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Insensitivity of cardiac delayed-rectifier I_{Kr} to tyrosine phosphorylation inhibitors and stimulators

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1 The rapidly activating delayed-rectifying K^+ current (I_{Kr}) in heart cells is an important determinant of repolarisation, and decreases in its density are implicated in acquired and inherited long QT syndromes. The objective of the present study on I_{Kr} in guinea-pig ventricular myocytes was to evaluate whether the current is acutely regulated by tyrosine phosphorylation.

2 Myocytes configured for ruptured-patch or perforated-patch voltage-clamp were depolarised with 200-ms steps to 0 mV for measurement of I_{Kr} tail amplitude on repolarisations to −40 mV.

3 I_{Kr} in both ruptured-patch and perforated-patch myocytes was only moderately (14–20%) decreased by 100 μM concentrations of protein tyrosine kinase (PTK) inhibitors tyrphostin A23, tyrphostin A25, and genistein. However, similar-sized decreases were induced by PTK-inactive analogues tyrphostin A1 and daidzein, suggesting that they were unrelated to inhibition of PTK.

4 Ruptured-patch and perforated-patch myocytes were also treated with promoters of tyrosine phosphorylation, including phosphotyrosyl phosphatase (PTP) inhibitor orthovanadate, exogenous c-Src PTK, and four receptor PTK activators (insulin, insulin-like growth factor-1, epidermal growth factor, and basic fibroblast growth factor). None of these treatments had a significant effect on the amplitude of I_{Kr}.

5 We conclude that Kr channels in guinea-pig ventricular myocytes are unlikely to be regulated by PTK and PTP.
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Abbreviations: bFGF1, basic fibroblast growth factor; DMSO, dimethyl sulphoxide; EGF, epidermal growth factor; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ERG, '*ether-a-go-go*'-related gene; ERK, extracellular-regulated kinase; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; IGF-1, insulin-like growth factor-1; I_{Kr}, rapidly activating delayed-rectifier K^+ current; I_{Ks}, slowly activating delayed-rectifier K^+ current; *I–V*, current–voltage; MAPK, mitogen-activated protein kinase; PTP, phosphotyrosyl phosphatase; PTK, protein tyrosine kinase

Introduction

The rapidly activating delayed-rectifier K^+ current (I_{Kr}) has a critical role in the electrical activity of cardiac ventricular myocytes. Outward I_{Kr} is a major factor in the termination of the plateau phase of the action potential and in driving repolarisation of the cell membrane (Sanguinetti & Jurkiewicz, 1990; Zeng *et al*., 1995; Jones *et al*., 1998). Downregulation of the channels that carry I_{Kr} contributes to the prolongation of action potential duration observed in failing heart cells (Priebe & Beuckelmann, 1998; Tomaselli & Marban, 1999), and dysfunction of the channels is implicated in certain forms of inherited and acquired long QT syndrome (Sanguinetti, 1999).

Cardiac I_{Kr} is regulated by intracellular serine/threonine phosphorylation systems. The current is negatively regulated by protein kinase A (Karle *et al*., 2002) and positively regulated by protein kinase C (Heath & Terrar, 2000). Although there is evidence that other cardiac currents regulated by these kinases are also acutely regulated by protein tyrosine kinase (PTK) (e.g., Wang & Lipsius, 1998; Ogura *et al*., 1999; Wang *et al*., 2003), there is no information (to our knowledge) on whether cardiac I_{Kr} is regulated by PTK. The objective of this study was to investigate that possibility by measuring I_{Kr} in guinea-pig ventricular myocytes that were exposed to inhibitors and stimulators of tyrosine phosphorylation. The compounds used to inhibit tyrosine phosphorylation were the broadspectrum PTK inhibitors tyrphostin A23, tyrphostin A25, and genistein (Gazit *et al*., 1989; Akiyama & Ogawara, 1991), whereas the compounds used to stimulate tyrosine phosphorylation were the phosphotyrosyl phosphatase (PTP) inhibitor orthovanadate (Swarup *et al*., 1982), exogenous c-Src PTK, and four activators of receptor PTK (insulin, insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF)).

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Methods

Preparation of myocytes

Adult guinea-pigs (225–275 g) were killed by cervical dislocation and exsanguination in accordance with the national and

university regulations on animal experimentation. Hearts were quickly removed, mounted on a Langendorff column, and perfused through the coronary artery for 10–15 min. The Ca^{2+}-free perfusate (37°C) contained (in mM) NaCl 125, KCl 5, $MgCl_2$ 1.2, taurine 20, glucose 20, and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) 5 (pH 7.4), as well as 0.08–0.12 mg ml^{-1} collagenase (Yakult Pharmaceutical Co., Tokyo, Japan). On completion of collagenase digestion, the heart tissue was minced, and myocytes dispersed in a high-K^+, nutrient-supplemented storage solution (22°C) that contained KCl 30, KOH 80, KH_2PO_4 30, $MgSO_4$ 3, glutamic acid 50, taurine 20, glucose 20, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) 0.5, and HEPES 10 (pH 7.4 with KOH).

