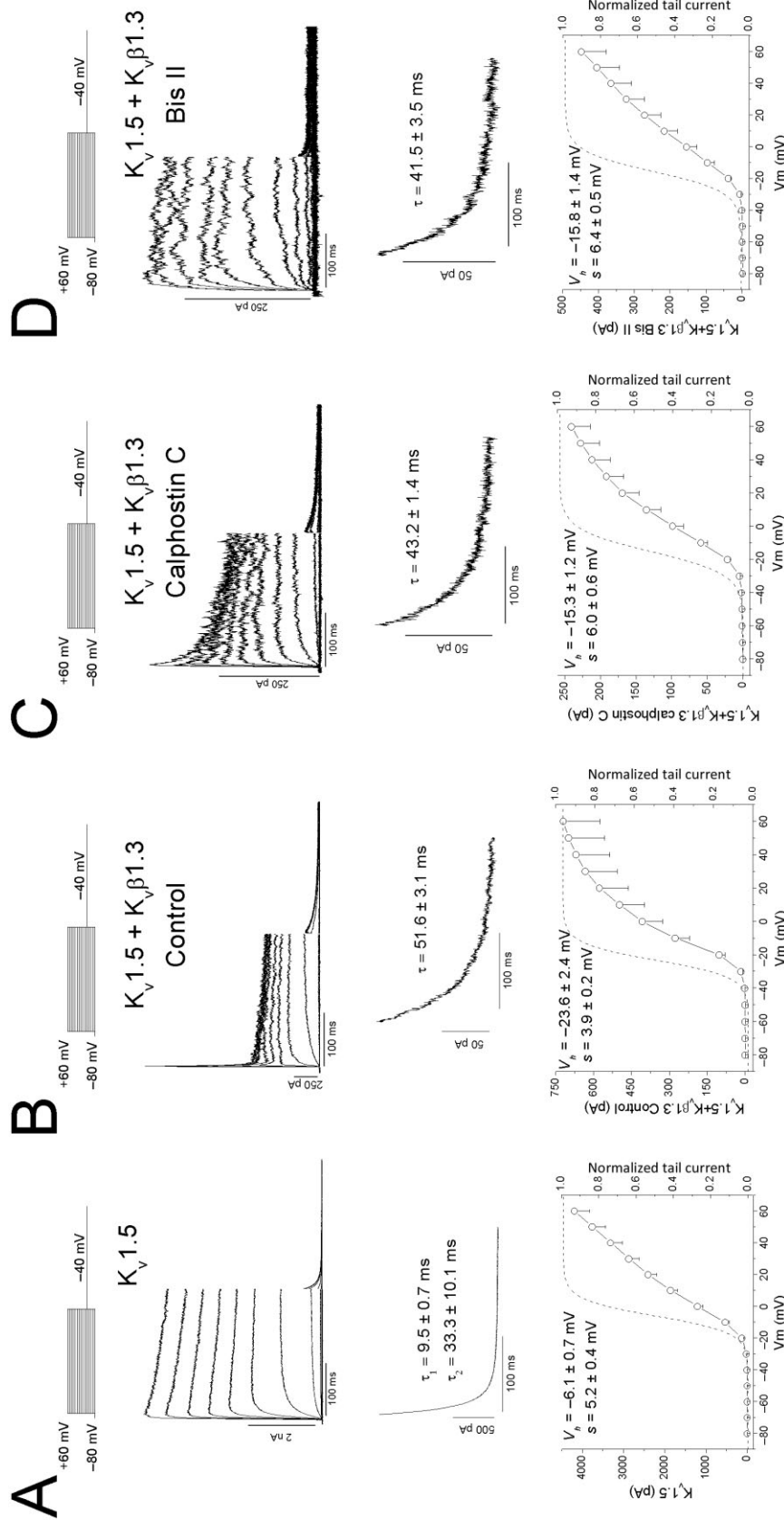
### *Drugs*

Racemic bupivacaine and quinidine (Sigma-Aldrich) were dissolved in distilled deionized water to yield stock solutions of 10 mM, from which further dilutions were made. Calphostin C, which was obtained from Calbiochem (Merck KGaA, Darmstadt, Germany), was dissolved in ethanol at a concentration of 100 µM for use as a stock solution. In all experiments, calphostin C was applied at a concentration of 3 µM for 2 h. Bisindolylmaleimide II (Sigma-Aldrich) was dissolved in DMSO at a concentration of 1 mM for use as a stock solution. In all experiments, bisindolylmaleimide II was applied at a concentration of 1 µM during 30 min. Hispidine from Calbiochem (Merck KGaA), was dissolved in DMSO at a concentration of 10 mM for use as a stock solution. In all experiments, hispidin was applied at a concentration of 5 µM for 30 min.

## Results

### *$K_v\beta1.3$ -induced fast inactivation is abolished by PKC inhibition*

Figure 1 shows the effects of the  $K_v\beta1.3$  subunit on the  $K_v1.5$  channel, which induced a fast and partial inactivation, a greater degree of slow inactivation, a slower deactivation process and a shift of the activation curve towards more



**Figure 1**

Original recordings obtained upon depolarization from a holding potential of  $-80$  to  $+60$  mV in  $10$  mV steps and upon repolarization to  $-40$  mV. (A) Current recordings obtained from the activation of  $K_v1.5$  channels. (B) Current recordings of  $K_v1.5$  channels in the presence of the  $K_v\beta1.3$  subunit. (C) Current recordings of  $K_v1.5$  channels in the presence of a  $K_v\beta1.3$  subunit in cells that had been previously treated with calphostin C. (D) Current recordings of  $K_v1.5$  channels in the presence of a  $K_v\beta1.3$  subunit in cells that had been previously treated with bisindolylmaleimide II. In all cases, the current (top), the deactivation process recorded at  $-40$  mV after a pulse to  $+60$  mV (middle) and the I-V relationship and activation curves (dashed line) obtained (bottom) are shown. When PKC is inhibited, the linearity of the I-V is recovered, and the activation process is shifted towards a more negative potential when the  $K_v\beta1.3$  subunit is present.

