The effects of monocarboxylic acid-derived Cl\textsuperscript{−} channel blockers on cardiac depolarization-activated K\textsuperscript{+} currents were investigated. Membrane currents in rat ventricular myocytes were recorded using the whole-cell configuration of the patch-clamp technique. 5-Nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and niflumic acid (NFA) induced an outward current at 0 mV. Both NPPB and NFA failed to induce any current when used intracellularly or after K\textsuperscript{+} in the bath and pipette solutions was replaced by equimolar Cs\textsuperscript{+}. Voltage pulse protocols revealed that NPPB and NFA enhanced the steady-state K\textsuperscript{+} current but inhibited the transient outward K\textsuperscript{+} current. Genistein, a tyrosine kinase (PTK) inhibitor, inhibited NPPB- and NFA-induced outward current. Another PTK inhibitor, lavendustin A, produced a comparable effect. In contrast, the inactive analogue of genistein, daidzein, was ineffective. Orthovanadate, a tyrosine phosphatase inhibitor, markedly slowed the deactivation of the outward current induced by NPPB and NFA. The protein kinase A (PKA) inhibitor H-89 inhibited NPPB-induced outward current at 0 mV. In contrast, the protein kinase C (PKC) inhibitor H-7 was without significant effect on the action of NPPB. Pretreatment of the myocytes with genistein or H-89 prevented the enhancing effect of NPPB. Increasing intracellular Cl\textsuperscript{−} from 22 to 132 mM slightly reduced NPPB-induced outward current at 0 mV. These results demonstrate that the monocarboxylic acid-derived Cl\textsuperscript{−} channel blockers NPPB and NFA enhance cardiac steady-state K\textsuperscript{+} current, and suggest that the enhancing effect of the Cl\textsuperscript{−} channel blockers is mediated by stimulation of PKA and PTK signalling pathways.

(Received 15 January 2007; accepted after revision 12 February 2007; first published online 15 February 2007)

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Cardiac action potentials reflect changes in membrane conductance governed by numerous different ion channels and electrogenic exchangers. Among the cardiac ion channels, depolarization-activated K\textsuperscript{+} channels play important roles in the repolarization of the action potential of cardiac myocytes. Changes in the function of depolarization-activated K\textsuperscript{+} channels may affect cardiac action potential repolarization and lead to the generation of life-threatening arrhythmias (Nerbonne & Kass, 2005). Therefore, depolarization-activated K\textsuperscript{+} channels are potent targets for drugs.

Chloride channel blockers are notorious for their non-specific effect, i.e. they can profoundly affect cation channels (Hume et al. 2000). In the heart, Cl\textsuperscript{−} channel blockers are found to inhibit Ca\textsuperscript{2+}-independent transient outward K\textsuperscript{+} current (I\textsubscript{to}; Lefevre et al. 1996), L-type Ca\textsuperscript{2+} current (I\textsubscript{Ca,L}; Conforti et al. 1994; Zhou et al. 2002) and Na\textsuperscript{+} current (Conforti et al. 1994; Zhou et al. 2005). However, how Cl\textsuperscript{−} channel blockers affect cation channels is still unclear. Monocarboxylic acid-derived Cl\textsuperscript{−} channel blockers, such as 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) and niflumic acid (NFA), are frequently used in the investigation of cardiac Cl\textsuperscript{−} channels. Both NPPB and NFA were found to inhibit cardiac I\textsubscript{Ca,L} (Zhou et al. 2002) and Na\textsuperscript{+} channels.

S-S. Zhou and L-B. Zhang contributed equally to this work.
Moreover, Cl⁻ substitution also has a profound influence on cardiac \( I_{\text{to}} \) \cite{Lefevre1996;Lai2004} and \( I_{\text{Ca,L}} \) \cite{Zhou2002;Lai2004}. Baker et al. \cite{Baker2004} recently found that a voltage-dependent anion-selective channel, located in the plasma membrane \cite{Lawen2005}, can function as an enzyme. This finding raises the possibility that the Cl⁻ channel per se may affect intracellular signalling pathways.

