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Regulation of swelling-activated Cl^- current by angiotensin II signalling and NADPH oxidase in rabbit ventricle

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Abstract

Aims—We assessed whether hypoosmotic swelling of cardiac myocytes activates volume-sensitive Cl^- current ($I_{Cl,swell}$) via the angiotensin II (AngII)-reactive oxygen species (ROS) signalling cascade. The AngII-ROS pathway previously was shown to elicit $I_{Cl,swell}$ upon mechanical stretch of β_{1D} integrin. Integrin stretch and osmotic swelling are, however, distinct stimuli. For example, blocking Src kinases stimulates swelling-induced but inhibits stretch-induced $I_{Cl,swell}$.

Methods and results—$I_{Cl,swell}$ was measured in rabbit ventricular myocytes by whole-cell voltage clamp. Swelling-induced $I_{Cl,swell}$ was completely blocked by losartan and eprosartan, AngII type I receptor (AT_1) antagonists. AT_1 stimulation transactivates epidermal growth factor receptor (EGFR) kinase. Blockade of EGFR kinase with AG1478 abolished both $I_{Cl,swell}$ and AngII-induced Cl^- current, whereas exogenous EGF evoked a Cl^- current that was suppressed by osmotic shrinkage. Phosphatidylinositol 3-kinase (PI-3K) is downstream of EGFR kinase, and PI-3K inhibitors LY294002 and wortmannin blocked $I_{Cl,swell}$. Ultimately, AngII signals via NADPH oxidase (NOX) and superoxide anion, $\bullet O_2^-$. NOX inhibitors, diphenyleneiodonium, apocynin and gp91ds-tat, eliminated $I_{Cl,swell}$, whereas scramb-tat, an inactive gp91ds-tat analogue, was ineffective. $\bullet O_2^-$ rapidly dismutates to H_2O_2. Consistent with H_2O_2 being a downstream effector, catalase inhibited $I_{Cl,swell}$, and exogenous H_2O_2 overcame suppression of $I_{Cl,swell}$ by AT_1 receptor, EGFR kinase, and PI-3K blockers. H_2O_2-induced current was not blocked by osmotic shrinkage, however.

Conclusion—Activation of $I_{Cl,swell}$ by osmotic swelling is controlled by the AngII-ROS cascade, the same pathway previously implicated in $I_{Cl,swell}$ activation by integrin stretch. This in part explains why $I_{Cl,swell}$ is persistently activated in several models of cardiac disease.

Keywords

Cl-channel; Angiotensin; NADPH oxidase; Signal transduction; Stretch/m-e coupling

1. Introduction

Volume-sensitive Cl^- current, $I_{Cl,swell}$, is elicited by osmotic swelling and isosmotic hydrostatic pressure-induced inflation of cardiac myocytes, and the current is persistently activated in myocytes isolated from ischaemic and non-ischaemic models of dilated cardiomyopathy in which angiotensin II (AngII) levels are elevated (for review,[1–3]).

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$I_{Cl,swell}$ influences cardiac electrical activity[4,5] and cell volume regulation[6] and may have a role in ischaemic preconditioning[7] and apoptosis.[8]

$I_{Cl,swell}$ also is activated by stretching β_{1D} integrins with paramagnetic beads coated with anti-β_{1D} monoclonal antibodies; integrin-bound beads are pulled towards an electromagnet placed above the cells.[9–11] Integrin stretch elicits $I_{Cl,swell}$ via Src, focal adhesion kinase (FAK), and the AngII AT_1 receptor signalling cascade. AT_1 signalling involves transactivation of epidermal growth factor receptor (EGFR) kinase, phosphatidylinositol 3-kinase (PI-3K), sarcolemmal NADPH oxidase (NOX), superoxide anion ($\bullet O_2^-$), and ultimately H_2O_2.[12–14] AngII is released by cardiac stretch, stretch causes AT_1-dependent hypertrophy,[15] and AngII activates NOX in cardiomyocytes[16–19] and the vasculature.[14,20] AngII and stretch also were recently found to destabilize Kv4.3 mRNA in neonatal rat myocytes via NOX-dependent $\bullet O_2^-$ production.[19]

Although $I_{Cl,swell}$ is evoked both by stretching β_{1D} integrins and by osmotic swelling, these are different stimuli and may signal by different pathways. Osmotic swelling dilutes the intracellular milieu and reduces its ionic strength, while integrin stretch is localized and does not alter the contents of the cytoplasm. Furthermore, PP2, an inhibitor of Src family tyrosine kinases, blocks $I_{Cl,swell}$ activation upon integrin stretch[9] consistent with its role as an upstream mediator of NOX activity,[12] whereas PP2 augments $I_{Cl,swell}$ in osmotically swollen myocytes.[21–23]

The goal of the present study was to determine whether osmotic control of $I_{Cl,swell}$ utilizes the same AngII signalling cascade engaged by β_{1D} integrin stretch, despite differences between the stimuli and observations that Src kinase inhibition has opposite effects on swelling- and stretch-induced $I_{Cl,swell}$. We found that activation of $I_{Cl,swell}$ by osmotic swelling was abrogated by inhibition of AT_1, EGFR kinase, PI-3K, or NOX and by scavenging H_2O_2. Moreover, exogenous epidermal growth factor (EGF) elicited $I_{Cl,swell}$ and exogenous H_2O_2 overcame block of AT_1 receptors, EGFR kinase, and PI-3K. In contrast, osmotic shrinkage failed to suppress H_2O_2-induced $I_{Cl,swell}$. These data argue that the AngII-ROS signaling cascade participates in the response of cardiomyocytes to osmotic swelling. AngII-dependent $I_{Cl,swell}$ activation may modulate electrical activity and cell volume in cardiac disease.

2. Methods

2.1. Ventricular myocytes

Studies conform to *Guide for the Care and Use of Laboratory Animals* (NIH Publication 85-23, revised 1996). Left ventricular myocytes were isolated from anesthetized New Zealand rabbits (~3–4 kg) using collagenase (type II) and pronase (type XIV).[11,22] Cardiomyocytes were washed twice and stored in modified Kraft–Brühe solution (pH 7.2; 295 mosmol/kg).[11] Rod-shaped quiescent cells with clear striations and no membrane blebs were studied within 8 h of isolation.

