Angiotensin II (AT1) Receptors and NADPH Oxidase Regulate Cl\textsuperscript{−} Current Elicited by β1 Integrin Stretch in Rabbit Ventricular Myocytes

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**ABSTRACT** Direct stretch of β1 integrin activates an outwardly rectifying, tamoxifen-sensitive Cl\textsuperscript{−} current (Cl\textsuperscript{−} SAC) via focal adhesion kinase (FAK) and/or Src. The characteristics of Cl\textsuperscript{−} SAC resemble those of the volume-sensitive Cl\textsuperscript{−} current, I\textsubscript{Cl,swell}. Because myocyte stretch releases angiotensin II (AngII), which binds AT1 receptors (AT1R) and stimulates FAK and Src in an autocrine-paracrine loop, we tested whether AT1R and their downstream signaling cascade participate in mechanotransduction. Paramagnetic beads coated with mAb for β1-integrin were applied to myocytes and pulled upward with an electromagnet while recording whole-cell anion current. Losartan (5 μM), an AT1R competitive antagonist, blocked Cl\textsuperscript{−} SAC but did not significantly alter the background Cl\textsuperscript{−} current in the absence of integrin stretch. AT1R signaling is mediated largely by H\textsubscript{2}O\textsubscript{2} produced from superoxide generated by sarcolemmal NADPH oxidase. Diphenyleneiodonium (DPI, 60 μM), a potent NADPH oxidase inhibitor, rapidly and completely blocked both Cl\textsuperscript{−} SAC elicited by stretch and the background Cl\textsuperscript{−} current. A structurally unrelated NADPH oxidase inhibitor, 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF, 0.5 and 2 mM), also rapidly and completely blocked Cl\textsuperscript{−} SAC as well as a large fraction of the background Cl\textsuperscript{−} current. With continuing integrin stretch, Cl\textsuperscript{−} SAC recovered upon washout of AEBSF (2 mM). In the absence of stretch, exogenous AngII (5 nM) activated an outwardly rectifying Cl\textsuperscript{−} current that was rapidly and completely blocked by DPI (60 μM). Moreover, exogenous H\textsubscript{2}O\textsubscript{2} (10, 100, and 500 μM), the eventual product of NADPH oxidase activity, also activated Cl\textsuperscript{−} SAC in the absence of stretch, whereas catalase (1,000 U/ml), an H\textsubscript{2}O\textsubscript{2} scavenger, attenuated the response to stretch. Application of H\textsubscript{2}O\textsubscript{2} during NADPH oxidase inhibition by either DPI (60 μM) or AEBSF (0.5 mM) did not fully reactivate Cl\textsuperscript{−} SAC, however. These results suggest that stretch of β1-integrin in cardiac myocytes elicits Cl\textsuperscript{−} SAC by activating AT1R and NADPH oxidase and, thereby, producing reactive oxygen species. In addition, NADPH oxidase may be intimately coupled to the channel responsible for Cl\textsuperscript{−} SAC, providing a second regulatory pathway.

**KEY WORDS:** stretch-activated channels • swelling-activated channels • arrhythmia • preconditioning • heart failure

**INTRODUCTION**

Integrins are heterodimeric receptors for the extracellular matrix that physiologically transmit forces from the extracellular matrix to the cytoskeleton and participate in signaling (Wang et al., 1993; Ross and Borg, 2001). We recently showed that direct stretch of β1-integrin using mAb-coated paramagnetic beads evokes an outwardly rectifying Cl\textsuperscript{−} current (Cl\textsuperscript{−} SAC) in rabbit ventricular myocytes (Browe and Baumgarten, 2003b), whereas several other forms of stretch do not stimulate Cl\textsuperscript{−} currents (Baumgarten and Clemo, 2003; Browe and Baumgarten, 2003b). Mechanotransduction involves protein tyrosine kinases (PTKs), specifically focal adhesion kinase (FAK) and/or Src, the principal upstream PTKs stimulated by both mechanical stretch (Sadoshima and Izumo, 1997) and integrin clustering (Parsons, 2003). Cl\textsuperscript{−} SAC resembles I\textsubscript{Cl,swell}, the volume-sensitive Cl\textsuperscript{−} current elicited in cardiac myocytes by osmotic swelling (Tseng, 1992; Sorota, 1992) or hydrostatic pressure-induced cell inflation (Hagiwara et al., 1992). Like I\textsubscript{Cl,swell}, Cl\textsuperscript{−} SAC activates slowly over several minutes, exhibits outward rectification, partially inactivates at positive potentials, and is blocked by tamoxifen (Browe and Baumgarten, 2003b). Furthermore, I\textsubscript{Cl,swell} is regulated by PTKs (Sorota, 1995), including Src (Lepple-Wienhues et al., 2000), and other signaling molecules activated by stretch or integrin clustering, such as PKC (Duan et al., 1999), protein phosphatases (Duan et al., 1999), phosphatidylinositol-3-kinase (PI-3K) (Shi et al., 2002), and small GTP-binding proteins (Tilly et al., 1996; Nilius et al., 1999).