In the present study, we further investigated the effects of monocarboxylic acid-derived Cl⁻ channel blockers on the depolarization-activated outward K⁺ currents. We found that the monocarboxylic acid-derived Cl⁻ channel blockers NPPB and NFA enhanced the steady-state component (\( I_{\text{SS}} \)) of the delayed rectifier K⁺ current (\( I_{\text{K1}} \)), but inhibited \( I_{\text{to}} \) in rat ventricular myocytes. The enhancing effect of NPPB and NFA was antagonized by the protein tyrosine kinase (PTK) inhibitors genistein and lavendustin A and by the protein kinase A (PKA) inhibitor H-89. Inhibition of protein tyrosine phosphatase (PTP) with sodium orthovanadate (VO₄) markedly slowed the deactivation of the enhanced outward K⁺ conductance after withdrawal of NPPB and NFA. Elevation of intracellular Cl⁻ concentration ([Cl⁻]ᵢ) slightly reduced NPPB-induced outward current at 0 mV. We conclude that NPPB and NFA activate cardiac \( I_{\text{SS}} \), probably by stimulating PKA and PTK signalling pathways.

Methods

Cell preparations

All experiments were conducted in accordance with the guidelines of the local ethics committee. Adult Sprague-Dawley rats (200–250 g) were anaesthetized with urethane (1.5 g kg⁻¹ i.p.). The trachea was intubated to permit artificial ventilation with room air using a ventilator (model I, Jiangwan, Shanghai, China). The chest was opened under artificial ventilation, and the aorta was cannulated in situ. The hearts were excised rapidly and retrogradely perfused at 37°C, using methods similar to those previously described \cite{Zhou2002}, with the following solutions in turn: Tyrode solution (5 min); \( \text{Ca}^{2+} \)-free Tyrode solution (5 min); \( \text{Ca}^{2+} \)-free Tyrode solution with 0.5 mg ml⁻¹ collagenase Type II (Gibco, Grand Island, NY, USA) and 1 mg ml⁻¹ bovine serum albumin (BSA; 35 min); and Kraftbrühe (KB; high-K⁺) solution (5 min). After dissociation and collection, the cells were kept in KB solution at room temperature (23–25°C) for electrophysiological recordings.

Whole-cell patch-clamp experiments

Aliquots of cell suspension were transferred into a perfusion chamber on the stage of an inverted microscope. Pipettes had tip resistances of 2.0–2.5 MΩ when filled with internal solution. Whole-cell recordings were performed at room temperature (23–25°C) using an EPC-10 patch-clamp amplifier and Pulse software (HEKA Elektronik, Lambrecht, Germany). The offset potentials between both electrodes were zeroed before the pipette touched the cell. The liquid junction potential between the pipette and the bath solutions was calculated by using the JPCalc program within Clampex 8.1 (Axon Instruments, Inc.) and corrected for each recording. The \( I_{\text{to}} \) was elicited by 300 ms pulses from a holding potential of −50 mV to test potentials ranging from −40 to +50 mV in 10 mV increments. Since currents activated from a holding potential of −20 mV reflect activation of \( I_{\text{SS}} \) in rat ventricular myocytes \cite{Himmel2002;Komukai2002}, in the present study, \( I_{\text{SS}} \) was activated from a holding potential of −20 mV to test potentials ranging from −70, −90 or −100 to +50 mV in 10 mV increments. In most experiments, single cardiac myocytes were voltage clamped at 0 mV to continuously monitor the effects of NPPB and NFA. The current signals were low-pass filtered at 5 kHz and stored in the hard disk of a compatible computer. Internal application of NPPB and NFA was performed by using the intrapipette perfusion technique as described in a previous study \cite{Zhou1997}.