2.2. Solutions and drugs

Bath solutions designed to isolate anion currents were isosmotic (1T; 300 mosmol/kg; T, times isosmotic), hypoosmotic (0.7T), or hyperosmotic (1.5T) and contained (mM): 90 *N*-methyl-D-glucamine-Cl, 3.0 $MgCl_2$, 10 HEPES, 10 glucose, 5 CsCl, 1 $BaCl_2$, 0.2 $CdCl_2$, 0–250 mannitol (pH 7.4, CsOH). These solutions suppressed the time-dependence of $I_{Cl,swell}$ at positive potentials[22] and allowed osmotic challenges at constant ionic strength. Osmolarity was verified by freezing-point depression.

Stock solutions of apocynin, AG1478, diphenyleneiodonium-Cl (DPI), LY294002, and wortmannin in DMSO and EGF, losartan-K, and eprosartan mesylate in H_2O were frozen (−20° C) in aliquots until use. A membrane-permeant fusion peptide inhibitor of NOX, gp91ds-tat, and a permeant but inactive scrambled analogue, scramb-tat, were synthesized.[24] The inhibitor is a 9-mer that blocks NOX assembly by mimicking the $gp91^{phox}$ (NOX2) docking site for $p47^{phox}$ joined to a tat 9-mer that drives transmembrane uptake. Peptide stocks (1.2 mg/ml) were made in 150 mM NaCl plus 10 mM acetic acid and frozen (−20°C) in aliquots until use. Final diluent concentrations, 0.1–0.5%, did not alter $I_{Cl,swell}$.

2.3. Electrophysiology

Myocytes were placed in a poly-L-lysine-coated chamber and super-fused at ~2 ml/min (21–22°C). Pipettes (2–3 MΩ) were filled with (mM): 110 Cs-aspartate, 20 CsCl or 20 TEA-Cl, 2.5 Mg-ATP, 8 Cs_2-EGTA, 0.15 $CaCl_2$, 10 HEPES (pH 7.1, CsOH; liquid junction potential, −11.5 ±0.7 mV, $n = 9$).[22] This gave a free-$[Ca^{2+}]_i$ of ~60 nM (WinMAXC 2.40; http://www.stanford.edu/~cpatton). Junction potentials were corrected, and ground was a 3-M KCl agar bridge. Seal resistances of 5–30 GΩ were achieved.

Myocytes were dialyzed for 10 min before data were taken. Whole-cell currents were recorded with an Axoclamp 200B and Digidata 1322A under pClamp 8. Currents were low-pass filtered (Bessel, 2 kHz) and digitized (5 kHz). Membrane capacitance was calculated from 5-mV steps. Successive 500-ms steps were made from −60 mV to test potentials between −100 and +60 mV in +10 mV increments. I–V relationships were obtained at 1-min intervals to track responses to interventions and were plotted from quasi steady-state currents. In preliminary studies, $I_{Cl,swell}$ fully activated in <5 min and was stable for at least 45 min. All agents were applied for sufficient time for currents to reach steady state.

Because myocytes were studied under whole-cell conditions, interventions that alter $I_{Cl,swell}$ are not expected to significantly alter cell volume.[21,25] This was confirmed in preliminary studies using video microscopy methods as previously described.[6]

2.4. Statistics

Percent block or activation (±SEM) were calculated using each myocyte as its own control and assessed with paired *t*-tests or, in one case, with a Wilcox rank sign test because the data were not normally distributed; *n* represents the number of cells, and $P < 0.05$ was taken as significant. Analysis of mean current densities (pA/pF) for each treatment group by repeated-measures ANOVA and Student–Newman–Keuls tests gave identical statistical conclusions.

3. Results

3.1. AT_1 signalling

Blocking AT_1 receptors with losartan inhibits $I_{Cl,swell}$ evoked by integrin stretch, and exogenous AngII elicits a Cl^- current attributed to $I_{Cl,swell}$.[10] Figure 1A–C shows that AT_1 receptors also are involved in $I_{Cl,swell}$ activation upon osmotic swelling in 0.7T. As expected, swelling turned on an outwardly rectifying current that reversed at −44 ± 1 mV, near the Cl^- equilibrium potential (E_{Cl}), −42 mV. Adding losartan (30 μM, 10–13 min) to 0.7T strongly suppressed $I_{Cl,swell}$. The losartan-sensitive difference current at +60 mV was 101 ±6% ($P < 0.001$, $n = 10$) of the current activated by swelling. Although currents were smaller at −100 than +60 mV, similar results were obtained at both voltages in this and subsequent protocols. Eprosartan (30 μM, 10 min), a carboxybenzylimidazol AT_1 antagonist with structural features distinct from losartan, a biphenylte-trazole,[26] also fully inhibited swelling-induced currents (98 ±2%; $P < 0.001$, $n = 9$) (Figure 1D and E). In contrast, block of $I_{Cl,swell}$ by a lower concentration of losartan (10 μM, 10 min) was incomplete (68 ±4%; $P < 0.001$, $n = 6$).

Integrin stretch elicits $I_{Cl,swell}$ in cardiomyocytes via AT_1 signalling and its downstream effector H_2O_2.[9,10] If the same pathway is stimulated by osmotic swelling, exogenous H_2O_2 (100 μM) should overcome block of $I_{Cl,swell}$ by losartan (Figure 1A–C). H_2O_2-induced current in the presence of losartan was 154 ±6% ($P < 0.001$, $n = 6$) of $I_{Cl,swell}$, confirming that ROS are downstream from AngII in the regulatory pathway.

