Differential Effects of Tyrosine Kinase Inhibitors on Volume-sensitive Chloride Current in Human Atrial Myocytes: Evidence for Dual Regulation by Src and EGFR Kinases

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ABSTRACT

To determine whether protein tyrosine kinase (PTK) modulates volume-sensitive chloride current (I \textsubscript{Cl,vol}) in human atrial myocytes and to identify the PTKs involved, we studied the effects of broad-spectrum and selective PTK inhibitors and the protein tyrosine phosphatase (PTP) inhibitor orthovanadate (VO\textsubscript{4}\textsuperscript{3-}). I \textsubscript{Cl,vol} evoked by hyposmotic bath solution (0.6-times isosmotic, 0.6T) was enhanced by genistein, a broad-spectrum PTK inhibitor, in a concentration-dependent manner (EC\textsubscript{50} = 22.4 μM); 100 μM genistein stimulated I \textsubscript{Cl,vol} by 122.4 ± 10.6%. The genistein-stimulated current was inhibited by DIDS (4,4′-disothiocyanostilbene-2,2′-disulfonic acid, 150 μM) and tamoxifen (20 μM), blockers of I \textsubscript{Cl,vol}. Moreover, the current augmented by genistein was volume dependent; it was abolished by hyperosmotic shrinkage in 1.4T, and genistein did not activate Cl\textsuperscript{-} current in 1T. In contrast to the stimulatory effects of genistein, 100 μM tyrphostin A23 (AG 18) and A25 (AG 82) inhibited I \textsubscript{Cl,vol} by 38.2 ± 4.9% and 40.9 ± 3.4%, respectively. The inactive analogs, daidzein and tyrphostin A63 (AG 43), did not alter I \textsubscript{Cl,vol}. In addition, the PTP inhibitor VO\textsubscript{4}\textsuperscript{3-} (1 mM) reduced I \textsubscript{Cl,vol} by 53.5 ± 4.5% (IC\textsubscript{50} = 249.6 μM). Pretreatment with VO\textsubscript{4}\textsuperscript{3-} antagonized genistein-induced augmentation and A23-or A25-induced suppression of I \textsubscript{Cl,vol}. Furthermore, the selective Src-family PTK inhibitor PP2 (5 μM) stimulated I \textsubscript{Cl,vol}, mimicking genistein, whereas the selective EGFR (ErbB-1) kinase inhibitor tyrphostin B56 (AG 556, 25 μM) reduced I \textsubscript{Cl,vol} mimicking A23 and A25. The effects of both PP2 and B56 also were substantially antagonized by pretreatment with VO\textsubscript{4}\textsuperscript{3-}. The results suggest that I \textsubscript{Cl,vol} is regulated in part by the balance between PTK and PTP activity. Regulation is complex, however. Src and EGFR kinases, distinct soluble and receptor-mediated PTK families, have opposing effects on I \textsubscript{Cl,vol} and multiple target proteins are likely to be involved.

KEY WORDS: cell volume • Src family kinases • EGFR kinase • protein tyrosine phosphatase • orthovanadate

INTRODUCTION

A volume-sensitive chloride current, termed I \textsubscript{Cl,vol} or I \textsubscript{Cl,swell}, is found in mammalian cardiac myocytes, including those from man (for reviews see Hiraoka et al., 1998; Sorota, 1999; Hume et al., 2000; Baumgarten and Clemo, 2003). The current is activated by cell swelling and/or membrane deformation. I \textsubscript{Cl,vol} regulates cell volume (Suleymanian et al., 1995), shortens action potential duration (Vandenberg et al., 1997), and depolarizes resting membrane potential (Du and Sorota, 1997), suggesting that I \textsubscript{Cl,vol} may play a role in cardiac electrophysiology and arrhythmogenesis in situ (Hiraoka et al., 1998).

Multiple signaling pathways have been postulated to control I \textsubscript{Cl,vol} in cardiac (Sorota, 1999; Hume et al., 2000; Baumgarten and Clemo, 2003) and noncardiac tissues (Nilius et al., 1997). One of these, protein tyrosine kinase (PTK), is immediately activated upon myocyte swelling (Sadoshima et al., 1996). Inhibitors of PTK, such as the isoflavone genistein (Akiyama and Ogawara, 1991) and several tyrphostins (Gazit et al., 1999), and inhibitors of protein tyrosine phosphatase (PTP), such as orthovanadate (VO\textsubscript{4}\textsuperscript{3-}) (Gordon, 1991), are widely used to identify regulation by tyrosine phosphorylation. Blocking PTKs with genistein inhibits I \textsubscript{Cl,vol} in dog atrial cells (Sorota, 1995), calf pulmonary artery endothelial cells (Voets et al., 1998), and rabbit ciliary epithelial cells (Shi et al., 2002). On the other hand, PTP inhibitors also suppress I \textsubscript{Cl,vol} in bovine chromaffin cells (Doroshenko, 1998) and mouse L-fibroblasts (Thoroed et al., 1999). These studies indicate that the

Abbreviations used in this paper: DIDS, 4,4′-disothiocyanostilbene-2,2′-disulfonic acid; EGFR, epidermal growth factor receptor; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; T, times isosmotic.
phosphorylation and dephosphorylation of tyrosine are involved in the modulation of $I_{Cl,vol}$. Manipulations with opposite consequences for tyrosine phosphorylation affected $I_{Cl,vol}$ identically, however, suggesting regulation may be tissue or species specific (Nilius et al., 1997; Okada, 1997). Such diversity may arise because tyrosine phosphorylation by specific PTKs controls distinct signaling cascades (Hunter, 1995; Pawson and Scott, 1997; Zhang et al., 2002). Moreover, PTK inhibitors previously have been shown to modulate both anion (Sorota, 1995; Gadsby and Nairn, 1999) and cation channels (Zhou et al., 1997) in heart and other tissues (Davis et al., 2001).