Solutions

The Tyrode solution contained (mm): NaCl, 143; KCl, 5.4; MgCl₂, 0.5; CaCl₂, 1.8; NaH₂PO₄, 0.3; glucose, 5; and Heps, 5 (pH was adjusted to 7.4 with NaOH). The nominally \( \text{Ca}^{2+} \)-free Tyrode solution was made by omitting CaCl₂ from the standard Tyrode solution. The KB solution contained (mm): potassium glutamate, 70; KCl, 25; taurine, 20; KH₂PO₄, 10; MgCl₂, 3; EGTA, 0.5; glucose, 10; and Heps, 10 (pH was adjusted to 7.35 with KOH). The standard pipette solution for recording cardiac K⁺ currents contained (mm) potassium aspartate, 110; KCl, 20; MgCl₂, 1; Na₂-phosphocreatine, 5; Mg-ATP, 5; EGTA, 5; and Heps, 10 (pH was adjusted to 7.2 with KOH). The bath solution contained (mm): NaCl, 140; KCl, 5; MgCl₂, 1; KH₂PO₄, 0.4; CaCl₂, 1.8; CdCl₂, 0.5; glucose, 10; and Heps, 5 (pH was adjusted to 7.4 with NaOH). Cadmium chloride was used to inhibit the \( I_{\text{Ca,L}} \) and the \( \text{Ca}^{2+} \)-activated Cl⁻ current, and Ba²⁺ (1 mm) was added to the bath solution to block inward rectifier K⁺ current (\( I_{\text{K1}} \); Zygmunt et al. 1997). In some experiments, K⁺ in the pipette and bath solutions was replaced by equimolar Cs⁺ to eliminate K⁺ currents, and aspartate in the standard pipette solution was replaced by equimolar Cl⁻.

Chemicals

All chemicals were purchased from Sigma (St Louis, MO, USA) except for H-7 (RBI, Natick, MA, USA) and
H-89 (Biomol Research Laboratory Inc., Plymouth Meeting, PA, USA). Stock solutions of NPPB (100 or 500 mM), NFA (100 mM), genistein (100 mM), daidzein (100 mM), lavendustin A (20 mM), H-89 (20 mM) and H-7 (40 mM) in DMSO were diluted to the desired final concentrations immediately before use. DMSO (0.2%) alone did not affect the cardiac membrane conductance.

**Statistical analysis**

The data are presented as means ± s.d. Statistical differences in the data were evaluated by Student’s paired t test or ANOVA as appropriate, and were considered significant at values of $P < 0.05$.

**Results**

**Effects of monocarboxylic acid-derived Cl$^-$ channel blockers on the membrane conductance of rat ventricular myocytes**

When the myocytes were held at 0 mV, bath application of monocarboxylic acid-derived Cl$^-$ channel blocker NPPB (25 μM) significantly increased the outward conductance from a basal level of 1.1 ± 0.5 pA pF$^{-1}$ to a peak of 50.3 ± 26.4 pA pF$^{-1}$ ($n = 6, P < 0.01$, Fig. 1A). A similar result was obtained by using another monocarboxylic acid-derived Cl$^-$ channel blocker, NFA (Fig. 1B). After exposure of the myocytes to 50 μM NFA, the outward conductance at 0 mV developed from a basal level of 2.1 ± 1.7 pA pF$^{-1}$ to a peak of 44.3 ± 22.8 pA pF$^{-1}$ ($n = 7, P < 0.01$). Moreover, internal application of high concentrations of NPPB (500 μM, $n = 5$, Fig. 1C) or NFA (100 μM, $n = 4$, Fig. 1D) did not induce any significant change in the membrane conductance. To identify the charge carrier of the current induced by NPPB and NFA, we replaced K$^+$ in the internal and external solutions with equimolar Cs$^+$. The present results showed that either NPPB ($n = 4$, Fig. 1E) or NFA ($n = 4$, Fig. 1F) did not induce any change in the membrane conductance at 0 mV after Cs$^+$ substitution for K$^+$. These data suggest that the outward current induced by NPPB and NFA is a K$^+$ current.