Table 1

Voltage-dependent activation and inactivation parameters of  $K_v1.5$ ,  $K_v1.5 + K_v\beta1.3$  and  $K_v1.5 + K_v\beta1.3$ -calphostin C channels obtained after applying 250 ms prepulses

	Activation		Inactivation		Degree of inactivation (%)
	$V_h$ (mV)	$s$ (mV)	$V_h$ (mV)	$s$ (mV)	
$K_v1.5$	$-6.0 \pm 0.7$	$5.2 \pm 0.4$	$-1.3 \pm 3.4$	$9.8 \pm 1.6$	$18.0 \pm 3.4$
$K_v1.5 + K_v\beta1.3$ control	$-23.6 \pm 2.4^a$	$3.9 \pm 0.2$	$-26.5$	4.2	$48.0 \pm 3.0$
$K_v1.5 + K_v\beta1.3$ -calphostin C	$-15.3 \pm 1.2^{b,c}$	$6.0 \pm 0.6^{b,c}$	$-6.5 \pm 1.8^b$	$10.1 \pm 1.1^b$	$33.8 \pm 2.2^{b,c}$

$s$ , slope of the Boltzmann equation;  $V_h$ , half-inactivation voltage. Data represent the mean  $\pm$  SEM of  $n > 7$  experiments.

<sup>a</sup>Statistically significant difference between  $K_v1.5 + K_v\beta1.3$  and  $K_v1.5$ .

<sup>b</sup>Statistically significant difference between  $K_v1.5 + K_v\beta1$ -calphostin C and  $K_v1.5 + K_v\beta1.3$ -control channels.

<sup>c</sup>Statistically significant difference between  $K_v1.5 + K_v\beta1.3$ -calphostin C and  $K_v1.5$  channels.

negative potentials (Figures 1A and B, and Table 1). PKC inhibition with calphostin C prevented  $K_v\beta1.3$ -induced fast inactivation of  $K_v1.5$  channels (Murray *et al.*, 1994; Kwak *et al.*, 1999a,b) as a consequence of a shift in the inactivation midpoint towards more positive potentials (David *et al.*, 2012). Under these experimental conditions,  $K_v1.5 + K_v\beta1.3$ -calphostin C channels exhibited an activation curve intermediate between that observed for the  $K_v1.5$  and  $K_v1.5 + K_v\beta1.3$  channels (Table 1), and similar deactivation kinetics to  $K_v1.5 + K_v\beta1.3$  channels (Figures 1B and C). Also, PKC inhibition induced by bisindolylmaleimide II prevented  $K_v\beta1.3$ -induced fast inactivation (Figure 1D), and like calphostin C, shifted the activation curve towards more positive potentials relative to  $K_v1.5 + K_v\beta1.3$  channels. A series of experiments were performed with hispidin (a selective PKC $\beta$ I and PKC $\beta$ II inhibitor). Hispidin only eliminated the  $K_v\beta1.3$ -induced fast inactivation in 30% of the cells analysed, consistent with previous studies (David *et al.*, 2012).

To analyse the electrophysiological characteristics of the inactivation process of  $K_v1.5 + K_v\beta1.3$  channels expressed in cells treated with calphostin C ( $K_v1.5 + K_v\beta1.3$ -calphostin C) we applied the double-pulse protocol shown at the top of Figure 2. Figure 2A shows the inactivation curves of  $K_v1.5$ ,  $K_v1.5 + K_v\beta1.3$ ,  $K_v1.5 + K_v\beta1.3$ -calphostin C. PKC inhibition induced by calphostin C shifted the inactivation curve towards more positive potentials, which was similar to the  $V_h$  observed in  $K_v1.5$  channels (Table 1). Although the degree of inactivation of  $K_v1.5 + K_v\beta1.3$ -calphostin C was decreased in comparison with  $K_v1.5 + K_v\beta1.3$  channels, it had not fully reverted back to that observed in  $K_v1.5$  channels (Figures 1 and 2, and Table 1). The degree of inactivation of  $K_v1.5 + K_v\beta1.3$  channels continued to increase as the membrane potential became more positive, indicating that  $K_v\beta1.3$ -mediated fast inactivation was voltage-dependent, as previously reported (Uebele *et al.*, 1998). However, the inactivation of  $K_v1.5 + K_v\beta1.3$ -calphostin C channels did not increase, similar to that observed with  $K_v1.5$  channels (Figure 2A).

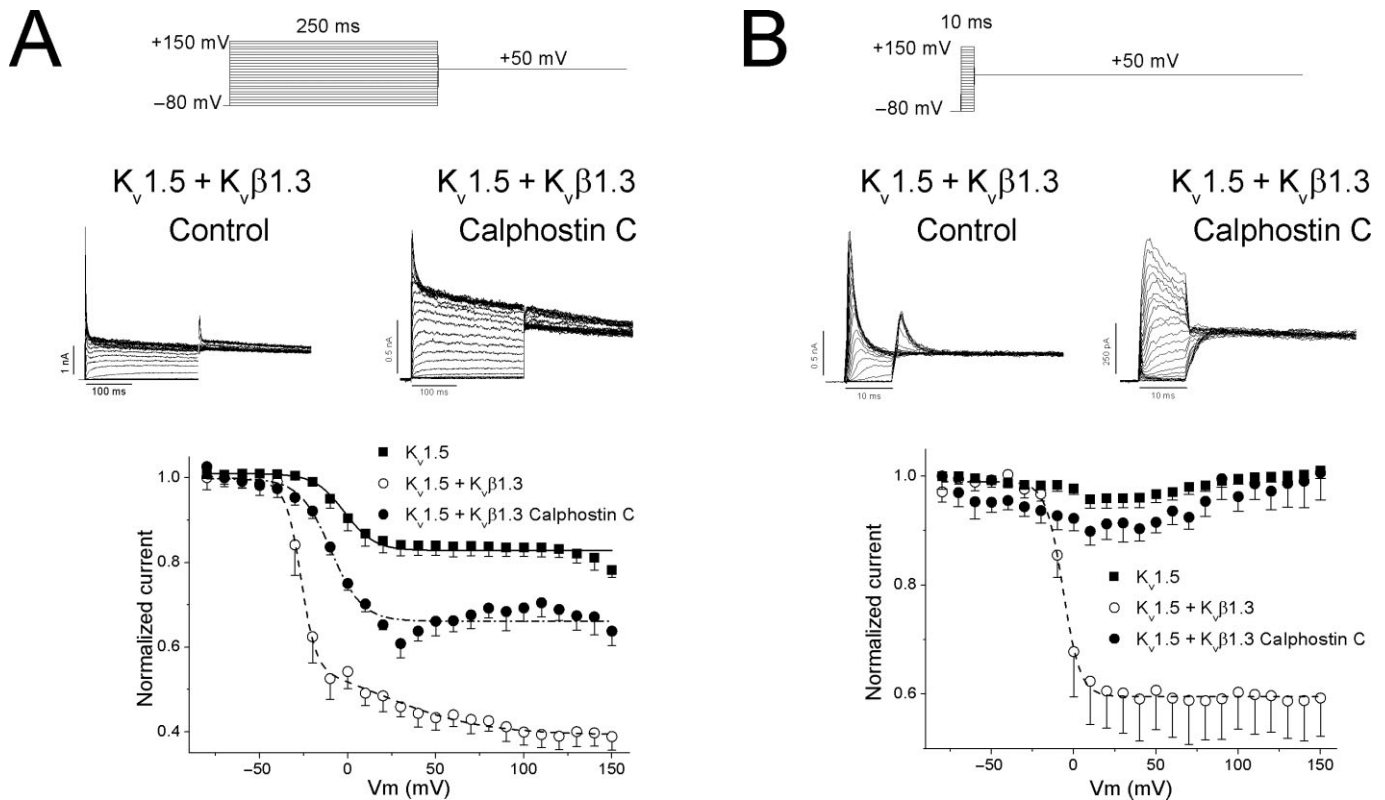
To isolate the fast component of the inactivation induced by the  $K_v\beta1.3$  subunit, a double-pulse protocol using a 10 ms prepulse from  $-80$  to  $+150$  mV in 10 mV steps was applied (Uebele *et al.*, 1998). Figure 2B shows the inactivation curve