3.2. EGFR kinase

EGFR kinase is a receptor protein tyrosine kinase that undergoes transactivation upon AT_1 occupancy and is downstream from AT_1 in the AngII-ROS cascade.[12,27] Figure 2A–C demonstrates that AG1478, a specific EGFR kinase blocker (IC_{50} = 0.003–0.9 μM), fully inhibited $I_{Cl,swell}$. Addition of AG1478 (1 μM, 10–13 min) to 0.7T suppressed 98 ±3% ($P < 0.001$, $n = 11$) of the swelling-induced current at +60 mV. Similar results were obtained in human atrial[21] and rabbit ventricular[22] myocytes using two other EGFR kinase blockers, AG556 and PD153035. As shown for losartan, block of $I_{Cl,swell}$ by AG1478 was overcome by exogenous H_2O_2. The H_2O_2-induced current in the presence of AG1478 was 150 ±15% ($P < 0.001$, $n = 6$) of $I_{Cl,swell}$, indicating that ROS are down-stream from EGFR kinase.

The putative role of EGFR kinase suggests that exogenous EGF should elicit a volume-sensitive Cl^- current under isosmotic conditions. Consistent with this prediction, Figure 2D and E shows that an outwardly rectifying Cl^-current was activated by 3.3 nM EGF in 1T (also see,[11]). EGF (10–15 min) increased the current at +60 mV by 137 ±19% ($P < 0.001$, $n = 6$) in the absence of osmotic swelling. Moreover, osmotically shrinking myocytes in hyperosmotic 1.5T bath solution inhibited 91±7% ($P < 0.001$, $n = 6$) of the EGF-induced current in the continued presence of EGF. Thus, the EGF-induced Cl^- current was volume-sensitive, and osmotic shrinkage must act at site(s) downstream to EGF.

Previously we reported that exogenous AngII elicits a tamoxifen-sensitive Cl^- current attributed to $I_{Cl,swell}$.[10] Figure 3 shows that AngII, like swelling, signals to Cl^- channels via EGFR kinase. Adding AngII (5 nM, 10 min) to 1T bathing media evoked an outwardly rectifying current that reversed near E_{Cl}, and AG1478 (1 μM, 10 min) in the continued presence of AngII inhibited 99 ±3% ($P < 0.001$, $n = 6$; +60 mV) of the AngII-induced current.

3.3. Phosphatidylinositol 3-kinase

PI-3K is downstream of EGFR kinase in the AngII-ROS signalling cascade and participates in NOX activation.[12,28] We tested whether PI-3K also regulates $I_{Cl,swell}$ using selective inhibitors, LY294002 (IC_{50} = 10 μM) and wortmannin (IC_{50} = 1−10 nM). Figure 4A–C illustrates that LY294002 (100 μM, 10–15 min) fully blocked $I_{Cl,swell}$. Addition of LY294002 to 0.7T inhibited the swelling-activated current by 95 ±3% ($P < 0.001$, $n = 8$) at +60 mV. As expected if H_2O_2 is downstream of PI-3K, the suppression of $I_{Cl,swell}$ was abrogated by exogenous H_2O_2. The H_2O_2-induced current in the presence of LY294002 was 147 ±13% ($P < 0.001$, $n = 5$) of the swelling-induced current.

Wortmannin possesses a distinct chemical structure and mechanism of action from those of LY294002.[29] After activating $I_{Cl,swell}$ in 0.7T, adding wortmannin (500 nM, 10–15 min) reduced the current by 60 ±2% ($P < 0.001$, $n = 6$) at +60 mV (Figure 4D and E).

3.4. NADPH oxidase

AT_1 activation ultimately generates ROS by initiating assembly of sarcolemmal NOX from cytoplasmic and membrane subunits,[28] and NOX and ROS participate in the activation of $I_{Cl,swell}$ in response to integrin stretch.[10] Therefore, we evaluated the role of NOX in the response to osmotic swelling. Apocynin (IC_{50} = 80 μM) prevents NOX assembly by conjugating thiol residues.[30] As shown in Figure 5A–C, apocynin (500 μM, 10 min) inhibited

84 ±12% ($P = 0.002$, $n = 5$) of $I_{Cl,swell}$ at +60 mV. Figure 5D and E demonstrates the effect of a structural distinct NOX inhibitor, DPI ($IC_{50} = 0.9$ μM), which binds to flavins and the haem b redox centres of gp91*phox* and, thereby, suppresses $^{\bullet}O_2^-$ production.[31] Addition of DPI (60 μM, 10–13 min) to 0.7T completely blocked $I_{Cl,swell}$ (116 ±16%; $P = 0.002$, $n = 5$).

Although block by DPI or apocynin is widely taken as sufficient evidence to infer participation of NOX, questions can be raised regarding specificity. To address this issue, we utilized gp91ds-tat, a membrane permeant fusion peptide that is regarded as a highly selective blocker of NOX assembly.[24] Figure 6A and B shows that exposure to gp91ds-tat also fully suppressed $I_{Cl,swell}$. Addition of gp91ds-tat (500 nM, 10–12 min) to 0.7T reduced $I_{Cl,swell}$ by 106 ±5% ($P < 0.001$, $n = 5$) at +60 mV. To rule out non-specific actions of the inhibitor peptide, the inactive peptide scramb-tat[24] was studied (Figure 6C). Exposure to scramb-tat (500 nM, 10–15 min) did not alter $I_{Cl,swell}$ (P = ns, $n = 4$).

3.5. Role of H_2O_2

NOX generates $^{\bullet}O_2^-$ by single e^- reduction of molecular O_2, and enzymatic and spontaneous dismutation forms H_2O_2, a membrane-permeant ROS, that is, a stronger oxidant.[28] H_2O_2 is a potent agonist of cardiac $I_{Cl,swell}$, participates in stretch-induced $I_{Cl,swell}$ activation,[10] and as shown in Figures 1, *2 and 4*, overcomes block of $I_{Cl,swell}$ by inhibitors of AT_1 receptors, EGFR kinase, and PI-3K. Therefore, we tested whether catalase, a H_2O_2 scavenger, would inhibit $I_{Cl,swell}$ after osmotic swelling (Figure 7). Addition of catalase (1000 U/ml, 10–15 min) to 0.7T blocked 90 ±11% ($P = 0.001$, $n = 5$) of $I_{Cl,swell}$ at +60 mV. This is consistent with the idea that H_2O_2 is a downstream mediator of $I_{Cl,swell}$ activation.

