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Antagonistic regulation of swelling-activated Cl^- current in rabbit ventricle by Src and EGFR protein tyrosine kinases

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Abstract

Regulation of swelling-activated Cl^- current ($I_{Cl,swell}$) is complex, and multiple signaling cascades are implicated. To determine whether protein tyrosine kinase (PTK) modulates $I_{Cl,swell}$ and to identify the PTK involved, we studied the effects of a broad-spectrum PTK inhibitor (genistein), selective inhibitors of Src (PP2, a pyrazolopyrimidine) and epidermal growth factor receptor (EGFR) kinase (PD-153035), and a protein tyrosine phosphatase (PTP) inhibitor (orthovanadate). $I_{Cl,swell}$ evoked by hyposmotic swelling was increased 181 ± 17% by 100 μM genistein, and the genistein-induced current was blocked by the selective I_{Cl},swell blocker tamoxifen (10 μM). Block of Src with PP2 (10 μM) stimulated tamoxifen-sensitive $I_{Cl,swell}$ by 234 ± 27%, mimicking genistein, whereas the inactive analog of PP2, PP3 (10 μM), had no effect. Moreover, block of PTP by orthovanadate (1 mM) inhibited $I_{Cl,swell}$ and prevented its stimulation by PP2. In contrast with block of Src, block of EGFR kinase with PD-153035 (20 nM) inhibited $I_{Cl,swell}$. Several lines of evidence argue that the PP2-stimulated current was $I_{Cl,swell}$: *1*) the stimulation was volume dependent, *2*) the current was blocked by tamoxifen, *3*) the current outwardly rectified with both symmetrical and physiological Cl^- gradients, and *4*) the current reversed near the Cl^- equilibrium potential. To rule out contributions of other currents, Cd^{2+} (0.2 mM) and Ba^{2+} (1 mM) were added to the bath. Surprisingly, Cd^{2+} suppressed the decay of Cd^{2+} plus Ba^{2+} eliminated time-dependent $I_{Cl,swell}$, and currents between −100 and −100 mV. Nevertheless, these divalent ions did not eliminate $I_{Cl,swell}$ or prevent its stimulation by PP2. The results indicate that tyrosine phosphorylation controls $I_{Cl,swell}$, and regulation of $I_{Cl,swell}$ by the Src and EGFR kinase families of PTK is antagonistic.

Keywords

volume-sensitive Cl^- current; AG 1879; PD-153035; orthovanadate; tamoxifen; genistein; epidermal growth factor receptor

OSMOTIC SWELLING OR HYDROSTATIC inflation of cardiac myocytes and numerous other tissues evokes the volume-sensitive Cl^- current $I_{Cl,swell}$. This current is outwardly rectifying, partially inactivated at positive voltages, and blocked by tamoxifen. These biophysical and pharmacological characteristics distinguish $I_{Cl,swell}$ from other Cl^- currents (for reviews, see Refs. 4 and 28). Under isosmotic conditions, $I_{Cl,swell}$ contributes to the background Cl^- current (17,18) and is activated in models of cardiac disease (12) and by

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stretching β_1-integrins (7,8). Functionally, the activation of $I_{Cl,swell}$ influences both cardiac electrical activity (16,30,49) and cell volume (11,12).

The signaling that underlies the activation of $I_{Cl,swell}$ is complex, and evidence implicates protein kinases C and A and protein tyrosine kinase (PTK) in its regulation in the heart (4, 28) and other tissues (32). PTK is activated by osmotic swelling of myocytes within 5 s (37, 38) and therefore is well positioned to be an early step in the signaling process. Although substantial evidence indicates that phosphorylation and dephosphorylation of tyrosine residues are involved in the control of $I_{Cl,swell}$, the details remain obscure. Studies with the broad-spectrum PTK inhibitor genistein found that blocking PTK inhibits $I_{Cl,swell}$ in dog atrial cells (44), calf pulmonary artery endothelial cells (51), and rabbit ciliary epithelial cells (40). On the other hand, genistein augments $I_{Cl,swell}$ in human atrial myocytes (15), and protein tyrosine phosphatase (PTP) inhibitors suppress $I_{Cl,swell}$ in bovine chromaffin cells (14) and mouse L-fibroblasts (45). Thus interventions that lead to both phosphorylation and dephosphorylation of tyrosine are capable of inhibiting $I_{Cl,swell}$.

The apparent inconsistency in the relationship between the phosphorylation state of tyrosine residues and the activity of $I_{Cl,swell}$ may indicate that regulatory processes are tissue or species specific as previously suggested (32,33). Another possibility is that $I_{Cl,swell}$ is differentially regulated by various families of PTK. Recently, studies on human atrial myocytes revealed that specific inhibition of Src leads to activation of $I_{Cl,swell}$, whereas specific inhibition of epidermal growth factor receptor (EGFR) kinase causes suppression of current (15).

Previous studies on heart focused on the role of PTK in atrial myocytes. The goal of the present study was to evaluate the role of the Src and EGFR kinase families of PTK in the egulation of $I_{Cl,swell}$ in ventricular cells. As in human (15) but not canine (44) atrial myocytes, specific inhibition of Src and inhibition of multiple PTKs by genistein stimulated $I_{Cl,swell}$ in hyposmotic bathing media, whereas specific inhibition of EGFR kinase suppressed $I_{Cl,swell}$. Moreover, the PTP inhibitor orthovanadate (21) reduced $I_{Cl,swell}$ and precluded its activation by Src inhibition. Src activity is not, however, the primary factor that controls the response of ventricular $I_{Cl,swell}$ to osmotic stress. Blocking of Src did not alter $I_{Cl,swell}$ under isosmotic conditions. Finally, we found that the time-dependent component of $I_{Cl,swell}$ at positive voltages could be inhibited without altering the regulation of time-independent $I_{Cl,swell}$ by Src. These data suggest that Src- and EGFR kinase-dependent tyrosine phosphorylation and PTP-dependent tyrosine dephosphorylation participate in the regulation of $I_{Cl,swell}$ in ventricular myocytes.

METHODS

Ventricular myocyte isolation.