obtained after applying this double-pulse protocol. After applying this short prepulse,  $K_v1.5$  channels were not inactivated, because they exhibited a slow inactivation with a time constant of  $\sim 200$  ms. In contrast,  $K_v1.5 + K_v\beta1.3$  channels that exhibited a fast inactivation with a time constant of  $\sim 3$  ms displayed an inactivation curve with a  $V_h = -5.2 \pm 2.5$  mV and  $s = 5.3 \pm 0.9$  mV ( $n = 4$ ). This inactivation curve only represents the voltage-dependence of  $K_v\beta1.3$ -induced fast inactivation; when cells were treated with calphostin C, this process was abolished. Figure 2B also shows that when this pulse protocol was applied, the fractional inactivation of  $K_v1.5 + K_v\beta1.3$  channels did not increase with membrane potential, exhibiting similar behaviour to that observed when a 250 ms in duration prepulse was applied in calphostin C-treated cells (Figure 2B and Table 1). However, when PKC was inhibited, the fast inactivation process was completely abolished, exhibiting the same phenotype as  $K_v1.5$  channels alone.

### Pharmacological properties of $K_v1.5 + K_v\beta1.3$ channels after calphostin C treatment mimic those in the absence of the $K_v\beta1.3$ subunit

In previous studies, we demonstrated that mutations in  $K_v1.5$  channel or their assembly with the  $K_v\beta1.3$  subunits modify the pharmacological characteristics of the channel (Franqueza *et al.*, 1997; González *et al.*, 2002; Arias *et al.*, 2007). Therefore, we studied the bupivacaine- and quinidine-induced blockade of  $K_v1.5 + K_v\beta1.3$  channels after PKC inhibition. Current recordings through  $K_v1.5 + K_v\beta1.3$ -calphostin C channels were obtained in the absence and presence of bupivacaine (50  $\mu$ M) or quinidine (30  $\mu$ M) (Figure 3A, B). The current-voltage ( $I$ - $V$ ) relationships were obtained after plotting the current amplitude measured at the end of 250 ms depolarizing pulses at different membrane potentials between  $-80$  and  $+60$  mV in the absence and presence of either drug (Figure 3). Both compounds decreased the magnitude of the current at all membrane potentials tested.

We also analysed the effects of bupivacaine and quinidine in  $K_v1.5 + K_v\beta1.3$  channels expressed in cells treated with bisindolylmaleimide II ( $K_v1.5 + K_v\beta1.3$ -bisindolylmaleimide II) and in cells in which hispidin suppressed the  $K_v\beta1.3$ -induced fast inactivation. Current recordings through  $K_v1.5 +$



**Figure 2**

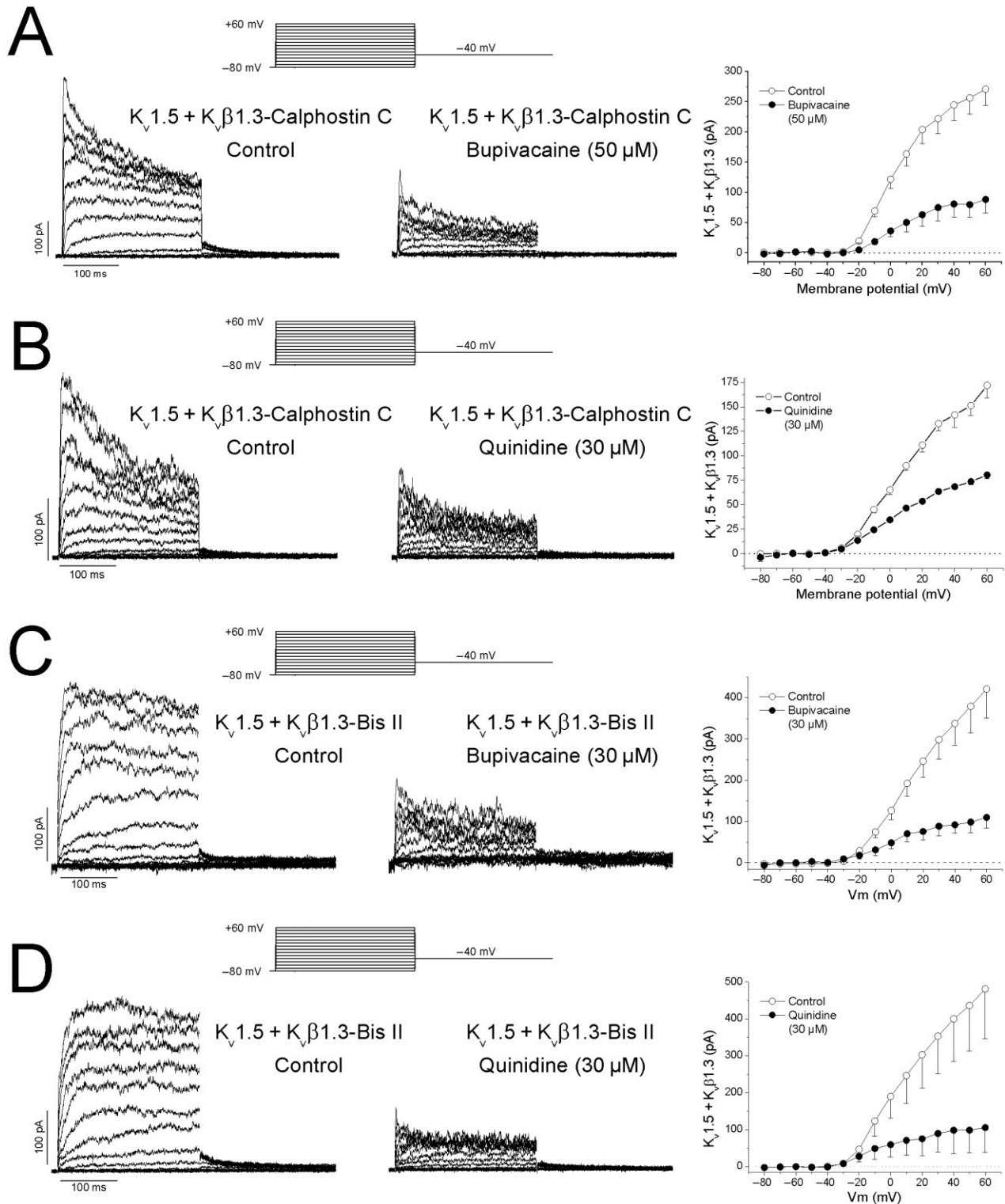
Voltage-dependence of K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channel inactivation before and after treatment with calphostin C. (A) Voltage-dependence of the inactivation following a 250 ms in duration prepulse. (B) Voltage-dependence of the inactivation following a 10 ms in duration prepulse.