To further characterize the site of action of H_2O_2, we tested whether H_2O_2-induced current was blocked by osmotic shrinkage. Exposure to 100 μM H_2O_2 in 1T increased outwardly rectifying current at +60 mV by 1.6 ±0.6 pA/pF ($n = 5$), but osmotic shrinkage in 1.5T in the continued presence of H_2O_2 failed to significantly alter the current (1.9 ±0.7 pA/pF, $P = 0.34$; data not shown). This suggests that H_2O_2 acts at sites distal to those regulated by osmotic shrinkage.

4. Discussion

We found that osmotic swelling activated $I_{Cl,swell}$ by means of the AngII AT_1 receptor signalling cascade that involves EGFR kinase, PI-3K, production of $^{\bullet}O_2^-$ by NOX, and its dismutation to H_2O_2.[12,14] Previously, we showed that the same pathway stimulates $I_{Cl,swell}$ when β_{1D}-integrins are stretched with paramagnetic beads.[10,11] A scheme illustrating the results is presented in Figure 8. As expected from this scheme, osmotic activation of $I_{Cl,swell}$ was blocked by inhibitors of AT_1 receptors, EGFR kinase, PI-3K, and NOX and by scavenging H_2O_2 with extracellular catalase, [10,11] whereas exogenous AngII, EGF, and H_2O_2 elicited $I_{Cl,swell}$. H_2O_2 must act late in the cascade because it overcame the suppression of $I_{Cl,swell}$ by AT_1 receptor, EGFR kinase, and PI-3K blockade. AT_1 receptor activation must be upstream from EGFR kinase because AG1478 blocked the AngII-induced current. The EGF-induced current was blocked by osmotic shrinkage, and we previously showed that it also is blocked by inhibitors of PI-3K and gp91ds-tat under different conditions.[11] These data imply that EGFR kinase is upstream from both PI-3K and NOX. While osmotic shrinkage blocked the EGF-induced current, it failed to affect H_2O_2-induced currents. Thus, osmotic shrinkage must regulate $I_{Cl,swell}$ at one or more sites between EGFR kinase and H_2O_2. Taken together, these data imply that osmotic shrinkage does not simply oppose the effect of osmotic swelling at its initial transduction site. Rather, shrinkage and swelling must be sensed by different signalling molecules.

The scheme presented here does not include other molecules that participate in AngII signalling in vasculature[32] and heart.[33] For example, a PKC-induced activation of NOX by AngII that is independent of EGFR kinase is found in vascular tissue.[32] Such a mechanism apparently does not significantly regulate $I_{Cl,swell}$ in ventricular myocytes because EGFR kinase inhibitors fully blocked $I_{Cl,swell}$. Nevertheless, a more complex interplay between components of the proposed cascade cannot be excluded. Interactions with other PKC- and PKA-dependent mechanisms that regulate $I_{Cl,swell}$ (for review,[1–3]) are unexplored.

Osmotic swelling also does not appear to stimulate $I_{Cl,swell}$ by activating integrin-dependent signalling mediated by FAK and Src. Block of the Src family by PP2 or FAK by over expression of the endogenous peptide inhibitor FRNK augments swelling-induced current in rabbit[22] and neonatal rat ventricular[23] and human atrial[21] myocytes, whereas PP2 suppresses stretch-induced $I_{Cl,swell}$.[9] Notably, PP2 prevents Src- and integrin-dependent FAK autophosphorylation at Tyr397 and Tyr577, whereas G protein-dependent phosphorylation at Tyr397 can occur by a PP2-insensitive, Src-independent pathway.[34] Src family members are differentially modulated by osmotic swelling,[35] and unique Src family members may dominate the responses to swelling and stretch.

4.1. AT_1 receptors

Autocrine-paracrine regulation of ventricular myocytes by stretch-induced AngII release has been described.[15] Although AngII secretion was not measured, it seems likely that AngII released from numerous myocytes in the chamber upon swelling initiated autocrine-paracrine AT_1 signalling, and $I_{Cl,swell}$ blockade by losartan and eprosartan is consistent with this idea. In contrast, Zou *et al*.[36] recently made a strong case that cardiomyocyte AT_1 receptors are mechanosensors and respond to stretch by an AngII-independent mechanism that, nonetheless, is blocked by the AT_1 antagonist candesartan. In the present study, complete block of $I_{Cl,swell}$ occurred with 30 μM but not 10 μM losartan. This implies an IC_{50} higher than the 31 nM reported for [^{125}I]-AngII binding to AT_1 receptors in rabbit ventricular membranes at low ionic strength,[37] but lower than that for AT_2 receptors, 81 μM.[38] Losartan is a non-competitive/surmountable antagonist,[26] and its IC_{50} for putative block of direct AT_1 activation by stretch is unknown. The involvement of AT_1 receptors is strongly supported by data implicating downstream components of its signalling cascade[12] in $I_{Cl,swell}$ regulation. Nevertheless, we cannot rigorously exclude the possibility that losartan and eprosartan act at another unidentified site.

4.2. EGFR kinase

AT_1 activation triggers transactivation of EGFR kinase[12,27] by stimulating membrane-bound metalloproteases that clip and release heparin-binding EGF, as well as by association of AT_1 receptors with EGFR kinase.[13] We found that the EGFR kinase inhibitor AG1478 blocked both $I_{Cl,swell}$ and the AngII induced Cl^- current, and Cl^- current elicited by exogenous EGF was inhibited by osmotic shrinkage. These data implicate EGFR kinase in the signalling pathway and further suggest that osmotic shrinkage must act at a distal site in the signalling cascade. Block of volume-sensitive, EGF-induced Cl^- current by tamoxifen, an $I_{Cl,swell}$ blocker, also was reported in studies under different experimental conditions.[11] Previously, we showed that block of EGFR kinase with PD153035 and AG556 inhibit $I_{Cl,swell}$ in rabbit ventricular[22] and human atrial[21] myocytes, respectively. In contrast, tyrphostin A51 (AG183), another EGFR kinase blocker, failed to suppress $I_{Cl,swell}$ in canine atrial myocytes.[25] The basis for this discrepancy is unknown. Consistent with the present results, activation of EGFR kinase by exogenous EGF or transfection with bovine papilloma virus recently were shown to potentiate $I_{Cl,swell}$ in C127 mammary cells,[39] and EGF also stimulates $I_{Cl,swell}$ in liver-derived HTC cells,[40] suggesting this is a common mode of $I_{Cl,swell}$ regulation in several tissues.