Although we (Li et al., 1996) and others (Oz and Sorota, 1995; Sakai et al., 1995; Sato and Koumi, 1998) identified $I_{Cl,vol}$ in human atrial myocytes, the modulation of $I_{Cl,vol}$ by signaling cascades is incompletely understood (Nilius et al., 1997; Sorota, 1999; Humé et al., 2000) and may be species and tissue dependent (Nilius et al., 1997; Okada, 1997). The present study demonstrated that protein tyrosine phosphorylation and dephosphorylation regulate $I_{Cl,vol}$ in human atrial myocytes and that inhibition of Src family and epidermal growth factor receptor (EGFR, ErbB-1) tyrosine kinases have opposing effects.

MATERIALS AND METHODS

Cell Isolation

Atrial myocytes were isolated from specimens of right atrial appendage obtained from patients (50.1 ± 8.2-yr-old, range, 35–74) undergoing coronary artery bypass. Procedures for obtaining the tissue with the patients’ written consent were approved by the Ethics Committee of the University of Hong Kong. No evidence for atrial arrhythmias or atrial dilation was found in any of the patients based on ECG and echocardiograms. After excision, samples were quickly immersed in oxygenated, nominally Ca$^{2+}$-free cardioplegic solution for transport to the laboratory. Atrial myocytes were enzymatically dissociated by a technique modified from that described previously (Li et al., 1996). Briefly, the myocardial tissue was sliced with a sharp blade, placed in a 15-ml tube containing 10 ml of Ca$^{2+}$-free Tyrode solution (36°C), and gently agitated by continuous bubbling with 100% O$_2$ for 15 min with transfer to fresh solution after 5 min. Then, the chunks were incubated for 50 min in a similar solution containing 150–200 U/ml collagenase (CLS II, Worthington Biochemical), 1.2 U/ml protease (type XXIV, Sigma-Aldrich), and 1 mg/ml bovine serum albumin (Sigma-Aldrich). Finally, the tissue was reincubated in a fresh enzyme solution without protease. The number and the quality of the isolated cells were determined by microscopic examination at 5–10 min intervals. When the yield appeared to be maximal, the tissue was suspended in a high K+ medium and gently pipetted. The isolated myocytes were kept at room temperature in the high K+ medium for at least 1 h before study.

A small aliquot of the solution containing the isolated cells was placed in an open perfusion chamber (1 ml) mounted on the stage of an inverted microscope (Leica DM IL). Myocytes were allowed to adhere to the bottom of the chamber for 5–10 min, and were then superfused at 2–3 ml/min with isosmotic 1.0T (T, times isosmotic), hypotonic 0.6T or hyperosmotic 1.4T Tyrode solution. Only quiescent rod-shaped cells showing clear cross- striations were used.

Solution and Drugs

Ca$^{2+}$-free cardioplegic solution for specimen transport contained (in mM): 50 KH$_2$PO$_4$, 8 MgSO$_4$, 5 adenosine, 10 HEPES, 140 glucose, 100 mannitol, 10 taurine, pH was adjusted to 7.3 with KOH. Standard Tyrode solution contained: 140 NaCl, 5.4 KCl, 1 MgCl$_2$, 1.8 CaCl$_2$, 0.33 NaH$_2$PO$_4$, 5 HEPES, 10 glucose, pH adjusted to 7.4 with NaOH. Ca$^{2+}$ was omitted from the Tyrode solutions used for enzymatic digestion and for washing the sliced atrial tissue. For osmotic swelling experiments, hypotonic 0.6T (~180 mosmol/L) Tyrode was made by reducing NaCl from 140 to 80 mM, and isosmotic 1T (~300 mosmol/L) and hyperosmotic 1.4T (~420 mosmol/L) Tyrode were prepared by adding 125 or 240 mM mannitol, respectively, to 0.6T. The pipette solution contained: 20 CsCl, 110 Cs-aspartate, 1.0 MgCl$_2$, 10 HEPES, 5 EGTA, 0.1 GTP, 5 Na$_2$-phosphocreatine, and 5 Mg-ATP, pH adjusted to 7.2 with CsOH (~295 mosmol/L). The high K+ storage medium contained: 10 KCl, 120 K-glutamate, 10 KH$_2$PO$_4$, 1.8 MgSO$_4$, 10 taurine, 10 HEPES, 0.5 EGTA, 20 glucose, 10 mannitol, pH adjusted to 7.3 with KOH. All experiments were done at room temperature, 21–22°C.

3-(4-Chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]-pyrimidin-4-amine (PP2) was purchased from Tocris. All other reagents were obtained from Sigma-Aldrich. Stock solutions were made with DMSO for genistein (100–200 mM), daidzein (100 mM), tyrphostin A23 (AG 18), A25 (AG 82), A63 (AG 43), B56 (AG 56) (100 mM), and tamoxifen (20 mM). The stocks were divided into aliquots and stored at −20°C. The maximum 0.1% final concentration of DMSO in bath solution did not affect $I_{Cl,vol}$.