Left ventricular myocytes were freshly isolated from New Zealand White rabbits (~3 kg body wt). Hearts were excised using methods approved by the Institutional Animal Care and Use Committee, mounted on a Langendorff apparatus, and initially perfused with 37°C oxygenated Tyrode solution that contained (in mM) 130 NaCl, 5 KCl, 3 $MgCl_2$, 1.8 $CaCl_2$, 0.4 KH_2PO_4, 5 HEPES, 15 taurine, 5 creatine, and 10 glucose, pH 7.25. After a 5-min perfusion with Ca^{2+}-free Tyrode solution that contained 0.1 mM Na_2-EGTA, the perfusate was switched to Ca^{2+}-free Tyrode solution that contained 0.4–0.5 mg/ml collagenase (type II; Worthington), 0.05 mg/ml pronase (type XIV; Sigma-Aldrich), and 1.5 mg/ml BSA (Sigma-Aldrich). At selected intervals (10–20 min), portions of the left ventricle were excised, cut into strips, placed in test tubes, and gently agitated. After filtration through nylon mesh to remove debris, myocytes were washed twice and stored in modified Kraft-Brühe solution that contained (in mM) 120 K-glutamate, 10 KCl, 10 KH_2PO_4, 1.8 $MgSO_4$, 0.5 K_2-EGTA, 10 taurine, 20 glucose, 10 mannitol, and 10 HEPES (pH 7.2; 295 mosM). Myocytes were used within 8 h of isolation,

and only rod-shaped quiescent cells with well-defined regular striations and no evidence of membrane blebbing were selected for study.

Experimental solutions and drugs.

Cells were placed in a poly-L-lysine-coated glass-bottomed chamber (~0.3 ml) mounted on an inverted microscope (Diaphot; Nikon) and were superfused with bathing solution ~22°C) at 2–3 ml/min; solution changes were complete within ~10 s. Anion currents were isolated by replacing Na^+ and K^+ in the bathing media with equimolar amounts of *N*-methyl-D-glucamine (NMDG) and adding Cs^+ to the bath and pipette solutions. Standard bathing solution contained (in mM) 90 NMDG-Cl, 3 $MgCl_2$, 4.63 $CaCl_2$, 5 Cs_2-EGTA, 10 HEPES, 10 glucose, and 0–100 mannitol (pH 7.4). This provides a free Ca^{2+} concentration of ~1.5 μM (WinMaxC 2.4; www.stanford.edu/~cpatton/maxc.html). In some experiments, 0.2 mM $CdCl_2$ or both 1 mM $BaCl_2$ and 0.2 mM $CdCl_2$ were added to the bath solution. This also necessitated omission of Cs_2-EGTA (replaced by 5 mM CsCl), and bath $CaCl_2$ was reduced to 0.1 mM. Bath solutions were designed to allow adjustment of osmolarity with mannitol at a constant ionic strength. Isosmotic (1T) solution was set as 300 mosM, and hypoosmotic (0.7T) solution was ~200 mosM. An osmometer (Osmette S; Precision Systems) was used to routinely verify solution composition.

Tamoxifen (10 mM), genistein (100 mM), the pyrazolopyrimidines PP2 (10 mM, also termed AG 1879) and PP3 (10 mM), and PD-153035 (250 μM) were dissolved in dimethyl sulfoxide (DMSO) at the indicated concentrations and kept frozen (−20°C) in aliquots until use. Cs_3VO_4 (orthovanadate) was added directly to the bath solution. Tamoxifen, orthovanadate, and DMSO were from Sigma-Aldrich, and the remaining agents were from Calbiochem.

Electrophysiological recordings.

Patch electrodes were made from thin-walled 7740 borosilicate glass (Sutter) and fire polished (initial resistance, 2–3 MΩ). Standard electrode-filling solution contained (in mM) 110 Cs-aspartate, 20 CsCl, 2.5 Mg-ATP, 8 Cs_2-EGTA, 0.15 $CaCl_2$, and 10 HEPES, pH 7.1 (liquid junction potential, −11.5 ± 0.7 mV; $n = 9$). For some experiments, a high- Cl^- pipette solution was used; it contained (in mM) 31.7 Cs-aspartate, 98.3 CsCl, 2.5 Mg-ATP, 8 Cs_2-EGTA, 0.15 $CaCl_2$, and 10 HEPES, pH 7.1 (liquid junction potential, −6.5 ± 0.5 mV; $n = 5$). This provided a free Ca^{2+} concentration of ~60 nM for both pipette solutions. A 3 M KCl agar bridge was used as ground. Seal resistances of 5–30 GΩ were typically achieved, and the measured junction potential was subtracted before seal formation.

Whole cell currents recorded with an Axoclamp 200A or 200B amplifier (Axon) were low pass filtered at 2 kHz (Bessel) and digitized at 5 kHz. Myocytes were dialyzed for 10 min before the recordings commenced. Voltage-clamp protocols and data acquisition were governed by a Digidata 1321A digitizer and pCLAMP 8.0 software (Axon). Successive 500-ms voltage steps were made from a holding potential of −60 mV to test potentials ranging from −100 to +60 or +100 mV in +10-mV increments. Current-voltage (*I-V*) relationships were plotted from the quasi-steady-state current except for in Figs. 1 and 2, which show currents at 25 ms. Capacitance was calculated with pCLAMP software using a 5-mV step.

Preliminary studies established that $I_{Cl,swell}$ fully activated in <5 min and remained stable for ≥45 min. All interventions were applied for a time sufficient for currents to reach a steady state as judged from *I-V* curves obtained at selected intervals (typically 1 or 2 min).

Statistics.

Data are expressed as means ± SE, and *n* refers to the number of cells. Mean currents are presented as current density (in pA/pF) to account for differences in myocyte surface membrane

area. Statistical analyses were done using SigmaStat 2.3 or 3.0 software (Systat). For multiple comparisons, a two-way repeated-measures ANOVA was used as appropriate, and the Student-Newman-Keuls test was performed to compare groups. Statistical significance was taken as $P < 0.05$.

RESULTS

Effects of genistein on $I_{Cl,swell}$.

To assess the effects of PTK on $I_{Cl,swell}$ in ventricular myocytes, the broad-spectrum PTK blocker genistein was applied after $I_{Cl,swell}$ was activated by osmotic swelling in solutions designed to isolate Cl^- currents. Figure 1 illustrates the *I-V* relationships obtained under each experimental condition and families of difference currents calculated by digital subtraction. As expected, osmotic swelling in 0.7T bath solution induced an outwardly rectifying Cl^- current that partially inactivated at positive potentials and reversed at −40.6 ± 2.4 mV, near the calculated Cl^- equilibrium potential (E_{Cl}) of −42 mV (Fig. 1, *A* and *B*). At +60 mV, for example, swelling significantly increased the Cl^- current from 1.3 ± 0.2 in 1T to 2.1 ± 0.3 pA/pF after 10 min in 0.7T solution ($n = 5$; $P < 0.001$). $I_{Cl,swell}$ was further enhanced by a 10-min exposure to 100 μM genistein in 0.7T solution. The genistein-induced difference current outwardly rectified, substantially inactivated at positive potentials, and reversed at the same potential as the swelling-induced current (Fig. 1, *A* and *C*). Addition of genistein to 0.7T solution increased the Cl^- current at +60 mV from 2.1 ± 0.3 to 2.7 ± 0.3 pA/pF ($n = 5$; $P < 0.001$). Thus the swelling-induced current with genistein was 163 ± 17% of the swelling-induced current without genistein in same-cell comparisons ($n = 5$; $P < 0.001$). Because genistein caused a much more prominent increase in the outward than the inward current with a physiological Cl^- gradient, its stimulation of $I_{Cl,swell}$ was not statistically significant at −100 mV.

