J Membrane Biol (2015) 248:7–18
DOI 10.1007/s00232-014-9723-3

ORIGINAL ARTICLES

Effects of α1-Adrenoceptor Agonist Phenylephrine on Swelling-Activated Chloride Currents in Human Atrial Myocytes

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Received: 10 March 2014 / Accepted: 14 August 2014 / Published online: 26 August 2014

Abstract Swelling-activated chloride currents ($I_{Cl.swell}$) play an important role in cardiac electrophysiology and arrhythmogenesis. However, the regulation of these currents has not been clarified to date. In this research, we focused on the function of phenylephrine, an α_1-adrenoceptor agonist, in the regulation of $I_{Cl.swell}$ in human atrial myocytes. We recorded $I_{Cl.swell}$ evoked by a hypotonic bath solution with the whole-cell patch-clamp technique. We found that $I_{Cl.swell}$ increased over time, and it was difficult to achieve absolute steady state. Phenylephrine potentiated $I_{Cl.swell}$ from -1.00 ± 0.51 pA/pF at -90 mV and 2.58 ± 1.17 pA/pF at $+40$ mV to -1.46 ± 0.70 and 3.84 ± 1.67 pA/pF, respectively ($P < 0.05$, $n = 6$), and the upward trend in $I_{Cl.swell}$ was slowed after washout. This effect was concentration-dependent, and the α_1-adrenoceptor antagonist prazosin shifted the dose–effect curve rightward. Addition of prazosin or the protein kinase C (PKC) inhibitor bisindolylmaleimide (BIM) attenuated the effect of phenylephrine. The PKC activator phorbol 12,13-dibutyrate (PDBu) activated $I_{Cl.swell}$ from -1.69 ± 1.67 pA/pF at -90 mV and 5.58 ± 6.36 pA/pF at $+40$ mV to -2.41 ± 1.95 pA/pF and 7.05 ± 6.99 pA/pF, respectively ($P < 0.01$ at -90 mV and $P < 0.05$ at $+40$ mV; $n = 6$). In conclusion, the α1-adrenoceptor agonist phenylephrine augmented $I_{Cl.swell,}$ a result that differs from previous reports in other animal species. The effect was attenuated by BIM and mimicked by PDBu, which indicates that phenylephrine might modulate $I_{Cl,swell}$ in a PKC-dependent manner.

Keywords Swelling-activated chloride current · Phenylephrine · α_1-Adrenoceptor agonist · Human atrial myocyte · Protein kinase C

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Introduction

In human atrial cells, several studies have identified the presence of a swelling-activated chloride current ($I_{Cl.swell}$) (Oz and Sorota 1995; Sakai et al. 1995; Li et al. 1996; Du et al. 2004; Demion et al. 2006). The current displays outward rectification and can be blocked by disulfonic stilbenes. The activation of $I_{Cl.swell}$ can cause depolarization of the resting membrane potential and shortening of action potential duration, suggesting that this current may play an important role in cardiac electrophysiology and could be a target for antiarrhythmic drug therapy (Du and Sorota 1997; Vandenberg et al. 1997; Hume et al. 2000; Baumgarten and Clemo 2003).

The regulation of $I_{Cl.swell}$ is not clearly understood, but seems to be species and cell type-dependent. A number of protein kinases, including protein kinase C (PKC) (Duan et al. 1995; Coca-Prados et al. 1996; Du and Sorota 1999; Zhong et al. 2002; Gong et al. 2004; Zhou et al. 2005), protein tyrosine kinase (PTK) (Tilly et al. 1993; Voets et al. 1998; Du et al. 2004), rho-associated kinase (Nilius et al. 1999), phosphatidylinositol 3-kinase (Feranchak et al. 1999), and protein kinase A (PKA) (Oz and Sorota 1995; Ellershaw et al. 2002), have been reported to play a role in the regulation of $I_{Cl.swell}$. Studies have shown that α_1-adrenoceptor agonists modulate $I_{Cl.swell}$ through a PKC-

dependent pathway (Duan et al. 1995). Phenylephrine, an α_1-adrenoceptor agonist, has previously been reported to have varying effects on $I_{Cl.swell}$ in different cell types. Phenylephrine inhibited $I_{Cl.swell}$ in a PKC-dependent manner in rabbit atrial myocytes (Duan et al. 1995), and via phosphatidylinositol 4,5-bisphosphate (PIP3) depletion brought about by phospholipase C (PLC)-dependent reduction of membrane phosphatidylinositol 4,5-diphosphate (PIP2) levels in mouse ventricular cells (Ichishima et al. 2010). Du and Sorota reported that in dog atrial cells phenylephrine did not affect $I_{Cl.swell}$ but PKC stimulated it (Du and Sorota 1999). To our knowledge, the effect of phenylephrine on $I_{Cl.swell}$ in human cardiac cells has not yet been studied. We have previously demonstrated that in human atrial cells $I_{Cl.swell}$ is regulated in part by the balance between PTK and protein tyrosine phosphatase (PTP) activity. Sarcoma gene (Src) and epidermal growth factor receptor (EGFR) kinases, distinct soluble and receptor-mediated PTK families, have opposing effects on $I_{Cl.swell}$ (Du et al. 2004). In the present research, we focused on phenylephrine regulation of $I_{Cl.swell}$ in human atrial myocytes.

Materials and Methods

Cell Isolation

Atrial myocytes were isolated from specimens of the right atrial appendage obtained from patients undergoing coronary artery bypass. Procedures for obtaining the tissues from patients who had given written consent were approved by the Ethics Committee of Wuhan Union Hospital, Huazhong University of Science and Technology. Based on ECG and echocardiograms, no evidence of atrial arrhythmias or atrial dilation was found in any patients.

After excision, samples were quickly immersed in oxygenated, nominally Ca^{2+}-free cardioplegic solution and transported to the laboratory. Atrial myocytes were enzymatically dissociated by a technique described previously (Du et al. 2004). Briefly, the myocardial tissue was sliced with a sharp blade, placed in a 15-mL tube containing 10 mL of Ca^{2+}-free Tyrode solution (36 °C), and gently agitated by continuous bubbling with 100 % O_2 for 15 min with transfer to fresh solution after 5 min. The chunks were then incubated for 50 min in a similar solution containing 150–200 U/mL collagenase (CLS II; Worthington Biochemical, Freehold, NJ, USA), 1.2 U/mL protease (type XXIV; Sigma-Aldrich, Oakville, Ontario, Canada), and 1 mg/mL bovine serum albumin (Sigma-Aldrich). Finally, the tissue was reincubated in fresh enzyme solution without protease. The number and quality of the isolated cells were determined by microscopic examination at 5- to 10-min intervals. When the yield appeared to be maximal, the tissue was suspended in a high-K^+ medium and gently pipetted. The isolated myocytes were kept at room temperature in the high-K^+ medium for at least 1 h before study. A small aliquot of the solution containing the isolated cells was placed in an open perfusion chamber (1 mL) mounted on the stage of an inverted microscope (Leica DM IL). Myocytes were allowed to adhere to the bottom of the chamber for 5–10 min and were then superfused at 2–3 mL/min with isosmotic 1.0 T (T, times isosmotic) or hyposmotic 0.6 T solution. Only quiescent rod-shaped cells showing clear cross-striations were used for recordings.