Data Acquisition and Analysis

Whole-cell currents were recorded using an EPC-9 amplifier and Pulse software (Heka), and signals were low-pass filtered (2 kHz) before 5 kHz digitization. Pipette resistance was 2–3 MΩ. gigaseals were >10 GΩ. Series resistance (5–8 MΩ) was compensated (60–80%) after membrane rupture, and a 3-M KCl-agar bridge was used as a reference electrode. In separate experiments, the liquid junction potential (bath – pipette) was measured as +12 mV and was not corrected.

Relative cell volume was determined during the whole-cell recording using each cell as its own control (Drewnowska and Baumgarten, 1991). Images of myocytes were captured with a digital camera (Leica DC300) at selected time points during the experiment. Cell area and width were measured with commercial software (Optimas, Media Cybernetics). Changes in cell width and thickness are proportional. Taking each cell as its own control (Drewnowska and Baumgarten, 1991). Images of myocytes were captured with a digital camera (Leica DC300) at selected time points during the experiment. Cell area and width were measured with commercial software (Optimas, Media Cybernetics). Changes in cell width and thickness are proportional. Taking each cell as its own control (Drewnowska and Baumgarten, 1991). Images of myocytes were captured with a digital camera (Leica DC300) at selected time points during the experiment. Cell area and width were measured with commercial software (Optimas, Media Cybernetics). Changes in cell width and thickness are proportional.
Stat 2.03, SPSS). P < 0.05 was considered to indicate statistical significance. Results are presented as mean ± SE.

RESULTS

Osmotic Swelling–induced Current

Fig. 1 A illustrates the time-course of swelling-induced changes in membrane current at +50 mV in a human atrial myocyte when bath solution was switched from isosmotic 1T to hyposmotic 0.6T solution and then back to 1T. Membrane current in 0.6T gradually increased to a new steady-state within 15 min and fully returned to control levels after reexposure to 1T. Similar results were obtained in five cells. The swelling-induced current is $I_{\text{Cl,vol}}$, as described previously (Li et al., 1996). This was confirmed by the application of the $I_{\text{Cl,vol}}$ blocker DIDS (Sorota, 1999; Hume et al., 2000; Baumgarten and Clemo, 2003).

Figure 1. $I_{\text{Cl,vol}}$ in human atrial myocytes. (A) Time course of activation of current at +50 mV on switching from isosmotic (1T) to hyposmotic (0.6T) bath solution and full recovery in 1T. Currents at time points a–c shown at right. Currents were elicited by 300-ms steps to +50 mV from −40 mV (inset). (B) Voltage-dependent currents in 1T control (a), 0.6T (b), and 0.6T with 150 μM DIDS (c). DIDS, a blocker of $I_{\text{Cl,vol}}$, substantially inhibited the swelling-induced current. Arrows indicate 0 current. Voltage protocol for B and C, 300-ms steps to between −100 and +60 mV from −40 mV (inset). (C) Current-voltage (I-V) relationships for the swelling-induced current before and after exposure to DIDS. $n = 6$. The swelling-induced current outwardly rectified and reversed at −28 mV (−40 mV after correction for the liquid junction potential), near the predicted $E_{\text{Cl}}$, −35 mV. DIDS almost completely inhibited the outward current, whereas inward current was inhibited by ~50%. These are characteristics of $I_{\text{Cl,vol}}$ and its voltage-dependent block by DIDS (Sorota, 1999; Hume et al., 2000; Baumgarten and Clemo, 2003).

Figure 2. Stimulation of $I_{\text{Cl,vol}}$ by genistein. (A) Time course of current at +50 mV on switching from isosmotic (1T) to hyposmotic (0.6T) bath solution and full recovery in 1T. Currents were elicited by 300-ms steps to +50 mV from −40 mV (inset). (B) Voltage-dependent currents in 1T control (a), 0.6T (b), and 0.6T with 100 μM genistein (c). Arrows indicate 0 current. Voltage protocols are shown in insets.
Effects of Genistein on $I_{\text{Cl,vol}}$

To study the effects of PTKs on $I_{\text{Cl,vol}}$, the broad-spectrum PTK blocker genistein was applied after activating $I_{\text{Cl,vol}}$ by osmotic swelling. Fig. 2 A shows the time-course of current at $V_{\text{H11001}} = 50 \text{ mV}$ as bathing solution was switched from 1T, to 0.6T, and to 0.6T with 100 $\mu$M genistein. $I_{\text{Cl,vol}}$ in 0.6T was significantly enhanced by genistein, and the stimulation was completely reversed upon washout. Similar results were obtained in seven cells. Families of currents obtained between $V_{\text{H11002}} = 100$ and $V_{\text{H11001}} = 60 \text{ mV}$ under the same conditions are presented in Fig. 2 B. The I-V relationships showed that genistein reversibly increased the amplitude of outwardly rectifying $I_{\text{Cl,vol}}$ in 0.6T at test potentials from $-100$ to $-50 \text{ mV}$ and from $+10$ to $+60 \text{ mV}$ (Fig. 2 C; $P < 0.05$ or $P < 0.01$, $n = 7$).

The concentration-response relationship for stimulation of $I_{\text{Cl,vol}}$ by genistein in 0.6T was evaluated at $+50 \text{ mV}$ (Fig. 2 D). Data were fitted with the equation: $E = E_{\text{max}} \left[1 + (EC_{50}/C)^b\right]$, where $E$ is the stimulation of $I_{\text{Cl,vol}}$ in percent at concentration $C$, $E_{\text{max}}$ is the maximum stimulation, $EC_{50}$ is the concentration for half-maximum action, and $b$ is the Hill coefficient. The best-fit parameters were $E_{\text{max}} = 134.4\%$, $EC_{50} = 22.4 \mu$M, and $b = 1.37$.