Tamoxifen blocks $I_{Cl,swell}$ but not cAMP-or Ca^{2+}-induced Cl^- currents ($I_{Cl,cAMP}$ or $I_{Cl,Ca}$, respectively) and can be used to distinguish between these currents under conditions that isolate anionic currents (48,5). To verify that the genistein-induced current was $I_{Cl,swell}$, myocytes were exposed to 10 μM tamoxifen for 10 min in the continued presence of genistein in 0.7T solution. The tamoxifen-sensitive currents and the *I-V* relationship after block by tamoxifen are shown (Fig. 1, *A* and *D*). Tamoxifen inhibited both the swelling- and genistein-induced currents but did not alter the reversal potential of the *I-V* curve. At +60 mV in 0.7T bath solution, tamoxifen reduced the Cl^- current from 2.7 ± 0.3 after stimulation by genistein to 1.3 ± 0.3 pA/pF ($n = 5$; $P < 0.001$), a value indistinguishable from that in 1T solution.

Stimulation of $I_{Cl,swell}$ by genistein in 0.7T bath solution was confirmed in an additional seven cells that were not exposed to tamoxifen. On the other hand, partial inhibition of Cl^- current by genistein was noted in 3 of 15 cells. In these cells, genistein decreased the current at +60 mV from 1.8 ± 0.1 to 1.3 ± 0.1 pA/pF ($n = 3$; $P < 0.02$).

Selective inhibition of Src.

Recently, it was reported that inhibition of Src family PTKs stimulates $I_{Cl,swell}$ in human atrial myocytes (15). To test the hypothesis that block of Src is responsible for the stimulation of $I_{Cl,swell}$ in rabbit ventricular myocytes, we used PP2, a selective inhibitor of the Src family (3,23). As before, osmotic swelling in 0.7T solution activated $I_{Cl,swell}$ (Fig. 2, *A* and *B*). Exposure to 10 μM PP2 for 10 min significantly stimulated $I_{Cl,swell}$ in 0.7T solution (Fig. 2*C*). PP2 augmented the current at +60 mV from 2.1 ± 0.3 to 2.9 ± 0.3 pA/pF ($n = 7$; $P < 0.001$); the swelling-activated current in PP2 was 206 ± 10% of the swelling-activated current without PP2. The effect of PP2 at −100 mV was not significant, however, as was the case with genistein. Both the PP2- and swelling-induced currents were sensitive to tamoxifen (Fig. 2*D*). Exposure to 10 μM tamoxifen for 10 min in the continued presence of PP2 reduced the Cl^- current at

+60 mV to 1.4 ± 0.2 pA/pF (n = 6; P = 0.001), a value indistinguishable from that in 1T, 1.4 ± 0.4 pA/pF. Thus inhibition of Src by PP2 augmented the tamoxifen-sensitive Cl^- current in ventricular myocytes and mimicked the usual effect of genistein.

To exclude the possibility that a nonspecific effect of PP2 was responsible for stimulation of $I_{Cl,swell}$, we applied PP3, an inactive analog of PP2 (3,46), in separate experiments. Treatment with 10 μM PP3 for 10 min did not alter the magnitude or time dependence of the $I_{Cl,swell}$ in 0.7T solution, and the resulting PP3-induced difference current was nil (Fig. 2*E*). In these cells, swelling increased the current at +60 mV from 1.6 ± 0.2 in 1T to 2.4 ± 0.2 pA/pF in 0.7T solution (n = 6; $P < 0.001$), but the current was unaffected [2.4 ± 0.3 pA/pF; n = 6; P = not significant (NS)] by addition of PP3 to 0.7T solution. Taken together, these results suggest that Src family PTKs play a critical role in the regulation of swelling-activated Cl^- channels in ventricular myocytes.

$I_{Cl,swell}$ is thought to contribute to the background Cl^- current (17,18). Therefore, it is important to distinguish whether inhibition of Src augments the response to cell swelling or simply activates $I_{Cl,swell}$ independent of cell volume. As shown in Fig. 3, PP2 (10 μM for 10 min) did not alter the *I-V* relationship for Cl^- current under 1T conditions, and the PP2-induced difference current in 1T solution was negligible (Fig. 3, *inset*). The current at +60 mV was 1.4 ± 0.2 pA/pF in both 1T solution and in 1T solution after treatment with PP2 (n = 7; P = NS). Thus blocking Src enhances activation of $I_{Cl,swell}$ in response to swelling but is insufficient to activate $I_{Cl,swell}$ or the background Cl^- current by itself.

Blocking time dependence of $I_{Cl,swell}$.

In several cells, inactivation of $I_{Cl,swell}$ at positive potentials appeared to deviate from an exponential decay. This raised the possibility that additional components contributed to the empirically defined $I_{Cl,swell}$. Consequently, we examined the effect of adding 0.2 mM Cd^{2+} to the bath solution. Figure 4 (*A–C* and *D–F)* shows the responses of two of the nine cells studied. As before, osmotic swelling in 0.7T solution evoked an outwardly rectifying Cl^- current (Fig. 4, *A* and *D*). Cd^{2+} largely blocked the rapidly inactivating component at positive potentials and had a smaller but variable effect on steady-state current (Fig. 4, *B* and *E*). Steady-state current in 0.7T solution with Cd^{2+} was 109 ± 9% of that without Cd^{2+} (n = 9; P = NS). The Cd^{2+}-sensitive current (Fig. 4, *C* and *F*) exhibited both inactivation at positive potentials and the outward-going rectification that are characteristic of $I_{Cl,swell}$. After suppression of the outward transient by Cd^{2+}, a delayed rectifier appeared to emerge. This is most obvious in Fig. 4*E*, which illustrates the myocyte with the strongest block of steady-state currents by Cd^{2+}.

