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Cardiac Na^+–Ca^{2+} exchanger current induced by tyrphostin tyrosine kinase inhibitors

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1 Tyrosine kinase (TK) inhibitors genistein and tyrphostin A23 (A23) inhibited Ca^{2+} currents in guinea-pig ventricular myocytes investigated under standard whole-cell conditions (K^+-free Tyrode's superfusate; EGTA-buffered (pCa–10.5) Cs^+ dialysate). However, the inhibitors (100 μM) also induced membrane currents that reversed between −40 and 0 mV, and the objective of the present study was to characterize these currents.

2 Genistein-induced current behaved like Cl^- current, and was unaffected by either the addition of divalent cations (0.5 mM Cd^{2+}; 3 mM Ni^{2+}) that block the Na^+–Ca^{2+} exchanger (NCX), or the removal of external Na^+ and Ca^{2+}.

3 A23-induced current was independent of Cl^- driving force, and strongly suppressed by addition of Cd^{2+} and Ni^{2+}, and by removal of either external Na^+ or Ca^{2+}. These and other results suggested that A23 activated an NCX current driven by submembrane Na^+ and Ca^{2+} concentrations higher than those in the bulk cytoplasm.

4 Improved control of intracellular Na^+ and Ca^{2+} concentrations was obtained by suppressing cation influx (10 μM verapamil) and raising dialysate Na^+ to 7 mM and dialysate pCa to 7. Under these conditions, stimulation by A23 was described by the Hill equation with EC_{50} 68 ± 4 μM and coefficient 1.1, tyrphostin A25 was as effective as A23, and TK-inactive tyrphostin A1 was ineffective. Phosphotyrosyl phosphatase inhibitor orthovanadate (1 mM) antagonized the action of 100 μM A23.

5 The results suggest that activation of cardiac NCX by A23 is due to inhibition of genistein-insensitive TK.

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Keywords: Guinea-pig; ventricular myocytes; genistein; tyrphostins A23, A25 and A1; orthovanadate; Cl^- current; Na^+–Ca^{2+} exchanger

Abbreviations: A1, tyrphostin A1; A23, tyrphostin A23; A25, tyrphostin A25; CFTR, cystic fibrosis transmembrane regulator; DMSO, dimethyl sulphoxide; E_{Cl}, Cl^- equilibrium potential; E_{Na}, Na^+ equilibrium potential; E_{rev}, reversal potential; EC_{50}, concentration that produces 50% of maximal response; EGTA, ethylene glycol-*bis*(b-aminoethyl)-*N*,*N*,*N*,*N*-tetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; *I–V*, current-voltage; $I_{Ca,L}$, L-type Ca^{2+} current; $I_{Cl(CFTR)}$, cystic fibrosis transmembrane regulator chloride current; I_h, holding current; I_{NCX}, Na^+–Ca^{2+} exchanger current; I_{200}, current at end of 200-ms pulse; K_D, dissociation constant; NCX, Na^+–Ca^{2+} exchanger; NMDG, *N*-methyl-D-glucamine; PKA, protein kinase A; PKC, protein kinase C; TK, tyrosine kinase

Introduction

Genistein, tyrphostin A23 (A23), and tyrphostin A25 (A25) are tyrosine kinase (TK) inhibitors (Akiyama *et al*., 1987; Gazit *et al*., 1989; Holmes *et al*., 1996) that are widely used to investigate the involvement of tyrosine phosphorylation in the regulation of ion channels (Cataldi *et al*., 1996; Wang *et al*., 1996; Ogura *et al*., 1999; Albert *et al*., 2001), pumps (Feraille *et al*., 1997), and exchangers (Wang *et al*., 1997; Linck *et al*., 1998; Kiang *et al*., 2003). The inhibitors are active on external application, and are typically used at concentrations up to 300 μM, with little effect on the activity of other kinases such as protein kinase A (PKA) and protein kinase C (PKC) (Akiyama *et al*., 1987; Enright & Booth, 1991; O'Dell *et al*., 1992). Nevertheless, it is commonly acknowledged that these drugs can have direct effects that are unrelated to their actions on TK (for a review, see Davis *et al*., 2001). In the case of genistein, the direct effects are believed to include activation of cystic fibrosis transmembrane regulator (CFTR) Cl^- channels (French *et al*., 1997; Weinreich *et al*., 1997; Zhou *et al*., 1998) and inhibition of various cation channels (Smirnov & Aaronson, 1995; Paillart *et al*., 1997; Washizuka *et al*., 1998; Albert *et al*., 2001).

In an earlier study on the action of TK inhibitors on L-type Ca^{2+} current ($I_{Ca,L}$) in guinea-pig ventricular myocytes (Ogura *et al*., 1999), we observed that both genistein and the structurally and mechanistically different A23 (Levitzki & Gazit, 1995) caused development of outward current at 0 mV. The current appeared to be CFTR Cl^- current ($I_{Cl(CFTR)}$), and was not further investigated. The results of the present study indicate that the genistein-induced current behaves like $I_{Cl(CFTR)}$, whereas the A23-induced current behaves like Na^+–Ca^{2+} exchanger (NCX) current (I_{NCX}).

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Methods

All procedures were carried out in accordance with national and university regulations on the care and treatment of laboratory animals.

Ventricular myocytes

Guinea-pigs (250–300 g) were killed by cervical dislocation. Hearts were quickly excised, mounted on a Langendorff column, and perfused through the aorta with Ca^{2+}-free Tyrode's solution (37°C) that contained collagenase (0.08–0.12 mg ml^{-1}; Yakult Pharmaceutical Co., Tokyo, Japan) for 10–15 min. The cells were dispersed and kept in a storage solution (22°C) that contained (mM) KOH 80, KCl 30, KH_2PO_4 30, $MgSO_4$ 3, glutamic acid 50, taurine 20, glucose 20, ethylene glycol-*bis*(b-aminoethyl)-*N*,*N*,*N*,*N*-tetraacetic acid (EGTA) 0.5, and *N*′-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) 10 (pH 7.4 with KOH).