Solutions and Drugs

The Ca^{2+}-free cardioplegic solution for specimen transport contained the following (in mM): 50 KH_2PO_4, 8 $MgSO_4$, 5 adenosine, 10 HEPES, 140 glucose, 100 mannitol, and 10 taurine; pH was adjusted to 7.3 with KOH. The standard Tyrode solution contained the following (in mM): 140 NaCl, 5.4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 0.33 NaH_2PO_4, 5 HEPES, and 10 glucose; pH was adjusted to 7.4 with NaOH. Ca^{2+} was omitted from the Tyrode solutions used for enzymatic digestion and for washing the sliced atrial tissue. For osmotic swelling experiments, hypotonic 0.6 T (180 mosmol/L) Tyrode was made by reducing NaCl from 140 to 80 mM, and isotonic 1T (300 mosmol/L) Tyrode was prepared by adding 125 mM mannitol to 0.6 T. The osmolality of all solutions was measured using a freezing-point depression osmometer (Osmomat 030; Gonotec, Berlin, Germany). The pipette solution contained the following (in mM): 20 CsCl, 110 Cs-aspartate, 1.0 $MgCl_2$, 10 HEPES, 5 EGTA, 0.1 GTP, 5 Na_2-phosphocreatine, and 5 Mg-ATP. PH was adjusted to 7.2 with CsOH (295 mosmol/L). The high-K^+ storage medium contained the following (in mM): 10 KCl, 120 K-glutamate, 10 KH_2PO_4, 1.8 $MgSO_4$, 10 taurine, 10 HEPES, 0.5 EGTA, 20 glucose, and 10 mannitol. pH was adjusted to 7.3 with KOH. All experiments were conducted at room temperature, 21–23 °C, in accordance with previous studies (Du et al. 2004; Gong et al. 2004; Zhou et al. 2005).

Propranolol (1 μM) was added to all superfusion solutions to block the β-adrenoceptor. 4-AP (4-amino-pyridine, 5 mM), $BaCl_2$ (200 μM), $CdCl_2$ (200 μM), and atropine (1 μM) were added to inhibit transient outward potassium current (I_{to}) and ultra-rapid delayed rectifier potassium current (I_{kur}), inward rectifier potassium current (I_{k1}), L-type calcium current ($I_{Ca.L}$), and acetylcholine-activated potassium current (I_{KACh}), respectively.

All reagents were purchased from Sigma-Aldrich. 4,4-diisothiocyanostilbene-2,2-disulfonic acid (DIDS) was freshly dissolved in the experimental solution. Phorbol-

12,13-dibutyrate (PDBu) and bisindolylmaleimide (BIM) were prepared as 1 or 2 mM stock solutions in dimethyl sulfoxide (DMSO). The maximum 0.1 % final concentration of DMSO in the bath solution did not affect $I_{Cl.swell}$. Stock solutions of the other drugs were prepared in distilled water and added to known volumes of the superfusion solution to obtain the desired concentrations.

Data Acquisition and Analysis

Whole-cell currents were recorded using an EPC-9 amplifier and Pulse software (Heka Electronik, Lambrecht, Germany), and signals were low-pass filtered (2 kHz) before 5-kHz digitization. Pipette resistance was 2–3 MΩ, and gigaseals were >10 GΩ. Series resistance (3–8 MΩ) was compensated (60–80 %) after membrane rupture, and a 3 M KCl-agar bridge was used as a reference electrode. In separate experiments, the liquid junction potential (bath pipette) was measured as 12 mV and was not corrected. Average cell membrane capacitance was 85.54 ± 9.23 pF. Currents were normalized to cell membrane capacitance prior to averaging (pA/pF). To obtain whole-cell I–V relations, 300-ms hyperpolarizing and depolarizing pulses were applied in +10 mV increments between −110 and +60 mV from a holding potential of −40 mV.

Relative cell volume was determined during the whole-cell recording using each cell as its own control according to our previous report (Du et al. 2004). Images of myocytes were captured with a digital camera (Leica DC300) at selected time points during the experiment.

All analyses were based on comparisons of currents in the presence of a drug with those recorded before drug superfusion in the same cell. Dose–response experiments were performed by superfusing the same cell with control solutions and then with each concentration of the drug to be tested. We initially planned to assess all effects after a superfusion interval long enough to achieve steady state, but we found it was difficult to achieve an absolute steady state of $I_{Cl.swell}$ in human atrial myocytes. In one cell, a steady state of $I_{Cl.swell}$ was still not obtained after more than 80 min. Therefore, in this study, after exposure to the hypotonic solution for more than about 20 min, either the assessment of $I_{Cl.swell}$ or the addition of a drug was performed, and when assessing the data, we took this phenomenon into consideration.

Nonlinear curve fitting was performed using SigmaPlot10.0. The concentration–response curve for phenylephrine was fitted to a logistic equation $Y - Y_{min} = (Y_{max} - Y_{min})/\{1 + (EC_{50}/\text{phenylephrine concentration})^n\}$, where Y is the ratio of the current in the presence of phenylephrine to the control current, the subscripts min and max indicate the minimum (set at 1) and maximum values of Y, EC_{50} is the concentration for a half-maximal effect, and n is the slope factor. The IC_{50} (half-maximal inhibitory concentration) of prazosin was calculated according to the formula shift factor = 1 + (antagonist concentration/antagonist affinity). The time courses of the currents were shown at +40 mV and the current densities were compared at +40 and −90 mV.

Data were presented as mean ± SE. Paired Student's t tests were used to evaluate differences between the two groups. A two-tailed probability of <5 % was taken to indicate statistical significance.

Results

Basal Chloride Current and $I_{Cl.swell}$ in Human Atrial Myocytes

Figure 1a, b shows the data obtained from all the cells that were perfused with 1.0 T and consequent 0.6 T bath, and for which the volumes were measured ($n = 23$). Under isotonic conditions, these cells possessed small basal currents with current densities of −0.58 ± 0.44 pA/pF at −90 mV and 0.97 ± 0.42 pA/pF at +40 mV. The basal current had a reversal potential of approximately −28.0 mV (−40 mV after correction for the liquid junction potential), which was near the calculated E_{Cl} (Cl^- equilibrium potential, −36 mV). Following perfusion with the hypotonic solution, the current densities increased to −1.46 ± 1.28 pA/pF at −90 mV and 4.05 ± 3.84 pA/pF at +40 mV ($P < 0.01$), and the relative cell volume in 0.6 T increased to 135.9 ± 3.6 % of that in 1.0 T ($P < 0.05$). The swelling-induced currents clearly displayed outward rectification and had a reversal potential of approximately −27.5 mV (−39.5 mV after correction for the liquid junction potential, near the calculated E_{Cl}).

We noticed that the chloride current inhibitor DIDS slightly attenuated the basal current in the three cells tested (Fig. 1c). Considering that the reversal potential was near the calculated E_{Cl}, the data suggested that the basal current was a chloride current.

Following re-immersion in 1.0 T, the current densities decreased from −1.02 ± 0.47 pA/pF at −90 mV and 2.45 ± 0.62 pA/pF at +40 mV to −0.66 ± 0.24 pA/pF and 1.16 ± 0.42 pA/pF, respectively (Fig. 2a, $P < 0.01$, $n = 6$). Application of DIDS (100 μM) voltage-dependently inhibited the currents by 68.97 ± 19.13 % at +40 mV and 35.89 ± 28.58 % at −90 mV (Fig. 2b, $n = 4$). Our data indicated that the currents evoked in the hypotonic solution were $I_{Cl.swell}$ (Hume et al. 2000; Baumgarten and Clemo 2003).