The remaining time-dependent component in 0.7T plus Cd^{2+} solution was Ba^{2+} sensitive. Figure 5 shows the effect of swelling a myocyte in bathing solutions containing 0.2 mM Cd^{2+} and 1 mM Ba^{2+}. Under these conditions, the current was essentially time independent over the entire voltage range studied. Nevertheless, osmotic swelling in 0.7T solution increased the Cl^- current at +60 mV from 1.5 ± 0.2 to 2.6 ± 0.3 pA/pF (n = 11; $P < 0.001$). Moreover, block of Src with 10 μM PP2 in 0.7T bath solution caused an additional increase in the outward current to 4.1 ± 0.4 pA/pF (n = 11; $P < 0.001$). Families of time-independent currents in 1T, 0.7T, and 0.7T plus PP2 solutions are shown in Fig. 5, *B-D*. Although the addition of Cd^{2+} and Ba^{2+} eliminated the time dependence of $I_{Cl,swell}$, the steady-state current densities in 1T and 0.7T solutions with and without these blockers were indistinguishable (cf. Figs. 2 and 5 at +60 mV). In four of these cells, we also verified that the time-independent swelling- and PP2-stimulated currents were blocked by 10 μM tamoxifen after 10 min, as was previously demonstrated for time-dependent currents in the absence of Cd^{2+} and Ba^{2+} (see Fig. 2). Addition of tamoxifen (Fig. 5*E*) significantly reduced the current in 0.7T plus PP2 solution from 3.6 ± 0.4 to 1.3 ± 0.2 pA/pF (n = 4; $P < 0.001$), a value indistinguishable from that in 1T

solution, 1.2 ± 0.3 pA/pF (n = 4; P = NS). As before, I-V curves after activation of $I_{Cl,swell}$, its stimulation, and its inhibition all crossed near E_{Cl}.

To determine whether Cd^{2+} and Ba^{2+} simply shifted the voltage dependence of the currents to more positive potentials (11,32), the voltage range studied was extended. $I_{Cl,swell}$ remained time independent to at least + 100 mV (n = 4) as shown in Fig. 6.

Symmetrical Cl^- gradient.

Both $I_{Cl,swell}$ and $I_{Cl,cAMP}$ undergo outward rectification with physiological Cl^- gradients such as the one used in the experiments described thus far {intracellular Cl^- concentration ($[Cl^-]_i$) = 20.3 mM; extracellular Cl^- concentration ($[Cl^-]_o$) = 98.6–105.3 mM}. In contrast, only $I_{Cl,swell}$ retains outward rectification in symmetrical high- Cl^- solutions (28). Figure 7 shows the effects of osmotic swelling in 0.7T and exposure to PP2 in symmetrical high- Cl^- solutions ($[Cl^-]_i = [Cl^-]_o$ = 98.6 mM). Swelling induced an outwardly rectifying current that reversed at −2.7 ± 0.3 mV (n = 6) near the expected reversal potential of 0 mV. The current at +60 mV increased from 0.6 ± 0.2 in 1T to 1.6 ± 0.2 pA/pF in 0.7T solution (n = 6; $P < 0.005$). PP2 additionally augmented the outwardly rectifying current in symmetrical high- Cl^- solutions to 2.8 ± 0.4 pA/pF (n = 6; $P < 0.001$) at +60 mV. In contrast to experiments with a physiological pipette Cl^-, a clear PP2-induced stimulation of inward current was detected with elevated pipette Cl^- (cf. Fig. 2). At −100 mV, for example, swelling in 0.7T solution increased the Cl^- current from −0.4 ± 0.1 to −0.9 ± 0.1 pA/pF (n = 6; $P < 0.025$), and PP2 additionally increased it to − 1.6 ± 0.2 pA/pF (n = 6; $P < 0.003$).

Roles of PTP and EGFR kinase.

If PP2 acts by blocking Src-dependent phosphorylation of a critical tyrosine residue, its action should be opposed by orthovanadate, which inhibits PTP (21) and thereby retains tyrosines in a phosphorylated state. Figure 8 shows a test of this prediction. After activation of $I_{Cl,swell}$ in 0.7T solution, myocytes first were exposed to 1 mM orthovanadate for 10 min in 0.7T solution and then were challenged with 10 μM PP2 for 10 min in the continued presence of orthovanadate. Orthovanadate alone reduced the current in 0.7T solution from 3.0 ± 0.2 to 1.8 ± 0.3 pA/pF (n = 4; $P < 0.033$), an action opposite to that of PP2. Moreover, PP2 failed to significantly stimulate $I_{Cl,swell}$ after pretreatment with orthovanadate. The current in PP2 plus orthovanadate was 2.2 ± 0.3 pA/pF, a value not different than that in orthovanadate alone (n = 4; P = NS).

In human atria, $I_{Cl,swell}$ is controlled by at least two families of PTKs (Src and EGFR kinase), which have opposite effects on $I_{Cl,swell}$ in osmotically swollen myocytes (15). Genistein usually stimulated $I_{Cl,swell}$ (see Fig. 1), but inhibition was observed in 20% of myocytes. Therefore, we tested whether EGFR also regulates $I_{Cl,swell}$ in the ventricle. Figure 9 illustrates the effects of PD-153035, a highly specific and potent blocker of EGFR kinase (19). As before, $I_{Cl,swell}$ was activated by swelling myocytes in 0.7T solution, and then cells were exposed to 20 nM PD-153035 in 0.7T media for 12–15 min. In contrast with the stimulatory effect of blocking Src, blocking EGFR kinase strongly inhibited $I_{Cl,swell}$. Swelling in 0.7T solution increased the current at +60 mV from 1.8 ± 0.4 to 4.0 ± 0.7 pA/pF (n = 4; P = 0.002), and PD-153035 reduced the current to 1.9 ± 0.4 pA/pF, a value indistinguishable from that in 1T solution (n = 4; P = ns). Thus inhibiting EGFR kinase suppressed ~95% of the swelling-induced current.

DISCUSSION

Previous studies on the heart focused on the role of PTK in the regulation of $I_{Cl,swell}$ in atria (15,44). The present study provides the first evidence that $I_{Cl,swell}$ in osmotically swollen ventricular myocytes is regulated in an opposing fashion by the Src and EGFR kinase families

of PTK and by PTP. PTKs are well placed to be sensors of cell volume and mechanical stretch. These signaling molecules interact with the cytoskeleton, integral membrane proteins, and sarcolemma (6), and tyrosine phosphorylation is among the earliest responses to osmotic swelling in cardiac myocytes and other cells (37,38).

Antagonistic regulation of $I_{Cl,swell}$ by Src and EGFR kinase.