Electrophysiology

Myocytes were voltage-clamped using either the standard ruptured-patch method or the nystatin perforated-patch method. Recording pipettes were fabricated from thick-walled borosilicate glass capillaries (H15/10/137, Jencons Scientific Ltd, Leighton Buzzard, U.K.), and had resistances of 2–3 MΩ when filled with dialysate. Pipette offsets were nulled before patch formation, and liquid-junction potentials (≈ −10 mV) were offset during data analysis. In the ruptured-patch experiments, series resistance ranged between 4 and 8 MΩ and was compensated by 60–80%. In the perforated-patch experiments, series resistance declined to 8–25 MΩ within 10–20 min of seal formation, and experiments were initiated when it was stable over a 10-min period. Series resistance compensation was used in most of the experiments such that uncompensated resistance was reduced below 10 MΩ (typically 2–6 MΩ). Membrane currents were recorded with an EPC-9 amplifier (HEKA Electronics, Mahone Bay, NS, Canada). The electrical signals were low-pass filtered at 3 kHz, and digitized with an A/D converter (Digidata 1200A, Axon Instruments, Foster City, CA, U.S.A.) and pCLAMP software (Axon Instruments) at a sampling rate of 8–10 kHz before analysis. All experiments were conducted at 36°C.

Superfusates and pipette solutions

The standard superfusate was a Tyrode's solution that contained (in mM) NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.2, glucose 10, and HEPES 5 (pH 7.4 with NaOH), as well as 3 μM glibenclamide (Sigma-Aldrich, Oakville, ON, Canada) to suppress any ATP-sensitive K^+ current that might arise following application of TK inhibitors (Harvey & Ashford, 1998; Stadnicka *et al*., 2002). In some experiments, the superfusate was a K^+-, Ca^{2+}-free Cd^{2+} solution that contained (in mM) NaCl 140, Cd^{2+} 0.2, $MgCl_2$ 1.2, glucose 10, and HEPES 5 (pH 7.4 with NaOH). The standard pipette solution used for ruptured-patch experiments contained (in mM) KCl 30, potassium aspartate 110, MgATP 5, EGTA 5, and HEPES 5 (pH 7.2 with KOH). The pipette solution used in the perforated-patch experiments contained (in mM) KCl 30, potassium aspartate 110, $MgCl_2$ 5, and HEPES 5 (pH 7.2 with KOH). Nystatin (Sigma-Aldrich) was added to this pipette-filling solution from a stock solution (100 mg ml^{-1} in dimethyl sulphoxide (DMSO)) to give a final concentration of 50–100 μg ml^{-1}. The nystatin stock solution was freshly prepared and used for up to 2 h.

Chemicals and drugs

All chemicals used in making solutions were purchased from Sigma-Aldrich and were of the highest purity grade available. Daidzein, genistein, glibenclamide, and tyrphostins A1, A23, A51, and A63 were purchased from Calbiochem (La Jolla, CA, U.S.A.), and nisoldipine was kindly provided by Bayer (Etobicoke, ON, Canada). These compounds were dissolved in DMSO (Sigma-Aldrich), stored in the dark at −20°C, and added to experimental solutions as required. The highest eventual concentration of DMSO in bathing solutions was 0.1%, a concentration that had no effect on I_{Kr}; nevertheless, when experiments were conducted with drug-containing bathing solution that had ⩾0.05% DMSO, the control bathing solution generally contained the same concentration of DMSO. Sodium orthovanadate (Fisher Scientific, Nepeon, ON, Canada) was freshly prepared in water before experiments, and added to superfusates whose pH was then adjusted with HCl. E4031 (Eisai, Tokyo, Japan) was added to the bathing solution, and active c-Src PTK (human, recombinant) (Upstate Biotechnology, Lake Placid, NY, U.S.A.) was added to the pipette solution. Insulin (human, recombinant), IGF-1 (human, recombinant), EGF (human, recombinant), and bFGF (recombinant) were obtained from Calbiochem and added to the bathing solution.

Statistics

Experimental data are expressed as means ± s.e.m.; *n* represents the number of experiments. Statistical comparisons were made using Student's paired or unpaired *t*-test. Differences were considered significant when $P<0.05$.