K<sub>v</sub>β1.3-bisindolylmaleimide II channels were obtained in the absence and presence of bupivacaine (30 μM) or quinidine (30 μM) (Figure 3C, D). The I–V relationships were obtained after plotting the current amplitude measured at the end of 250 ms depolarizing pulses at different membrane potentials between -80 and +60 mV in the absence and presence of either drug (Figure 3). Both compounds decreased the magnitude of the current at all membrane potentials tested.

The concentration–response curves obtained for the inhibitory effects of bupivacaine and quinidine on K<sub>v</sub>1.5, K<sub>v</sub>1.5 + K<sub>v</sub>β1.3, K<sub>v</sub>1.5 + K<sub>v</sub>β1.3-calphostin C, K<sub>v</sub>1.5 + K<sub>v</sub>β1.3-bisindolylmaleimide II and K<sub>v</sub>1.5 + K<sub>v</sub>β1.3-hispidin channels are shown in Figure 4 and Supporting Information Fig. S1. The suppression of the current at the end of 50 or 250 ms depolarizing pulses to +60 mV was used as an index of block (Figure 4, Supporting Information Figs S1–S4). In contrast to the blockade previously reported for K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels (González *et al.*, 2002), bupivacaine-induced blockade of K<sub>v</sub>1.5 + K<sub>v</sub>β1.3-calphostin C channels showed a biphasic curve, with an IC<sub>50(1)</sub> of 0.2 ± 0.1 nM and an IC<sub>50(2)</sub> of 21.4 ± 4.1 μM (*n* = 41). Note that the IC<sub>50(2)</sub> was similar to that of K<sub>v</sub>1.5 channels (González *et al.*, 2002). PKC inhibition did not modify the *n*<sub>H</sub> value (0.74 ± 0.06 vs. 0.71 ± 0.08 for bupivacaine-produced blockade of K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 and K<sub>v</sub>1.5 + K<sub>v</sub>β1.3-calphostin C channels, respectively) (González *et al.*, 2002) (Figure 4A). Similarly, quinidine-induced blockade of

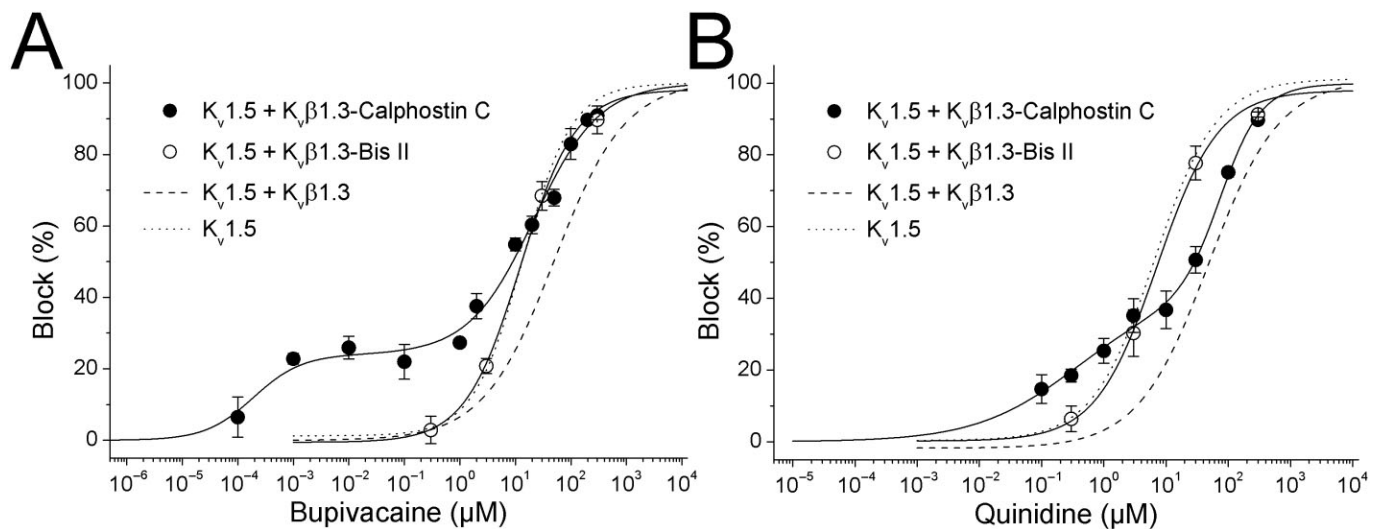
K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels was modified by calphostin C treatment (Figure 4B). Indeed, the concentration–response curve was also a biphasic concentration–response curve, with IC<sub>50(1)</sub> and IC<sub>50(2)</sub> values of 3.4 ± 5.4 μM and 70.4 ± 15.4 μM (*n* = 50) respectively. Interestingly, the IC<sub>50(1)</sub> value was similar to that reported for the quinidine-induced blockade of K<sub>v</sub>1.5 channels (Yeola *et al.*, 1996), whereas the IC<sub>50(2)</sub> value was similar to the value reported for blockade of K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels (González *et al.*, 2002). The *n*<sub>H(1)</sub> and *n*<sub>H(2)</sub> values for this concentration–response curve were 0.34 ± 0.07 and 1.36 ± 0.46 respectively. The *n*<sub>H(2)</sub> value was similar to that observed in the concentration–response curve of quinidine-induced blockade of K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels (González *et al.*, 2002). In contrast to that observed in K<sub>v</sub>1.5 + K<sub>v</sub>β1.3-calphostin C, the concentration–response curves for bupivacaine and quinidine of K<sub>v</sub>1.5 + K<sub>v</sub>β1.3-bisindolylmaleimide II channels were monophasic with IC<sub>50</sub> values of 12.4 ± 1.8 μM and 7.2 ± 1.2 μM respectively. These values resemble those previously reported for bupivacaine and quinidine to block K<sub>v</sub>1.5 channels (13.5 ± 1.4 μM and 4 μM, respectively). Intermediate results were obtained after inhibition of PKC with hispidin (Supporting Information Fig. S1). IC<sub>50</sub> values were similar when block was measured after 50 ms or 250 ms of depolarizing pulses under all experimental conditions (Supporting Information Figs S1–S3).