Stretch of cardiac myocytes causes the rapid release of angiotensin II (AngII), which stimulates the G pro-
tein–coupled AT1 receptor in an autocrine–paracrine loop (Sadoshima et al., 1993). Subsequently, AT1 receptors initiate the activation of FAK, Src, PKC, protein phosphatases, PI-3K, and small GTP-binding proteins (Seshiah et al., 2002; Touyz, 2002). These are the same signaling molecules that are activated by integrin clustering, and in turn, regulate Cl− SAC and/or IClswell. Furthermore, AngII elicits an outwardly rectifying Cl− current in rabbit ventricular (Morita et al., 1995) and sino-atrial node (Bescond et al., 1994) myocytes. The AngII-stimulated Cl− current in sino-atrial node is regulated by PKC and blocked by losartan, a nonpeptide specific AT1 receptor antagonist (Bescond et al., 1994). Taken together, these data raise the possibility that AT1 receptors are involved in the activation of Cl− SAC by β1-integrin stretch.

AngII-induced signaling is mediated largely by reactive oxygen species (ROS) generated by sarcosomal NADPH oxidase, a heteromeric enzyme complex broadly distributed throughout cardiovascular and other tissues (Griendling et al., 2000; Vignais, 2002). Cardiac myocytes express all of the components of a phagocyte-like NADPH oxidase: a transmembrane flavocytochrome b558 complex consisting of a large gp91phox (Nox2) and a smaller p22phox subunit, cytosolic p47phox and p67phox subunits, and the small GTP-binding protein Rac (Li et al., 2002; Xiao et al., 2002; Heymes et al., 2003). Nox4, a gp91phox homologue, recently was found to be expressed as well (Byrne et al., 2003). Translocation of the cytosolic subunits and Rac to the membrane and their assembly with gp91phox and p22phox involves PKC, Src and other PTKs, and PI-3K (Yamaguchi et al., 1996; Bokoch and Diebold, 2002; Seshiah et al., 2002; Vignais, 2002). Once activated, the phagocyte-type NADPH oxidase uses intracellular NADPH and NADH as electron donors to catalyze the single electron reduction of extracellular molecular oxygen to superoxide anion (O2−•) (Griendling et al., 2000; Vignais, 2002). O2−• is unstable and is rapidly converted by superoxide dismutase (SOD) to H2O2, a more stable, membrane-permeant ROS that widely participates in signaling (Griendling and Ushio-Fukai, 2000) and directly activates NADPH oxidase (Grandvaux et al., 2001; Li et al., 2001). In ventricular myocytes, dismutation is performed by membrane-bound extracellular-facing SOD (ecSOD), as well as cytoplasmic CuZn and mitochondrial Mn isoforms of SOD (Brahmajothi and Campbell, 1999).

The aim of the present study was to test the hypothesis that the AT1 receptor-NADPH oxidase-H2O2 signaling pathway participates in the activation of Cl− SAC by stretch of β1-integrin in ventricular myocytes. Paramagnetic beads coated with anti-β1-integrin mAb were employed to specifically stretch integrins. Block of either the AT1 receptor or NADPH oxidase and also enzymatic scavenging of H2O2 during stretch inhibit Cl− SAC. Furthermore, either AngII or H2O2 applied in the absence of stretch activate Cl− SAC. Preliminary reports appeared previously (Browe and Baumgarten, 2003a, 2004).

MATERIALS AND METHODS

Ventricular Myocyte Isolation

Left ventricular myocytes were freshly isolated from adult New Zealand white rabbits (~3 kg) by a pronase-collagenase II enzymatic dissociation procedure as described previously (Browe and Baumgarten, 2003b) and stored in a modified KB medium. All membrane current recordings were made within 10 h after myocyte isolation. Single myocytes chosen for study were rod-shaped, quiescent, displayed clear striations, and were free of membrane blebs or other morphological irregularities.

Tyrode solution for cell isolation contained (in mM) 130 NaCl, 5 KCl, 1.8 CaCl2, 0.4 KH2PO4, 5 MgCl2, 5 HEPES, 5 tauro, 5 creatine, 10 glucose, pH 7.25. For Ca-free Tyrode solution, CaCl2 was replaced with 0.1 mM Na2EGTA. For enzyme solution, 1.5–1.75 mg/ml BSA (Sigma-Aldrich), 0.5 mg/ml collagenase (type II; Worthington), and 0.05 mg/ml pronase (type XIV; Sigma-Aldrich) were added to Ca- and EGTA-free Tyrode. KB solution contained (in mM) 120 K-glutamate, 10 KCl, 10 H2PO4, 0.5 K2EGTA, 10 tauro, 1.8 MgSO4, 10 HEPES, 20 glucose, 10 manitol, pH 7.2.

Experimental Solutions and Drugs

Single ventricular myocytes were scattered on a polystyrene-coated, glass-bottomed chamber and placed on the stage of an inverted microscope (Diaphot; Nikon). Hoffman modulation optics (×40; NA = 0.55) and a high resolution TV camera (CCD72; Dage-MTI) were used to visualize myocytes. Bath solution designed to isolate current signals was superfused at 2–3 ml/min and contained (in mM) 145 N-methyl-d-glucamine (NMDG)-Cl, 4.3 MgCl2, 10 HEPES, 5 glucose, pH 7.4. The pipette solution contained (in mM) 110 Cs-aspartate, 20 Cs2SO4, 2.5 MgATP, 8 CsEGTA, 0.1 CaCl2, 10 HEPES, pH 7.1 (liquid junction potential, ~13.2 mV). Pipette free-Ca2+ was estimated as ~35 mM (WinMAXC ver 2.4; www.stanford.edu/~cpatton/maxc.html). All recordings were made at room temperature (22–23°C).