To rule out possible nonspecific effects of genistein, daidzein, an analogue of genistein that does not inhibit PTK, was studied in five myocytes. Fig. 3 shows that daidzein (100 $\mu$M) did not affect the magnitude or the time-independence of the current at $+50 \text{ mV}$, whereas genistein (100 $\mu$M) reversibly stimulated the current by $\geq 60\%$ in the same myocyte. I-V relationships confirmed that daidzein did not affect $I_{\text{Cl,vol}}$ over the entire voltage range examined (Fig. 3 C) even though genistein enhanced the current in each cell studied.

Effects of $I_{\text{Cl,vol}}$ Blockers and Cell Volume on Genistein-enhanced Current

To verify the identity of the genistein-induced increase of $\text{Cl}^-$ current, DIDS and tamoxifen, which block $I_{\text{Cl,vol}}$ but not $I_{\text{Cl,cAMP}}$ in heart (Vandenberg et al., 1994; Sorota, 1999; Hume et al., 2000; Baumgarten and Clemo, 2003), were used. Currents were recorded in 1T, after swelling in 0.6T, after stimulation of current by 100 $\mu$M genistein in 0.6T reached a steady-state, and after exposure to $I_{\text{Cl,vol}}$ blockers in 0.6T in the continued presence of genistein. At $+50 \text{ mV}$, DIDS (150 $\mu$M) decreased the
ICl.vol by 91.2 ± 2.3% (n = 4, unpublished data). In addition, tamoxifen (20 μM) blocked ~95% of the current at +50 mV (n = 3) in the continued presence of genistein (Fig. 4). Thus, two structural distinct I_{Cl.vol} blockers inhibited both the current augmented by genistein and the current activated by cell swelling.

Another means of identifying the genistein-augmented current is to examine its dependence on cell volume. Genistein (100 μM) did not alter membrane conductance when it was applied in 1T (n = 5, unpublished data). Furthermore, both the 0.6T- and genistein-dependent activation of current promptly was reversed by cell shrinkage in 1.4T in the continued presence of genistein (Fig. 5). Together, these data indicate that genistein modulates a Cl\(^{-}\) current that is turned on in swollen myocytes and turned off by cell shrinkage.

**Effect of Orthovanadate on I_{Cl.vol}**

The PTP inhibitor VO_4\(^{3-}\) was used to determine whether tyrosine dephosphorylation is involved in the activation of I_{Cl.vol} in human atrial myocytes. Fig. 6 A shows that VO_4\(^{3-}\) (1 mM) substantially inhibited I_{Cl.vol} in 0.6T within 5 min, and the effect rapidly and fully reversed on washout. I-V relationships in the absence and presence of VO_4\(^{3-}\) indicated that the current blocked by VO_4\(^{3-}\) was outwardly rectifying and reversed near the reversal potential for I_{Cl.vol} (Fig. 6 B; n = 7). In contrast, VO_4\(^{3-}\) (1 mM) did not alter currents in 1T, confirming that the VO_4\(^{3-}\)-sensitive current was I_{Cl.vol} (n = 5; unpublished data).

The concentration-response relationship for block of I_{Cl.vol} by VO_4\(^{3-}\) at +50 mV is displayed in Fig. 6 C. Data were fitted as described above, except for inhibition. The best-fit parameters were E_{max} = 49.5%, I_{50} = 249.6 μM, and b = 1.71.

**Influence of Orthovanadate on Genistein**

If stimulation of I_{Cl.vol} by genistein is due to block of tyrosine phosphorylation, inhibiting PTP-dependent tyrosine dephosphorylation should antagonize its effect. A test of this prediction is illustrated in Fig. 7 A. At +50 mV, VO_4\(^{3-}\) significantly reduced I_{Cl.vol} in 0.6T and substantially prevented stimulation of I_{Cl.vol} by genistein.
Modulation of Cardiac I_{Cl,vol} by Tyrosine Kinases

Fig. 7 B illustrates families of I_{Cl,vol} obtained by subtracting the current in 1T from those in 0.6T after the application of 1 mM VO_4^-/H_11002^3 alone or VO_4^-/H_11002^3 with 100 μM genistein, and VO_4^-/H_11002^3 with 100 μM genistein plus VO_4^-/H_11002^3. Pretreatment with VO_4^-/H_11002^3 significantly diminished stimulation of I_{Cl,vol} by genistein. Currents are at right. (B) Voltage-dependent I_{Cl,vol} obtained by digital subtraction of the current in 0.6T, after 1 mM VO_4^-/H_11002^3, and VO_4^-/H_11002^3 with 100 μM genistein from that in 1T. (C) IV relationships of I_{Cl,vol} obtained by digital subtraction of currents before and after the application of VO_4^-/H_11002^3 (C), and VO_4^-/H_11002^3 with 100 μM genistein ( ). Although pretreatment with VO_4^-/H_11002^3 significantly reduced stimulation of I_{Cl,vol} by genistein (compare with Fig. 2; P < 0.01), a small but significant stimulation was observed, 16.9 ± 2.5 and 18.9 ± 4.7% at −90 and +50 mV (P < 0.01, n = 7). Voltage protocols are shown in insets.