Bupivacaine- and quinidine-produced blockade of K<sub>v</sub>1.5 + K<sub>v</sub>β1.3-calphostin C channels was time-dependent, as



**Figure 3**

Effects of bupivacaine (50  $\mu$ M) and quinidine (30  $\mu$ M) on  $K_v1.5 + K_v\beta1.3$  channels in cells that have been treated with calphostin C or bisindolylmaleimide II. (A) Current recordings and I-V relationships obtained in the absence and presence of bupivacaine in cells pretreated with calphostin C. (B) Current recordings and I-V relationships obtained in the absence and presence of quinidine in cells pretreated with calphostin C. (C) Current recordings and I-V relationships obtained in the absence and presence of bupivacaine in cells pretreated with bisindolylmaleimide II. (D) Current recordings and I-V relationships obtained in the absence and presence of quinidine in cells pretreated with bisindolylmaleimide II. The blockade induced by both drugs was measured at the end of the pulse at +60 mV. Each point represents the mean  $\pm$  SEM of six and five experiments respectively.



**Figure 4**

Concentration-dependence of bupivacaine-induced (A) and quinidine-induced (B) blockade of calphostin C- or bisindolylmaleimide II-treated K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels. The dotted and dashed lines represent the dose–response curves obtained for the bupivacaine- and quinidine-produced blockade of K<sub>v</sub>1.5 channels alone and K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels, respectively (taken from González *et al.*, 2002). The reduction in the current (relative to the control) at the end of depolarizing steps from –80 to +60 mV was used as an index of blockade. Concentration–response curves for bupivacaine and quinidine in calphostin C-treated K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels and in bisindolylmaleimide II-treated K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels are shown. Each point represents the mean  $\pm$  SEM of three to eight experiments. The continuous line represents the fit of the experimental data to a biphasic or monophasic Hill equation.