Results

The effects of modulators of tyrosine phosphorylation on I_{Kr} were investigated in myocytes that were bathed with standard Tyrode's solution, and pretreated with 1 μM nisoldipine to suppress L-type Ca^{2+} current. The myocytes were pulsed with short (200 ms) low-amplitude (–40 to 0 mV) steps that selectively activate I_{Kr} over I_{Ks} (Sanguinetti & Jurkiewicz, 1990; Jones *et al*., 2000), and changes in I_{Kr} were monitored by measuring the amplitude of tail currents on repolarisations to −40 mV. Under control conditions, the I_{Kr} tails in eight ruptured-patch myocytes deactivated in a biexponential fashion ($\tau_1 = 299 \pm 21$ ms, amplitude 76 ± 9 pA; $\tau_2 = 3155 \pm 262$ ms, amplitude 66 ± 5 pA) as described in earlier studies on guinea-pig (Heath & Terrar, 1996) and canine (Liu & Antzelevitch, 1995; Varró *et al*., 2000) ventricular myocytes.

Effects of PTK inhibitors and their inactive analogues on I_{Kr}

Experiments on ruptured-patch myocytes Figure 1a shows the data obtained from a ruptured-patch myocyte that was exposed to PTK inhibitor tyrphostin A25 (50 μM). The amplitude of I_{Kr} was only slightly reduced by the inhibitor, whereas it was reduced to almost zero by subsequent addition of 5 μM E4031, a specific inhibitor of cardiac I_{Kr} (Sanguinetti & Jurkiewicz, 1990). On average, application of 50 μM tyrphostin A25 for ≈ 10 min reduced the amplitude of the current by

16±6% ($n=4$), and application of 100 μM reduced it by 20±4% ($n=5$) ($P<0.01$) (Figure 1c). Similar-sized reductions in the amplitude of I_{Kr} were measured in myocytes that were exposed for 10 min to PTK inhibitor tyrphostin A23 (100 μM) (14±6%, $n=5$) ($P<0.05$). For comparison with these results, myocytes were exposed to tyrphostin A1 (100 μM), a PTK-inactive analogue of tyrphostins A23 and A25 (Gazit *et al*., 1989). The analogue reduced the amplitude of I_{Kr} by 13±5% ($n=6$) ($P<0.05$) (Figure 1c).

Similar experiments were conducted with genistein, a PTK inhibitor that is structurally and mechanistically different than the tyrphostins used here (Gazit *et al*., 1989; Davis *et al*., 2001). As illustrated by the results obtained from a myocyte exposed to 100 μM genistein for 10 min (Figure 2a), the inhibitor had relatively small effects on I_{Kr}. Overall, genistein (20, 50, and 100 μM) decreased the amplitude of the I_{Kr} tail by 1±5% ($n=9$), 12±4% ($n=14$) ($P<0.01$), and 17±4% ($n=8$) ($P<0.01$), respectively (Figure 2c). As a negative control for the involvement of PTK in the inhibitory action of genistein, we evaluated the effects of daidzein (20, 50, and 100 μM). Like genistein, the inactive analogue had a modest concentration-dependent inhibitory effect on I_{Kr}; at 100 μM, the current amplitude was reduced by 15±5% ($n=6$) ($P<0.05$) (Figure 2b and c).

Experiments on perforated-patch myocytes The 'true' responses of whole-cell I_{Kr} to kinase modulators may be better preserved in myocytes investigated using the perforated-patch technique than in those investigated using the ruptured-patch technique (Heath & Terrar, 2000). To determine whether this was the case in the present study, we patched myocytes with pipettes that were filled with a nystatin solution (Korn & Horn, 1989; Missan *et al*., 2004). The representative data and summary presented in Figure 3 indicate that the effects of 100 μM concentrations of tyrphostin A23, tyrphostin A25, and genistein on I_{Kr} in perforated-patch myocytes were similar to those observed in ruptured-patch myocytes.

A test of the 'activity' of PTK inhibitors The relatively small effects of PTK inhibitors on I_{Kr} raised the question of whether these agents were indeed 'active', that is, did they modulate other membrane currents in a manner expected from the results of earlier studies? To address that question, we investigated the effects of PTK inhibitors on slowly-activating I_{Ks} because it has been suggested that at least part of the rapid inhibitory effect of 50 μM genistein on canine (Zhou *et al*.,