**Effects of NPPB and NFA on depolarization-activated outward currents**

We then observed the effect of NPPB and NFA on depolarization-activated outward currents by using a depolarizing step-pulse protocol. The data showed that NPPB ($n = 6$) and NFA ($n = 4$) abolished $I_{to}$ but enhanced the sustained component of outward K$^+$ current (Fig. 2). To further identify the current enhanced by NPPB and NFA, we observed the effect of the Cl$^-$ channel blockers on the outward current after $I_{to}$ was inactivated by using a holding potential of $-20$ mV (see references in Komukai et al. 2002). The data showed that NPPB increased the outward current from 7.1 ± 6.3 to 53.6 ± 19.4 pA pF$^{-1}$ at +50 mV ($n = 6, P < 0.01$, Fig. 3A), and that NFA enhanced the outward current...
from $2.2 \pm 1.0$ to $45.2 \pm 25.2$ pA pF$^{-1}$ at $+50$ mV ($n = 6$, $P < 0.01$, Fig. 3B). The enhanced current produced by NPPB and NFA exhibited an outward rectifying property (Fig. 3Ad and Bd). Substitution of $K^+$ in the bath and pipette solutions with equimolar Cs$^+$ abolished the enhancing effect of NPPB ($n = 4$, Fig. 3Cb) and NFA ($n = 4$, Fig. 3Cc) on the outward conductance. We further examined the shift of the reversal potential of NPPB- and NFA-enhanced current recorded in different intracellular $K^+$ concentrations ([K$^+$]$_i$). In control conditions ([K$^+$]$_i$ = 130 mM, [K$^+$]$_o$ = 5.4 mM), NPPB- and NFA-sensitive currents reversed at $-76.2 \pm 9.5$ mV ($n = 9$) and $-75.7 \pm 3.6$ mV ($n = 6$), respectively (Fig. 4E and F), which were near the $K^+$ equilibrium potential given by the Nernst equation ($-82.2$ mV). Reduction of [K$^+$]$_i$, resulted in a reduction of NPPB- and NFA-induced current, with a rightward shift of the reversal potential. After intracellular $K^+$ was decreased to 65 or 30 mM by partly replacing intracellular $K^+$ with Cs$^+$, NPPB-sensitive current reversed at $-55.5 \pm 3.5$ (n = 12) and $-33.6 \pm 4.7$ mV (n = 5), respectively (Fig. 4A, B and E), and NFA-sensitive current reversed at $-54.2 \pm 4.2$ (n = 7) and $-37.8 \pm 2.8$ mV (n = 5), respectively (Fig. 4C, D and F), which were close to the predicted $K^+$ equilibrium potentials ($-64.3$ mV in 65 mM [K$^+$]$_i$ and $-44.3$ mV in 30 mM [K$^+$]$_i$). These data indicate that the outward current sensitive to NPPB and NFA is $I_{SS}$.

### Effects of PTK and PTP inhibitors on the current induced by NPPB and NFA

To explore the mechanism of action of the Cl$^-$ channel blockers, we observed whether protein tyrosine phosphorylation was involved in the process. The results showed that genestein, a PTK inhibitor, decreased NPPB- and NFA-induced outward current at 0 mV by $87.7 \pm 2.4$ ($n = 5$, $P < 0.01$, Fig. 5A) and $78.7 \pm 3.2$% ($n = 5$, $P < 0.01$, Fig. 5C), respectively. In contrast, the inactive analogue of genistein, daidzein, was without significant effect ($n = 4$, Fig. 5B). Pretreating the myocytes with the PTK inhibitor genistatin (100 $\mu$m) prevented the enhancing effect of NPPB (Figs 5D, n = 3). To further examine the involvement of PTK, we tested the effect of another PTK inhibitor, lavendustin A. The results showed that lavendustin A (100 $\mu$m) reduced NPPB- and NFA-enhanced outward current by $83.8 \pm 12.5$% ($n = 4, P < 0.01$, Fig. 6A) and $71.7 \pm 17.4$% ($n = 5, P < 0.01$, Fig. 6B), respectively. It seems that the enhancing effect of NPPB and NFA on $I_{SS}$ is mediated by PTK activation. If that is the case, inhibition of PTP may affect the deactivation of the enhanced $K^+$ conductance after withdrawal of the compounds. To test this possibility, we observed the effect of PTP inhibitor orthovandate (VO$^4$) on the deactivation of NPPB- and NFA-induced outward current at 0 mV. In control conditions, the enhanced outward $K^+$ conductance...
Chloride channel blockers affect cardiac outward potassium currents

returned to the basal level within 3 min after washout of NPPB or NFA (Fig. 1A and B). In contrast, in the presence of extracellular and intracellular VO4 (1 mM), the deactivation of the K+ current became incomplete after washout of NPPB and NFA (Fig. 7). These data suggest that a PTK signalling pathway may be involved in the enhancement of the outward current induced by the Cl− channel blockers.