4.3. Phosphatidylinositol 3-kinase

PI-3K is downstream of EGFR kinase[12,28] and is stimulated by osmotic swelling.[41,42] In turn, PI-3K produce phosphoinositol-3,4,5-triphosphate and phosphoinositol-3,4-bisphosphate,[29] which activate NOX both by binding to the PX domain of p47*phox*[28] and by augmenting Rac-mediated nucleotide exchange.[12,29] Block of $I_{Cl,swell}$ by LY294002 and wortmannin argue that PI-3K participates in the regulation of cardiomyocyte $I_{Cl,swell}$. We did not investigate why block by 500 nM wortmannin was incomplete. One possibility is involvement of monomeric class II PI-3K-C2α, a cardiac isoform that is more resistant to wortmannin (IC_{50} = 400 nM) than dimeric PI-3K (IC_{50} = 1−10 nM).[29] Inhibition of PI-3K also suppresses $I_{Cl,swell}$ in hepatocytes[43] and pulmonary artery smooth muscle[42] and swelling-induced Na^+-K^+ pump stimulation in rabbit ventricular myocytes.[44]

4.4. Reactive oxygen species

A major component of AT_1 signalling depends on generation of $^{\bullet}O_2^-$ by NOX.[14,33] The involvement of NOX and ROS in hypoosmotic activation of $I_{Cl,swell}$ is strongly supported by findings that three distinct NOX inhibitors all blocked $I_{Cl,swell}$. DPI is promiscuous in binding to FAD-containing enzymes[45,46] and does not distinguish between mitochondrial, microsomal, and sarcolemmal sources of ROS. Apocynin does not alter $^{\bullet}O_2^-$ production by cardiac mitochondria,[47] but it inhibits P450 monooxygenase in endothelial cells.[48] In contrast, gp91ds-tat is thought to be highly NOX selective because of specific interactions between the 9-mer docking sequence and p47*phox* or its homologues (e.g. NOXO1, assembles with NOX1).[24] NOX2 (gp91*phox*) and NOX4 are expressed in cardiomyocytes.[17,49] The present experiments do not establish which isoform is responsible, but two factors favour involvement of NOX2. First, knockout of NOX2 fully abrogates AngII-induced, NOX-dependent, $^{\bullet}O_2^-$ production in cardiomyocytes, indicating that NOX2 rather than NOX4 is the AngII-responsive isoform.[17,50] Second, NOX4 activity in native cells and heterologous expression systems does not depend on cytoplasmic subunits including p47*phox* and its homologues.[49] Thus, gp91ds-tat binding to p47*phox*-like proteins should not have suppressed NOX4 activity.

NOX transfers e^- from intracellular NADPH to extracellular O_2 via FAD bound to its cytoplasmic C-terminal domain and two haem complexes in its membrane-spanning domains.[28] Consequently, NOX produces $^{\bullet}O_2^-$ at the extracellular membrane face, where it rapidly undergoes dismutation to H_2O_2 by extracellular SOD (SOD-3) anchored to proteoglycans.[51] H_2O_2 is a longer-lived species and stronger oxidant than $^{\bullet}O_2^-$ and is membrane permeant.[28] Consistent with a role for H_2O_2, scavenging H_2O_2 by adding catalase to the bath suppressed $I_{Cl,swell}$ in osmotically swollen myocytes. Previously, we showed that tamoxifen-sensitive $I_{Cl,swell}$ is activated by exogenous H_2O_2 (EC_{50} = 8 μM) in rabbit ventricular myocytes.[10] Recent reports indicate that exogenous H_2O_2 also elicits $I_{Cl,swell}$ in HeLa (200–500 μM) and HTC (EC_{50} = 100 μM) cell lines.[40,52] Both of these groups detected ROS production attributed to NOX upon swelling. Expression of the dominant negative p47^{S379A} NOX subunit suppresses activation of $I_{Cl,swell}$ in HeLa cells,[40] as does the non-selective flavin-inhibitor DPI in both HeLa and HTC cells.[40,52] It remains to be seen whether the ultimate target of H_2O_2 is the Cl^- channel itself or redox-sensitive signalling pathways.[33,53] Cysteines with an acidic pK_a are particularly sensitive to oxidation by H_2O_2. Such cysteines are found in the active site of all protein tyrosine phosphatases, including PTEN, which opposes PI-3K by dephosphorylating inositols.[53] Nevertheless, we cannot rule out the possibility that other reactive species contribute to the regulation of $I_{Cl,swell}$.

4.5. Implications

$I_{Cl,swell}$ is persistently activated in models of dilated cardiomyopathy,[2] but the underlying mechanism is obscure. The present results suggest that upregulation of the AngII cascade,

which occurs in ventricular hypertrophy, heart failure, hypertension, atherosclerotic coronary artery disease, hypercholesterolemia, and diabetes, may in part explain persistent activation of $I_{Cl,swell}$ in dilated cardiomyopathy and predicts similar findings in other settings with elevated AngII or NOX activity. For example, NOX is upregulated by subpressor doses of AngII and aortic banding,[16,17] after myocardial infarction,[54] during the progression from hypertrophy to failure,[55] and in atrial fibrillation.[56] Because $I_{Cl,swell}$ outwardly rectifies, its upregulation in cardiac disease promotes reduction of action potential duration, which favours reentrant tachyarrhythmias by decreasing the minimum wavelength for a reentrant circuit.