In our experiments, we found that $I_{Cl.swell}$ increased over time in most of the cells tested. It was difficult to achieve an

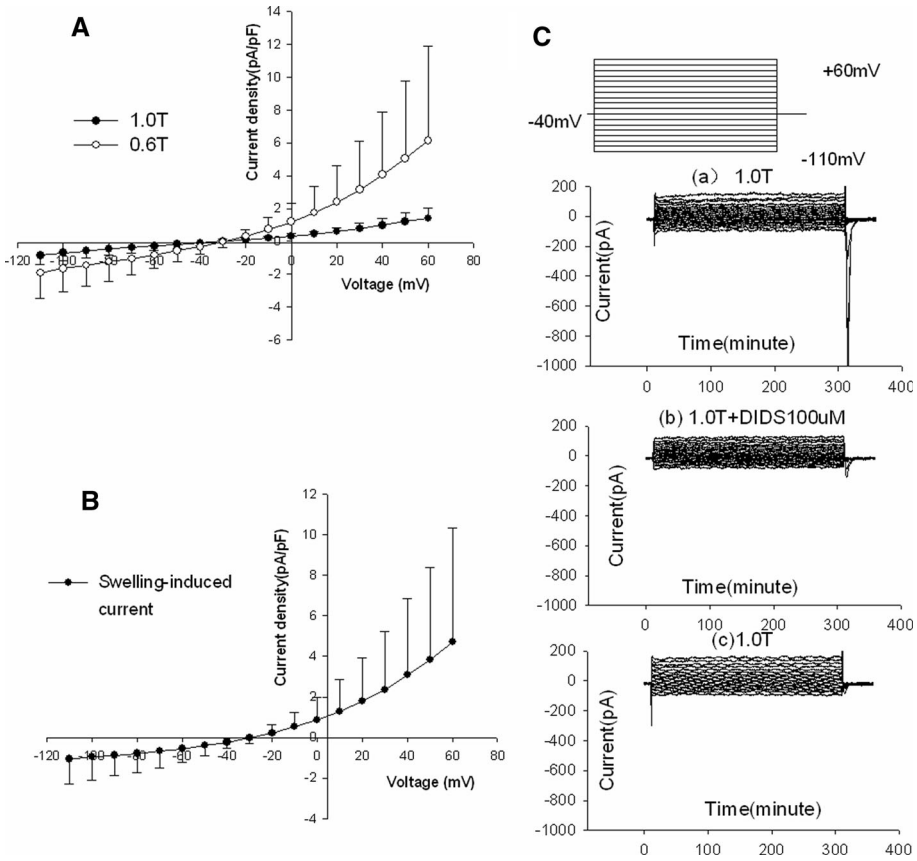


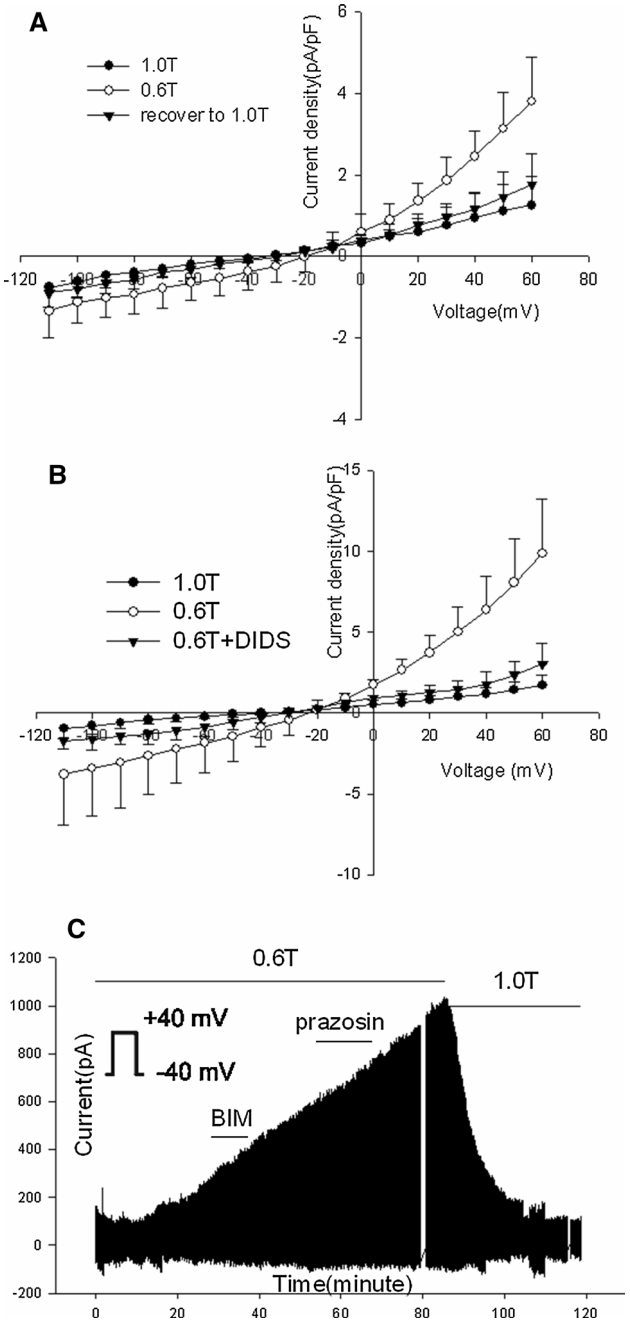
Fig. 1 The swelling-activated chloride current ($I_{Cl.swell}$). **a** The I–V relationships obtained by perfusing human atrial myocytes successively with 1.0 T (*filled circle*) and 0.6 T (*open circle*) bath. $n = 23$. **b** The I–V relationships of the swelling-induced currents. Swelling-induced currents were obtained by subtracting the current in 1.0 T from that in 0.6 T. The swelling-induced currents displayed outward rectification and had a reversal potential of approximately −27.5 mV. $n = 23$. **c** The current recorded successively in 1.0 T (**a**), 1.0 T with DIDS (**b**) and washout with 1.0 T (**c**) from a representative cell. DIDS slightly attenuated the basal current, and washout reversed the effect of DIDS. Similar phenomena were observed in another two cells tested. In this and subsequent figures illustrating original currents, the voltage protocol is 300-ms steps from −40 mV to between −110 and +60 mV (*inset*)

absolutely steady state. In one cell, even after 85.45 min, the current was still increasing over time (Fig. 2c). This phenomenon could not be due to leak currents or the activation of other currents because the swelling-induced currents could be reversed almost completely by re-immersing in 1.0 T or the addition of DIDS (Fig. 2a, b). Neither an α1-adrenoceptor antagonist (prazosin) nor a PKC inhibitor (BIM) changed the trend, which indicated that prazosin or BIM alone did not affect $I_{Cl.swell}$ (Fig. 2c, $n = 3$).