Tamoxifen (20 mM; Sigma-Aldrich) was prepared as a stock solution in DMSO and kept frozen (~4°C) in small aliquots until use. Diphenyleciodinium chloride (DPI; Sigma-Aldrich) was dissolved by warming in DMSO and added to bath solution. The final concentration of DMSO was 0.1%. Losartan-K (Merck), 4-(2-aminoethyl)-benzenesulfonyl fluoride HCl (AEBSF; Sigma-Aldrich), and catalase (Sigma-Aldrich) were dissolved directly in bath solution. Human AngII (Calbiochem) was dissolved in 5% HAc, but its addition to bath solution did not significantly alter pH. H2O2-containing solutions were freshly prepared by diluting 30% (wt/wt) H2O2 (Fisher Scientific) to make a 10 mM stock that was added to bath solution.

Paramagnetic Bead Method

As previously described (Browe and Baumgarten, 2003b), stretch was applied directly and specifically to β1-integrins with mAb-coated paramagnetic beads and an electromagnet. IgG, mAb for the β1 subunit of integrin (MAB2250; Chemicon) was attached by an anti-pan IgG mAb to the surface of uniform 4.5 ± 0.2 μm diameter (mean ± SD) superparamagnetic beads containing iron oxides (Dynabeads M-50 Pan Mouse IgG; Dynal Biotech).
Anti-β1-integrin mAb–coated beads were added to myocytes in the experimental bath and permitted to randomly settle on myocytes from above while the flow of bath solution was turned off. After ~5 min, unbound beads were washed away by restoring bath flow. Myocytes chosen for study typically had three to five coated beads on their surface, and presumably, each bead was bound to multiple β1 integrins.

An electromagnet was placed directly on top of the experimental bath, and patch pipettes were passed through an elliptical opening at its base. Coil current was set to generate a magnetic field density of 35 Gauss (G) and a magnetic flux density gradient of 2,400 G/m that was uniform in the x–y plane occupied by the myocytes (5080 Gauss meter; F.W. Bell). The resulting force vector imposed on each bead was directed upwards toward the coil, perpendicular to the long axis of the myocyte, and was estimated to have a magnitude of 1.2 pN/bead (Browe and Baumgarten, 2003b).

**Electrophysiological Recordings**

Pipettes were pulled from 7740 thin-walled borosilicate glass capillary tubing and then fire polished to give a final tip diameter of 3–6 μm and a resistance in bath solution of 2–3 MΩ. Membrane currents were recorded with an EPC-7 amplifier (List-Medical) using the whole cell configuration of the patch clamp technique. A 150 mM KCl agar bridge served as the ground electrode during recordings. Seal resistances of 5–30 GΩ were typically obtained. Membrane potential was corrected for the measured liquid junction potential before forming a seal. The membrane patch was ruptured by a brief, 500-mV zapping pulse, and myocytes were dialyzed for 10 min before recordings commenced. Voltage clamp protocols and data acquisition were governed by a Digidata 1200B A/D board and pClamp 8.0 (Axon Instruments). Successive 500-ms voltage steps were taken from a holding potential of −60 mV to test potentials ranging from −100 to +40 mV in +10 mV increments. Membrane currents were low-pass filtered at 2 kHz (8-pole Bessel 902; Frequency Devices) and digitized at 10 kHz. For presentation, selected recordings were filtered at 50 Hz, Cl− SAC exhibited strong voltage-dependent inactivation, and isochronal IV curves were plotted based on the average current recorded 20–35 ms after the onset of the voltage step.

**Statistics**

Data are reported as mean ± SEM; n denotes the number of cells. Mean currents are expressed as current density (pA/pF) to account for differences in myocyte surface membrane area. For multiple comparisons, a repeated measures or one-way ANOVA was performed, and the Student-Newman-Keuls or the Bonferroni t test was employed to compare groups. For comparisons of two groups, a one-tailed paired Student’s t test was conducted. Statistical analyses were performed by SigmaStat 2.03 (Systat), and P < 0.05 was taken as significant.

**RESULTS**

**AT1 Receptors Participate in the Activation of Cl− SAC by β1-Integrin Stretch**

Mechanical stretch of myocytes releases AngII, which binds to AT1 receptors and activates FAK and Src in an autocrine–paracrine loop (Sadoshima et al., 1993). Therefore, losartan, a selective AT1 receptor competitive antagonist (Chung and Unger, 1998), was used to test whether AT1 receptors participate in the FAK-stretch-induced activation of Cl− SAC upon β1-integrin stretch (Browe and Baumgarten, 2003b).

Fig. 1 shows an example of families of currents obtained upon stepping voltages to between −100 and +40 mV for 500 ms, and the corresponding I–V relationships. Under control conditions in solutions designed to isolate anion currents, membrane current families recorded before (A, Control) and after (B, Stretch) 8 min of integrin stretch, and following application of 5 μM losartan for 30 min while maintaining integrin stretch (C, + Losartan). Horizontal bar denotes 0 current. (D) I–V relationships for A–C. Each reversed near ECl, At +40 mV, the stretch-activated current was 1.15 ± 0.22 pA/pF, and losartan blocked 72 ± 3% (n = 4) of Cl− SAC. Inset, chemical structure of losartan.

![Figure 1](image_url)

Overall, 5 μM losartan applied for 30–32 min with continued stretch inhibited 72 ± 3% (n = 4; P = 0.002) of the Cl− SAC at +40 mV. Stretch significantly
increased the current at +40 mV from 1.97 ± 0.15 to 3.12 ± 0.29 pA/pF, and after block of AT1 receptors, the current was reduced to 2.30 ± 0.18 pA/pF, a value not significantly different from control (P = 0.08, n = 4). At −100 mV, the stretch-induced inward currents were much smaller than the outward currents, and the control, stretch, and stretch plus losartan currents at −100 mV were not significantly different from each other (P = 0.363).