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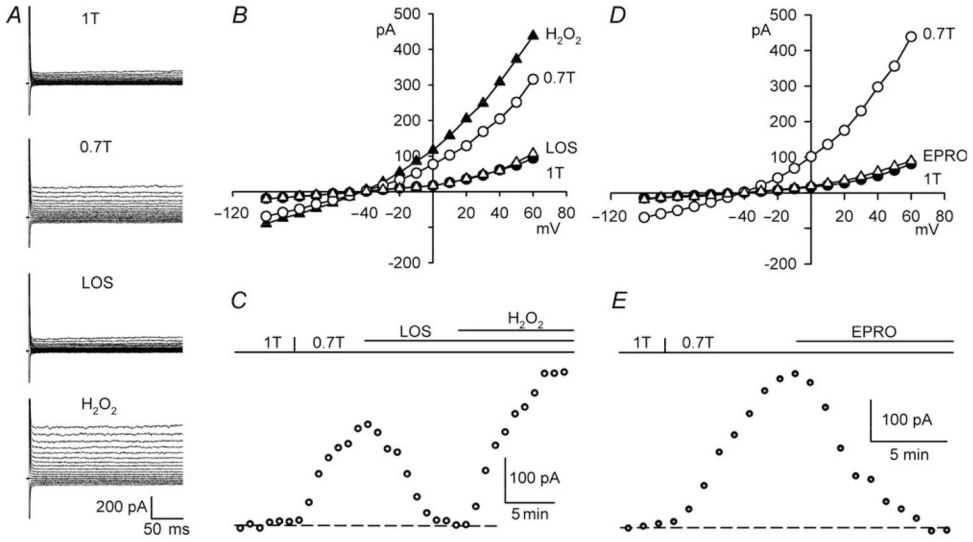

Figure 1.

AT_1 blockers inhibit $I_{Cl,swell}$ during osmotic swelling, and block is reversed by H_2O_2. (*A*) Currents and (*B*) I–V relationships in 1T, 0.7T (10 min), after losartan (30 μM, 10 min) in 0.7T (LOS), and after H_2O_2 (100 μM, 10 min) in presence of losartan (H_2O_2). (*C*) Time course of $I_{Cl,swell}$ at +60 mV; dash line, control current in 1T. (*D* and *E*) Analogous experiments with eprosartan (EPRO; 30 μM, 10 min). $I_{Cl,swell}$ is time-independent because bath contained Cd^{2+} and Ba^{2+}.[22]

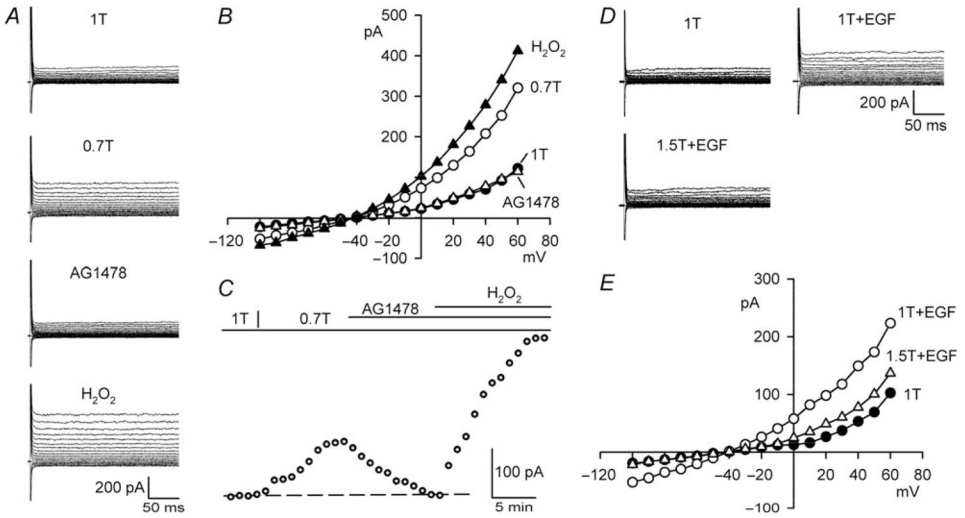

Figure 2.

Block of EGFR kinase suppresses $I_{Cl,swell}$ and is reversed by exogenous H_2O_2; exogenous EGF activates $I_{Cl,swell}$. (*A*) Currents and (*B*) I–V relationships in 1T, 0.7T (10 min), after AG1478 in 0.7T (1 μM, 10 min), and after H_2O_2 (100 μM, 10 min) in presence of AG1478. (*C*) Time course of $I_{Cl,swell}$ at +60 mV. (*D*) Currents and (*E*) I–V relationships in 1T, after EGF in 1T (3.3 nM, 10 min; 1T+EGF), and after osmotic shrinkage in 1.5T (10 min) in presence of EGF (1.5T+EGF).

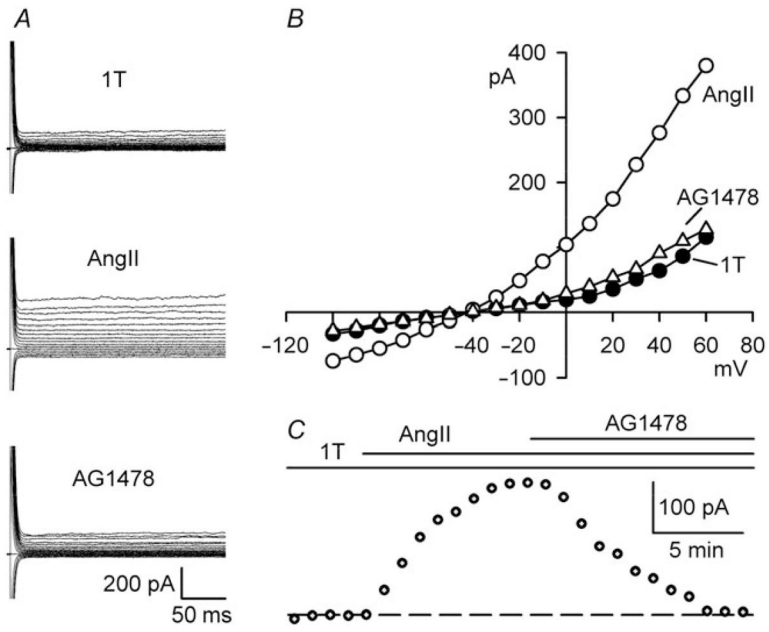

Figure 3.