A few drops of the cell suspension were placed in a 0.3-ml perfusion chamber mounted on an inverted microscope stage. After the cells had settled to the bottom, the chamber was perfused ($\approx$3 ml min^{-1}) with Tyrode's solution at 36°C. Whole-cell membrane currents were recorded using an EPC-7 amplifier (List Electronic, Darmstadt, Germany). Recording pipettes were fabricated from thick-walled borosilicate glass capillaries (H15/10/137, Jencons Scientific Ltd, Bedfordshire, U.K.), and had resistances of 1.5–2.5 MΩ when filled with pipette solution. Voltages were corrected by −10 and −5 mV to account for junction potentials between external solution and low- and high-Cl^- pipette solutions, respectively. The series resistance ranged between 3 and 7 MΩ, and was compensated by 60–80%. Leakage compensation was not used. The current 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 kHz prior to analysis.

Myocytes were initially superfused with normal Tyrode's solution that contained (mM) NaCl 143, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.2, glucose 10, and HEPES 5 (pH 7.4 with NaOH). Thereafter, the myocytes were superfused with standard K^+-free Tyrode's solution (KCl omitted), or with a K^+-free Tyrode's solution that was either Na^+-free (Na^+ replaced by *N*-methyl-D-glucamine ($NMDG^+$)) or Ca^{2+}-free ($CaCl_2$ replaced by $MgSO_4$).

Myocytes were dialysed with one of the following K^+-free Cs^+ pipette solutions: standard solution that contained (mM) CsCl 40, CsOH 106, aspartic acid 106, MgATP 5, EGTA 5, and HEPES 5 (pH 7.2 with CsOH) (calculated pCa 10.5); high-Cl^- solution (standard solution that contained 140 mM Cl^- (aspartate replaced)); pCa 7 solution (standard solution with added $CaCl_2$); and 7 mM Na^+-pCa 7 solution (pCa 7 solution that contained 7 mM Na^+ (Cs^+ replaced) and either 40 or 65 mM Cl^- (aspartate replaced)).

Drugs

Genistein (Sigma-Aldrich, Oakville, ON, Canada) and tyrphostins A1, A23 and A25 (Calbiochem, San Diego, CA, U.S.A.) were prepared as 100-mM stock solutions in dimethyl sulphoxide (DMSO). Appropriate amounts of stock solutions were added to external solutions, and mixtures sonicated as required to ensure proper dispersion of drug. Corresponding amounts of DMSO ($\leqslant$0.3% v v^{-1}) were also added to the control external solutions. These concentrations of DMSO have little effect on membrane currents in guinea-pig ventricular myocytes (Ogura *et al*., 1995). Verapamil (Sigma-Aldrich) was dissolved in water and diluted in the bathing solution. Aqueous stock solutions (100 mM) of Na_3VO_4 (orthovanadate) (Fisher Scientific, Nepean, ON, Canada) were freshly prepared, and the pH was adjusted to $\sim$10 (Gordon, 1991; Shuba *et al*., 1996). Appropriate amounts of stock solution were added to the superfusate just before use, and the pH was readjusted to 7.4 with NaOH.

Statistics

Results are expressed as means$\pm$s.e.m.; *n* indicates the number of experiments. Comparisons were made using Student's *t*-test, and differences were considered significant when $P<0.05$.

Results

Differential effect of Cd^{2+} on membrane currents induced by genistein and tyrphostin A23

Myocytes superfused with standard K^+-free Tyrode's solution were dialysed with standard Cs^+ solution to suppress K^+ currents, and held at –40 mV to inactivate Na^+ and T-type Ca^{2+} currents. Under these conditions, 200-ms depolarizations to 0 mV elicited inward $I_{Ca,L}$ that rapidly reached a peak and then inactivated. As previously reported (Ogura *et al*., 1999), $I_{Ca,L}$ was reversibly inhibited by 100 μM genistein and 100 μM A23 (Figure 1a). These drugs also had reversible effects on holding current at –40 mV (I_h) and end-of-pulse current (I_{200}) at 0 mV (Figure 1a). On average ($n=10$), 100 μM genistein shifted I_h by -0.12 ± 0.06 pA pF^{-1} ($P<0.05$) and I_{200}(0 mV) by 0.72 ± 0.2pA pF^{-1} ($P<0.01$), whereas 100 μM A23 shifted I_h by -0.21 ± 0.07 pA pF^{-1} ($P<0.01$) and I_{200}(0 mV) by 0.55 ± 0.14 pA pF^{-1} ($P<0.01$). A plausible explanation for the shifts is that both genistein and A23 induced currents that had a reversal potential (E_{rev}) between –40 and 0 mV.

The first indication that the ionic basis of the genistein-induced current was different from that of the A23-induced current was provided by the responses to Cd^{2+}, a Ca^{2+}-channel blocker that also inhibits NCX activity (Iwamoto & Shigekawa, 1998). When myocytes bathed and dialysed with standard solutions were pretreated with 0.5 mM Cd^{2+}, application of 100 μM genistein alone ($n=4$) or with 100 μM A23 ($n=3$) shifted the membrane current as described above (e.g., by 0.81 ± 0.13 pA pF^{-1} ($n=7$) ($P<0.01$) at 0 mV), whereas application of 100–200 μM A23 was without effect (e.g., shift of -0.05 ± 0.08 pA pF^{-1} ($n=6$) at 0 mV). To quantify this differential response, 0.5 mM Cd^{2+} was applied to myocytes that had been treated with 100 μM genistein or A23 for 5 min. Cd^{2+} had little effect on genistein-induced shifts in I_h and I_{200}(0 mV), but suppressed the A23-induced shifts (Figure 1b). Overall, Cd^{2+} inhibited 5 ± 4% of the genistein-induced shift in I_{200}(0 mV) ($n=4$), and 93 ± 6% of the A23-induced shift ($n=8$). Ni^{2+} (3 mM) had similar differential effects on drug-induced shifts ($n=3$ each).