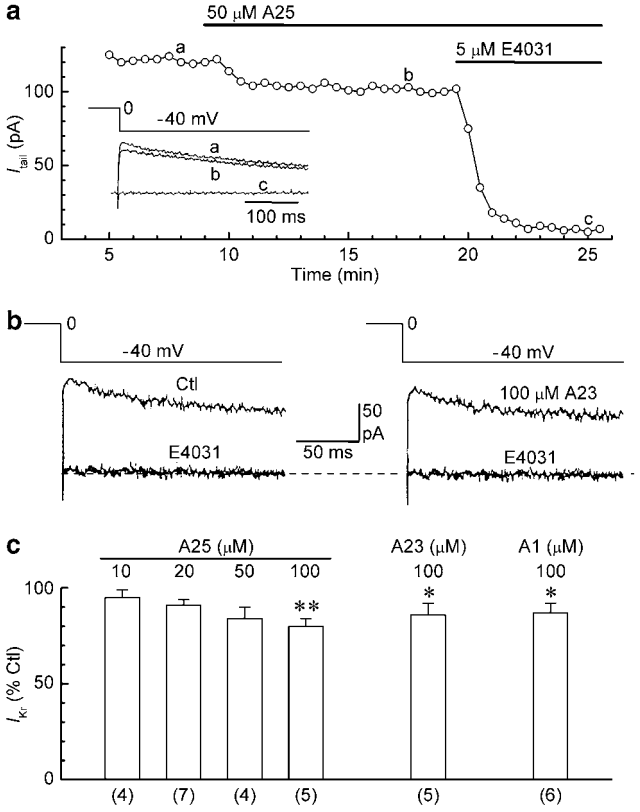

Figure 1 Effects of tyrphostins A25, A23, and A1 on I_{Kr} in ruptured-patch myocytes. The myocytes were pulsed with 200-ms steps from −40 to 0 mV for measurement of the amplitude of the I_{Kr} tail on repolarisation to −40 mV. (a) Effects of 50 μM tyrphostin A25 and subsequent 5 μM E4031 on I_{Kr} tail amplitude in a representative myocyte. Inset: superimposed current records obtained at the times indicated on the plot. (b) Tail current records obtained before (Ctl) (left) and 10 min after application of 100 μM tyrphostin A23 (A23) (right) to a representative myocyte. Two traces obtained after addition of E4031 (as in panel a) are superimposed on the control and A23 records. (c) Summary of the results obtained with the three tyrphostin compounds. *$P<0.05$, **$P<0.01$, *versus* predrug control amplitude. Numbers of myocytes in parentheses.

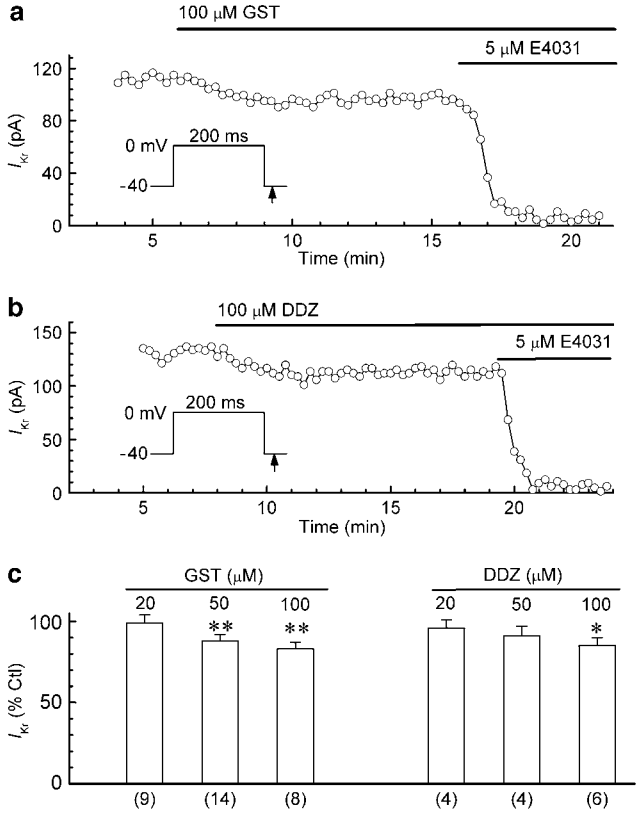

Figure 2 Effects of genistein (GST) and daidzein (DDZ) on I_{Kr} in ruptured-patch myocytes. The myocytes were pulsed with 200-ms steps from −40 to 0 mV for measurement of the amplitude of the I_{Kr} tail on repolarisation to −40 mV. (a, b) Effects of 100 μM GST and 100 μM DDZ on I_{Kr} tail amplitude in representative myocytes. (c) Summary of the results obtained with 8–10 min applications of the drugs. *$P<0.05$, **$P<0.01$ *versus* predrug control amplitude. Numbers of myocytes in parentheses.