To verify that losartan was principally inhibiting the stretch-induced current rather than the background current, 100 μM losartan was applied for 12–15 min to unstretched myocytes that had bound anti-β1-integrin mAb-coated beads. Blockade of AT1 receptors under these conditions did not significantly alter the membrane current (n = 4; unpublished data).

**NADPH Oxidase and H$_2$O$_2$ Participate in the Activation of Cl$^-$ SAC**

Activation of AT1 receptors generates ROS primarily by stimulation of the sarcolemmal NADPH oxidase (Seshiah et al., 2002). Moreover, mechanical stretch or integrin clustering can also generate ROS via activation of NADPH oxidase (Howard et al., 1997; Löffgren et al., 1999; Pimentel et al., 2001; Oeckler et al., 2003). Therefore, we tested the idea that the NADPH oxidase-mediated generation of ROS regulates the Cl$^-$ SAC elicited by integrin stretch.

Fig. 2 shows the effect of DPI, a potent inhibitor of O$_2^-$ production by NADPH oxidase that binds to the flavin and heme b redox centers of gp91phox (O’Donnell et al., 1993; Doussiere et al., 1999). As before, a small, outwardly rectifying background Cl$^-$ current was present before stretch (Fig. 2, A and D), and 8 min of integrin stretch greatly increased the Cl$^-$ current (Fig. 2, B and D). In the continued presence of integrin stretch, exposure to 60 μM DPI for 5 min completely blocked both the Cl$^-$ SAC and nearly all of the outwardly rectifying background Cl$^-$ current. The current remaining after block by DPI (Fig. 2, C and D) was very small in amplitude and exhibited a linear I–V relationship.

DPI profoundly inhibited the Cl$^-$ SAC as well as the background Cl$^-$ current in each myocyte studied. At +40 mV, 60 μM DPI inhibited 156 ± 9% (n = 6, P < 0.001) of the Cl$^-$ SAC after 5 min. In these myocytes, integrin stretch significantly increased the Cl$^-$ current from 0.97 ± 0.07 to 2.04 ± 0.12 pA/pF, and DPI markedly decreased the current to 0.44 ± 0.08 pA/pF in the continued presence of integrin stretch. The current after DPI was significantly less than the background Cl$^-$ current before stretch (n = 6, P < 0.001). At −100 mV, integrin stretch increased the Cl$^-$ current from −0.08 ± 0.02 to −0.29 ± 0.17 pA/pF, and DPI decreased the current to −0.07 ± 0.03 pA/pF. The inward currents were small and were statistically indistinguishable, however. These results suggest that NADPH oxidase is required for both the Cl$^-$ SAC and the background Cl$^-$ current.

To verify that NADPH oxidase is involved in the regulation of Cl$^-$ SAC, the effect of AEBSF, a second NADPH oxidase inhibitor, was examined at two concentrations, 500 μM and 2 mM. AEBSF is structurally distinct from DPI and interferes with the assembly of the active NADPH oxidase complex (Diatchuk et al., 1997).

Experiments with AEBSF are illustrated in Fig. 3. As shown previously, the outwardly rectifying background Cl$^-$ current present (Fig. 3, A and D) was increased by 5 min of integrin stretch (Fig. 3, B and D). Treatment with AEBSF (500 μM, 6 min) while maintaining stretch restored the current to its control level (Fig. 3, C and D). At +40 mV, 500 μM AEBSF (5–6 min) blocked 106 ± 7% (n = 3, P = 0.001) of the Cl$^-$ SAC. Stretch significantly increased the outward current from 1.47 ± 0.31 to 2.49 ± 0.52 pA/pF, and 500 μM AEBSF reduced the outward current to 1.43 ± 0.35 pA/pF, a value not different from control. At −100 mV, the effects of integrin stretch and 500 μM AEBSF were small and not statistically significant.

A higher concentration of AEBSF had a more pronounced effect on the Cl$^-$ current that was similar to that seen with 60 μM DPI. After the activation of Cl$^-$

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**Figure 2.** Diphenyleneiodonium (DPI), a potent inhibitor of electron transport within the NADPH oxidase complex, completely blocks both Cl$^-$ SAC and background Cl$^-$ current. Currents before (A, Control) and after (B, Stretch) activation of Cl$^-$ SAC by 8 min of integrin stretch, and after application of 60 μM DPI for 5 min with continued stretch (C, + DPI). (D) I–V relationships for A–C, with each reversing at about −50 mV. At +40 mV, DPI blocked 156 ± 9% (n = 6) of the integrin stretch–induced current. The membrane current after DPI exhibited a linear I–V relationship and was significantly less than the background Cl$^-$ current (P < 0.001 at +40 mV). Inset, chemical structure of DPI.
SAC with 5 min of integrin stretch, myocytes were exposed to 2 mM AEBSF for 6 min with continued stretch. This resulted in the complete inhibition of Cl\textsuperscript{−} SAC as well as most of the background Cl\textsuperscript{−} current (Fig. 3 E). At +40 mV, 2 mM AEBSF (5–6 min) reduced the Cl\textsuperscript{−} SAC by 139 ± 28% (n = 3, P = 0.006). In these myocytes, integrin stretch significantly increased the current from 1.22 ± 0.23 to 2.20 ± 0.26 pA/pF, and then 2 mM AEBSF decreased the current to 0.76 ± 0.24 pA/pF.