The Effect of Phenylephrine

After exposure to the hypotonic solution for more than 20 min, subsequent addition of 100 μM phenylephrine caused distinct enhancement of $I_{Cl.swell}$, and the current density increased from -1.00 ± 0.51 pA/pF at −90 mV and 2.58 ± 1.17 pA/pF at +40 mV to -1.46 ± 0.70 pA/pF and 3.84 ± 1.67 pA/pF, respectively, in 9.34 ± 2.41 min (Fig. 3b, c, $P < 0.05$, $n = 6$). Subsequent washout of phenylephrine for 20.88 ± 25.85 min did not significantly change the currents levels reached in the presence of the agonist ($P = 0.23$ at −90 mV and 0.16 at +40 mV, $n = 6$; Fig. 3a). At +40 mV, phenylephrine potentiated $I_{Cl.swell}$ at a rate of 0.1917 ± 0.1017 pA/pF.min. After washout, the rate of change decreased to 0.0365 ± 0.0745 pA/pF.min ($P < 0.05$, $n = 6$). In two cells, the washout reversed the effect of phenylephrine, and in the remaining four cells it did not, but the upward trend slowed down and at the terminal time, the currents had begun to decrease. This phenomenon might be due to the continuous increasing of $I_{Cl.swell}$. The enhancement of $I_{Cl.swell}$ by phenylephrine was distinct from what has been previously reported in rabbits (Duan et al. 1995), rats (Ichishima et al. 2010), or canines (Du and Sorota 1999). In the presence of 100 μM phenylephrine, relative cell volume was 103.8 ± 4.4 % of that in 0.6 T. Phenylephrine did not affect the relative cell volume ($n = 17$, $P = 0.40$ vs. in 0.6 T). To exclude the possibility that the effect of phenylephrine was due to the action on the basal chloride current, further experiments were performed. In all of the three cells tested, phenylephrine did not potentiate, but slightly attenuated the basal chloride current (Fig. 3d).

To confirm that the current enhanced by phenylephrine was $I_{Cl,swell}$, we tested whether restoring to isotonic solution or the addition of a chloride current inhibitor could affect the action of phenylephrine. After the current had been increased by phenylephrine, subsequent restoration to isotonic solution or addition of DIDS caused a marked decrease in $I_{Cl,swell}$ (Fig. 4, $n = 3$).



◀**Fig. 2** $I_{Cl.swell}$ was inhibited by isotonic solution or DIDS. **a** The I–V relationships of currents recorded sequentially in isotonic (1.0 T) solution (*filled circle*), hypotonic (0.6 T) solution (*open circle*) and 1.0 T (*inverted filled triangle*). Each point is the mean ± SE of six cells. **b** The I–V relationships for currents recorded sequentially in 1.0 T (*filled circle*), 0.6 T (*open circle*), and 0.6 T with 100 μM DIDS (*inverted filled triangle*). Each point is the mean ± SE of four cells. **c** Representative time course of current at +40 mV from one cell in **a**. In this and subsequent figures illustrating the time course, currents were elicited by 300-ms steps to 40 mV from −40 mV (*inset*). In this cell, the current was still increasing after 85.45 min in 0.6 T solution, and this was reversed by 1.0 T solution. Similar phenomena were observed in four of six cells tested, and the average time of perfusion in 0.6 T was 48.52 ± 35.65 min. $I_{Cl.swell}$ did reach absolute steady state in two cells, and for these cells the perfusion times in 0.6 T were 35.21 and 56.75 min. In all six cells, 1 μM prazosin or 100 nM BIM did not change the trend of $I_{Cl.swell}$

The present experiments were performed at room temperature, 21–23 °C. We also tested the effects at higher temperatures. After exposure to the hypotonic solution at room temperature and $I_{Cl,swell}$ was evoked, subsequent heating resulted in death of all the cells tested ($n = 5$, data not shown). If the solution was heated to the set temperature (35.8 °C) from the start of the procedure, phenylephrine augmented $I_{Cl,swell}$ in the surviving cells as much as at room temperature ($n = 3$, data not shown), but the cells did not survive long enough to undertake a long-term procedure.

Effects of Selective α_1-Receptor Antagonists on Phenylephrine Action

It was shown above in Fig. 2c that prazosin (1 μM), an α1-adrenoceptor antagonist, did not change the upward trend of $I_{Cl.swell}$. Figure 5 ($n = 6$) illustrates the effect of prazosin on the action of phenylephrine. When exposed to 100 μM phenylephrine, the current density of $I_{Cl.swell}$ increased to −1.38 ± 0.11 pA/pF at −90 mV and 3.37 ± 1.04 pA/pF at +40 mV in 10.61 ± 3.09 min ($P < 0.01$). After incubation with prazosin for 19.63 ± 10.28 min, the current densities at −90 and +40 mV were altered to −1.40 ± 0.10 pA/pF and 3.42 ± 1.02 pA/pF, respectively, and the changes were not statistically significant ($P = 0.57$ at −90 mV and 0.46 at +40 mV). At +40 mV, phenylephrine potentiated $I_{Cl.swell}$ at a rate of 0.2056 ± 0.1135 pA/pF min. After addition of prazosin, the rate of change decreased to 0.0213 ± 0.0587 pA/pF min ($P < 0.05$). $I_{Cl.swell}$ decreased in two cells and increased in the remaining four cells. Subsequent exposure to a superfusate containing the same concentration of phenylephrine in the absence of prazosin resulted in increases at both −90 and +40 mV, up to −1.56 ± 0.24 and 4.32 ± 0.70 pA/pF ($P < 0.01$), respectively (Fig. 5a, b). Application of phenylephrine in the presence of 1 μM prazosin still enhanced $I_{Cl.swell}$ concentration dependently, but the concentration–response curve was shifted rightward ($E_{max} = 2.02 \pm 0.13$, $EC_{50} = 17.03 \pm 9.97$ μM without prazosin and $E_{max} = 2.36 \pm 1.30$, $EC_{50} = 331.89 \pm 103.45$ μM in the presence of prazosin). The calculated IC_{50} of prazosin was 0.05 μM (Fig. 5c). The α_1-adrenoceptor antagonist prazosin attenuated the effect of phenylephrine, which supported the hypothesis that phenylephrine regulated $I_{Cl.swell}$ by activating α1-adrenoceptors.

Effects of PKC Inhibitor

Because many of the effects of α_1-adrenergic agonists are linked to the activation of PKC, we tested whether inhibition of endogenous PKC would block the effect of