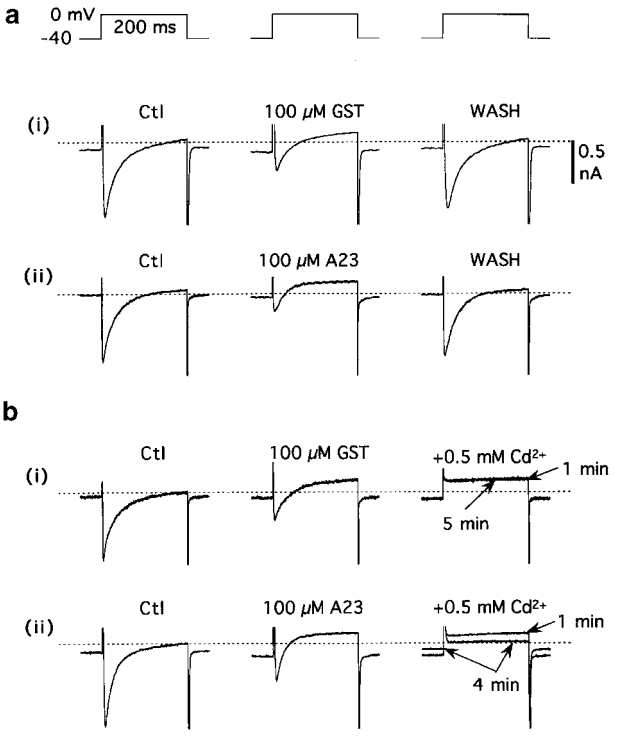

Figure 1 Differential effect of 0.5 mM Cd^{2+} on membrane currents induced by 100 μM genistein and 100 μM A23. The myocytes were dialysed with standard Cs^+ pipette solution, superfused with standard K^+-free Tyrode's solution, and depolarized from –40 to 0 mV for 200 ms at 0.2 Hz. (a) Reversible effects of (i) genistein (GST), and (ii) A23. In addition to inhibiting inward $I_{Ca,L}$ at 0 mV, both genistein and A23 induced an inward shift in the holding current (I_h) at –40 mV and an outward shift in the end-of-pulse current (I_{200}) at 0 mV. The records were obtained before (Ctl), 5 min after drug application, and $\approx$ 5 min after drug removal. (b) Effects of 0.5 mM Cd^{2+} on the shifts in current induced by (i) 100 μM genistein and (ii) 100 μM A23. Cd^{2+} rapidly abolished $I_{Ca,L}$ in both myocytes, and gradually reversed the current shifts in the A23-treated myocyte. The dashed lines indicate zero-current levels in these and subsequent records.

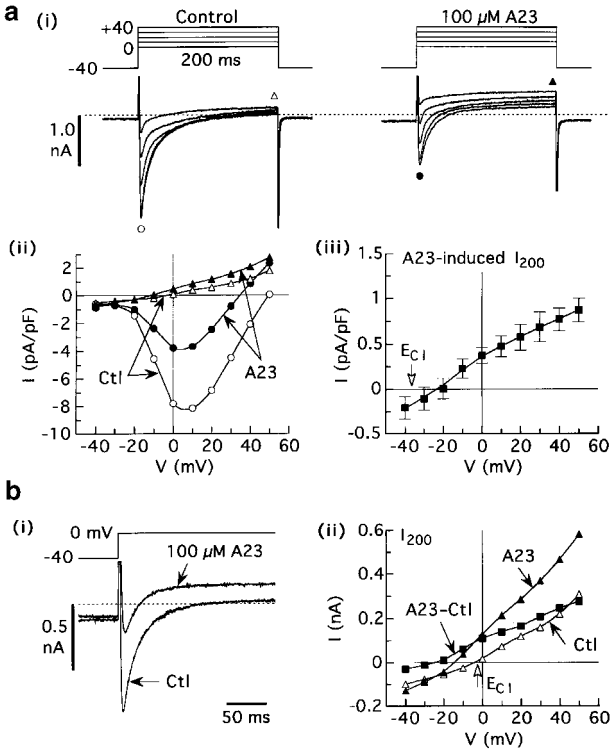

Figure 2 Lack of effect of dialysate Cl^- concentration on the reversal potential of A23-induced current. Myocytes superfused with standard solution were dialysed with Cs^+ solution that contained either 40 or 140 mM Cl^-, and depolarized with 200-ms pulses from holding potential –40 mV. (a) Data obtained from myocytes dialysed with 40 mM Cl^- solution. (i, ii) Current records and *I–V* relationships of peak inward current (circles) and I_{200} (triangles) obtained before and 5–6 min after application of 100 μM A23 to a representative myocyte. (iii) Average *I–V* relationship of A23-induced (A23 minus control) I_{200}. E_{rev} estimated by linear interpolation was –22 $\pm$ 4 mV (n = 7). (b) Data obtained from a myocyte dialysed with 140 mM Cl^- solution. (i) Example records obtained before (Ctl) and 4 min after addition of 100 μM A23. (ii) *I–V* relationships of I_{200}, illustrating that the E_{rev} of the A23-induced current (–24 mV) was similar to that measured in myocytes dialysed with 40 mM Cl^- solution, despite the + 33-mV shift in calculated E_{Cl} (vertical arrow).