EGFR kinase inhibition blocks AngII-induced $I_{Cl,swell}$. (*A*) Currents and (*B*) I–V relationships in 1T, after AngII (5 nM, 10 min), and after AG1478 (1 μM, 10 min) in presence of AngII (AG1478). (*C*) Time course of $I_{Cl,swell}$ at +60 mV.

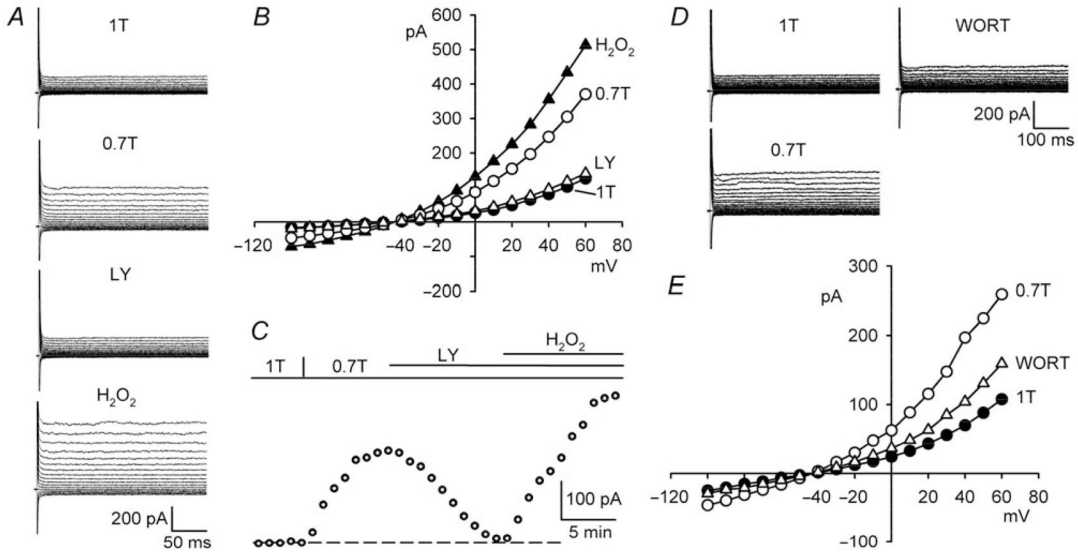

Figure 4.

PI-3K blockade inhibits $I_{Cl,swell}$ and is reversed by exogenous H_2O_2. (*A*) Currents and (*B*) I–V relationships in 1T, 0.7T (10 min), after LY294002 in 0.7T (100 μM, 10 min; LY), and after H_2O_2 (100 μM, 10 min) in presence of LY294002 (H_2O_2). (*C*) Time course of $I_{Cl,swell}$ at +60 mV. (*D* and *E*) Analogous experiments with wortmannin (500 nM, 13 min; WORT).

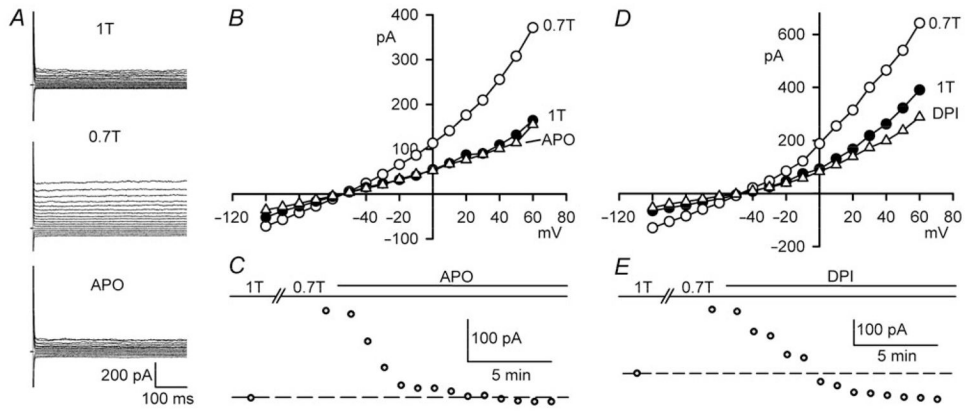

Figure 5.

NOX inhibition blocks $I_{Cl,swell}$. (*A*) Currents and (*B*) I–V relationships in 1T, 0.7T (10 min), after apocynin in 0.7T (500 μM, 10 min; APO). (*C*) Time course $I_{Cl,swell}$ at +60 mV. (*D* and *E*) Analogous experiments with DPI (60 μM, 13 min).

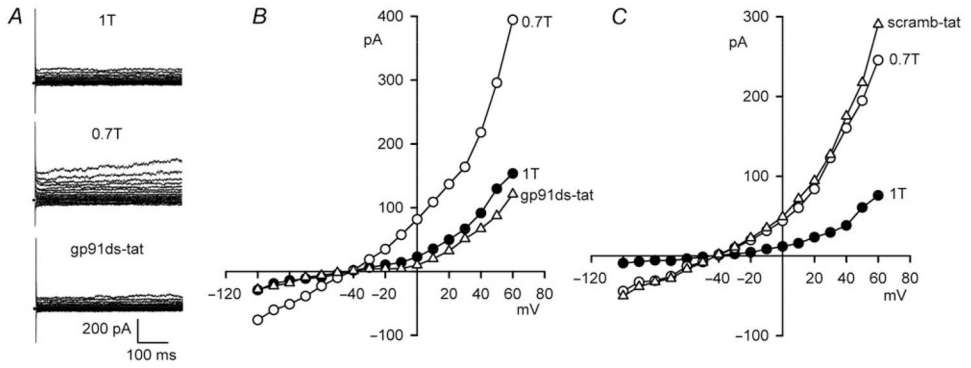

Figure 6.

Block of NOX with gp91ds-tat inhibits $I_{Cl,swell}$. (*A*) Currents and (*B*) I–V relationships in 1T, 0.7T (10 min), and after gp91ds-tat in 0.7T (500 nM, 10 min). (*C*) Analogous experiment with scramb-tat (500 nM, 10 min), a membrane permeant but inactive peptide.