$I_{Cl,swell}$ was enhanced by the selective Src family inhibitor PP2. Stimulation of $I_{Cl,swell}$ is unlikely to be due to nonspecific effects of PP2, because its inactive analog, PP3, did not alter the magnitude or the time independence of the current. Moreover, as expected for a process that depends on the phosphorylation of tyrosine residues, blocking PTP and thereby tyrosine dephosphorylation with orthovanadate inhibited $I_{Cl,swell}$ in 0.7T solution, an effect opposite to that obtained by blocking Src-dependent tyrosine phosphorylation. Ultimately, stimulation of $I_{Cl,swell}$ upon blocking Src must result from accumulation of critical tyrosine residues in the dephosphorylated state. Consistent with this idea, suppressing the rate of dephosphorylation by PTP with orthovanadate also precluded $I_{Cl,swell}$ stimulation by PP2. Because orthovanadate associates with a variety of phosphate-binding sites, nonspecific effects of this agent cannot be rigorously ruled out.

Regulation of $I_{Cl,swell}$ also critically depended on EGFR kinase, a second distinct family of PTK. PD-153035, an extremely potent and selective inhibitor of EGFR kinase (19), completely suppressed $I_{Cl,swell}$ in 0.7T solution. AG-1478, the less-potent chloro derivative of PD-153035, also fully inhibited $I_{Cl,swell}$ in rabbit ventricular myocytes (unpublished observations). The antagonistic effect of inhibiting Src and EGFR kinase PTK families suggests that at least two distinct tyrosine residues that are phosphorylated by Src and EGFR kinase, respectively, must be involved in the regulation of $I_{Cl,swell}$ in rabbit ventricles as we previously proposed for human atria (15). Orthovanadate inhibited $I_{Cl,swell}$, which is expected if the PTP inhibitor primarily opposed the action of Src rather than EGFR kinase. This suggests that the Src family PTK site may be dominant or that orthovanadate differentially modulates the dephosphorylation of the targets of these two PTK families (15).

An antagonistic regulation of $I_{Cl,swell}$ by distinct PTK families may in part explain the inconsistent effects of the broad-spectrum PTK inhibitor genistein, which usually stimulated $I_{Cl,swell}$ but inhibited the current in 20% of the cells examined. These divergent responses might reflect differences in the activities of Src and EGFR kinase in individual myocytes. The 50% inhibitory concentration of genistein for v-Src and EGFR kinase are quite similar, 26 and 22 μM, respectively, based on in vitro phosphorylation of exogenous substrates (1), and, therefore, 100 μM of genistein should have largely inhibited both PTK families.

Osmotic swelling of neonatal rat ventricular myocytes leads to activation of PTK and tyrosine phosphorylation of target proteins within 5 s, although the PTK involved was not established (37,38). The present observation that blocking Src stimulates $I_{Cl,swell}$ after osmotic swelling but has no effect under isosmotic conditions argues that a swelling-induced activation of Src is unlikely to be responsible for the activation of $I_{Cl,swell}$. On the other hand, blocking EGFR kinase inhibited $I_{Cl,swell}$ in 0.7T solution. This raises the possibility that stimulation of EGFR kinase by swelling could contribute to the activation of current seen under these conditions. Consistent with this idea, exogenous EGF activates an outwardly rectifying, tamoxifen-sensitive Cl^- current with the characteristics of $I_{Cl,swell}$ in rabbit ventricular myocytes (9).

Regulation of swelling-activated Cl^- current by PTK appears to be different in rabbit ventricular and canine atrial myocytes. Sorota (44) reported that $I_{Cl,swell}$ is inhibited by pretreatment with genistein, whereas acute application of tyrphostin A51, an EGFR kinase inhibitor, and herbimycin A, a Src inhibitor, have no effect. $I_{Cl,swell}$ activated by mechanical stretch of rabbit ventricular myocytes is suppressed by acute application of either genistein or

PP2 (7). On the other hand, genistein and PP2 stimulate swelling-induced $I_{Cl,swell}$ in human atrial myocytes (15), but genistein inhibits $I_{Cl,swell}$ in cultured embryonic chick heart (56). Thus the regulation of $I_{Cl,swell}$ is likely to depend on the method of stimulation (e.g., swelling vs. stretch) and the particular tissue studied. Interventions that favor tyrosine phosphorylation diminish $I_{Cl,swell}$ in bovine chromaffin cells (14) and mouse L-fibroblasts (45), whereas those that suppress tyrosine phosphorylation augment $I_{Cl,swell}$ in calf pulmonary artery endothelial cells (51) and rabbit ciliary epithelial cells (40). Moreover, it is apparent that several different molecules act as volume-sensitive anion channels and/or channel regulators and that certain properties of $I_{Cl,swell}$ in different tissues are distinct (4). Finally, differences in experimental solutions and conditions (e.g., temperature) may affect the regulation of $I_{Cl,swell}$ by signaling pathways.

Time dependence of $I_{Cl,swell}$.

$I_{Cl,swell}$ in the heart is usually described as an inactivating current at positive potentials, although in some cases little inactivation is observed (4,28). The time-dependent genistein- and PP2-stimulated currents recorded here (see Figs. 1 and 2) were consistent with $I_{Cl,swell}$ in that they were volume sensitive, outwardly rectifying, and inhibited by tamoxifen. To our surprise, the addition of Cd^{2+} or Cd^{2+} and Ba^{2+} eliminated the time dependence. A 50% higher concentration of Cd^{2+} (0.3 mM) failed to block the time-dependent, outwardly-rectifying, tamoxifen-sensitive $I_{Cl,swell}$ found in human intestinal T84 cells (5), and a time-dependent $I_{Cl,swell}$ also was reported in guinea pig myocytes with 0.1– 0.2 mM Cd^{2+} in the bath solution (43). Nevertheless, in the presence of these divalent ion blockers, PP2 augmented a volume- and tamoxifen-sensitive outwardly rectifying current that reversed at E_{Cl} with both physiological and symmetrical Cl^- gradients. These characteristics are diagnostic for $I_{Cl,swell}$ (4,28), and thus we attribute both the time-dependent and time-independent components to $I_{Cl,swell}$. One possible mechanism for the block of current decay is a shift in the voltage dependence of $I_{Cl,swell}$ to more positive voltages. Extending the range of test voltages to +100 mV did not elicit time dependence in the presence of Cd^{2+} and Ba^{2+}, whereas current decay was evident at +20 mV in their absence. These findings argue against but do not rigorously exclude a rightward shift in voltage dependence; a shift of >80 mV, which seems unlikely, would be required to explain the data. We also cannot exclude the possibility that distinct Cd^{2+}-sensitive and -insensitive Cl^- channels contribute to $I_{Cl,swell}$. If this is the case, both meet the phenomenological definition of $I_{Cl,swell}$.