Effects of PKA and PKC inhibitors on NPPB- and NFA-sensitive cardiac K+ currents

To determine whether other protein kinases play a role in the action of NPPB, we examined the effect of H-89, a potent PKA inhibitor, on the action of NPPB and NFA. The present results showed that H-89 significantly antagonized the enhancing effect of NPPB (Fig. 8A) and NFA (Fig. 8C). The H-89 (20 μM) reduced the amplitude of NPPB- and NFA-induced outward current at 0 mV by 87.2 ± 9.9% (n = 4, P < 0.01) and 95.2 ± 1.2% (n = 4, P < 0.01), respectively. In contrast, bath application of H-89, a PKC inhibitor, neither significantly inhibited NPPB-induced outward current at 0 mV (n = 4, Fig. 8D), nor prevented the enhancing effect of NPPB (n = 3, Fig. 8E). Moreover, NPPB failed to induce any significant effect on the membrane conductance at 0 mV after pretreatment of the myocytes with H-89. In contrast, withdrawal of H-89 unmasked the enhancing effect of NPPB (n = 4, Fig. 8B).

Effect of intracellular Cl− on the action of NPPB

Accumulating evidence indicates that Cl− may modify ion channels (Pusch et al. 1999; Yuan et al. 2000; Bekar et al. 2005). Since blockade of the Cl− channel may alter [Cl−]i, we tested whether [Cl−]i may play a role in the effect of NPPB. The present results showed that NPPB-induced outward current at 0 mV in high-Cl− (132 mM)-dialysed myocytes (39.8 ± 15.8 pA pF−1, n = 34) was slightly reduced compared with those in normal-Cl− (22 mM)-dialysed myocytes (55.7 ± 23.8 pA pF−1, n = 34; P < 0.05, Fig. 9).

Figure 3. Effects of NPPB and NFA on Ik

Ik was elicited by 300 (A) or 400 ms depolarizing pulses (B and C) from a holding potential of −20 mV to test potentials ranging from −90 (A) or −70 mV (B and C) to +50 mV in 10 mV (A and B) or 20 mV increments (C). Ik1 was blocked by Ba2+ (1 mM). A, representative current traces recorded in the absence (a) and presence of NPPB (b) and after washout of NPPB (c); d, current–voltage relationship measured at the end of test pulses for recordings shown in Aa and Ab. B, representative current traces recorded before application of NFA (a), in the presence of NFA (b) and after removal of NFA (c); d, current–voltage relationship measured at the end of test pulses for recordings shown in Ba and Bb. The dashed lines in A and B indicate the zero current level. C, an example showing that NPPB (b) and NFA (c) were ineffective on the membrane conductance after K+ in the pipette and bath solutions was replaced by equimolar Cs+: a, control; b, after application of NPPB; and c, in the presence of NFA. The data in A, B and C represent 6, 6 and 4 experiments, respectively.
Discussion

The present study demonstrates that: (1) monocarboxylic acid-derived Cl\(^-\) channel blockers NPPB and NFA inhibit \(I_{\text{to}}\) but stimulate \(I_{\text{K}}\); (2) the enhancing effect of NPPB and NFA on \(I_{\text{K}}\) is suppressed by the PTK inhibitors genistein and lavendustin A and by the PKA inhibitor H-89; (3) the PTP inhibitor VO\(_4\) slows the deactivation of the enhanced \(I_{\text{K}}\) after washout of NPPB and NFA; (4) pretreatment of cardiac myocytes with genistein or H-89 prevents the enhancing effect of NPPB on \(I_{\text{K}}\); and (5) elevation of [Cl\(^-\)]\(_i\) slightly reduces the action of NPPB on \(I_{\text{K}}\).

Effects of monocarboxylic acid-derived Cl\(^-\) channel blockers on depolarization-activated K\(^+\) currents

Several lines of evidence indicate that Cl\(^-\) channel blockers have profound influences on a variety of K\(^+\) currents. For example, NFA is found to enhance the presynaptic voltage-activated K\(^+\) current (Miralles et al. 1996), Ca\(^{2+}\)-activated K\(^+\) current (Miralles et al. 1996), human ERG (ether-a-go-go related gene) current (Malykhina et al. 2002) and lipid-sensitive mechano-gated 2P domain K\(^+\) current (Takahira et al. 2005). In the present study, we found that the monocarboxylic acid-derived Cl\(^-\) channel blockers NPPB and NFA activated a macroscopic outward current at 0 mV in rat ventricular myocytes. The NPPB- and NFA-sensitive current reversed near the predicted K\(^+\) equilibrium potential, suggesting that the current is a K\(^+\) current.