Block of Cl\textsuperscript{−} current by 2 mM AEBSF was almost completely reversed after 6 min of washout of the drug in the continued presence of integrin stretch (Fig. 3 F). The Cl\textsuperscript{−} current blocked by 2 mM AEBSF at +40 mV recovered by 91 ± 16% (n = 3, P = 0.007), returning to 2.01 ± 0.46 pA/pF. There was no significant difference between the stretch-induced current before treatment with AEBSF and after washout (P = 0.496). These results strongly support the idea that NADPH oxidase is required for both the activation of Cl\textsuperscript{−} SAC by integrin stretch and the background Cl\textsuperscript{−} current.

Fig. 4 illustrates the time course of Cl\textsuperscript{−} current block by AEBSF (500 μM and 2 mM) and DPI (60 μM) at +40 mV obtained from I–V curves taken at 1-min intervals. Each is well described by a single exponential. The time constant for Cl\textsuperscript{−} current block by 2 mM AEBSF was 0.39 ± 0.03 min (n = 3), approximately fourfold faster than that for the fourfold lower concentration of AEBSF, 1.48 ± 0.17 min (n = 3). Block of Cl\textsuperscript{−} current by DPI (60 μM) proceeded with a time constant of 0.55 ± 0.03 min (n = 3). The rapid kinetics of block of both the Cl\textsuperscript{−} SAC and the background Cl\textsuperscript{−} current, ~90% block occurred within 1 min with 2 mM AEBSF and 60 μM DPI, suggests a close coupling between NADPH oxidase activity and gating of the Cl\textsuperscript{−} channels.

Experiments with NADPH oxidase blockers suggest that up-regulation of NADPH oxidase and ultimately the production of O\textsubscript{2}\textsuperscript{−} and its dismutation to H\textsubscript{2}O\textsubscript{2} are critical for the activation of Cl\textsuperscript{−} SAC. If this idea is correct, degradation of the H\textsubscript{2}O\textsubscript{2} produced by myocyte stretch should abrogate the response to stretch. Fig. 5 shows the effect of catalase, which rapidly converts H\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O\textsubscript{4} on activation of Cl\textsuperscript{−} SAC by integrin stretch. Following a 15-min exposure of the myocyte to 1,000 U/ml catalase in the bath solution, a control current exhibiting modest outward rectification typical of the background Cl\textsuperscript{−} current was recorded (see Fig. 1 D). Integrin stretch was then applied for 6 min in the continued presence of catalase, a time sufficient to activate significant Cl\textsuperscript{−} SAC, but virtually no change in the SAC.
Cl\textsuperscript{−} current was observed. Subsequent washout of catalase for 5 min resulted in full activation of Cl\textsuperscript{−} SAC, documenting the ability of the myocyte to respond to stretch. At +40 mV, in the presence of 1,000 U/ml extracellular catalase, the current after stretch, $1.97 \pm 0.42 \text{ pA/pF}$, was not significantly different than that before stretch, $1.77 \pm 0.40 \text{ pA/pF}$ ($n = 4$, $P = 0.393$). Following washout of catalase, the current markedly increased to $2.86 \pm 0.46 \text{ pA/pF}$. The small increment of current activated in the presence of catalase, $0.19 \pm 0.08 \text{ pA/pF}$, represented only $17 \pm 7\%$ of total Cl\textsuperscript{−} SAC activated by the end of the catalase washout period.

**Angiotensin II Activates a DPI-sensitive, Outwardly Rectifying Cl\textsuperscript{−} Current**

If stretch of β1 integrin releases AngII, which stimulates AT1 receptors in an autocrine–paracrine loop to elicit Cl\textsuperscript{−} SAC, then exogenous AngII should rapidly elicit a Cl\textsuperscript{−} current in the absence of stretch. Myocytes were treated with 5 nM AngII for 6 min, a concentration sufficient to activate AT1 ($K_d = 420 \text{ pM}$) but not AT2 ($K_d = 100 \text{ nM}$) receptors in rabbit ventricle (Wright et al., 1983). As shown in Fig. 6 (A, B, and D), exogenous AngII elicited an outwardly rectifying Cl\textsuperscript{−} current in the absence of stretch that partially inactivated at positive potentials. Moreover, the AngII-induced current was fully inhibited by the NADPH oxidase blocker DPI (60 μM; Fig. 6, C and D), as previously shown for the stretch-induced Cl\textsuperscript{−} SAC. At +40 mV, AngII increased the current by $0.85 \pm 0.03 \text{ pA/pF}$, from $1.18 \pm 0.25$ to $2.04 \pm 0.27 \text{ pA/pF}$ ($n = 4$, $P = 0.01$), and DPI blocked $149 \pm 30\%$ of the AngII-induced current ($n = 4$, $P < 0.005$), reducing the current to $0.75 \pm 0.13 \text{ pA/pF}$. There was no significant difference between the background current before AngII and the current after treatment with DPI ($P = 0.11$).