Fig. 7 B illustrates families of I_{Cl,vol} obtained by subtracting the current in 1T from those in 0.6T after the application of 1 mM VO_4^-/H_11002^3 alone or VO_4^-/H_11002^3 with 100 μM genistein, and Fig. 7 C displays the I-V relationships of I_{Cl,vol} after the addition of 1 mM VO_4^-/H_11002^3 and VO_4^-/H_11002^3 with 100 μM genistein. Pretreatment with VO_4^-/H_11002^3 significantly antagonized the genistein-induced increase of I_{Cl,vol} over the entire voltage range studied as compared with genistein alone (see Fig. 2). The results suggest that regulation of I_{Cl,vol} depends at least in part on the balance of tyrosine phosphorylation by PTKs and dephosphorylation by PTPs. Both genistein- and osmotic swelling–induced stimulation of I_{Cl,vol} were opposed by blocking dephosphorylation of tyrosine.

Selective Inhibition of Src

Although genistein is a broad-spectrum PTK inhibitor that blocks both receptor and nonreceptor PTK, its ED_{50} for stimulation of I_{Cl,vol} was 22.4 μM (see Fig. 2 D) was almost identical to its IC_{50} for block of pp60^{src}, 25.9 or 29.6 μM (Akiyama and Ogawara, 1991). This suggested that inhibition of Src family PTKs might be responsible for the stimulation of I_{Cl,vol}. To test this hypothesis, we used PP2, a selective inhibitor of Src family PTKs (Hanke et al., 1996). Fig. 8 A shows I_{Cl,vol} in 0.6T before and after application of 5 μM PP2 and after its washout. PP2 gradually increased I_{Cl,vol}, reaching a steady-state after 10 min, and nearly complete recovery was obtained after a prolonged (>30 min) washout of the drug. As previously shown for genistein (Fig. 6), stimulation of I_{Cl,vol} by PP2 was antagonized by pretreatment with VO_4^-/H_11002^3 (Fig. 8 B). At +50 mV, for example, PP2 increased I_{Cl,vol} by 67.5 ± 15.2% (n = 9, P < 0.01), whereas stimulation...
Effect of Tyrphostins on $I_{\text{Cl,vol}}$

Another important class of PTK inhibitors is the tyrphostins. To study effects of these nonisoflavone PTK inhibitors on $I_{\text{Cl,vol}}$, tyrphostin A23 (100 μM) was applied after activation of $I_{\text{Cl,vol}}$ in 0.6T. In contrast to the stimulatory effect of genistein and PP2, A23 reversibly inhibited $I_{\text{Cl,vol}}$ (Fig. 9 A). I-V relationships show that A23 significantly decreased the amplitude of $I_{\text{Cl,vol}}$ at test potentials from $-100$ to $-50$ mV and 0 to $+60$ mV ($n = 7$, $P < 0.05$ or $P < 0.01$; Fig. 9 B). The concentration-response relationship for inhibition of $I_{\text{Cl,vol}}$ by A23, $IC_{50}$ was 27.5 μM; number of cells in parentheses.

Another tyrphostin compound, A25, produced a similar inhibition of $I_{\text{Cl,vol}}$ as A23 ($n = 6$, unpublished data). To confirm the tyrphostin-sensitive currents were volume sensitive, A23 and A25 were studied in 1T. No change in currents was observed (100 μM, $n = 4$ each; unpublished data). Thus, tyrphostin PTK inhibitors, which are structurally distinct from genistein and PP2, produce the opposite effect on $I_{\text{Cl,vol}}$.

Tyrphostin A63, an inactive analogue of A23 and A25, was examined to exclude nonspecific effects of tyrphostins on $I_{\text{Cl,vol}}$. $I_{\text{Cl,vol}}$ was not affected by the application of 100 μM A63 for 8 min but was blocked by A23 in the same cell (Fig. 10 A and B). The I-V relationships confirmed that A63 did not alter $I_{\text{Cl,vol}}$ over the entire voltage range studied (Fig. 10 C; $n = 5$). The results suggest that suppression of $I_{\text{Cl,vol}}$ by tyrphostin A23 and A25 was related to inhibition of tyrphostin-sensitive PTKs.

Influence of Orthovanadate on Tyrphostins

The effect of block of PTP on the modulation of $I_{\text{Cl,vol}}$ by tyrphostin A23 is illustrated in Fig. 11. As shown previously (Fig. 6), $V_{3}^{-}$ (1 mM) substantially inhibited $I_{\text{Cl,vol}}$ at $+50$ mV in 0.6T. After block of PTP by $V_{3}^{-}$, A23 (100 μM; Fig. 10 A) caused only a slight further suppression of $I_{\text{Cl,vol}}$. Fig. 11 C summarizes the data for A23, $I_{\text{Cl,vol}}$
The tyrphostin A23. (A) Time course of current at produced by swelling in 0.6T. **P diminished the block of I Cl.vol by A23. Protocol is in inset. (B) Currents at time points a–d in A. (C) Histograms summarize effect of results suggest that the inhibition of ICl.vol by A23 and A25 significantly precluded by VO4

6, P

0.05 vs. 0.6T with VO4

100

M A23 (d) in 0.6T. Pretreatment with VO4

3, A23 produced only a slight, 11.1

n

3 plus 100

M A23 on ICl.vol in-duced by swelling in 0.6T. **P < 0.01 vs. 0.6T-induced ICl.vol. *P < 0.05 vs. 0.6T with VO4

3. VO4

3.1% by A23 (n = 7; P < 0.01; data from Fig. 9 C), whereas 1 mM VO4

3 inhibited ICl.vol by 45.6 ± 3.1% (P = 0.001), whereas 1 mM VO4

3 inhibited ICl.vol by 45.6 ± 5.7% (n = 9, P < 0.001).