Although genistein is a popular tool for identifying the involvement of PTK, it previously was found to stimulate $I_{Cl,cAMP}$ by a mechanism that is independent of protein phosphorylation (10,52; cf. 41 and 42), and it also modifies the behavior of gramicidin channels in planar bilayers by altering the energetics of the hydrophobic interaction between the channels and the bilayer (29). Because the cardiac isoform of the cystic fibrosis transmembrane conductance regulator is expressed in ventricular myocytes (27,58), this raises the possibility that genistein-sensitive $I_{Cl,cAMP}$ might contribute to the genistein-sensitive current. Cardiac $I_{Cl,cAMP}$ is, however, a time-independent current at all voltages (28,43); it exhibits a linear *I-V* relationship in symmetrical high- Cl^- solutions (2,34), and it is insensitive to tamoxifen (50). Moreover, we are unaware of evidence suggesting that PP2 modulates $I_{Cl,cAMP}$. Thus the characteristics of current elicited by genistein and PP2 are inconsistent with $I_{Cl,cAMP}$. It is also unlikely that the current is $I_{Cl,Ca}$. Activation of $I_{Cl,Ca}$ requires a Ca^{2+} transient to elevate the cytoplasmic Ca^{2+} concentration. In the present studies, cytoplasmic Ca^{2+} was buffered at ~60 nM with 8 mM EGTA, and bath Ca^{2+} was either reduced to ~1.5 μM to limit Ca^{2+} entry, or Ca^{2+} channels were blocked by Cd^{2+}. These conditions should preclude the occurrence of the Ca^{2+} transient that is required to elicit a transient outwardcurrent (I_{to}) due to $I_{Cl,Ca}$ (47,61). In addition, the biophysical characteristics of the genistein- and PP2-induced currents are inconsistent with $I_{Cl,Ca}$. A bell-shaped *I-V* relationship and inactivation at positive voltages are expected for

$I_{Cl,Ca}$ with physiological Ca^{2+} regulation (47,61), whereas $I_{Cl,Ca}$ is a linear and time-independent current when cytoplasmic Ca^{2+} is fixed at an elevated concentration (60).

We, as others, utilized replacement of K^+ and Na^+ with internal Cs^+ and external NMDG to block cation currents and thereby isolate Cl^- currents. It is well known, however, that Cs^+ is slightly permeant through a variety of cation channels (22,24), and the possibility that time-dependent outward currents attributed to Cl^- were in fact permeation of Cs^+ through cation channels also must be considered. One possibility is Cs^+ efflux via I_{to}, which in rabbit ventricular myocytes is due to Kv1.4 rather than Kv4.x channels (53,57). Cd^{2+} block of I_{to} in rabbit ventricular myocytes and of Kv1.4 expressed in *Xenopus* oocytes is inconsistent with the present results, however. Cd^{2+} (10 –500 μM) does not inhibit Kv1.4 currents or I_{to}; 500 μM Cd^{2+} shifts the voltage dependence of inactivation rightward by only 12 and 13 mV, respectively, and 100 μM Cd^{2+} does not significantly shift inactivation of I_{to} (57). Another possibility is the efflux of Cs^+ via L-type Ca^{2+} channels ($I_{Ca\text{-}L}$). This seems unlikely for several reasons. First, osmotic swelling causes partial inhibition of $I_{Ca\text{-}L}$ in rabbit ventricular myocytes (31), whereas a prolonged swelling-induced stimulation of Ca^{2+} channels would be required to explain the present data. Second, 50 μM Cd^{2+} only partially blocked the transient Cl^- current (unpublished observations), but should have blocked ~90% of current through L-type Ca^{2+} channels, which exhibit an apparent dissociation constant (*K*d) of 2.14 μM and a Hill coefficient of 0.74 for Cd^{2+} in rabbit ventricular myocytes (26). Furthermore, previous studies of tail currents recorded during the inactivation of $I_{Cl,swell}$ demonstrate reactivation of the underlying conductance on stepping from +80 to −80 mV (43), behavior that is inconsistent with Ca^{2+} channels.

With Cd^{2+}, a small, slowly activating outward current remained. It is likely that the slow component of delayed rectifier current (I_{Ks}) contributes to this time-dependent current. I_{Ks} is stimulated by osmotic swelling of myocytes (36,39,54,59), and delayed rectifiers are more permeant to Cs^+ than most other K^+ channels (the permeability ratio, P_{Cs}/P_K, is ~0.16; Ref. 22; also see Ref. 24). Moreover, Cd^{2+} can stimulate both I_{Ks} (13) and the rapid component of delayed rectifier current (I_{Kr}; Ref. 35) by shifting their voltage dependence. Such a stimulation of delayed rectifier current and suppression of the superimposed $I_{Cl,swell}$ transient both are likely to explain why a delayed rectifier emerged after Cd^{2+} was added to 0.7T bath solution. Extracellular Ba^{2+} blocks delayed rectifier K^+ current in heart (25) as well as currents through KCNQ1 (20), the pore-containing subunit of I_{Ks}. The combination of Cd^{2+} and Ba^{2+} eliminated the slowly activating outward current observed with Cd^{2+} alone. Of course, Ba^{2+} also blocks a number of other K^+ channels (24) including the I_{Kr} due to human *ether-á-go-go*-related gene (55), but I_{Kr} is not enhanced by cell swelling (36,54).

In summary, blocking Src family PTKs leads to activation of $I_{Cl,swell}$ in rabbit ventricular myocytes after osmotic swelling but not under isosmotic conditions, and blocking PTP suppresses $I_{Cl,swell}$ and precludes its activation by inhibition of Src. On the other hand, blocking EGFR kinase PTK inhibited $I_{Cl,swell}$. Thus $I_{Cl,swell}$ is regulated antagonistically by two distinct PTK families as well as by other signaling cascades. Moreover, the transient component of $I_{Cl,swell}$ is sensitive to Cd^{2+}.

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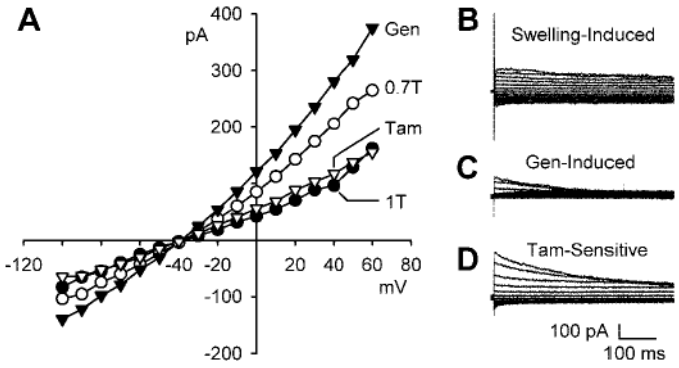

Fig. 1.