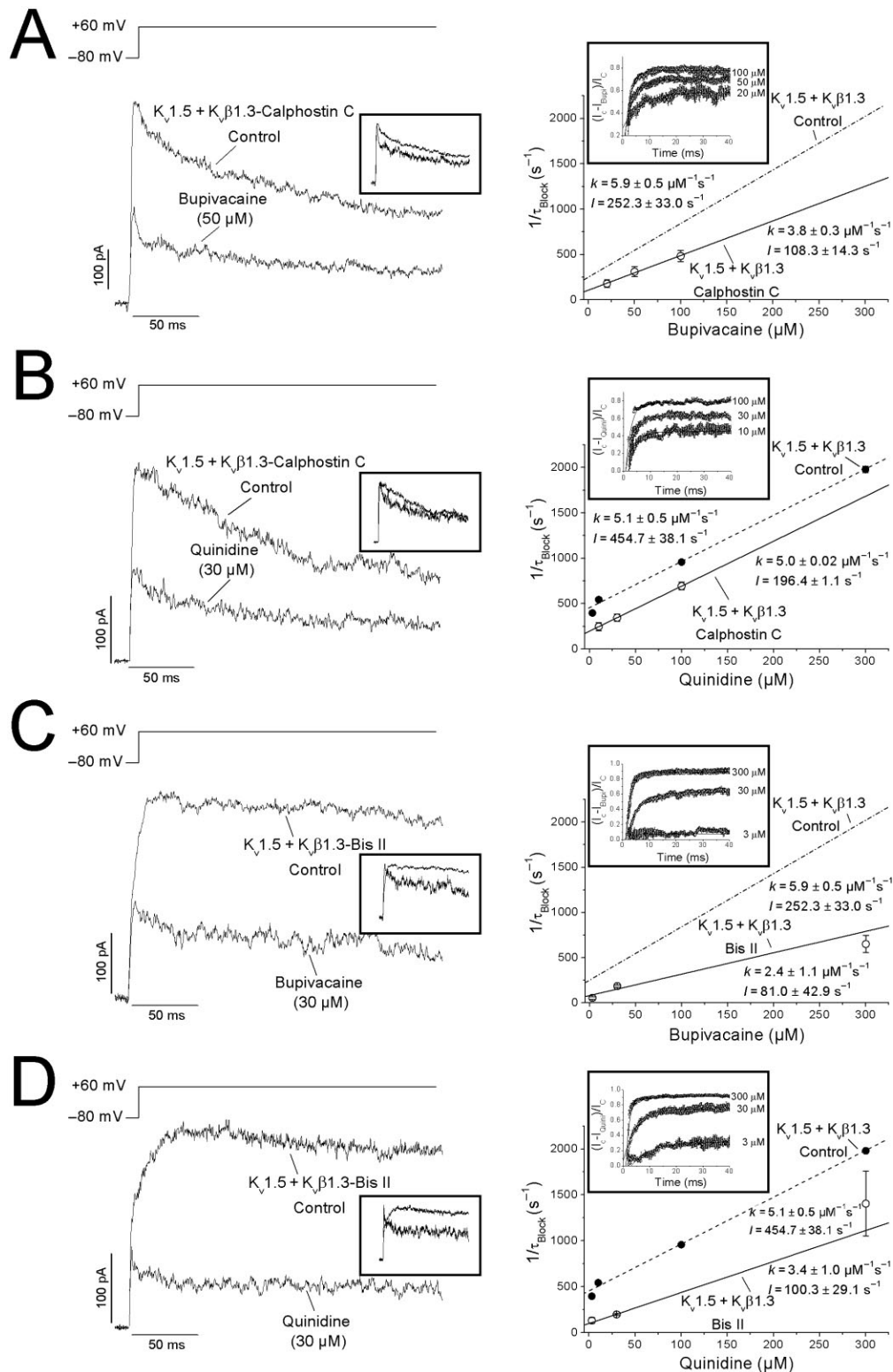
indicated by the normalized current in the insets of the left panels of Figure 5A and B. To analyse the kinetics of blockade induced by both drugs, we plotted the ratio of the drug-sensitive current and the current under control conditions  $[(I_{\text{control}} - I_{\text{drug}})/I_{\text{control}}]$  during the first 40 ms in the presence of 20, 50 and 100  $\mu\text{M}$  bupivacaine and 10, 30 and 100  $\mu\text{M}$  quinidine (right panel of Figure 5A and B, inset). The blockade exponentially increased during depolarization, and the time constant of this process was faster at higher drug concentrations. Thus, the time constant of this process was considered to be a good index of the development of blockade ( $\tau_{\text{block}}$ ). Based on the  $\tau_{\text{block}}$  values obtained at different bupivacaine concentrations, the association ( $k$ ) and dissociation ( $l$ ) rate constants were derived, averaging  $5.9 \pm 0.5 \mu\text{M}^{-1}\cdot\text{s}^{-1}$  and  $252.3 \pm 33.0 \text{ s}^{-1}$  versus  $3.8 \pm 0.3 \mu\text{M}^{-1}\cdot\text{s}^{-1}$  and  $108.3 \pm 14.3 \text{ s}^{-1}$  ( $n = 14$ ) in K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 and K<sub>v</sub>1.5 + K<sub>v</sub>β1.3-calphostin C channels, respectively (Figure 5A) (González *et al.*, 2002). In the case of quinidine, the  $k$  and  $l$  values for K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels were  $5.1 \pm 0.51 \mu\text{M}^{-1}\cdot\text{s}^{-1}$  and  $454.7 \pm 38.05 \text{ s}^{-1}$ , while the  $k$  and  $l$  values for K<sub>v</sub>1.5 + K<sub>v</sub>β1.3-calphostin C channels were  $5.0 \pm 0.02 \mu\text{M}^{-1}\cdot\text{s}^{-1}$  and  $196.4 \pm 1.1 \text{ s}^{-1}$  ( $n = 11$ ) (Figure 5B). Similar changes in the association and dissociation rate constants were observed when the bupivacaine and quinidine kinetics of block were studied in K<sub>v</sub>1.5 + K<sub>v</sub>β1.3-bisindolylmaleimide II channels (Figure 5C, D). Although the blockade induced by both drugs was time-dependent, as observed during depolarization pulses from –80 to +60 mV, this time dependence was not evident in the tail currents recorded at –40 mV under any experimental condition, probably because of the changes in the association and dissociation kinetics and/or to changes in the gating of the channel (Figure 6).

## Discussion and conclusions

In the present study, we analysed the electrophysiological and pharmacological consequences of PKC inhibition using calphostin C and bisindolylmaleimide II in K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels. We demonstrated that the voltage-dependence of K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channel inactivation after PKC inhibition resembles that observed in K<sub>v</sub>1.5 channels alone. Inhibition of PKC using calphostin C or bisindolylmaleimide II partially reversed the changes in the pharmacological properties induced by the assembly of the K<sub>v</sub>β1.3 subunit with K<sub>v</sub>1.5 channels (González *et al.*, 2002; Decher *et al.*, 2005) in such a way that the pharmacology of these channels in cells pre-treated with calphostin C or bisindolylmaleimide II resembled that exhibited by K<sub>v</sub>1.5 channels in the absence of the K<sub>v</sub>β1.3 subunit (Snyders *et al.*, 1992; Valenzuela *et al.*, 1995).