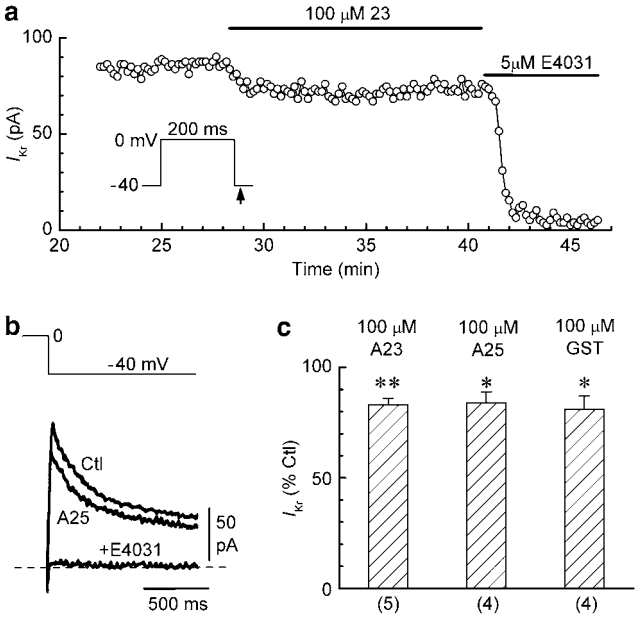

Figure 3 Effects of TK inhibitors on I_{Kr} in perforated-patch myocytes. (a) Moderate reduction of I_{Kr} amplitude during application of 100 μM tyrphostin A23 to a representative myocyte. (b) Superimposed records of I_{Kr} tails obtained from a myocyte before (Ctl), 12 min after the addition of 100 μM tyrphostin A25, and 4 min after the subsequent addition of 5 μM E4031. (c) Summary of the results obtained with the tyrphostins and genistein. $^*P<0.05$, $^{**}P<0.01$ *versus* predrug control amplitude. Numbers of myocytes in parentheses.

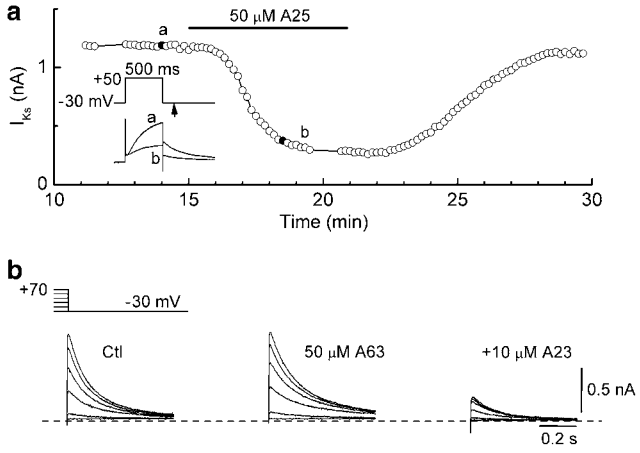

Figure 4 Effects of tyrphostin compounds on slowly-activating I_{Ks} in two ruptured-patch myocytes. The myocytes were bathed in a K^+-free Cd^{2+} solution and depolarised from -30 mV at 0.1 Hz. (a) Time plot of the amplitude of I_{Ks} tails following 500-ms depolarisations to $+50$ mV in a myocyte exposed to 50 μM tyrphostin A25. Inset: records obtained at the times indicated in the plot. (b) Families of I_{Ks} tails following 2-s depolarisations from -30 mV. The tails were recorded before drug treatment (Ctl), after 10-min exposure to PTK-inactive tyrphostin A63 (50 μM) (middle), and after a subsequent 10-min exposure to combined tyrphostin A63 and tyrphostin A23 (10 μM).

1997) and guinea-pig (Hool *et al*., 1998) ventricular I_{Ks} is related to an inhibition of PTK. The experiments on I_{Ks} were performed on ruptured-patch myocytes under conditions (K^+-free Cd^{2+} external solution; long depolarisations) that enhance I_{Ks} and minimise I_{Kr} (Sanguinetti & Jurkiewicz, 1990; Missan *et al*., 2003). The myocytes were depolarised from -30 to $+50$ mV for 500 ms at 0.1 Hz except for sequences of 2-s depolarisations at appropriate intervals. Figure 4a shows the time course of the amplitude of the I_{Ks} tail (-30 mV) recorded from a myocyte that was exposed to 50 μM tyrphostin A25. The inhibitor lowered the amplitude by $\approx$75%, and this action was fully reversed during the 10-min washout period. In five experiments of this type, the I_{Ks} tail after 2-s depolarisations to $+70$ mV was reduced to 23 ± 5% of its control amplitude ($P<0.001$). This action of tyrphostin A25 may well be related to an inhibition of PTK because I_{Ks} was insensitive to PTK-inactive tyrphostin A63 (50 μM) but strongly suppressed by 10 μM tyrphostin A23 ($n=3$; e.g., Figure 4b).