Genistein, a broad-spectrum protein tyrosine kinase (PTK) blocker, augmented swelling-activated Cl^- current ($I_{Cl,swell}$). *A*: current-voltage (*I-V*) relationship in isosmotic (1T) solution, after swelling in hypoosmotic (0.7T) solution for 10 min, after exposure to 100 μM genistein in 0.7T solution for 10 min (Gen), and after addition of 10 μM tamoxifen (Tam), a selective $I_{Cl,swell}$ blocker, to 0.7T plus genistein solution. *I-V* curves cross near the Cl^- equilibrium potential (E_{Cl}), which is −42 mV. *B*: families of swelling-induced difference currents. $I_{Cl,swell}$ partially inactivated at positive potentials. *C*: genistein-induced difference currents in 0.7T solution. *D*: tamoxifen-sensitive difference currents in 0.7T plus genistein solution. Swelling activated outwardly rectifying $I_{Cl,swell}$ that reversed near E_{Cl} and was stimulated by genistein. Tamoxifen blocked both the swelling- and genistein-induced components. Calibrations apply to all current records.

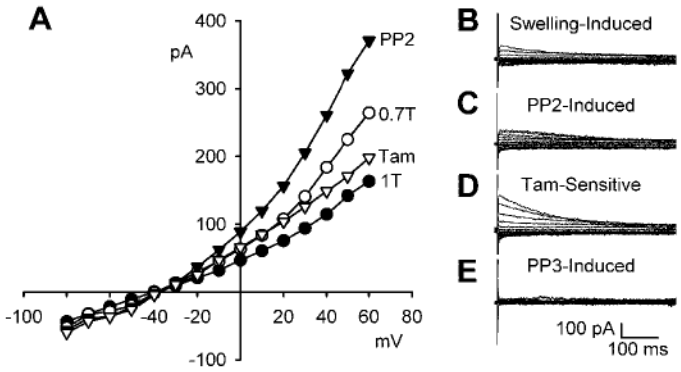

Fig. 2.

$I_{Cl,swell}$ is stimulated by PP2, a selective inhibitor of Src family PTKs, but not by its inactive analog, PP3. *A: I-V* relationships in 1T solution, after swelling in 0.7T solution for 10 min, after exposure to 10 μM PP2 in 0.7T solution for 10 min (PP2), and after addition of 10 μM tamoxifen to 0.7T solution plus PP2. *B*: families of swelling-induced difference currents. *C*: PP2-induced difference currents in 0.7T solution. *D*: tamoxifen-sensitive difference currents in 0.7T plus PP2 solution. Both the PP2- and swelling-induced currents were sensitive to 10 μM tamoxifen. Selective inhibition of Src by PP2 mimicked the effect of genistein. In contrast, PP3 (an inactive analog of PP2; 10 μM for 10 min) did not alter the magnitude or time course of membrane currents in 0.7T solution. *E*: PP3-induced difference currents in 0.7T solution. These records were obtained in a different cell than for *A-D*.

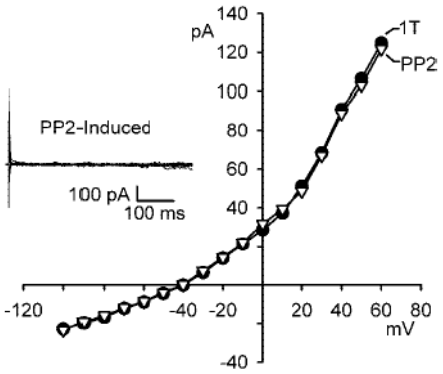

Fig. 3.

Stimulation of $I_{Cl,swell}$ by PP2 requires cell swelling. *I-V* relationships in 1T and after exposure to 10 μM PP2 in 1T solution for 10 min are shown. *Inset*: families of PP2-induced difference currents in 1T solution. PP2 did not alter the outwardly rectifying background Cl^- current attributed at least in part to $I_{Cl,swell}$ under isosmotic conditions. Note the change in scale of *I-V* relationship compared with previous figures.

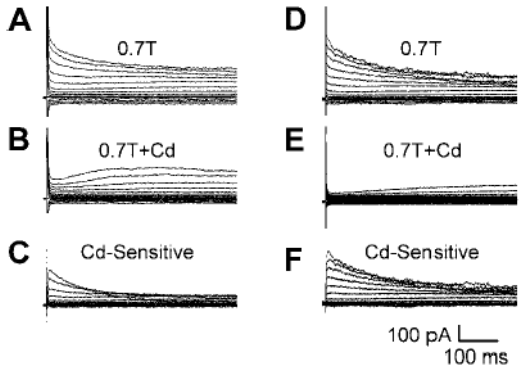

Fig. 4.

Cd^{2+} (0.2 mM) suppressed time dependence of $I_{Cl,swell}$ at positive potentials and revealed a delayed rectifier current. Examples from two myocytes are shown. *A, B, C:* data from one myocyte. *D, E, F:* data from a second myocyte. Families of currents after swelling in 0.7T solution (*A* and *D)*, after exposure to Cd^{2+} (*B* and *E)*, and Cd^{2+} -sensitive current (*C* and *F)* are shown. Cd^{2+} inhibited the time dependence of $I_{Cl,swell}$ at positive potentials and had a variable effect on the steady-state current. After Cd^{2+} block, a slowly activating outward component of current was apparent. Block of $I_{Cl,swell}$ was greatest in the second myocyte (*D-F)*.

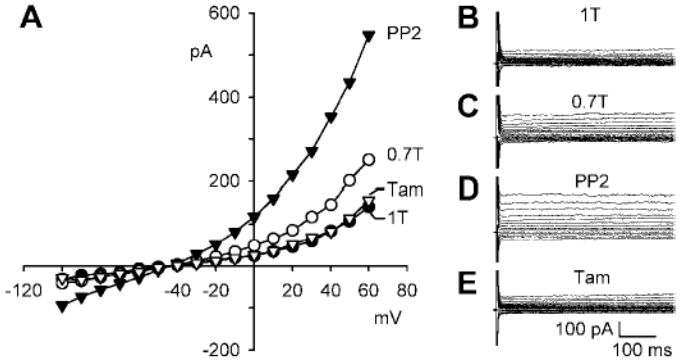

Fig. 5.