A concern with the outward shifts of I_{200} (0 mV) caused by genistein and A23 under $I_{Ca,L}$-recording conditions is that they were conceivably due to inhibition of residual inward $I_{Ca,L}$ at 200 ms. A minor contribution from this source is not ruled out, but a major contribution can be discounted based on the marked inward shift in I_{200} (0 mV) caused by Cd^{2+} in A23 (but not genistein)-treated myocytes (e.g., Figure 1b), and on results obtained in the absence of $I_{Ca,L}$ (see below).

Differential effect of dialysate Cl^- on currents induced by A23 and genistein

The involvement of Cl^- conductance in A23 action was evaluated by determining the effects of altered Cl^- distribution on drug-induced current. Current–voltage (*I–V*) relationships were obtained from myocytes that were bathed with standard superfusate (149 mM Cl^-) and dialysed with either standard (40 mM) or high (140 mM) Cl^- pipette solution to set calculated E_{Cl} at –35 or –2 mV. In the myocytes dialysed with standard solution, the I_{200} induced by 100 μM A23 (A23 minus control) had an E_{rev} (–22 $\pm$ 4 mV, n = 7) that was significantly more positive than calculated E_{Cl} (Figure 2a). The effects of A23 were unaffected by 140-mM Cl^- dialysate; in particular, the E_{rev} of A23-induced I_{200} was unchanged at –23 $\pm$ 2 mV (n = 9) (e.g., Figure 2b). In marked contrast, the E_{rev} of genistein-activated current was sensitive to elevated-Cl^- pipette solution, shifting from –33 $\pm$ 3 mV (n = 5) in myocytes dialysed with 40 mM Cl^- solution (e.g., Figure 4b) to –21 $\pm$ 2 mV (n = 6) (Figure 6e), –2 $\pm$ 2 mV (Shuba & McDonald, 1997), and –1 $\pm$ 3 mV (n = 5) (this study) in myocytes dialysed with 65, 130, and 140 mM Cl^- solution, respectively.

Lack of involvement of nonselective cation channels in A23 action

A possible explanation for the effects of A23 on I_{200} was that the drug activates a Cd^{2+}-sensitive, nonselective cation current with $E_{rev} \approx$ –22 mV under standard conditions. In that case, replacement of standard (Na^+) superfusate with Na^+-free ($NMDG^+$) superfusate should affect the voltage dependence of the A23-induced current by abolishing the inward component, enhancing the outward component, and shifting

E_{rev} to a more negative potential. To test this prediction, myocytes treated with 100 μM A23 were superfused with Na^+ solution and then with Na^+-free solution. The results from a representative experiment (Figure 3a) indicate that the removal of Na^+ had two reversible effects on A23-induced I_{200}; it rapidly suppressed the inward component, and slowly suppressed the outward component. The slow suppression of outward current is inconsistent with involvement of nonselective cation channels in A23 action.

The *I–V* relationship of the early Na^+-sensitive component (measured after 1.5 min Na^+-free exposure) was inwardly rectifying with E_{rev} near +50 mV, and the *I–V* relationship of the late Na^+-sensitive component (measured after $\approx$7 min exposure) was slightly outwardly rectifying, with an E_{rev} of -26 ± 3 mV ($n = 5$) (Figure 3b). In marked contrast to these findings, removal of Na^+ had neither an early nor late effect on genistein-modified current ($n = 4$) (e.g., Figure 3c).

Evidence for involvement of the NCX in A23-induced current

The effects of Cd^{2+}, Ni^{2+}, and Na^+ removal pointed to involvement of NCX in A23-induced current. To investigate this possibility in more detail, experiments were conducted on myocytes with modified transmembrane distributions of Ca^{2+} and Na^+.

Experiments on myocytes with modified Ca^{2+} distribution Superfusion with Ca^{2+}-free solution is expected to be permissive for inward but not outward I_{NCX}. To determine whether this was the case with A23-induced current, myocytes dialysed with standard solution were superfused with Ca^{2+}-free solution and then treated with A23. The results of these experiments were inconclusive because the drug failed to induce any current at all (Figure 4a); in nine myocytes, 100–250 μM A23 changed the current levels at 0 and −80 mV by negligible 0.03 ± 0.04 and -0.02 ± 0.02 pA pF^{-1}, respectively. By contrast, 100 μM genistein induced an outwardly rectifying current with E_{rev} near E_{Cl} (Figure 4b).

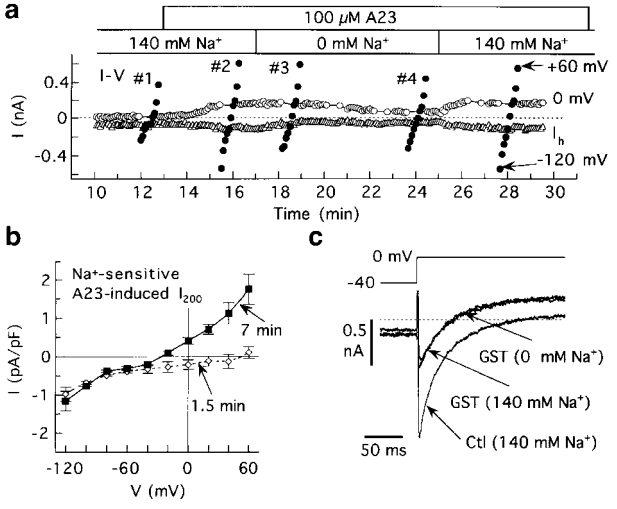

Figure 3 Differential effects of Na^+-free external solution on membrane currents induced by A23 and genistein. Myocytes dialysed with standard Cs^+ solution were superfused with standard 140 mM Na^+ or 0 mM Na^+ solution, and depolarized from –40 to 0 mV for 200 ms at 0.2 Hz, except for determinations of *I–V* relationships. (a) Reversible early and late effects of Na^+-free solution on I_{200} in a myocyte treated with 100 μM A23. The filled circles indicate the amplitudes of I_{200} on the *I–V* runs. Time markers indicate time after patch-breakthrough. (b) Na^+-sensitive A23-induced I_{200} determined at $\approx$1.5 and 7 min after replacement of standard solution with Na^+-free solution ($n = 5$). The current was measured by subtracting I_{200} (A23, 0 mM Na^+) from I_{200} (A23, 140 mM Na^+) (e.g., in (a): #3 from #2 (1.5 min), and #4 from #2 (7 min)). (c) Superimposed records illustrating the lack of effect of an 8-min exposure to Na^+-free solution on genistein-modified current.