Lack of effect of PTP inhibitor orthovanadate on I_{Kr}

The foregoing results suggested that I_{Kr} was little affected by treatments aimed at lowering the basal activity of PTK. To evaluate whether the current was affected by an inhibition of basal PTP activity, myocytes were treated with 1 mM orthovanadate for 10 min. These treatments had no significant effect on the amplitude of I_{Kr} in either ruptured-patch myocytes (increase of 2 ± 3%; $n=15$) or perforated-patch myocytes (increase of 5 ± 3%; $n=8$) (Figure 5a and b). In some of the experiments on ruptured-patch myocytes, the orthovanadate pretreatments were followed by cotreatments with 100 μM tyrphostin A25 or 100 μM genistein to determine whether the inhibition of PTP blocked the inhibition of I_{Kr} by the PTK inhibitors. As indicated by the data in Figure 5a and c, pretreatment with orthovanadate had no effect on the inhibitory actions of tyrphostin A25 and genistein.

Effects of stimulators of tyrosine phosphorylation on I_{Kr}

The first approach used to stimulate tyrosine phosphorylation was to dialyse ruptured-patch myocytes with pipette solution that contained 15 or 50 U ml^{-1} c-Src PTK. From earlier whole-cell studies on the role of c-Src in the regulation of other ion channel types (e.g., Caraiscos *et al*., 2002; Strauss *et al*., 2002), it seemed likely that maximal effects of the kinase would be observed in 5–10 min. In the present study, myocytes were dialysed with the kinase solution for 10–15 min before the addition of 5 μM E4031. As indicated by the data shown in Figure 6a and c, the kinase dialysate had no detectable effect on the amplitude of I_{Kr}.

The second approach used to stimulate tyrosine phosphorylation was to treat myocytes with ligands that on binding their receptors cause activation of receptor PTK. In the heart, these receptors include those that bind insulin (Eckel *et al*., 1985; Gupta *et al*., 1989), IGF-1 (Engelmann *et al*., 1989), EGF (Rabkin *et al*., 1987; Yu *et al*., 1992), and bFGF (Schneider & Parker, 1990; Akiyama *et al*., 1999). The 10–15 min duration of the treatments with these four ligands was as long or longer than that required for full development of acute responses in earlier studies on cardiac preparations (e.g., Cittadini *et al*., 1998; Quintaje *et al*., 1998; Aulbach *et al*., 1999; Wu *et al*., 2000). The experiments were conducted on both ruptured-patch and perforated-patch (e.g., Figure 6b) myocytes, and the results are summarised in Figure 6c. Neither insulin (1 μM), IGF-1 (1 μM) nor bFGF (50 ng ml^{-1}) had any significant effect on the amplitude of I_{Kr} in ruptured-patch myocytes. Similarly,

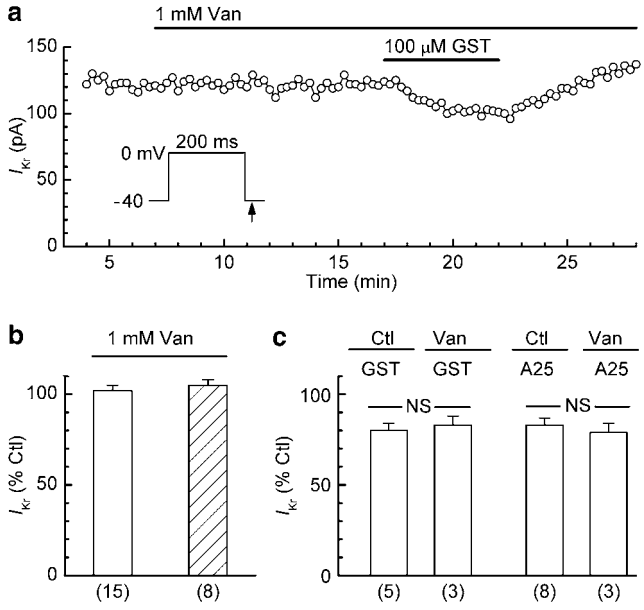

Figure 5 Effects of orthovanadate and PTK inhibitors on I_{Kr}. The myocytes were pulsed with 200-ms steps from −40 to 0 mV. (a) Time plot of the amplitude of the I_{Kr} tail in a ruptured-patch myocyte that was treated with 1 mM orthovanadate (Van) and then co-treated with 100 μM genistein (GST). The pretreatment did not prevent inhibition by GST. (b) Lack of effect of 1 mM orthovanadate on the amplitude of I_{Kr} in control experiments on ruptured-patch (open bar) and perforated-patch (hatched bar) myocytes. (c) Lack of effect of orthovanadate pretreatment on the responses of I_{Kr} to 100 μM tyrphostin A25 and 100 μM GST in ruptured-patch myocytes. NS: no significant difference between the two groups (unpaired *t*-test). The Van data obtained from myocytes in the Van/A25 and Van/GST experiments are included in the Van (1 mM) data of panel b. The control A25 and GST data are from Figures 1c and 2c, respectively. Numbers of myocytes in parentheses.