Cd^{2+} plus Ba^{2+} eliminated the time dependence of membrane currents but not the stimulation of $I_{Cl,swell}$ by PP2. *A: I-V* relationships in the presence of 0.2 mM Cd^{2+} and 1 mM Ba^{2+} in 1T solution, after swelling in 0.7T solution for 10 min, after exposure to 10 μM PP2 for 10 min in 0.7T solution, and after addition of 10 μM tamoxifen for 10 min to block $I_{Cl,swell}$. *B, C, D,* and *E*: families of currents in 1T solution, 0.7T solution, 0.7T solution plus PP2, and 0.7T solution plus PP2 and tamoxifen, respectively. PP2 stimulated an outwardly rectifying, tamoxifen-sensitive current that reversed near E_{Cl}.

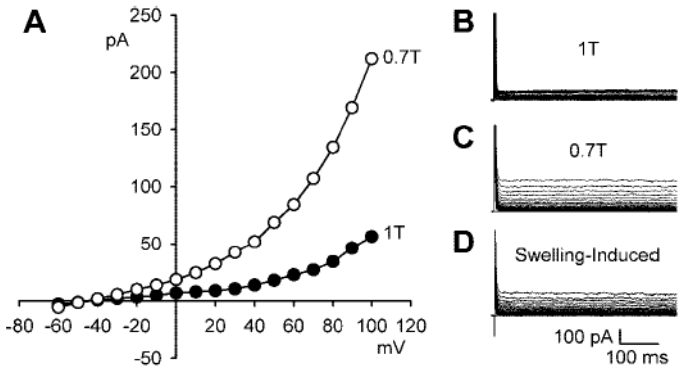

Fig. 6.

Cd^{2+} plus Ba^{2+} eliminated the time dependence of $I_{Cl,swell}$ at strongly positive potentials. To test whether Cd^{2+} (0.2 mM) and Ba^{2+} (1 mM) shifted the onset of $I_{Cl,swell}$ inactivation to more positive voltages, the membrane voltage was stepped from −60 mV to potentials between −60 and +100 mV. *A: I-V* relationships in 1T solution and after 10 min of swelling in 0.7T solution. *B* and *C:* families of currents in 1T and 0.7T solutions, respectively. *D*: swelling-induced difference current. $I_{Cl,swell}$ remained time independent to at least +100 mV.

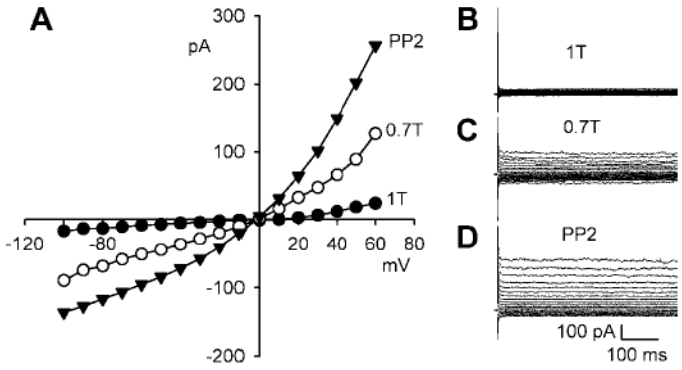

Fig. 7.

Cl^- currents in symmetrical high- Cl^- solutions (intracellular and extracellular Cl^- concentrations = 98.6 mM). *A: I-V* relationships for currents in 1T solution, after 10 min of swelling in 0.7T solution, and after 10 min of exposure to 10 μM PP2 in 0.7T solution. *B, C,* and *D*: families of currents in 1T, 0.7T, and 0.7T plus PP2 solution, respectively. Swelling and PP2 stimulated outwardly rectifying, time-independent currents that reversed near 0 mV in symmetrical Cl^- solutions with 0.2 mM Cd^{2+} and 1 mM Ba^{2+} in the bath.

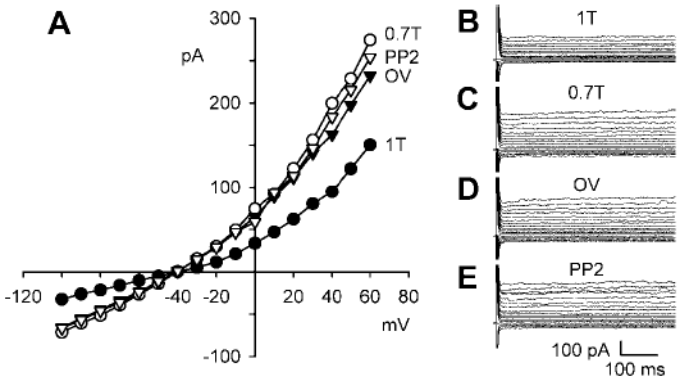

Fig. 8.

Block of protein tyrosine phosphatase (PTP) by orthovanadate partially inhibits $I_{Cl,swell}$ and prevents stimulation of $I_{Cl,swell}$ upon block of Src by PP2. *A: I-V* relationships for currents in 1T solution, after 10 min of swelling in 0.7T solution, after exposure to 1 mM orthovanadate in 0.7T solution for 10 min (OV), and after addition of 10 μM PP2 for 10 min. *B*, *C*, *D*, and *E*: families of currents in 1T, 0.7T, 0.7T plus orthovanadate, and 0.7T plus orthovanadate and PP2 solution, respectively.

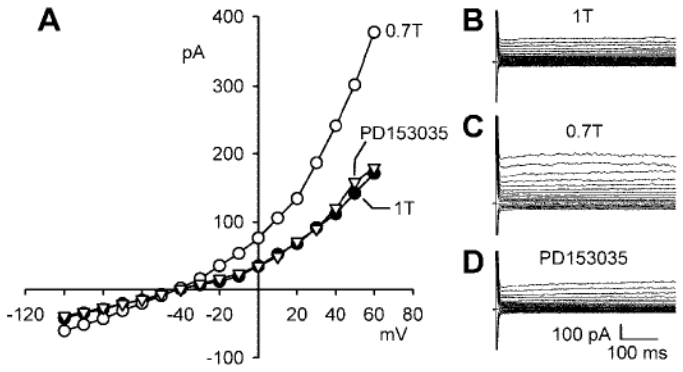

Fig. 9.

Block of epidermal growth factor receptor (EGFR) kinase by PD-153035 inhibits $I_{Cl,swell}$. *A: I-V* relationships for currents in 1T solution, after 10 min of swelling in 0.7T solution, and after exposure to 20 nM PD-153035 in 0.7T solution for 12–15 min. *B, C*, and *D*: families of currents in 1T, 0.7T, and 0.7T plus PD-153035 solution, respectively.