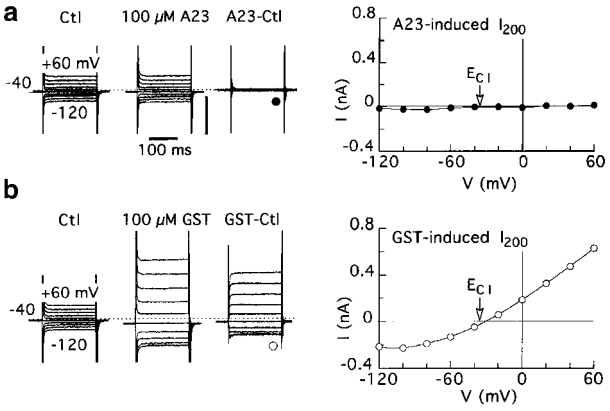

Figure 4 Effects of A23 and genistein on membrane currents in myocytes superfused with Ca^{2+}-free solution and dialysed with standard pCa 10.5 solution. The myocytes were pulsed from –40 mV to other voltages for 200 ms at 0.2 Hz before and 5 min after application of 100 μM A23 or genistein. (a) Lack of effect of A23. Left: original records and difference (A23-induced) current; right: *I–V* relationship of the A23-induced I_{200}. (b) Activation of outwardly rectifying current by genistein. The current reversed near calculated E_{Cl} (−35 mV). The calibration bar indicating 0.5 nA applies to all records.

The failure of A23 to induce inward current in the foregoing trials was tentatively attributed to the ancillary effects of Ca^{2+}-free superfusion, including suppression of Ca^{2+} entry and consequent lowering of subsarcolemmal Ca^{2+} essential for NCX activity. To stabilize intracellular Ca^{2+} in myocytes superfused with Ca^{2+}-free solution, dialysate Ca^{2+} was raised from standard pCa 10.5 to pCa 7. Under these conditions, 100 μM A23 caused a reversible increase in current amplitude at negative potentials (Figure 5a). The *I–V* relationship of the A23-induced current had the properties of a forward-mode I_{NCX} (i.e., inwardly rectifying with poorly defined positive E_{rev}) (Figure 5b), whereas the *I–V* relationship of genistein-induced current had the usual outwardly rectifying form (Figure 5c). In agreement with the view that the A23-induced current was in large measure due to NCX activity, the current was undetectable in myocytes that were superfused with Ca^{2+}-free solution that contained 5 mM Ni^{2+} (e.g., Figure 5d). In six experiments, the current induced by 100 μM A23 at −100 mV was -0.06 ± 0.05 pA pF^{-1} (*versus* -0.51 ± 0.07 pA pF^{-1} in the absence of Ni^{2+} (Figure 5b)).

Experiments on myocytes having near-physiological distributions of Ca^{2+} and Na^+ To determine the *I–V* relationship of A23-induced current under near-physiological ionic conditions, myocytes were dialysed with a 7 mM Na^+ pipette solution and superfused with K^+-free Tyrode's solution that was supplemented with 10 μM verapamil to block

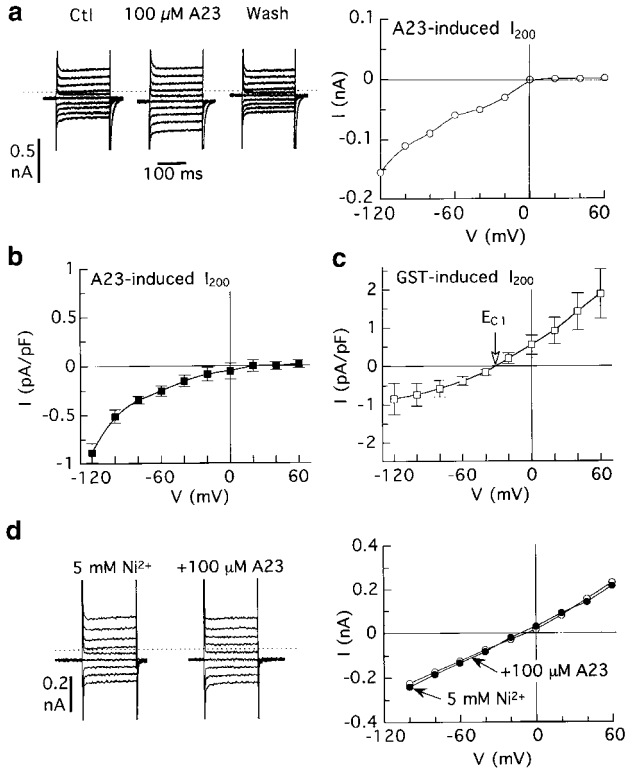

Figure 5 Effects of A23, genistein, and Ni^{2+} on membrane currents in myocytes superfused with Ca^{2+}-free solution and dialysed with pCa 7 solution. The myocytes were pulsed from −40 mV to other voltages for 200 ms at 0.2 Hz before, 5 min after application of 100 μM A23 or genistein, and 5–7 min after drug washout. (a) Reversible effects of A23 on membrane current in a representative myocyte. (b) The voltage dependence of A23-induced I_{200}; $n=9$ myocytes. (c) The voltage dependence of genistein-induced I_{200}; $n=4$. E_{rev} was near the calculated E_{Cl} of −32 mV. (d) Inhibition of A23-induced current by pretreatment with 5 mM Ni^{2+}.