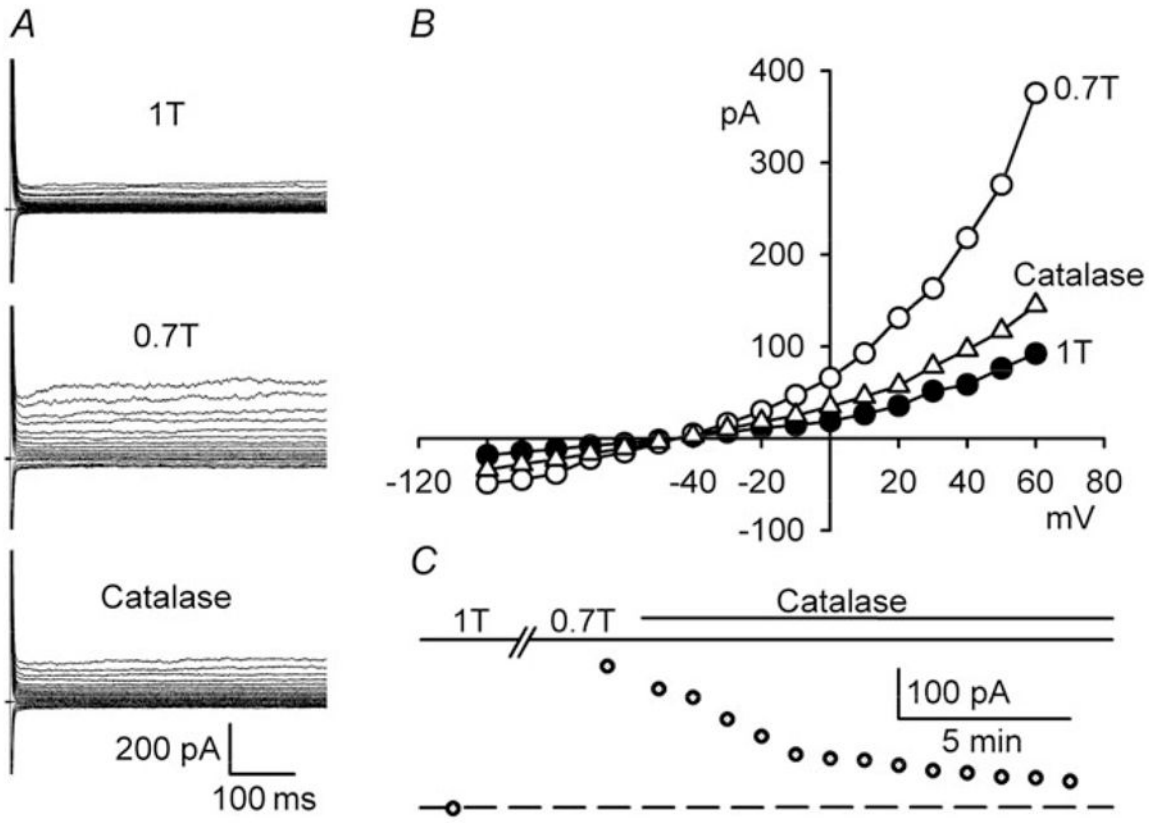

Figure 7.

Scavenging of H_2O_2 by catalase inhibits $I_{Cl,swell}$. (*A*) Currents and (*B*) I–V relationships in 1T, 0.7 T (10 min), and after 1000 U/ml catalase to 0.7 T (10 min, Catalase). (*C*) Time course of $I_{Cl,swell}$ at +60 mV.

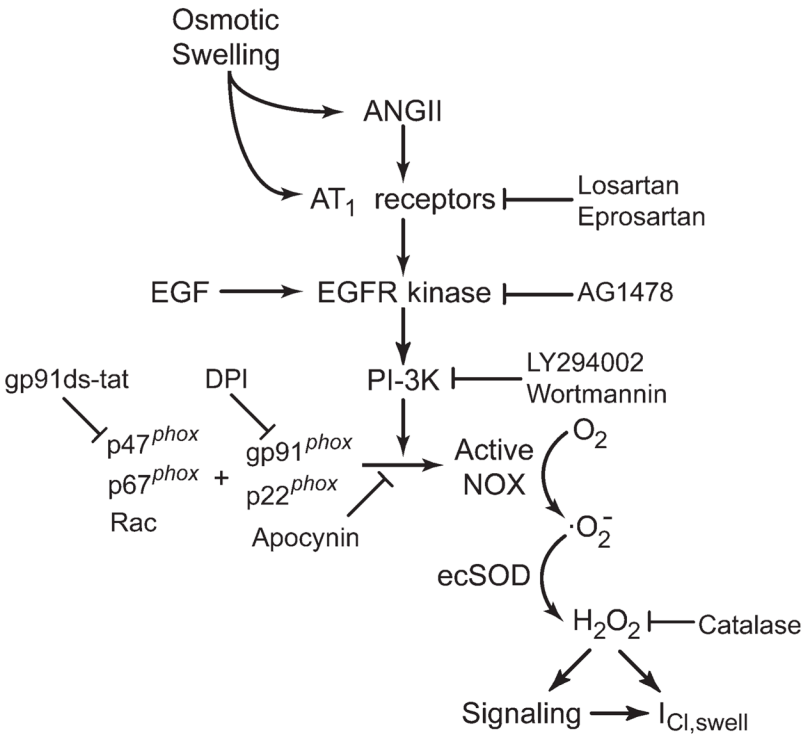

Figure 8.

Proposed scheme for regulation of $I_{Cl,swell}$ by AngII-ROS signalling. Osmotic swelling releases AngII and/or directly activates AT_1 receptors. AT_1 activation evokes downstream signalling via EGFR kinase and PI-3K, causing assembly of NOX from membrane-bound ($gp91^{phox}$, $p22^{phox}$) and cytoplasmic ($p47^{phox}$, $p67^{phox}$, Rac) components. NOX produces $\bullet O_2^-$, which rapidly undergoes dismutation by ecSOD to membrane permeant H_2O_2. H_2O_2 may modulate $I_{Cl,swell}$ directly or via a variety of redox sensitive kinases and phosphatases.