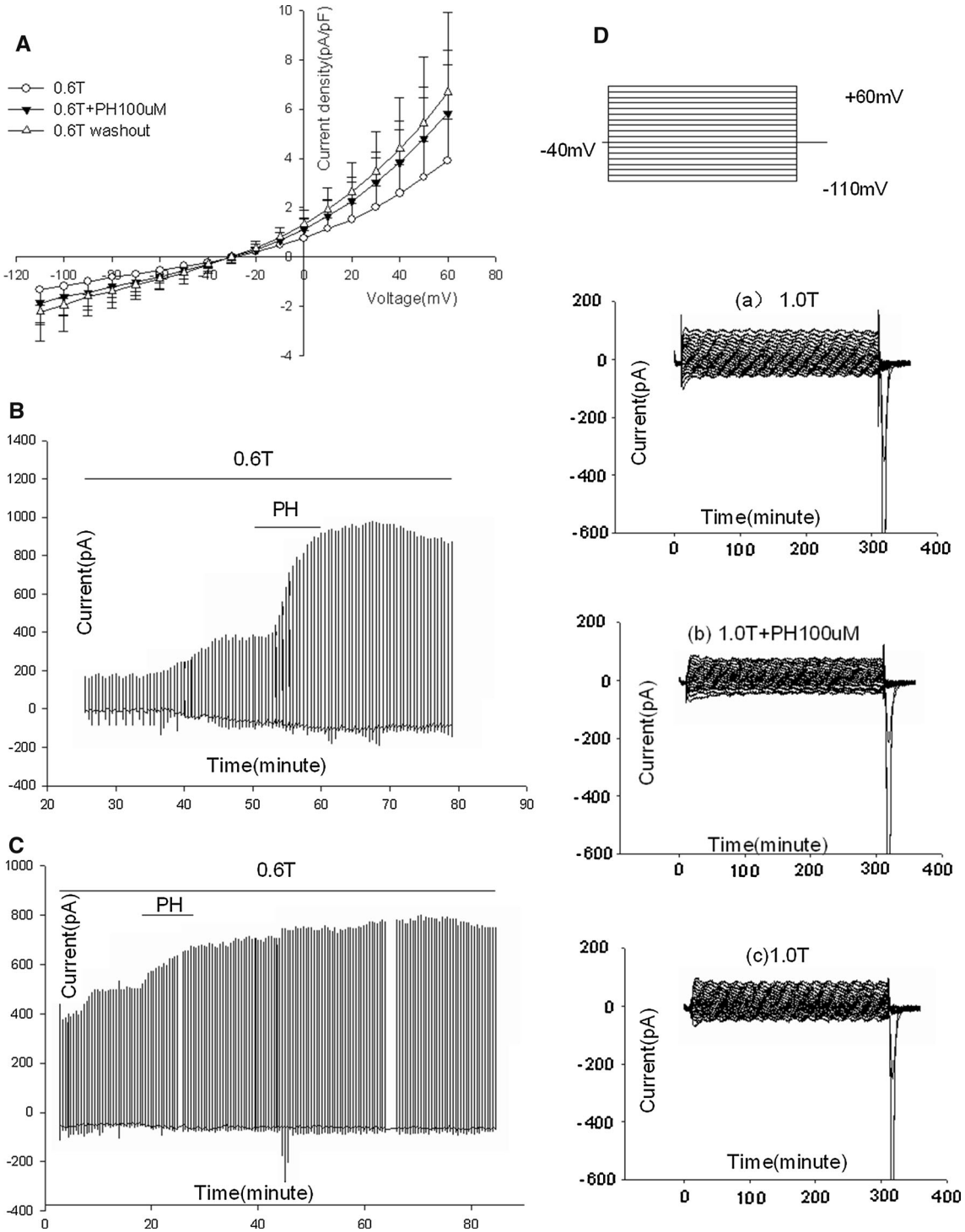


Fig. 3 Effect of phenylephrine. **a** I–V relationships for currents recorded during sequential infusion with 0.6 T (*open circle*), 0.6 T with 100 μM phenylephrine (PH, *inverted filled triangle*) and after washout (*open triangle*). Each point is the mean ± SE of six cells. **b**, **c** Time course of current at +40 mV. **b** is from a representative cell in which washout reversed the effect of PH (similar phenomena were observed in two of six cells). **c** is from a cell in which washout did not reverse the effect. Notice that in C the upward trend slowed down after washout, and at the terminal time the current had begun to decrease. Similar phenomena were observed in four cells. **d** The original currents recorded sequentially in 1.0 T (**a**), 1.0 T with 100 μM PH (**b**), and washout with 1.0 T (**c**) from a representative cell. PH slightly attenuated the basal current, and washout did not reverse the effect of PH. Similar phenomena were observed in two other cells

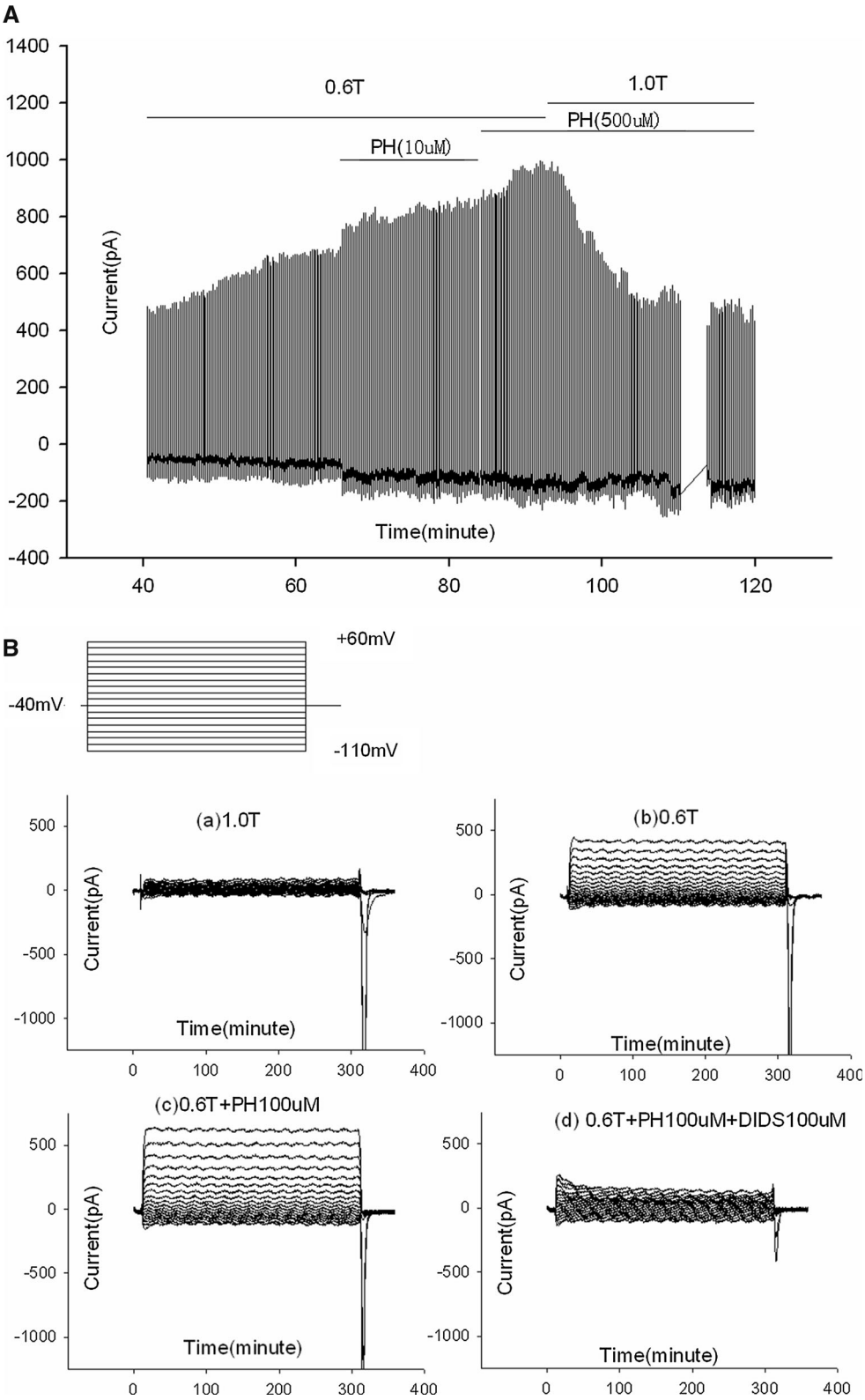
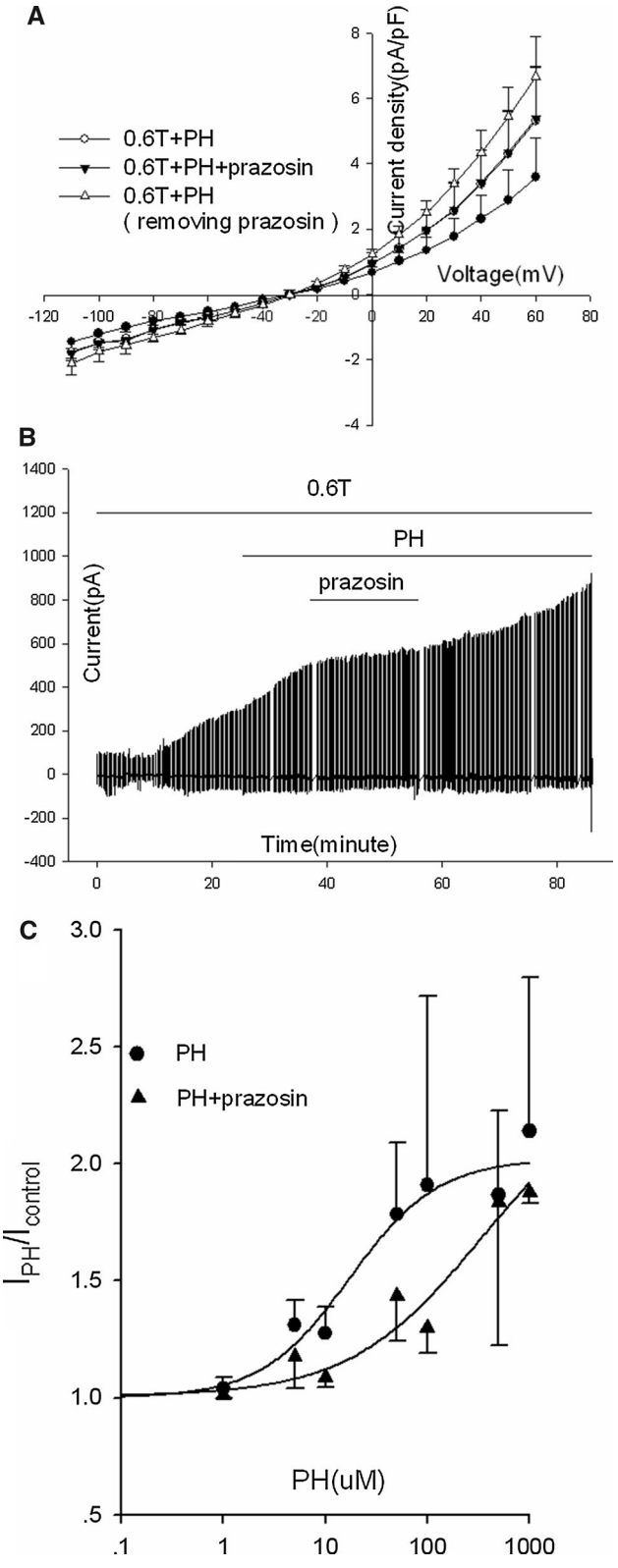