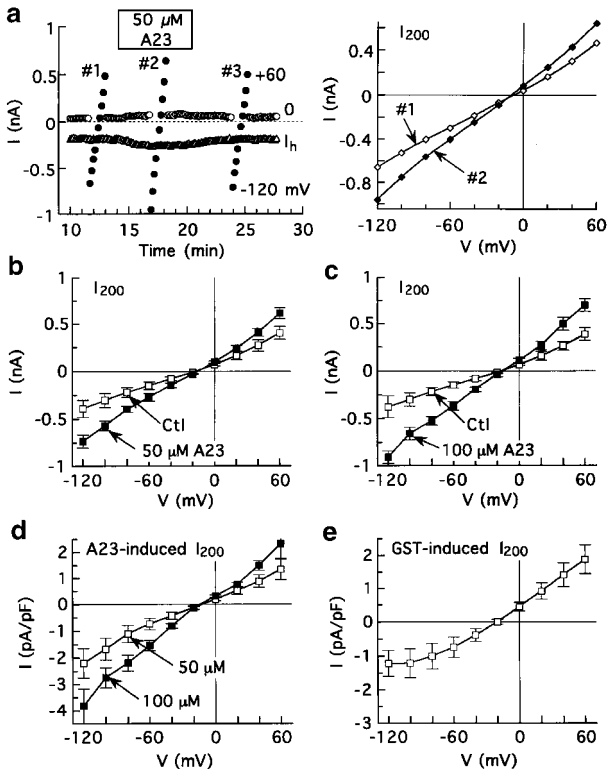

Figure 6 Effects of A23 and genistein on membrane currents in myocytes having near-physiological transmembrane gradients of Na^+ and Ca^{2+}. The myocytes were superfused with standard solution that contained 10 μM verapamil, dialysed with pCa 7 solution that contained 7 mM Na^+ and 65 mM Cl^-, and depolarized from –40 to 0 mV for 200 ms at 0.2 Hz except for determinations of *I–V* relationships. (a) Effects of 50 μM A23 in a representative experiment. Left: time course of changes in I_{200} amplitudes. Right: I_{200}–*V* relationships determined before and after addition of A23. (b, c) I_{200} measured before and after addition of 50 μM A23 (b, $n=5$) or 100 μM A23 (c, $n=6$). (d) A23-induced I_{200}; same myocytes as in (b, c). (e) I_{200} induced by 100 μM genistein; $n=6$ myocytes.

Ca^{2+} (and Na^+) channels with minimal inhibition of NCX (see Van Amsterdam & Zaagsma, 1986). Under these conditions, 50 μM A23 reversibly induced a current that had an E_{rev} near −15 mV (Figure 5a). In five myocytes treated with 50 μM A23, the drug-induced current reversed at -13 ± 5 mV (Figure 5b), a value that is close to the E_{rev} of I_{NCX} (−18.7 mV) estimated as $3E_{Na}$–$2E_{Ca}$ (where E_{Na} is the Na^+ equilibrium potential, and E_{Ca} is the Ca^{2+} equilibrium potential). The current induced by 100 μM A23 was larger than that induced by 50 μM, and reversed at -15 ± 3 mV ($n=6$) (Figure 6c,d). For comparison, the current induced by genistein was outwardly rectifying and reversed at -21 ± 2 mV ($n=6$) (calculated E_{Cl} –22 mV) (Figure 6e).

In myocytes with near-physiological distributions of Na^+ and Ca^{2+}, the reversal of the effects of higher ($\geqslant$100 μM) A23 (and A25, see below) was not always as satisfactory as it was with lower concentrations or with higher concentrations under standard conditions. Reversal was >85% complete in ≈30% of myocytes treated with high A23, and >60% complete in another 30%; in the remaining myocytes, recovery was interrupted by development of hypercontracture. The data in Figures 7 and 8 (see below) are from all myocytes in which membrane currents were relatively stable before and 5–7 min after addition of a tyrphostin.

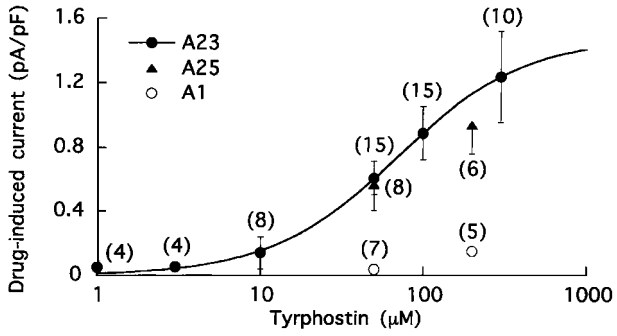

Figure 7 Concentration-dependent effects of A23, A25 and A1. The myocytes were superfused with standard solution that contained 10 μM verapamil, dialysed with pCa 7 solution that contained 7 mM Na^+ and 40 mM Cl^-, and depolarized from −40 to 0 mV for 200 ms at 0.1 Hz. The amplitude of the voltage-averaged current between –40 and 0 mV (I_{200}(0 mV) minus I_h(−40 mV)) was measured before and 5–7 min after drug application to obtain the drug-induced current. The data obtained with A23 (filled circles) are described by the Hill equation with an EC_{50} of 68 ± 4 μM and coefficient of 1.1. Also shown on the plot are data obtained from myocytes that were treated with A25 (triangles) and A1 (open circles). Numbers of myocytes in parentheses.