The \(I_{\text{K}}\) is composed of multiple components, including \(I_{\text{Kr}}\) (\(I_{\text{K,rapid}}\), \(I_{\text{K,slow}}\), \(I_{\text{Kur}}\) (\(I_{\text{K,ultrarapid}}\) and \(I_{\text{SS}}\). Evidence has revealed that the Kv1.5 probably encodes \(I_{\text{Kur}}\) and \(I_{\text{K,slow1}}\), and that the ERG encodes \(I_{\text{Kr}}\) (Nerbonne & Kass, 2005). Studies indicate that Kv1.5 and human ERG channels show significant Cs\(^+\) permeability (Fedida et al.)

**Figure 4. Effects of NPPB and NFA on membrane conductance in different intracellular K\(^+\) concentrations**

Currents were elicited by 400 ms depolarizing pulses from a holding potential of −20 mV to test potentials ranging from −100 to +50 mV in 10 mV increments. \(I_{\text{K}}\) was blocked by Ba\(^{2+}\) (1 mM). A and C, representative current traces recorded in the absence and presence of NPPB and NFA, respectively, in 65 mM [K\(^+\)]. Intracellular K\(^+\) was partly replaced by Cs\(^+\) ([K\(^+\)];[Cs\(^+\)] = 65:65 mM). B and D, representative current traces recorded before and after application of NPPB and NFA, respectively, in 30 mM [K\(^+\)]; [K\(^+\)];[Cs\(^+\)] = 30:100 mM). The dashed lines in A–D indicate the zero current level. The data in A, B, C and D represent 12, 5, 7 and 5 experiments, respectively. E and F, current–voltage relationships showing the shift of reversal potential of NPPB- and NFA-enhanced currents under different intracellular K\(^+\) concentrations.
If the channel activated by NPPB and NFA is $I_{Kur}$ or $I_{Kr}$, there should be a Cs$^+$ current after replacement of intracellular and extracellular K$^+$ with Cs$^+$. However, the present study found that Cs$^+$ substitution for K$^+$ completely abolished the enhancing effect of NPPB and NFA. Therefore, it seems unlikely that NPPB and NFA target these two channels.

In rat ventricular myocytes, currents activated from a holding potential of $-20$ mV reflect activation of $I_{SS}$, the non-inactivating component of $I_K$ that is sensitive to blockade by Cs$^+$ (Himmel et al. 1999; Komukai et al. 2002). The present results indicated that, after replacement of intracellular and extracellular K$^+$ with Cs$^+$, NPPB and NFA neither induced any significant effect on the membrane conductance at 0 mV, nor enhanced the outward current activated from a holding potential of $-20$ mV (see Figs 1D and 3C). These results suggest that NPPB- and NFA-induced enhancement of $I_K$ is a result of activation of the $I_{SS}$ component. To our knowledge, this study provides the first evidence that Cl$^-$ channel blockers NPPB and NFA can simulate cardiac $I_{SS}$.

**Figure 5. Effect of genistein and daidzein on NPPB- and NFA-elicited outward current**

Whole-cell current at 0 mV was continuously monitored. A and C, representative current traces showing the inhibitory effect of genistein on the outward current induced by NPPB (A) and NFA (C). B, representative current trace showing that daidzein is without significant effect on NPPB-induced current. D, an example showing the effect of pre-application of genistein on the enhancing action of NPPB. The data in A, B, C and D represent 5, 4, 5 and 3 experiments, respectively.

**Involvement of protein kinases in the effects of NPPB and NFA on cardiac K$^+$ currents**

Protein phosphorylation is a common mechanism for regulating K$^+$ channel activity (Levitan, 1994; Davis et al. 2001). It is becoming apparent that tyrosine phosphorylation may modulate the activity of a variety of K$^+$ channels (Davis et al. 2001), including $I_K$ channels in cardiac myocytes (Gao et al. 2004; Missan et al. 2006). The present study found that the stimulating effect of NPPB and NFA on the outward current was markedly inhibited by the PTK inhibitors genistein and lavendustin A, but not by the inactive analogue of genistein, daidzein. Moreover, inhibition of PTP with VO$_4$ significantly slowed the deactivation of NPPB- and NFA-induced outward current at 0 mV (Fig. 6). So it seems that the effect of NPPB and NFA on $I_{SS}$ involves the PTK signalling pathway.