After pretreatment with VO4

3, B56 produced a slight but significant further inhibition of ICl.vol, 9.9 ± 5.3% of ICl.vol in 0.6T (P < 0.05 vs. after VO4

3, n = 9). The percent reduction of ICl.vol by VO4

3 plus B56, 54.4 ± 6.5%, was less than that with B56 alone, 72.6 ± 3.1% (P < 0.01). Thus, selective inhibition of EGRF kinase by B56 and its interaction with VO4

3 recapitulated the observations made with the broad-spectrum tyrphostins, A23 and A25.

Relative Cell Volume

To quantify the swelling of human atrial myocyte under the present experimental conditions, relative cell volume was determined using each cell as its own control while the cell was voltage clamped. Relative cell volume increased in 0.6T to 132.4 ± 2.5% of that in 1T (n = 25). Although genistein, VO4

3, and tyrphostin A23 and A25 substantially modulated ICl.vol, they did not significantly affect relative cell volume. Relative cell volume was 134.3 ± 3.4, 131.7 ± 8.3, 133.6 ± 5.7, and 135.6 ± 4.2%, respectively, in the presence of 100 µM genistein, A23, or A25 or 1 mM VO4

3 (n = 4–7; P = NS vs. 0.6T).

DISCUSSION

This study provides the first evidence that ICl.vol in human atrial myocytes is regulated in part by the interplay of PTK and PTP and that Src and EGRF kinases, distinct PTK families, have opposing effects ICl.vol. ICl.vol was enhanced by the broad-spectrum isoflavone PTK inhibitor genistein and the selective Src family inhibitor PP2, whereas broad-spectrum tyrphostin PTK inhibitors A23 and A25, and the highly selective EGRF kinase inhibitor B56 diminished ICl.vol. ICl.vol also was suppressed by the PTP inhibitor VO4

3. Observations that isoflavone
and tyrphostin PTK inhibitors modulate $I_{\text{Cl,vol}}$ in opposite directions imply that more than one PTK and more than one tyrosine phosphorylation site contributes to regulation of $I_{\text{Cl,vol}}$. Moreover, genistein did not stimulate $I_{\text{Cl,vol}}$ in isosmotic 1T solution and did not maintain the current after osmotic shrinkage in 1.4T. This suggests that tyrosine phosphorylation modulates the effects of other signaling cascades rather than directly controlling channel gating. Src and EGFR kinase are well placed to be sensors of cell volume and mechanical stretch. These signaling molecules interact with cytoskeleton, integral membrane proteins, and the sarcolemma, and tyrosine phosphorylation is among the earliest responses to osmotic swelling in cardiac myocytes (Sadoshima et al., 1996) and other cells (Tilly et al., 1993).

Genistein, tyrphostins, and $\text{VO}_4^{3-}$ are widely employed to ascertain whether PTKs and PTPs participate in signaling cascades, but these agents also might act by nonspecific mechanisms (Davis et al., 2001). Two lines of evidence support the conclusion that PTK and PTP inhibitors acted via phosphotyrosine in this study. First, daidzein and tyrphostin A63, inactive analogs of genistein and tyrphostins, respectively, were without effect. Second, as expected for phosphorylation-dependent processes, inhibition of PTP by $\text{VO}_4^{3-}$ countered inhibition of PTK. This observation makes it unlikely that genistein acted directly on the $I_{\text{Cl,vol}}$ channel, for example, by altering its volume set point. Alternatively, one might argue that the tyrphostins A23, A25, and B56 act as antioxidants (Sagara et al., 2002) rather than PTK inhibitors. The antioxidant hypothesis does not explain why $\text{VO}_4^{3-}$ suppressed the action of tyrosinase, however. One also might posit that the $\text{VO}_4^{3-}$ anion suppressed $I_{\text{Cl,cAMP}}$ (e.g., Fig. 6) by blocking the $\text{Cl}^-$ channel directly. This idea seems unlikely because the fractional block of $\text{Cl}^-$ current by $\text{VO}_4^{3-}$ strongly depended on experimental conditions (1T vs. 0.6T; stimulated by genistein or PP2 vs. inhibited by tyrphostin A23, A25, or B56); if $\text{VO}_4^{3-}$ simply blocked the pore, fractional block should have been independent of interventions that increased or decreased $I_{\text{Cl,vol}}$. Moreover, block by $\text{VO}_4^{3-}$ did not appear to be voltage dependent. On the other hand, because of its high affinity for phosphate binding sites, $\text{VO}_4^{3-}$ interferes with several phosphorylation-dependent processes besides PTP. The possibility that nonspecific effects of PTK inhibitors and $\text{VO}_4^{3-}$ contributed to the results cannot be rigorously excluded.