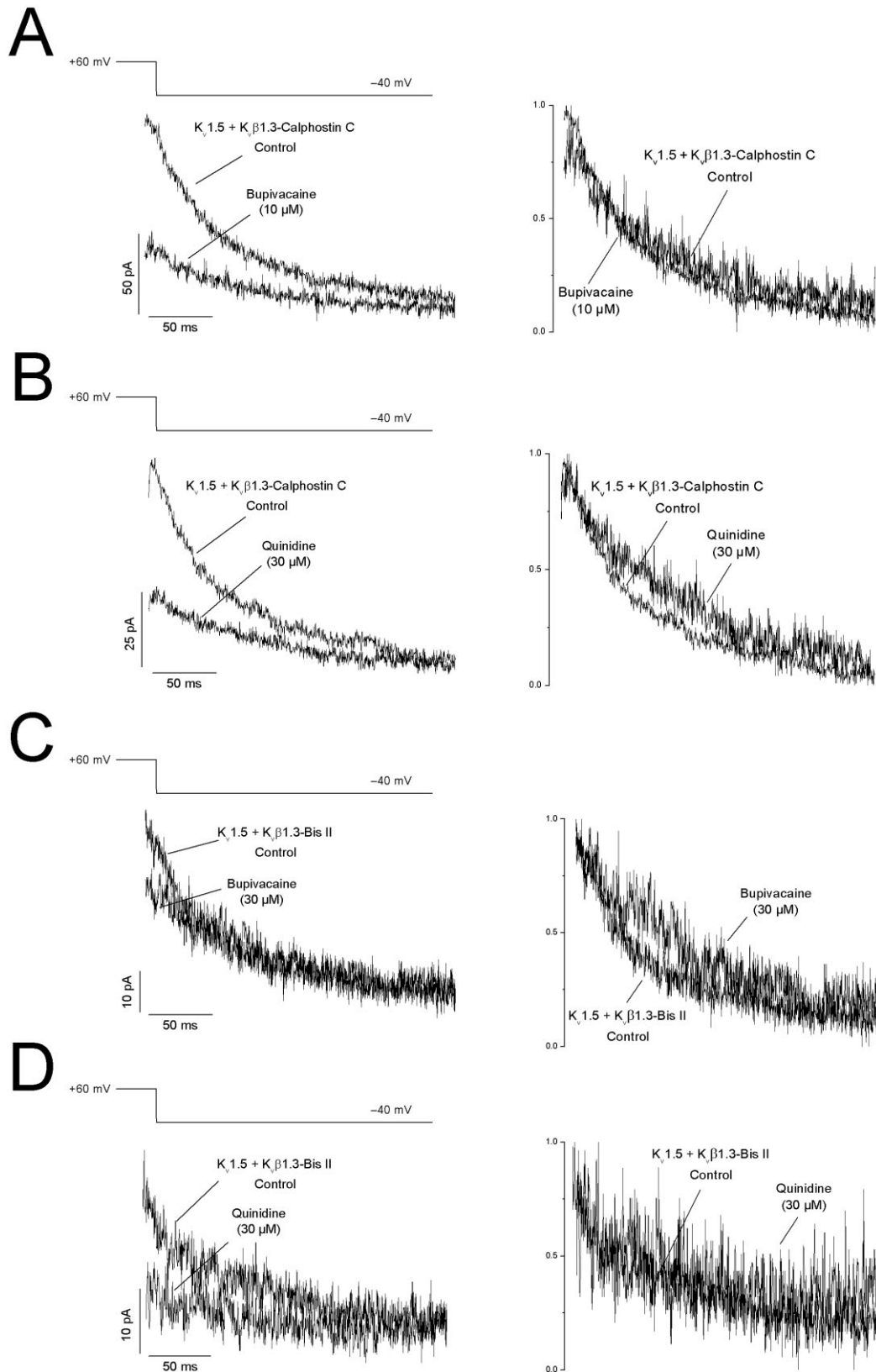
Calphostin C-mediated PKC inhibition results in the abolishment of K<sub>v</sub>β1.3-induced fast inactivation because of a shift in the inactivation midpoint of the K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels (David *et al.*, 2012). Calphostin C produces a marked down-regulation that can be due to an abnormal recycling of the K<sub>v</sub>1.5 channels to the membrane. Surprisingly, the voltage-dependence of inactivation of K<sub>v</sub>1.5 + K<sub>v</sub>β1.3-calphostin C and that of K<sub>v</sub>1.5 channels were similar. These results suggest not only that the voltage-dependent inactivation induced by this  $\beta$  subunit is abolished, but also that the complex behaves as if the K<sub>v</sub>β1.3 subunit was not assembled with the K<sub>v</sub>1.5 channels. The voltage-dependence of K<sub>v</sub>β1.3-mediated fast inactivation differs from that of N-type inactivation in *Shaker* channels and with the fast inactivation conferred by K<sub>v</sub>β1.2 on K<sub>v</sub>1.5 channels, both of which are insensitive to





**Figure 5**

Time-dependence of bupivacaine- and quinidine-induced blockade of calphostin C- and bisindolylmaleimide II-treated K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels. *Left:* Superimposed traces for the steps from -80 to +60 mV and those normalized to the control values (inset). *Right:* Relationship between  $1/\tau_{\text{Block}}$  values at different concentrations of bupivacaine (A, C) and quinidine (B, D). Each point represents the mean  $\pm$  SEM of three to six experiments. For a first-order blocking scheme, a relationship is expected:  $1/\tau_{\text{Block}} = k \times [\text{Drug}] + l$ . The lines represent the fit from which the apparent binding ( $k$ ) and unbinding ( $l$ ) rate constants were obtained under control (dashed) and PKC-inhibited (solid) conditions. The relationship between the sensitive current and depolarizing time  $[(I_{\text{control}} - I_{\text{drug}})/I_{\text{control}}]$  at three different drug concentrations is plotted in the inset of each panel.



**Figure 6**

(A,C) Bupivacaine- and (B, D) quinidine-induced effects on the deactivation kinetics of (A, B) calphostin C- and (C, D) bisindolylmaleimide II-treated K<sub>v</sub>1.5 + K<sub>v</sub>β1.3 channels. The currents obtained after applying the protocol outlined at the top of the figure are shown. The tails were superimposed (top) and normalized (bottom) to show the similar deactivation kinetics in the absence and presence of the drugs.

membrane potential (Zagotta *et al.*, 1989; De Biasi *et al.*, 1997; Uebele *et al.*, 1998). The voltage dependence of  $K_v\beta 1.3$ -mediated inactivation may be due to the charge within the blocking particle sensing the transmembrane potential as well as to some voltage-dependent changes in the  $K_v1.5$  channel that are involved in the interaction with the  $K_v\beta 1.3$  subunit. To discriminate between these possibilities, experiments using a pulse protocol with 10 ms depolarizing pre-pulses were performed. These experiments allowed us to isolate the fast inactivation induced by the  $K_v\beta 1.3$  subunit from the slow inactivation that is characteristic of  $K_v1.5$  channels. Indeed, inactivation curves obtained from  $K_v1.5 + K_v\beta 1.3$  and  $K_v1.5 + K_v\beta 1.3$ -calphostin C channels were similar after applying 10 and 250 ms pre-pulse protocols respectively. Taken together, these data suggest that the voltage-dependence of  $K_v\beta 1.3$ -mediated inactivation is independent of the properties of the  $K_v1.5$  channel. However, we cannot rule out the possibility that a conformational change in  $K_v\beta 1.3$ , due to dephosphorylation, occurs and could be transmitted to the S6 in  $K_v1.5$  subunit, changing the interaction between the receptor site at the  $K_v1.5$  and the  $K_v\beta 1.3$  inactivation 'ball'.