It is well known that PKA-dependent phosphorylation plays an important role in regulation of a variety of cardiac K$^+$ channels. This PKA-dependent phosphorylation can upregulate $I_K$ (Walsh & Kass, 1988; Huang et al. 1994). In
the present study, we found that the enhancing effect of NPPB and NFA on the outward current was also inhibited by the PKA inhibitor H-89, but not by the PKC inhibitor H-7. These results suggest that, besides PTK, PKA may also be involved in the action of NPPB and NFA on $I_{SS}$. The effect of NPPB and NFA on $I_{SS}$ is most likely to result from enhancement of PTK and PKA activity. Moreover, the present results demonstrate that either presuppression of PTK with genistein (Fig. 4D) or pre-inhibition of PKA with H-89 (Fig. 7B) prevented the enhancing effect of NPPB on the outward current. Therefore, it seems that a cross-talk between PKA and PTK pathways is required for the enhancing effect of NPPB. Although there are clues to suggest that PKA may regulate the activity of PTKs and PTPs (Wilson & Kaczmarek, 1993; Park et al. 2000), the relationship between these two signalling pathways in the regulation of cardiac $I_{SS}$ is not known from the present study.

It is known that both PKA and PTK are involved in modulation of cardiac $I_{Ca,L}$. Activation of PKA can upregulate $I_{Ca,L}$ (van der Heyden et al. 2005). If NPPB and NFA can stimulate the PKA signal pathway, they may also enhance $I_{Ca,L}$. Thus, it seems that our previous study indicating that NPPB and NFA inhibit $I_{Ca,L}$ (Zhou et al. 2002) is inconsistent with the present finding of activation of the PKA signalling pathway by NPPB and NFA. However, it should be noted from the present study that NPPB and NFA seem also to stimulate the PTK signalling pathway, which is known to downregulate $I_{Ca,L}$ (Boixel et al. 2000; Schroder et al. 2004; van der Heyden et al. 2005). In this case, the response of $I_{Ca,L}$ to NPPB and NFA may depend on which kinase plays a dominant role in modulating the $I_{Ca,L}$ channel. Therefore, it is not strange that NPPB and NFA may induce $I_{Ca,L}$ inhibition.

It has long been known that $\alpha_1$-adrenoceptor stimulation can inhibit $I_{to}$ (Apkon & Nerbonne, 1988; Ravens et al. 1989). A recent study reveals that $\alpha_1$-adrenoceptor stimulation inhibits $I_{to}$ via a cAMP/PKA-mediated pathway (Gallego et al. 2005). It is generally accepted that the voltage-gated $K^+$ channels Kv4.2 and Kv4.3 are the most likely molecular correlates for $I_{to}$ (Dixon et al. 1996; Nerbonne & Kass, 2005). Recent evidence...
Figure 8. Effect of PKA and PKC inhibitors on NPPB action
A, an example of an original current trace showing the inhibitory effect of H-89 on NPPB-induced outward current at 0 mV.
B, an example showing that NPPB produced no significant effect on the membrane conductance monitored at 0 mV after the myocyte was pretreated with 20 μM H-89; in contrast, removal of H-89 unmasked the enhancing effect of NPPB. C, an example of a typical current trace showing the inhibitory effect of H-89 on NFA-induced outward current at 0 mV. D and E, representative current traces showing that H-7 neither significantly inhibited the NPPB-induced outward current at 0 mV, nor prevented the simulating action of NPPB.
The data in A, B, C, D and E represent 4, 4, 4, 4 and 3 experiments, respectively.

Figure 9. Effect of [Cl\textsuperscript{−}]. on the action of NPPB
Histogram showing the mean density of NPPB-induced outward current at 0 mV with standard [Cl\textsuperscript{−}] (22 mM, open bar, n = 34) or high [Cl\textsuperscript{−}] (132 mM, hatched bar, n = 34) in the pipette solution. *P < 0.05 versus standard [Cl\textsuperscript{−}]. The data were from 6 hearts. Equal numbers of normal [Cl\textsuperscript{−}]- and high [Cl\textsuperscript{−}]-dialysed myocytes were observed in each heart.