Fig. 4 Effects of phenylephrine were reversed by restoring to isotonic solution or addition of DIDS. **a** Time course of currents at +40 mV from a representative cell in which the effect of phenylephrine (PH) was reversed after restoring to isotonic solution. **b** The original currents recorded sequentially in 1.0 T (**a**), 0.6 T (**b**), and 0.6 T with 100 μM PH in the absence (**c**) or presence (**d**) of 100 μM DIDS. DIDS almost completely reversed the effect of PH. Similar phenomena were observed in two other cells

phenylephrine. It was shown in Fig. 2c that BIM, a PKC inhibitor, did not change the upward trend of $I_{Cl.swell}$. In Fig. 6 ($n = 5$), after phenylephrine (100 μM) induced a substantial increase of $I_{Cl,swell}$ ($P < 0.01$) in 9.25 ± 1.70 min, subsequent application of BIM (100 nM) attenuated the current density in 11.51 ± 2.89 min from −2.85 ± 1.86 pA/pF at



◀**Fig. 5** The effects of prazosin on phenylephrine action. **a** I–V relationships for currents recorded during sequential infusion of 0.6 T with 100 μM phenylephrine (PH) alone (*open circle*) or plus 1 μM prazosin (*inverted filled triangle*), and 0.6 T with 100 μM PH removing prazosin (*open triangle*). Each point is the mean ± SE of six cells. **b** Time course of currents at +40 mV from a representative cell in which prazosin (1 μM) did not reverse, but attenuated the effect of PH (100 μM). **c** Concentration–response curves for PH at +40 mV in the absence (*filled circle*, seven cells) or presence (*filled triangle*, four cells) of 1 μM prazosin. $I_{PH}/I_{control}$ is the ratio of the current in the presence of PH to the control current without PH. PH concentration-dependently enhanced $I_{Cl.swell}$ ($E_{max} = 2.02 \pm 0.13$ and $EC_{50} = 17.03 \pm 9.97$ μM). Prazosin (1 μM) shifted the dose–effect curve rightward ($E_{max} = 2.36 \pm 1.30$, $EC_{50} = 331.89 \pm 103.45$ μM). The IC_{50} of prazosin was 0.05 μM

−90 mV and 6.83 ± 3.12 pA/pF at +40 mV to −2.68 ± 1.50 pA/pF and 6.38 ± 2.78 pA/pF, respectively. The changes were not statistically significant ($P = 0.21$ at −90 mV and $P = 0.09$ at +40 mV). At +40 mV, phenylephrine potentiated $I_{Cl.swell}$ at a rate of 0.2731 ± 0.1550 pA/pF min. After addition of BIM, the rate of change decreased to −0.1124 ± 0.0932 pA/pF min ($P < 0.05$). In four cells, BIM attenuated the effect of phenylephrine, and in a fifth cell, the currents increased moderately, but the upward trend slowed down and at the terminal time the current had begun to decrease. Subsequent exposure to a superfusate containing the same concentration of phenylephrine in the absence of BIM resulted in an increase of $I_{Cl,swell}$ up to −3.19 ± 1.79 pA/pF at −90 mV and 7.65 ± 3.08 pA/pF at +40 mV ($P < 0.05$; Fig. 6a, b). Our data suggested that BIM, an inhibitor of PKC, could attenuate the effect of phenylephrine.