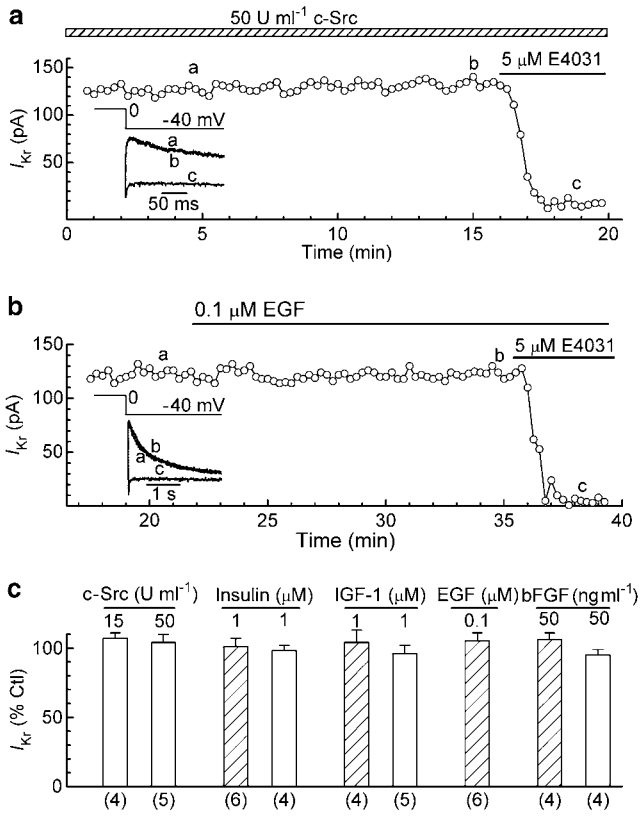

Figure 6 Effects of stimulators of tyrosine phosphorylation on I_{Kr} in ruptured-patch and perforated-patch myocytes. The myocytes were pulsed from −40 to 0 mV for 200 ms for measurement of the amplitude of the I_{Kr} tail on repolarisation to −40 mV before (control) and 10–15 min after the initiation of treatment. (a) I_{Kr} in a ruptured-patch myocyte dialysed with pipette solution that contained 50 U ml^{-1} c-Src. Inset: superimposed records obtained at the times indicated in the plot. (b) I_{Kr} in a perforated-patch myocyte treated with 0.1 μM EGF. Inset: superimposed records obtained at the post-pipette-attachment times indicated in the plot. (c) Summary of the data obtained on ruptured-patch (open bars) and perforated-patch (hatched bars) myocytes. In the experiments with c-Src dialysate, the amplitude of I_{Kr} measured during the first minute of dialysis was taken as the control amplitude. Numbers of myocytes in parentheses.

neither these ligands nor EGF (0.1 μM) had any significant effect on the current in perforated-patch myocytes.

Discussion

It is now well established that a wide variety of ion channels are acutely regulated by PTK (for reviews, see Davis *et al*., 2001; Cohen, 2005). We have investigated the possible involvement of PTK in the acute regulation of Kr channels in guinea-pig ventricular myocytes by measuring the responses of I_{Kr} to applications of broadspectrum PTK inhibitors, PTK-inactive analogues, PTP inhibitor orthovanadate, pipette c-Src PTK, insulin, and growth factors IGF-1, EGF, and bFGF. As discussed below, the results suggest that PTK does not have a significant role in the regulation of these channels.

We used 10–100 μM concentrations of tyrphostin A23, tyrphostin A25, and genistein to evaluate whether inhibition of basal PTK activity had an effect on I_{Kr}. Similar concentrations of these inhibitors have previously been employed to evaluate PTK regulation of L-type Ca^{2+} channels (Wijetunge & Hughes, 1995; Ogura *et al*., 1999; Wijetunge *et al*., 2000), Na^+ channels (Wang *et al*., 2003), hyperpolarisation-activated cation channels (Wu & Cohen, 1997; Wu *et al*., 2000), and swelling-activated Cl^- channels (Du *et al*., 2004). We found that the PTK inhibitors reduced the amplitude of I_{Kr} in a concentration-dependent manner, but that the degree of inhibition was small, that is, 100 μM concentrations only reduced the current by 14–20%. However, even this small reduction in I_{Kr} is more likely to have been due to low-affinity channel block than to an inhibition of PTK because (i) similar-sized reductions were observed with 100 μM concentrations of PTK-inactive analogues tyrphostin A1 and daidzein and (ii) pretreatment of myocytes with PTP inhibitor orthovanadate had no significant effect on the reduction of I_{Kr} by PTK inhibitors.