**H\textsubscript{2}O\textsubscript{2} Activates a Tamoxifen-sensitive, Outwardly Rectifying Cl\textsuperscript{−} Current**

If stretch-induced activation of AT1 receptors and NADPH oxidase stimulates Cl\textsuperscript{−} current via ROS, exogenous ROS might be expected to mimic the effects of stretch. Fig. 7 illustrates a test of this prediction with H\textsubscript{2}O\textsubscript{2} as the ROS. A typical background current was observed under control conditions (Fig. 7, A and E). Addition of 500 μM H\textsubscript{2}O\textsubscript{2} to the bath solution leads to the substantial activation of outward Cl\textsuperscript{−} current in the absence of stretch. The current recorded after a 7-min exposure to H\textsubscript{2}O\textsubscript{2} was outwardly rectifying, reversed at $-50 \text{ mV}$, and partially inactivated at potentials positive to +10 mV (Fig. 7, B and E), as were shown for the control current. A family of H\textsubscript{2}O\textsubscript{2}-induced difference currents, calculated by digitally subtracting the control currents from those after application of H\textsubscript{2}O\textsubscript{2}, and the resulting I-V relationship are shown in Fig. 7 (D and F). H\textsubscript{2}O\textsubscript{2} evoked a much greater increase in outward current than inward current, as previously found for integrin stretch, and the difference current reversed at $-50 \text{ mV}$, close to the calculated $E_{Cl}$ of $-52 \text{ mV}$. Thus,
Tamoxifen inhibits $I_{\text{Cl,swell}}$ but does not affect CFTR or $\text{Ca}^{2+}$-activated $I_{\text{Cl}}$. Tamoxifen inhibits both $I_{\text{Cl}}$ and a large fraction of the background $I_{\text{Cl}}$ during integrin stretch (Browe and Baumgarten, 2003b). To further identify the $I_{\text{Cl,swell}}$-induced current, we tested its sensitivity to tamoxifen. Fig. 7 shows that 10 $\mu$M tamoxifen, applied for 6 min in the continued presence of 500 $\mu$M $H_2O_2$, completely blocked the $I_{\text{Cl}}$ elicited by $H_2O_2$, as well as virtually all of the background $I_{\text{Cl}}$. An $H_2O_2$-induced, outwardly rectifying $I_{\text{Cl}}$ that was blocked by 10 $\mu$M tamoxifen was observed in each myocyte tested. $H_2O_2$ (500 $\mu$M), in the absence of integrin stretch, significantly increased outward $I_{\text{Cl}}$ current at +40 mV by 1.00 ± 0.15 pA/pF ($n = 4$, $P < 0.01$), from 1.24 ± 0.21 to 2.27 ± 0.23 pA/pF. Tamoxifen (10 $\mu$M, 6–8 min) in the continued presence of $H_2O_2$ significantly decreased the current at +40 mV to 1.06 ± 0.26 pA/pF, representing a block of 121 ± 15% ($P = 0.002$) of the current evoked by $H_2O_2$. There was no significant difference between the control current and the current after tamoxifen block ($P = 0.349$). The inward $Cl^-$ current at −100 mV was significantly increased 0.36 ± 0.13 pA/pF ($n = 4$, $P < 0.05$) by $H_2O_2$, from −0.22 ± 0.08 to −0.59 ± 0.21 pA/pF. Tamoxifen decreased the current at −100 mV to −0.45 ± 0.16 pA/pF in the continued presence of $H_2O_2$, although block at −100 mV was not statistically significant ($P = 0.2$).

Although 500 $\mu$M exogenous $H_2O_2$ often is used to demonstrate effects of ROS, this concentration may be higher than the local concentration produced by stretch in situ. Fig. 8 compares the activation of outwardly rectifying $Cl^-$ current by different concentrations of exogenous $H_2O_2$ in the absence of integrin stretch. Exposure to 100 $\mu$M $H_2O_2$ for 7 min increased the outward $Cl^-$ current (Fig. 8 A) to the same degree as seen with 500 $\mu$M, whereas exposure to 10 $\mu$M $H_2O_2$ for 7 min elicited a smaller current (Fig. 8 B). At +40 mV, 100 $\mu$M $H_2O_2$ increased $Cl^-$ current by 1.04 ± 0.08 pA/pF, from 1.74 ± 0.15 to 2.78 ± 0.16 pA/pF ($n = 4$; $P < 0.0005$), but 10 $\mu$M $H_2O_2$ stimulated the current by 0.65 ± 0.04 pA/pF ($n = 4$; $P < 0.0005$), from 1.88 ± 0.29 to 2.52 ± 0.33 pA/pF. The current densities at +40 mV for the $Cl^-$ currents activated by 10, 100, and 500 $\mu$M $H_2O_2$ and by integrin stretch are illustrated in Fig. 8 C. The magnitude of the currents evoked by integrin stretch and 100 or 500 $\mu$M $H_2O_2$ were not statistically distinguishable, whereas the $Cl^-$ current elicited by 10 $\mu$M $H_2O_2$ was significantly smaller than the stretch-induced $I_{\text{Cl,swell}}$ ($P = 0.026$).