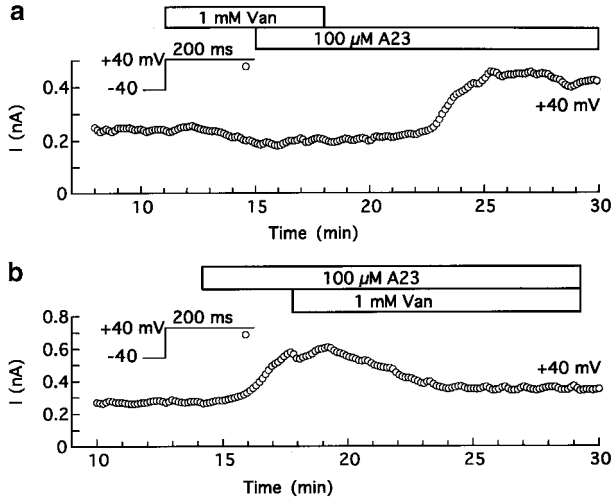

Figure 8 Antagonism of A23 action by orthovanadate. The myocytes were superfused with standard solution that contained 10 μM verapamil, dialysed with 7 mM Na^+, pCa 7 solution, and depolarized from −40 to +40 mV for 200 ms at 0.133 Hz. (a) Reversible antagonism of A23 action on I_{200} (+40 mV) by 1 mM orthovanadate (Van). (b) Inhibition of A23-induced current by 1 mM orthovanadate.

Relative effectiveness of A23 and two other tyrphostins

To determine the concentration dependence of A23 action, myocytes dialysed with 7 mM Na^+–pCa 7 solution and superfused with verapamil solution were treated with a concentration of A23 between 1 and 300 μM. The myocytes were pulsed from –40 to 0 mV, and a voltage-averaged current (I_{200}(0 mV) minus I_h(−40 mV)) was measured before and 5 min after application of A23. A plot of A23-induced current *versus* A23 concentration is shown in Figure 7, and the Hill equation fitting the data has an EC_{50} of 68±4 μM and a coefficient of 1.1. The plot also shows the results of trials with two other tyrphostins, TK-inhibitor A25 and TK-inactive A1. The current induced by 50 μM A25 (0.57±0.14 pA pF^{-1}; $n=8$; $P<0.001$) was as large as the current induced by 50 μM A23 (0.61±0.1 pA pF^{-1}; $n=15$), and considerably larger ($P<0.005$) than that induced by 50 μM A1 (0.04±0.03 pA pF^{-1}; $n=7$). A similar discrepancy between A23 and A1 effectiveness was observed when 200 μM concentrations were applied.

Antagonism of A23 action by orthovanadate

The data in Figure 7 indicate that TK-inhibiting tyrphostins induce a current with properties like I_{NCX}. To investigate the possible involvement of tyrosine phosphorylation in the action of the TK inhibitors, we looked for antagonism of A23 action by orthovanadate, an established inhibitor of phosphotyrosyl phosphatase (PTP) (Swarup *et al*., 1982; Davis *et al*., 2001). Two types of experiments were performed on myocytes with near-physiological distributions of Na^+ and Ca^{2+}. In the first series, myocytes were pretreated with 1 mM orthovanadate and then treated with 100 μM A23; in the second, myocytes were pretreated with 100 μM A23 and then treated with 1 mM orthovanadate.

Figure 8a shows that pretreatment with orthovanadate caused a small reduction in outward current monitored at +40 mV, and prevented the increase in amplitude usually observed after addition of A23. However, subsequent washout of the PTP inhibitor led to a typical A23 response in this and two similar experiments. Representative data from the second series of experiments are shown in Figure 8b. A23 (100 μM) induced an outward current at +40 mV, and addition of 1 mM orthovanadate resulted in a partial inhibition of the current. In six myocytes, the A23-induced current was reduced by 63±8% ($P<0.01$).

Discussion

NCX plays a major role in the function of cardiac cells. When operating in its forward mode (Ca^{2+} efflux), the exchanger contributes to diastolic relaxation and counterbalances Ca^{2+} entry *via* Ca^{2+} channels (Carmeliet, 1992; Bers *et al*., 2003); when operating in its reverse mode, it promotes a Ca^{2+} influx that contributes to Ca^{2+} loading of the sarcoplasmic reticulum and/or Ca^{2+} release from the organelle (Crespo *et al*., 1990; Leblanc & Hume, 1990; Lipp & Niggli, 1994; Litwin *et al*., 1998; Meme *et al*., 2001; Bers *et al*., 2003). In either mode, I_{NCX} affects the configuration of the action potential (Mitchell *et al*., 1984; Noble *et al*., 1991; Janvier & Boyett, 1996; Wei *et al*., 2003).

NCX is upregulated by PKC (Iwamoto *et al*., 1996) and PKA (Ruknudin & Schulze, 2002; Schulze *et al*., 2003), but there is little information on a possible role for TK (see below). In the present study, we have investigated the effects of TK inhibitors A23 and genistein on cardiac membrane currents, and found that A23 induced a current that behaved like I_{NCX}. We discuss the properties of this current, and consider possible underlying mechanisms.

Properties of the current induced by A23

Under standard $I_{Ca,L}$ recording conditions, both genistein and A23 induced reversible outward shifts in late current at 0 mV. The genistein-induced shifts were almost certainly due to activation of outwardly rectifying $I_{Cl(CFTR)}$ as previously reported (Shuba *et al*., 1996; Chiang *et al*., 1997; Shuba & McDonald, 1997), whereas the A23-induced shifts were unrelated to Cl^- pathways because they were unaffected by changes in dialysate Cl^- concentration. A further distinction between genistein- and A23-induced current was that A23-induced current was strongly inhibited by Cd^{2+}. The latter result suggested that Cd^{2+}-sensitive nonselective cation conductance and/or Cd^{2+}-sensitive NCX were involved in the A23 response. However, a major contribution by nonselective channels seems unlikely on several grounds, including the finding that removal of external Na^+ or Ca^{2+} inhibited the outward component of A23-induced current. Conversely, the activation of Ni^{2+}-sensitive inwardly rectifying current by A23 in myocytes bathed with Ca^{2+}-free solution and dialysed with pCa 7 pipette solution suggests the involvement of NCX.