Chloride has been found to play roles in cell function. It affects the gating of some Cl\textsuperscript{−} channels (Pusch et al. 1999; Pusch, 2004), interferes with non-chloride channels (Yuan et al. 2000; Bekar et al. 2005), and even influences protein phosphorylation (Yang et al. 2000). Thus, there is a possibility that the effect of the Cl\textsuperscript{−} channel blockers NPPB and NFA on cardiac K\textsuperscript{+} channels may be a Cl\textsuperscript{−}-mediated reaction, since blockade of the Cl\textsuperscript{−} channel may alter [Cl\textsuperscript{−}]. The present results indicated that increasing [Cl\textsuperscript{−}] from 22 to 132 mM slightly attenuated the action of NPPB on the outward current. It seems that the change in [Cl\textsuperscript{−}], is not the main reason for the effect of NPPB.
It is well established that the action potential of cardiac myocytes is the result of harmonious activity of dozens of channels (Nerbonne & Kass, 2005). However, the relationship among the ion channels is still unclear. Lefèvre et al. (1996) compared the effect of Cl\(^-\) substitution with that of Cl\(^-\) channel blockers, and found that the effect of the Cl\(^-\) channel blocker SITS (4-acetamido-4\'-isothiocyanatostilbene-2,2\'-disulphonic acid) on \(I_{t0}\) was similar to that substitution of the less permeant anion aspartate for Cl\(^-\). A comparable effect of Cl\(^-\) channel blockers and less permeant anions on cardiac \(I_{Ca,L}\) has also been observed in rat ventricular myocytes (Zhou et al. 2002). Based on these observations, we therefore speculate that the Cl\(^-\) channel may be involved in the non-specific effect of less permeable anions and Cl\(^-\) channel blockers on cardiac cation channels (Zhou et al. 2002; Lai et al. 2004). Indeed, Baker et al. (2004) recently found that voltage-dependent anion-selective channel 1, located in the mitochondrial outer membrane and the plasma membrane, can function as an enzyme (Lawn et al. 2005). This finding raises the possibility that the Cl\(^-\) channel per se may play a direct role in modulating intracellular signalling events. Thus, it seems possible that any factors (including Cl\(^-\) channel blockers) that affect the Cl\(^-\) channel may alter the channel-mediated responses, such as intracellular signalling. The present data showed that intracellular application of NPPB and NFA did not have any effect on the outward current. These results demonstrate that NPPB and NFA influence the outward current from the outside of the cell membrane, and suggest that it is unlikely that these Cl\(^-\) channel blockers stimulate \(I_{SS}\) through a direct action on PKA or cytoplasmic PTKs. Thus, there is a possibility that NPPB and NFA may exert a direct action on cardiac \(I_{SS}\) channels. However, the present study found that the enhancing effect of the Cl\(^-\) channel blockers on \(I_{SS}\) is antagonized by the inhibitors of either PKA or PTK. Therefore, it is also unlikely that NPPB and NFA act directly on \(I_{SS}\) channels. Chloride may affect the gating of Cl\(^-\) channels (Pusch et al. 1999; Pusch, 2004), and Cl\(^-\) channel blockers may alter Cl\(^-\) flux through Cl\(^-\) channels. Thus, these factors may change the Cl\(^-\) channel activity and consequently Cl\(^-\) channel-mediated intracellular signalling. Therefore, the possibility of involvement of the Cl\(^-\) channel in the effect of the Cl\(^-\) channel blockers on \(I_{SS}\) cannot be ruled out.

In conclusion, the present study demonstrates that the monocarboxylic acid-derived Cl\(^-\) channel blockers NPPB and NFA can stimulate cardiac steady-state K\(^+\) current, which is probably mediated by stimulation of the PKA and PTK signalling pathways.

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**Acknowledgements**

This research was supported by the National Natural Science Foundation of China (no. 30570665). The authors thank Qing Chi for technical assistance and Ms Li-Xia Wang for secretarial assistance.