The Effect of PKC Activators on $I_{Cl.swell}$

To further assess whether the phenylephrine-induced increase in $I_{Cl.swell}$ was mediated by the activation of PKC, we examined the effects of PDBu, a PKC activator. As shown in Fig. 7a, b ($n = 6$), the current densities before and after application of PDBu (500 nM) were −1.69 ± 1.67 and −2.41 ± 1.95 pA/pF at −90 mV, and 5.58 ± 6.36 and 7.05 ± 6.99 pA/pF at +40 mV. PDBu induced a substantial increase of $I_{Cl.swell}$ during a period of 10.99 ± 3.58 min ($P < 0.01$ at −90 mV and $P < 0.05$ at +40 mV). After 14.26 ± 9.35-min washout, the current densities were altered to −2.68 ± 1.30 pA/pF at −90 mV and 6.84 ± 3.31 pA/pF at +40 mV, and the changes were not statistically significant. At +40 mV, PDBu potentiated $I_{Cl.swell}$ at a rate of 0.1980 ± 0.0852 pA/pF min. After washout, the rate of change decreased to −0.0215 ± 0.0071 pA/pF min ($P < 0.05$). Following perfusion with PDBu once more, the current density of $I_{Cl,swell}$ increased to −3.34 ± 1.51 pA/pF at −90 mV and 7.75 ± 3.53 pA/pF at +40 mV ($P < 0.05$). Therefore, in the present study, PDBu, an activator of PKC, mimicked the effect of phenylephrine.

In the subsequent experiment (Fig. 7c, $n = 3$), we found that PDBu did not potentiate, but slightly attenuated the basal chloride current in all of the three cells tested, which

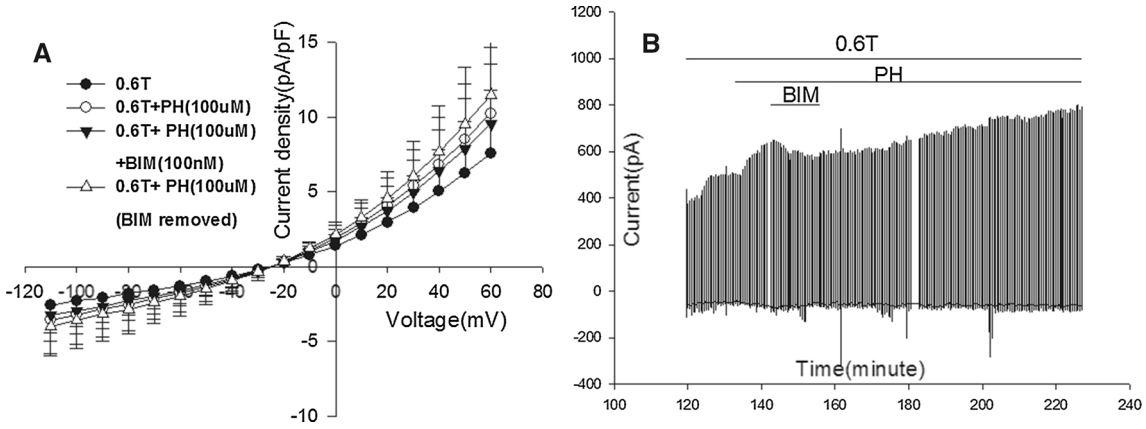


Fig. 6 Effects of BIM on the action of phenylephrine. **a** I–V relationships for currents recorded sequentially in 0.6 T (*filled circle*), 0.6 T with 100 μM phenylephrine (PH, *open circle*) or PH plus 100 nM BIM (*inverted filled triangle*), and 100 μM PH without BIM in 0.6 T (*open triangle*). Each point is the mean ± SE of five cells. **b** Time course of currents at +40 mV from a representative cell in which BIM (100 nM) attenuated the effect of PH (100 μM)

excludes the possibility that the effect of PDBu on $I_{Cl.swell}$ was the result of its action on the basal chloride current.

Discussion

Continuous Increase of $I_{Cl,swell}$ in Human Atrial Myocytes

In our study, it was difficult to achieve an absolute steady state of $I_{Cl.swell}$ in human atrial myocytes. $I_{Cl.swell}$ increased over time in most of the cells tested. This made it difficult to assess the effects of drugs. We measured the rate of change in the current and the modification of this rate by the drugs, and then perfused the cells once more with drug-free solutions. However, in some cases, such as the assessment of the concentration–response curve, there was no way to take into account the deviation of the current over time.

Phenylephrine Enhanced $I_{Cl.swell}$ in Human Atrial Myocytes

There have been inconsistent reports about the effect of phenylephrine in cardiac cells. Studies have shown that phenylephrine inhibits $I_{Cl.swell}$ in rabbit atrial myocytes (Duan et al. 1995) and in mouse ventricular cells (Ichishima et al. 2010), but does not affect $I_{Cl.swell}$ in canine atrial cells (Du and Sorota 1999). In the present study, phenylephrine potentiated $I_{Cl,swell}$ in human atrial myocytes by activating the α1-adrenoceptor. The reason for these discrepancies is unknown and the differences in tissues and species could be a factor, but differences in methodologies might also contribute to the phenomenon.

In the previous studies, the recording temperatures were 30 ± 1 °C for rabbit atrial myocytes (Duan et al. 1995), 36 ± 1 °C for canine atrial cells (Du and Sorota 1999) and 36.5 ± 0.5 °C for mouse ventricular cells (Ichishima et al. 2010). The present experiments were performed at room temperature, 21–23 °C. At a higher temperature (35.8 °C), we managed to test the effect of phenylephrine in three cells, and found that phenylephrine augmented $I_{Cl,swell}$ as well as it did at room temperature, but the cells did not survive long enough to enable longer-term experiments to be carried out.

In addition, it was reported that in rabbit portal vein myocytes noradrenaline enhanced $I_{Cl.swell}$ by acting on α1-adrenoceptors and reduced $I_{Cl.swell}$ by stimulating β-adrenoceptors (Ellershaw et al. 2002). In some reports, the high concentration of phenylephrine [100 μM in canine atrial cells (Du and Sorota 1999) and mouse ventricular cells (Ichishima et al. 2010), mostly 800 μM in rabbit atrial myocytes (Duan et al. 1995), might have effectively competed with the relatively low concentration of propranolol (1 μM in rabbit atrial myocytes (Duan et al. 1995) and mouse ventricular cells (Ichishima et al. 2010), 300 nM in canine atrial cells (Du and Sorota 1999)]. It is possible that the activation of β-adrenoceptors by high concentrations of phenylephrine might inhibit $I_{Cl.swell}$, which might be the reason that phenylephrine could inhibit $I_{Cl.swell}$ in some reports.

In our experiments, we found that $I_{Cl.swell}$ increased over time, but the effect of phenylephrine could not be due to the sustained increasing of $I_{Cl.swell}$, because the current increased notably and the washout slowed down the upward trend, and at the terminal time the currents began to decrease. Furthermore, an α1-receptor antagonist and PKC inhibitor attenuated the effect of phenylephrine, which