Bupivacaine- and quinidine-induced blockade of  $K_v1.5$  channels result from their binding to external and internal receptor sites (Yeola *et al.*, 1996; Franqueza *et al.*, 1997; Longobardo *et al.*, 2000; 2001). Although the molecular determinants of the external binding site remain unknown, both drugs share a common internal receptor site, which is located at the S6 segment and involves a polar interaction with T507 and two hydrophobic interactions with L510 and V514 (Yeola *et al.*, 1996; Franqueza *et al.*, 1997; Arias *et al.*, 2007). Moreover, the bupivacaine binding site on  $K_v1.5$  channels overlaps with the binding site of the  $K_v\beta 1.3$  subunit in such a way that bupivacaine blocks the  $K_v1.5 + K_v\beta 1.3$  channel to a lesser extent than  $K_v1.5$  channels alone (Arias *et al.*, 2007). The pharmacology of the  $K_v1.5 + K_v\beta 1.3$  channels was modified after PKC inhibition, decreasing the  $IC_{50}$  values for both bupivacaine and quinidine. These results can be explained by the decrease in the dissociation rate values obtained in  $K_v1.5 + K_v\beta 1.3$ -calphostin C and  $K_v1.5 + K_v\beta 1.3$ -bisindolylmaleimide II channels with bupivacaine and quinidine, which indicates that the drug-channel complex is more stable than in  $K_v1.5 + K_v\beta 1.3$  channels, but less stable than that observed in  $K_v1.5$  channels. However, the association rate constants remained nearly unchanged in comparison with those reported for  $K_v1.5$  and  $K_v1.5 + K_v\beta 1.3$  channels (González *et al.*, 2002; Arias *et al.*, 2007). This result also corroborates the finding that the phenotype of  $K_v1.5 + K_v\beta 1.3$ -calphostin C or  $K_v1.5 + K_v\beta 1.3$ -bisindolylmaleimide II channels is closer to that of  $K_v1.5$  channels alone (Arias *et al.*, 2007). This gain of potency is a very important result if we keep in mind that the expression levels of  $\alpha$ - and  $\beta$ -adrenoceptors, as well as the release of catecholamines, can be modified in several cardiac pathologies (Schlaich *et al.*, 2003; 2005), and may have important consequences in cardiac pharmacology (Longobardo *et al.*, 1998; González *et al.*, 2002; 2010; Ravens and Wettwer, 2011). Interestingly, the concentration–response curves for bupivacaine- and quinidine-induced block in cells in which PKC activity was inhibited with calphostin C was biphasic, while these curves were monophasic when bisindolylmalinimide II was used to

inhibit PKC. If drug binding at the site with highest affinity saturates at around 25%, then we have to assume that drug binding only partially blocks ionic flow, somewhat similar to the 'turret' block of  $K_v11.1$  channels by some toxins, like CnErg1 (Hill *et al.*, 2007). Therefore, block induced by very low drug concentrations can be attributed to rapid fluctuations between the drug-blocked channel and a full conductance drug-channel encounter complex. In fact, similar to the block produced by CnErg1 that induces an incomplete block at very high concentrations, bupivacaine and quinidine were not able to completely abolish the current even at very high concentrations (Hill *et al.*, 2007).

In summary, we have characterized the functional contribution of PKC activity to the modulation of the  $K_v1.5$  channelosome from an electrophysiological and pharmacological perspective. Importantly, PKC inhibition changes the electrophysiological and pharmacological characteristics of  $K_v1.5 + K_v\beta 1.3$  channels, causing them to be more similar to those observed in the absence of the  $K_v\beta 1.3$  subunit. This functional characterization of the  $K_v1.5$  channelosome opens up a variety of possible mechanisms that may elucidate the variations underlying the development of cardiac hypertrophy or any other cardiovascular disease that involves modifications in PKC activity.

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## Author contributions

C. V. designed research. A. M., A. P., A. dIc. and D. A. P. performed and analysed experiments. A. M., T. G., M. M. T. and C. V. analysed the data. C. V. and A. M. wrote the paper.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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**Figure S1** Concentration dependence of bupivacaine-induced blockade of hispidin-treated Kv1.5 + Kv $\beta$ 1.3 channels. (A) The dashed and dotted lines represent the

dose–response curves obtained for the bupivacaine-induced blockade of Kv1.5 and Kv1.5 + Kv $\beta$ 1.3 channels, respectively (taken from González *et al.*, 2002). Reduction in the current (relative to the control) at the end of depolarizing steps from –80 to +60 mV was used as an index of blockade. (●): Concentration–response curves for bupivacaine in bisindolylmaleimide II-treated Kv1.5 + Kv $\beta$ 1.3 channels and in hispidin-treated Kv1.5 + Kv $\beta$ 1.3 channels (○). (B) Reduction in the current (relative to the control) at 50 ms (○) and at 250 ms (●) depolarizing steps from –80 to +60 mV. Each point represents the mean  $\pm$  SEM of three to four experiments. The lines represent the fit of the experimental data to a monophasic Hill equation.

**Figure S2** Concentration dependence of bupivacaine- (A) and quinidine-induced (B) blockade of calphostine C-treated Kv1.5 + Kv $\beta$ 1.3 channels. The dashed and continuous lines represent the dose–response curves obtained for the bupivacaine- or quinidine-induced blockade at 50 ms (○) and at 250 ms (●) depolarizing steps from –80 to +60 mV respectively. Each point represents the mean  $\pm$  SEM of three to eight experiments. The continuous line represents the fit of the experimental data to a biphasic Hill equation.

**Figure S3** Concentration dependence of bupivacaine- (A) and quinidine-induced (B) blockade of bisindolylmaleimide II-treated Kv1.5 + Kv $\beta$ 1.3 channels. The dashed and continuous lines represent the dose–response curves obtained for the bupivacaine- or quinidine-induced blockade at 50 ms (○) and at 250 ms (●) depolarizing steps from –80 to +60 mV respectively. Each point represents the mean  $\pm$  SEM of three to eight experiments. The continuous line represents the fit of the experimental data to a monophasic Hill equation.

**Figure S4** Absolute values for bupivacaine- and quinidine-blockade of calphostin C- and bisindolylmaleimide II-treated Kv1.5 + Kv $\beta$ 1.3 channels. Magnitude of calphostin C-Kv1.5 + Kv $\beta$ 1.3 currents in the absence and in the presence of different bupivacaine (A) and quinidine (B) concentrations. Magnitude of bisindolylmaleimide II-Kv1.5 + Kv $\beta$ 1.3 currents in the absence and in the presence of different bupivacaine (C) and quinidine (D) concentrations. Each point represents the mean  $\pm$  SEM of three to eight experiments.

**Table S1** IC<sub>50</sub> and n<sub>H</sub> from the of the concentration–response curves to bupivacaine in Kv1.5 + Kv $\beta$ 1.3 channels expressed in cells treated with hispidin and bisindolylmaleimide II in comparison with those obtained of bupivacaine on Kv1.5 and Kv $\beta$ 1.3 channels.