Another concern is that genistein augments $I_{\text{Cl,cAMP}}$ the CFTR current, in heart, cell lines, and expression systems (Shuba et al., 1996; Zhou et al., 1998; Gadsby and Nairn, 1999). This raises a question about the origin of the genistein-stimulated current. Identification of $I_{\text{Cl,vol}}$ was based on biophysical and pharmacological criteria, including outward rectification, volume sensitivity, reversal potential, and inhibition by DIDS and tamoxifen, blockers of $I_{\text{Cl,vol}}$ (Vandenberg et al., 1994; Sorota, 1999; Hume et al., 2000; Baumgarten and Clemo, 2003). $I_{\text{Cl,cAMP}}$ can be confused with $I_{\text{Cl,vol}}$ in certain situations but was ruled out here; cardiac $I_{\text{Cl,cAMP}}$ is insensitive to both DIDS and tamoxifen (Vandenberg et al., 1994; Hume et al., 2000; Baumgarten and Clemo, 2003). Most workers agree that $I_{\text{Cl,cAMP}}$ is not detectable in human atrial myocytes (Oz and Sorota, 1995; Sakai et al., 1995; Li et al., 1996; Sato and Koumi, 1998), although one group reported $I_{\text{Cl,cAMP}}$ in a fraction (<20%) of human atrial cells and coexpression of exon 5+ and 5− splice variants of CFTR (Warth et al., 1996). Moreover, genistein could not stimulate membrane current under 1T conditions. Finally, contrary to the present results, stimulation of $I_{\text{Cl,cAMP}}$ by genistein is not reversed by inhibiting PTP (Zhou et al., 1998; Gadsby and Nairn, 1999). The Ca$^{2+}$-activated Cl$^-$ cur-
rent, $I_{Cl}$ appears to be different in human atrial than in canine atrial and rabbit ventricular myocytes. Sorota (1995) reported that $I_{Cl}$ was inhibited by pretreatment with 50–80 $\mu$M genistein and that acute application of tyrphostin A51, an EGFR kinase inhibitor, and herbimycin A, a Src inhibitor, had no effect. $I_{Cl}$ activated by mechanical stretch also was suppressed by the acute application of 100 $\mu$M genistein or 10 $\mu$M PP2 (Browe and Baumgarten, 2003). It remains unclear, however, whether these apparent differences in the regulation of $I_{Cl}$ arise from the intrinsic characteristics of the cells studied or experimental details such as the timing of genistein exposure, means of activating $I_{Cl}$, or temperature at which the study was done. Furthermore, enhancement of $125^I$ efflux in cultured neonatal rat myocytes by pervanadate has been attributed to inhibition of PTP and activation of $I_{Cl}$ (Tilly et al., 1996), but the efflux pathway was not identified by direct means.

**Control of $I_{Cl}$ by Tyrosine Phosphorylation in Human Atria**

The simplest explanation for the actions of genistein, PP2, VO$_4^{3-}$, and their interactions is that dephosphorylation of a critical tyrosine residue on the channel or a signaling molecule directly or indirectly augments $I_{Cl}$. In this model (Fig. 13), inhibition of Src family PTK by genistein or PP2 allows unopposed dephosphorylation of a tyrosine (Tyr1) by PTP, whereas inhibition of PTP blocks $I_{Cl}$ by allowing its unopposed phosphorylation by the same PTK. This also explains why pretreatment with VO$_4^{3-}$ precluded the effect of genistein and PP2. Once Tyr1 becomes phosphorylated during VO$_4^{3-}$ pretreatment, blocking PTKs will have little effect because there is no efficient means of dephosphorylating the residue. It is important to note, however, that genistein did not stimulate $I_{Cl}$ in 1T, and stimulation of $I_{Cl}$ was reversed by osmotic shrinkage in 1.4T. Thus, phosphorylation of the genistein-sensitive site by Src does not appear to be sufficient to activate or maintain $I_{Cl}$ in the face of contrary cell volume-dependent signaling. Such volume-dependent signaling has been postulated to include one or more Ser/Thr kinases (PKC, PKA, and MAPK) and phosphatases (Nilius et al., 1997; Sorota, 1999; Hume et al., 2000), and the activity of Ser/Thr kinases and phosphatases are modulated by tyrosine phosphorylation (Hunter, 1995; Pawson and Scott, 1997; Zhang et al., 2002).

A second tyrphostin-sensitive PTK and a second phosphorylation site, Tyr2 (Fig. 13), also must be present and regulate $I_{Cl}$ in the opposite manner as Tyr1 because $I_{Cl}$ was inhibited when EGFR kinase was blocked by tyrphostin B36, A23, or A25. Recently, activation of $I_{Cl}$ by EGF peptides and inhibition by tyrphostin B46, an inhibitor of EGFR tyrosine kinase, was reported in murine mammary C127 cells (Abdullaev et al., 2003). The proposed involvement of EGFR kinase in the regulation of cardiac $I_{Cl}$ does not necessarily require the presence of its ligand, however. Transactivation of EGFR in myocytes and other types of cell can be accomplished by multiple signaling molecules, oxidants, and hyperosmotic shock (Zwick et al., 1999; Shah and Catt, 2003). Moreover, EGFR activity is regulated by autophosphorylation and Src-dependent tyrosine phosphorylation, and Src may act directly on EGFR and downstream. Opposing effects of specific PTK families on molecular and cellular function is a known regulatory motif (Pawson and Scott, 1997; Yoon et al., 1998; Zhang et al., 2002), but such dual regulation has not been reported previously for $I_{Cl}$ or other channels (Davis et al., 2001).

Although both Src and EGFR kinases regulate $I_{Cl}$ control by Src appeared to be dominant under present conditions. The net effect of inhibiting PTP with VO$_4^{3-}$