The responses of I_{Kr} to PTK inhibitors, PTK-inactive analogues, and PTK inhibitors in the presence of orthovanadate, all suggested that a lowering of basal tyrosine phosphorylation has little effect on the activity of Kr channels in guinea-pig ventricular myocytes. However, the primary results were obtained on ruptured-patch myocytes, and this raised the possibility that the effects of the signalling probes had been distorted by the washout of a critical cytoplasmic constituent. This appears not to have been the case, because the effects

of the three PTK inhibitors and orthovanadate on I_{Kr} in perforated-patch myocytes (minimal washout) were not different than those in ruptured-patch myocytes.

To our knowledge, there have been no previous reports on the effects of PTK inhibitors on cardiac I_{Kr}. However, it is of interest to compare the present results with those obtained in two studies on the role of PTK in the acute regulation of '*ether-a-go-go*' related gene (ERG) K^+ channels. Cayabyab & Schlichter (2002) recorded native ERG current in a rat microglial cell line (MLS-9), and found that 50 μM genistein reduced current amplitude by $\approx$60%, whereas 50 μM daidzein only reduced it by an insignificant 19$\pm$8% (see also Schlichter *et al*., 2006). On the other hand, Schledermann *et al*. (2001) recorded currents carried by rat ERG1 α-subunits expressed in GH_3/B_6 cells and found that acute application of 100 μM tyrphostin A23 only reduced the maximal amplitude of the current by about 18%. Our results with 100 μM tyrphostin A23 are in agreement with those of the Schledermann *et al*. (2001) study.

An important objective of the present study was to determine whether pharmacological treatments designed to produce a stimulation of tyrosine phosphorylation caused a change in the amplitude of I_{Kr}. The compounds used for this purpose included recombinant c-Src PTK and four activators of receptor PTK (insulin, IGF-1, EGF, and bFGF). c-Src PTK was supplied *via* the pipette at strengths (15 and 50 U ml^{-1}) similar to those (20–30 U ml^{-1}) used in earlier whole-cell studies on ion channel regulation (e.g., Caraiscos *et al*., 2002; Strauss *et al*., 2002; Feranchak *et al*., 2003). However, dialysis of the kinase for up to 15 min caused little change in I_{Kr}. The trials with insulin and IGF-1 were conducted with a concentration (1 μM) that rapidly stimulated cardiac contraction and L-type Ca^{2+} current (Strömer *et al*., 1996; Cittadini *et al*., 1998; Aulbach *et al*., 1999), whereas those with bFGF used a concentration (50 ng ml^{-1}) equal to or higher than those which rapidly stimulated Ca^{2+} currents in glial and neuronal cells (Puro & Mano, 1991; Koike *et al*., 1993) and mitogen-activated protein kinase (MAPK) activity in cardiomyocytes (Eppenberger-Eberhardt *et al*., 1997). The outcome of these trials on ruptured-patch and perforated-patch myocytes was an unchanged I_{Kr}. EGF was applied at a maximally effective concentration of 0.1 μM (Lorita *et al*., 2002) (see also Wu *et al*., 2000) and, like the other receptor-PTK activators, had no significant effect on I_{Kr} in perforated-patch myocytes.

A common action of insulin and the three growth factors studied here is they stimulate the MAPK pathway (Pawson & Scott, 1997; Quintaje *et al*., 1998; Siddle *et al*., 2001). There is accumulating evidence that activation of two terminal MAPKs, ERK (extracellular-regulated kinase) 1 and 2, has a stimulatory effect on an array of ion channel types, including Ca^{2+} channels (Ma *et al*., 1996), volume-sensitive Cl^- channels (Crepel *et al*., 1998), ATP-sensitive K^+ channels (O'Malley *et al*., 2003), large conductance Ca^{2+}-activated K^+ channels (O'Malley *et al*., 2003; O'Malley & Harvey, 2004), and Kv4.2 channels (Schrader *et al*., 2005). The lack of effect of insulin and growth factors on myocyte I_{Kr} suggests that in the absence of other perturbations, ERK1 and 2 have limited involvement in the regulation of cardiac Kr channels.

Recent studies on the effects of tyrosine phosphorylation modulators on cardiac myocytes suggest that hyperpolarisation-activated pacemaker current (Yu *et al*., 2000, 2004), L-type Ca^{2+} current (Hool *et al*., 1998; Wang & Lipsius, 1998; Ogura *et al*., 1999), Na^+ current (Wang *et al*., 2003), transient outward current (Wang *et al*., 2002), and volume-sensitive Cl^- current (Du *et al*., 2004; Ren & Baumgarten, 2005) are under the acute regulatory influence of PTK. The results of the present study indicate that this is unlikely to be the case for I_{Kr}.

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