The foregoing analysis points to I_{NCX} as the identity of A23-induced current. An apparent difficulty with this interpretation concerns the availability of intracellular Na^+ to carry the A23-induced outward current recorded under standard experimental conditions (Na^+-free pipette solution). However, it seems

highly likely that Na^+ ions were present in submembrane regions (Carmeliet, 1992) due to technical limitations in controlling intracellular ion concentrations (Pusch & Neher, 1988; Mathias *et al*., 1990), especially in the face of continuous influx *via* noninactivated Na^+ channels, 'background' Na^+ channels, nonselective cation channels, and forward-mode NCX activity. It has been estimated that this influx can raise global cytoplasmic Na^+ by $\approx 1\,mM\,min^{-1}$ in nonpatched myocytes when, as here, Na^+ pump sites are inhibited by K^+-free external conditions (Eisner *et al*., 1981; Désilets & Baumgarten, 1986; Bers *et al*., 2003). Although continuous diffusion of cytoplasmic Na^+ into the pipette will have attenuated elevation of global Na^+ in our patched myocytes, it is probable that submillimolar concentrations of subsarcolemmal Na^+ were only attained after a period of restricted Na^+ influx. In this regard, superfusion with Na^+-free solution had a biphasic effect on A23-modified current, an early reduction of inward current that we attribute to depletion of external Na^+, and a secondary reduction of outward current that we attribute to depletion of subsarcolemmal Na^+.

It is also probable that subsarcolemmal Ca^{2+} concentration was considerably higher than cytoplasmic Ca^{2+} concentration in myocytes patched with standard (pCa 10.5) pipette solution, especially during pulses that elicited $I_{Ca,L}$ (You *et al*., 1997; Weber *et al*., 2002). The data in Figure 2a(iii) indicate that the A23-induced current reversed at about –22 mV, an E_{rev} for I_{NCX} that would be consistent with respective subsarcolemmal Na^+ and Ca^{2+} concentrations of 3.6 mM and 10 nM (for example). In the absence of additional modulation, a Ca^{2+} concentration of this magnitude would only secure a mild activation of cardiac NCX *via* binding of the divalent cation to the allosteric activation site (K_D 125–300 nM: (Weber *et al*., 2001; Reeves & Condrescu, 2003).

In myocytes configured for measurement of $I_{Ca,L}$, 0.5 mM Cd^{2+} completely suppressed both $I_{Ca,L}$ and A23-induced current (Figure 1b). The two events were linked in so far as suppression of $I_{Ca,L}$ suppressed Ca^{2+} influx, lowered submembrane Ca^{2+} concentration, and inhibited Ca^{2+}-mediated activation of NCX. However, it is probable that Cd^{2+} also suppressed A23-induced current by competing with Ca^{2+} for external NCX sites. Cd^{2+} has been shown to inhibit smooth muscle NCX (Smith *et al*., 1987), cardiac NCX (Bers *et al*., 1980; Trosper & Philipson, 1983), and cloned NCX1 (Iwamoto & Shigekawa, 1998) with $K_D \leqslant 33\,\mu M$, and has been used (0.2–1 mM) as a prophylactic measure to block NCX in recent studies on cardiomyocytes (Feraille *et al*., 1997; Gao *et al*., 2002). Application of Ni^{2+} (3 mM), a weaker inhibitor of $I_{Ca,L}$ (McDonald *et al*., 1994) and NCX1 (Iwamoto & Shigekawa, 1998) than is Cd^{2+}, also suppressed A23-induced current.

Earlier findings and possible mechanisms

The effects of 100–200 μM genistein on NCX have been examined in three earlier studies, with one reaching the conclusion that the drug inhibited NCX in cultured neuronal cells (Wang *et al*., 1997), and the others that it had no effect on the activity of NCX1 expressed in fibroblasts (Condrescu *et al*., 1996; Linck *et al*., 1998). In the present study, genistein-induced current was insensitive to application of Cd^{2+} and modifications of Na^+ and Ca^{2+} concentrations. These results, and the failure of genistein to induce current at any E_{Cl} between −35 and −1 mV, lead to the conclusion that (in agreement with the earlier studies) genistein does not stimulate NCX activity.

To our knowledge, there are no previous data on the effects of tyrphostin compounds on the activity of either native or expressed NCX. The present results with A23 are consistent with a stimulatory effect on NCX1 in guinea-pig ventricular myocytes, perhaps by affecting the activatory action of intracellular Ca^{2+} (see above). A key question is whether the tyrphostin acted by influencing tyrosine phosphorylation. In that regard, it is known that NCX1 has consensus sites for phosphorylation by TK (Quednau *et al*., 1997), and that tyrosine phosphorylation of NCX regulatory protein can modulate basal NCX activity (Kiang *et al*., 2003). Evidence in favour of a phosphorylation-related mechanism is that a second TK inhibitor (A25) was as effective as A23, the TK-inactive analogue (A1) was substantially less effective, and PTP inhibitor orthovanadate antagonized the action of A23. On the other hand, the ineffectiveness of TK inhibitor genistein weakens the case for such a mechanism. However, it is important to note that even though both A23 and genistein are classified as broad-spectrum TK inhibitors, they may preferentially interact with one TK family over another (Akiyama & Ogawara, 1991; Ramdas *et al*., 1994). Since it is well established that specific TK families can have opposing effects on cellular processes (Zhang *et al*., 2002), it is entirely possible that A23 and genistein have TK-related differential actions on I_{NCX}. In that regard, differential action on specific TK families has been held responsible for the divergent effects of 100 μM A23 (ca. 40% inhibition) and 100 μM genistein (120% stimulation) on swelling-activated Cl^- current in human atrial myocytes (Du *et al*., 2004).

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