**Figure 13.** Schematic model of biphasic regulation of $I_{Cl}$ by Src family and EGFR PTKs and PTPs. Two tyrosine phosphorylation sites, Tyr1 and Tyr2, on the $I_{Cl}$ channel or signaling molecules are postulated. Tyr1 is phosphorylated by a PP2- and genistein-sensitive Src family kinase and dephosphorylated by a VO$_4^{3-}$-sensitive PTP (PTP$_a$). Phosphorylation of Tyr1 by one or more steps (dashed line) favors $I_{Cl}$ channel closure, whereas dephosphorylation favors opening. Tyr2 is phosphorylated by tyrphostin-sensitive EGFR kinase and dephosphorylated by a VO$_4^{3-}$-sensitive PTP (PTP$_b$). In contrast to Tyr1, phosphorylation of Tyr2 favors channel opening, whereas dephosphorylation favors closure. The effect of VO$_4^{3-}$ on Tyr1 (thick lines) was dominant over that on Tyr2 (thin lines). On the other hand, genistein did not activate $I_{Cl}$ in 1T or maintain its activation after osmotic shrinkage (thick solid lines). Therefore, tyrosine phosphorylation appears to modulate channel activity rather than directly control gating.
was suppression of current, as expected from phosphorylation of Tyr1, rather than the stimulation expected from phosphorylation of Tyr2. The outcome of inhibiting PTPs with opposing actions might reflect differing sensitivities of two PTPs to VO$_4^{-3}$, differing basal activities of Src and EGFR kinase, or the functional consequences of phosphorylation at Tyr1 and Tyr2 and their position in the signaling cascade. The effect of temperature (21–22°C) on the balance between these PTKs was not evaluated. Finally, our simplified model assumes that inhibition of PTKs and PTPs are independent. To the contrary, PTK positively regulates PTP and PTP negatively regulates PTK in certain cases (Lammers et al., 1993; Vogel et al., 1993).

Although genistein and both tyrphostin A23 and A25 are considered broad-spectrum PTK inhibitors, details of their pharmacology support the conclusions reached with the specific PTK inhibitors, PP2 and B56. Genistein binds to the ATP site on PTK and blocks pp60$^v$src with an IC$_{50}$ of 25.9 or 29.6 μM (Akiyama and Ogawara, 1991). This is in good agreement with the EC$_{50}$, 22.4 μM, for genistein-dependent stimulation of I$_{Cl,vol}$ and 100 μM genistein should substantially inhibit Src. This notion is supported by the results with the Src family PTK inhibitor PP2. On the other hand, A23 and A25 bind to the substrate site (Gazit et al., 1989) and block Src with IC$_{50}$ of 440 and 150 μM, respectively (Ramdas et al., 1994). Thus, only partial inhibition of Src should have occurred with the 100 μM tyrphostin A23 or A25 used here.

Regulation of I$_{Cl,vol}$ by Tyrosine Phosphorylation in Other Tissues

Consistent with the present results with genistein and VO$_4^{-3}$, inhibition of PTP, which favors tyrosine phosphorylation, blocks swelling-induced activation of I$_{Cl,vol}$ in bovine chromaffin cells (Doroshenko, 1998) and mouse L-fibroblasts (Thoroed et al., 1999). Genistein and tyrphostin B46 do not alter I$_{Cl,vol}$ in chromaffin cells, however (Doroshenko, 1998).

Evidence in several systems indicates tyrosine phosphorylation can stimulate I$_{Cl,vol}$. Expression of p56$^lck$, an Src family PTK, was sufficient to activate I$_{Cl,vol}$ in human T lymphocytes in the absence of swelling (Lepple-Wienhues et al., 1998). In ciliary epithelial cells, swelling-induced I$_{Cl,vol}$ was augmented by a peptide that binds to the SH2 domain and up-regulates c-Src (Shi et al., 2002). Moreover, PTK inhibitors suppress and PTP inhibitors stimulate I$_{Cl,vol}$ in calf pulmonary artery endothelial (Voets et al., 1998) and human prostate cancer epithelial cells (Shuba et al., 2000). Finally, genistein also blocks I$_{Cl,vol}$ in rat astrocytes (Crepel et al., 1998).

Significance

Regulation of I$_{Cl,vol}$ by phosphorylation is variously reported to depend on PKC, PKA, PTK, and MAPK in different cell types and species (Nilius et al., 1997; Sorota, 1999; Hume et al., 2000). Involvement of PTK was first proposed based on $^{125}$I flux studies in human intestinal 407 cells (Tilly et al., 1993), and the present studies establish the importance of tyrosine phosphorylation in this process in human atrial cells. There are multiple sites of cross-talk between PTKs and PTPs and the PKC, PKA, and MAPK cascades (Baumgarten and Clemo, 2003), and each will need to be explored to fully understand the regulation of I$_{Cl,vol}$ in heart.

Both the molecular identity of I$_{Cl,vol}$ and the signaling systems responsible for its control are controversial (Nilius et al., 1997; Okada, 1997; Sorota, 1999; Hume et al., 2000; Jentsch et al., 2002). Some of the conflicts in the literature might be rationalized if multiple distinct molecules produce the current empirically defined as I$_{Cl,vol}$ or if the volume sensor or regulatory signaling cascades are tissue or species specific. Evidence for the participation of multiple pore-forming molecules includes the widely varying unitary conductance of swelling-activated Cl$^-$ channels; unitary conductance is 9.6 pS in human prostate (Shuba et al., 2000), 31 pS in lymphocytes (Lepple-Wienhues et al., 1998), and 105 pS in ciliary epithelium (Zhang and Jacob, 1997). Moreover, multiple swelling activated unitary Cl$^-$ currents are present in several cells (Banderali and Ehrenfeld, 1996; Zhang and Jacob, 1997). In rabbit atrial myocytes, a DIDS-sensitive, ~60-pS outwardly rectifying channel was described (Duan and Nattel, 1994) and a 28-pS channel was observed in other experiments (Duan et al., 1997). In contrast, an 8.6-pS DIDS- and stretch-sensitive Cl$^-$ channel was found in human atrial myocytes (Sato and Koumi, 1998). Thus, it also is possible that multiple channel proteins controlled by distinct signaling pathways contribute to I$_{Cl,vol}$ in heart.

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