Fig. 9 compares the kinetics of activation of $Cl^-$ current by 500 $\mu$M $H_2O_2$ and integrin stretch. In both cases, stimulation occurred over several minutes, but differences in the kinetics were notable. The time course for activation of the $H_2O_2$-induced $Cl^-$ current at +40 mV (Fig. 9 A, filled circles) was well-described by a single exponential with a time constant of 1.79 ± 0.13 min ($n = 4$), equivalent to a $t_{1/2}$ of 1.24 ± 0.09 min. In contrast, the activation of $I_{\text{Cl}}$ at +40 mV (Fig. 9 A, open circles) is sigmoidal with a $t_{1/2}$ of 3.5 ± 0.1 min ($n = 5$) (Browe and Baumgarten, 2003b). The more rapid activation of $Cl^-$ current by $H_2O_2$ than by stretch is consistent with the idea that $H_2O_2$ is an intermediate in the process. Moreover, the I–V relationships for the $H_2O_2$-induced $Cl^-$ current and $I_{\text{Cl,swell}}$ determined after reaching steady-state activation (Fig. 9 B) nearly superimposed.
If the action of NADPH oxidase blockers is solely to prevent generation of $\text{O}_2^- \text{ and ultimately } \text{H}_2\text{O}_2$, exogenous $\text{H}_2\text{O}_2$ should be sufficient to reactivate the stretch-induced $\text{Cl}^-$ current in the presence of NADPH oxidase blockade. Fig. 10 (A and B) illustrates experiments testing this idea. First, myocytes were stretched for 5 min to elicit the $\text{Cl}^-$ SAC. Then, either 60 $\mu$M DPI (Fig. 10 A) or 500 $\mu$M AEBSF (Fig. 10 B) was applied for 5–6 min to block the $\text{Cl}^-$ SAC in the continued presence of stretch. Finally, myocytes were exposed to 500 $\mu$M $\text{H}_2\text{O}_2$ for 10 min in the presence of both the NADPH oxidase blocker and stretch. At $+40 \text{ mV}$, $\text{H}_2\text{O}_2$ reactivated only 4 ± 2% ($n = 3$) and 31 ± 2% ($n = 3$) of the $\text{Cl}^-$ current blocked by DPI and AEBSF, respectively. There was not a significant difference between the current after $\text{H}_2\text{O}_2$ addition and the current after either DPI or AEBSF block, however. Thus, block of NADPH oxidase prevented full activation of $\text{Cl}^-$ channels by a concentration of exogenous $\text{H}_2\text{O}_2$ normally sufficient to fully activate $\text{Cl}^-$ SAC.

Tamoxifen blocks both the $\text{H}_2\text{O}_2$-induced $\text{Cl}^-$ current (Fig. 7) and the stretch-activated $\text{Cl}^-$ SAC (Browe and Baumgarten 2003b). Provided that the same channel protein is responsible for both currents, a model assuming block results from binding of tamoxifen to the channel predicts that the kinetics of block of both of these currents will be identical. A test of this prediction
induced Cl− current and Cl− SAC were well described by single exponential functions, the kinetics of block were different. The time constant for tamoxifen block of the H2O2-induced Cl− current, 4.15 ± 0.49 min (n = 4), was significantly slower than that of the Cl− SAC, 2.07 ± 0.25 min (n = 4; P < 0.01).

**DISCUSSION**

We previously demonstrated that direct and specific stretch of β1-integrin activates an outwardly rectifying, tamoxifen-sensitive Cl− SAC in ventricular myocytes via FAK and/or Src and that Cl− SAC resembles the volume-sensitive Cl− current, IC_{Cl,swell} (Browe and Baumgarten, 2003b). The present results suggest that integrin stretch-induced activation of Cl− SAC requires release of AngII and subsequent stimulation of both AT1 receptors and sarcolemmal NADPH oxidase. NADPH oxidase directly produces O$_2^-$, which is rapidly converted to membrane-permeant H$_2$O$_2$ by dismutation (Brahmajothi and Campbell, 1999), and H$_2$O$_2$ and possibly other ROS participate in the activation of Cl− SAC. As expected from the proposed mechanism depicted in Fig. 12, inhibition of AT1 receptors by losartan, inhibition of NADPH oxidase by DPI and AEBSF, and scavenging of H$_2$O$_2$ by catalase suppressed Cl− SAC. In addition, exogenous AngII and H$_2$O$_2$ each elicited a Cl− current that resembled Cl− SAC. The H$_2$O$_2$-induced Cl− current was suppressed by tamoxifen, a blocker of

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**Figure 11.** Time course of tamoxifen block of H$_2$O$_2$- and stretch-induced Cl− currents. Currents at +40 mV were recorded at 1-min intervals after application of 10 μM tamoxifen and were normalized by the initial current. In both cases, the time course of block was exponential, but the time constant for block of H$_2$O$_2$-induced current, 4.15 ± 0.49 min (n = 4), was slower than for stretch-induced current, 2.07 ± 0.25 min (n = 4; P < 0.01). This unexpected discrepancy can be accounted for by an H$_2$O$_2$-mediated breakdown of tamoxifen.

**Figure 12.** Simplified proposed model of the mechanotransduction process coupling β1 integrin stretch to activation of Cl− SAC in ventricular myocytes. Integrin stretch triggers the phosphorylation and activation of focal adhesion kinase (FAK) and Src, and the release of Ang II from secretory vesicles. Ang II binds to the AT1 receptor (AT1R) and activates the AT1R signaling cascade. Components of the AT1R signaling cascade, possibly in concert with components of integrin signaling, induce the activation of p47$^{phox}$, p67$^{phox}$, and rac, which translocate to the membrane and assemble with gp91$^{phox}$ and p22$^{phox}$ to form the active NADPH oxidase complex. NADPH oxidase recruits NAD(P)H as an electron donor and catalyzes the transmembrane transfer of electrons to molecular O$_2$ to form superoxide (O$_2^-$). Extracellular O$_2^-$ is rapidly converted to membrane-permeant H$_2$O$_2$ by ecSOD. H$_2$O$_2$ may activate Cl− SAC either directly or via ROS-sensitive signaling pathways. The idea that NADPH oxidase may be a closely coupled regulator of the Cl− SAC channel is not illustrated.
Cl\(^{-}\) SAC and I\(_{\text{Cl\text{\_swell}}}\), and the AngII-induced current was blocked by inhibition of NADPH oxidase. Moreover, the background Cl\(^{-}\) current also was blocked by inhibition of NADPH oxidase but not by an AT1 receptor antagonist. To our knowledge, this is the first report that cardiac Cl\(^{-}\) currents are regulated by H\(_2\)O\(_2\) or other ROS and the first evidence that NADPH oxidase modulates cardiac electrical activity.