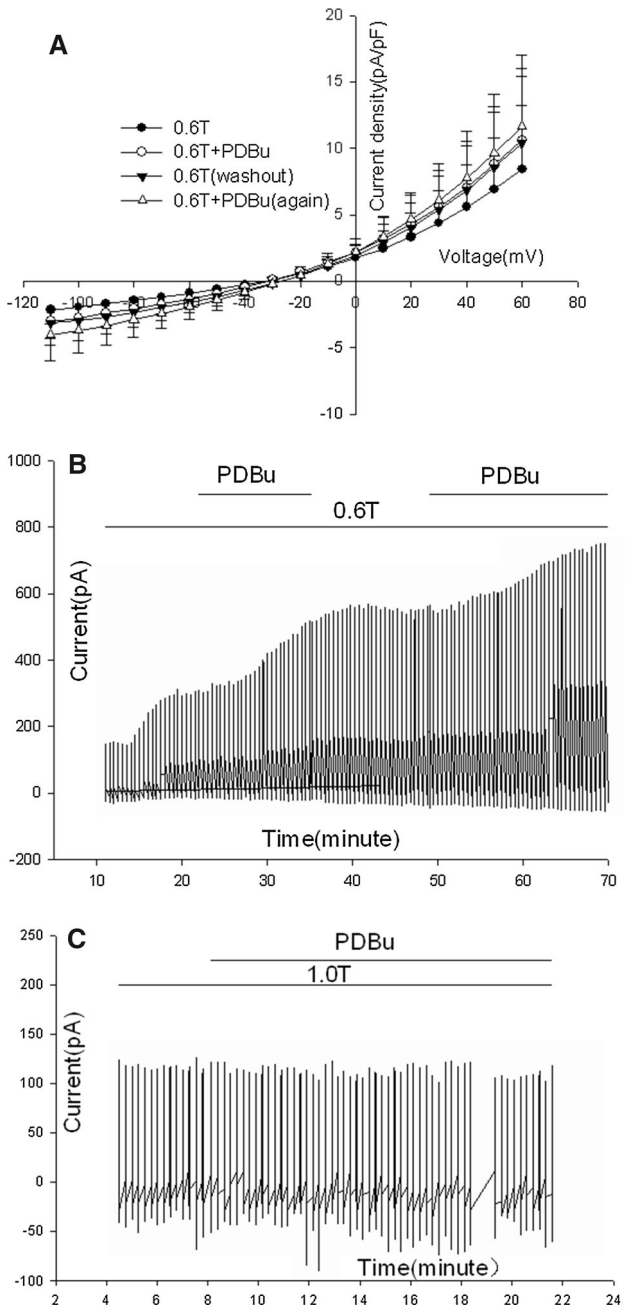


Fig. 7 The effects of PDBu. **a** I–V relationships for currents recorded during sequential infusion with 0.6 T (*filled circle*), 0.6 T with 500 nM PDBu (*open circle*) and after washout (*filled inverted triangle*), and 0.6 T with PDBu once more (*open triangle*). Each point is the mean ± SE of recordings from six cells. **b** Time course of currents at +40 mV from a representative cell in which PDBu (500 nM) enhanced $I_{Cl.swell}$, which decreased after washout and increased again when superfused again with PDBu. **c** Time course of currents at +40 mV from a representative cell where PDBu did not potentiate, but instead slightly attenuated the basal chloride current. Similar phenomena were observed in another two cells tested

should have increased the current more obviously if the continuous increasing of $I_{Cl.swell}$ accounted for the phenomenon, whereas in fact phenylephrine inhibited $I_{Cl.swell}$.

Considering the E_{Cl} value in cardiac cells under physiological conditions, $I_{Cl.swell}$ induces an outward current at depolarized potentials and an inward current at resting potential. Because $I_{Cl.swell}$ displays marked outward rectification, its activation may induce a much more profound effect on action potential duration (APD) compared with its effects on the resting membrane potential. The activation of $I_{Cl.swell}$ may perpetuate re-entry by shortening the refractory period. In principle, this favors the development of atrial or ventricular fibrillation, which depends on the presence of multiple re-entrant circuits or rotating spiral waves (Du and Sorota 1997; Vandenberg et al. 1997; Hume et al. 2000; Baumgarten and Clemo 2003). During acute myocardial ischemia and heart failure, cell swelling and/or stretch can activate $I_{Cl.swell}$ and catecholamine concentrations increase. Under these circumstances, atrial or ventricular fibrillation often occurs and α1-adrenergic activation of $I_{Cl.swell}$ may play a role in this.

Phenylephrine Stimulates $I_{Cl,swell}$ in a PKC-dependent Manner

In the heart, the most extensively documented signaling responses to α1-adrenoceptor stimulation are mediated via PLC-induced hydrolysis of PIP2, giving rise to a variety of potential second messengers, including inositol trisphosphate (IP-3), and 1,2-diacylglycerol (DAG) (Brown et al. 1985; Steinberg et al. 1985; Otani et al. 1988; Fedida et al. 1993), which is thought to be an endogenous activator of PKC (Exton 1988; Fedida et al. 1993). In the present study, the effect of phenylephrine was attenuated by prazosin or BIM, and could be mimicked by the PKC activator, PDBu. The data, therefore, indicated that phenylephrine might mediate $I_{Cl,swell}$ in a PKC-dependent manner in human atrial myocytes.

Demion et al. reported that PKC activators [PDBu or phorbol 12-myristate 13-acetate (PMA)] increased $I_{Cl,swell}$ in human atrial myocytes (Demion et al. 2006), which is consistent with our results. Duan et al. reported that in rabbit atrial cells, phenylephrine inhibits $I_{Cl,swell}$ by interacting with an α_1-adrenoceptor mechanism that is coupled to PKC via a pertussis toxin-sensitive G-protein (Duan et al. 1995). All of the above support the hypothesis that phenylephrine might regulate $I_{Cl,swell}$ through a PKC-dependent pathway in the heart.

However, the effect of phenylephrine is not always consistent with activation of PKC. In mouse ventricular cells, phenylephrine inhibited $I_{Cl,swell}$ by activating PLC through a Gq protein-dependent process (Ichishima et al. 2010), whereas PKC stimulates $I_{Cl,swell}$ in the same species (Gong et al. 2004). In dog atrial cells, phenylephrine did not affect $I_{Cl.swell}$ but PKC stimulated it (Du and Sorota 1999). It was indicated that α_1-agonist activated the novel

PKC isoforms, such as PKC-δ and PKC-ε, in mouse cardiac myocytes (Bogoyevitch et al. 1993; Rohde et al. 2000). In contrast, PDBu or PMA could activate conventional PKC isoforms such as α, in addition to the δ and ε isoforms (Rybin and Steinberg 1994). The lack of effect of phenylephrine on $I_{Cl,swell}$ in some species could indicate either that PKC-δ or PKC-ε did not stimulate $I_{Cl,swell}$ or that the duration or extent of α-agonist-induced PKC activation was insufficient to stimulate $I_{Cl,swell}$. As a result, α1-adrenoceptors could induce a PKC-dependent stimulatory effect that was counteracted by the inhibitory signal transduction pathway. It is possible that the divergent effects of phenylephrine are because of the different complements of PKC isoforms present in different tissue and species.

Conclusion

In our experiments, phenylephrine increased $I_{Cl,swell}$ in human atrial myocytes by activating α1-adrenoceptors, a result, which was distinct from previous reports in other species. Differences in the species studied and the methodologies used might explain the discrepancies. The effect was attenuated by BIM, and could be mimicked by PDBu, which indicated that phenylephrine might mediate $I_{Cl,swell}$ in a PKC-dependent manner.

Acknowledgments This work was supported by the Scientific Research Foundation for Returned Scholars, Ministry of Education of China, (2004) No. 527 and the Project on Social Development, Department of Science and Technology of Guizhou Province, China, (2011) No. 040.

Conflict of interest Authors Yetao Li and Xinling Du declare that they have no conflict of interest.

Human Studies Consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (the Ethics Committee of Wuhan Union Hospital, Huazhong University of Science and Technology, China) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

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