**Autocrine/Paracrine Regulation of Cl\(^{-}\) SAC by Angiotensin II**

AngII is stored in secretory vesicles in myocytes and released within 1 min by mechanical stretch of cultured rat myocytes grown on an elastic substrate (Sadoshima et al., 1993). Stretch-induced AngII release is sufficient to activate AT1 receptors in an autocrine–paracrine loop and induce gene expression and hypertrophy. Block of Cl\(^{-}\) SAC by losartan, a highly selective AT1 antagonist with an IC\(_{50}\) of 20 nM (Lambert et al., 1995; Chung and Unger, 1998), activation of Cl\(^{-}\) current by exogenous AngII (5 nM), and the much greater AngII affinity of AT1 than AT2 receptors (Wright et al., 1983) strongly argue for involvement of AngII and AT1 receptors in the transduction of integrin stretch. The amount of AngII released was not determined. It is important to note, however, that a large number of myocytes bound to β1-integrin mAb-coated beads covered the chamber floor. All myocytes were stretched simultaneously, and presumably all released AngII. Therefore the voltage-clamped cell was exposed not only to locally released AngII, but also to AngII delivered by diffusion and solution flow from other cells.

Morita et al. (1995) previously reported that exogenous AngII evokes an outwardly rectifying Cl\(^{-}\) current in rabbit ventricular myocytes. They found the current is blocked by sarasolin and eliminated when cytoplasmic free-Ca\(^{2+}\) is driven to vanishing levels with Ca\(^{2+}\)-free pipette solutions containing 10 mM EGTA. In our study, free-Ca\(^{2+}\) was ~35 nM. In rabbit SA node, AngII induces an outwardly rectifying Cl\(^{-}\) current that is PKC dependent and blocked by losartan (Bescond et al., 1994). On the other hand, I\(_{\text{CFTR\text{\_cardiac}}}\) is strongly inhibited by AngII via AT1 receptors and inhibition of adenylate cyclase (Oyabashi et al., 1997). AngII also activates a Ca\(^{2+}\)-dependent Cl\(^{-}\) current in mesangial (Marerro et al., 1996) and adrenal zona fasciculata cells (Chorvatova et al., 1998). In addition to effects on Cl\(^{-}\) currents, AngII modulates a number of cation currents (for review see Chorvatova et al., 1996), and thus, integrin stretch-induced activation of AT1 receptors also may affect cardiac cation currents (Browe and Baumgarten, 2003b).

A coordination of stretch and AngII receptor activation is implicated in a variety of cardiac responses in addition to hypertrophy and gene expression (Sadoshima et al., 1993). Losartan and/or the AngII converting enzyme inhibitor captopril suppress stretch-induced phosphatidylinositol hydrolysis, PKC translocation (Paul et al., 1997), and atrial natriuretic peptide secretion (Ruskoaho et al., 1997). AngII also mediates stretch-induced activation of the Na\(^{+}/H^{+}\) exchanger and changes in contractility in cardiac myocytes (Dostal and Baker, 1998; Cingolani et al., 2001).

**Regulation of Cl\(^{-}\) SAC by NADPH Oxidase and ROS**

It is proposed that activation of Cl\(^{-}\) SAC by integrin stretch is due to the activation of NADPH oxidase and production of ROS. Involvement of NADPH oxidase and ROS in the stimulation of Cl\(^{-}\) current is supported by three lines of evidence. First, two structurally distinct blockers of NADPH oxidase, DPI and AEBSF, rapidly and completely inhibited Cl\(^{-}\) SAC. DPI acts by displacing FAD from the electron transfer chain (O’Donnell et al., 1993; Doussiere et al., 1999), making it a potent NADPH oxidase inhibitor; its IC\(_{50}\) for O\(_{2}\)\(^{\bullet}\) production is 0.9 and 5.6 μM in intact macrophages (Hancock and Jones, 1987) and neutrophils (O’Donnell et al., 1993), respectively. DPI also inhibits other flavoprotein-containing enzymes, however, including nitric oxide synthase (Stuehr et al., 1991). AEBSF prevents assembly of the NADPH oxidase active complex and blocks O\(_{2}\)\(^{\bullet}\) production with an IC\(_{50}\) of 1 mM (Diatchuk et al., 1997), but there is no evidence that AEBSF interacts with nitric oxide synthase. Moreover, DPI and AEBSF inhibit ROS-dependent ERK activation in ventricular myocytes (Xiao et al., 2002). Second, activation of Cl\(^{-}\) SAC by integrin stretch was strongly attenuated by extracellular catalase, which rapidly breaks down H\(_{2}\)O\(_2\). This implies that integrin stretch must lead to H\(_{2}\)O\(_2\) production and that H\(_{2}\)O\(_2\) is required for Cl\(^{-}\) SAC activation. The effect of extracellular catalase is consistent with the topology of gp91\(^{\text{phox}}\) (Nox2), the prototypic phagocyte-type NADPH oxidase found in heart. Nox2 produces O\(_{2}\)\(^{\bullet}\) at the extracellular face of the sarcolemma (Griendling et al., 2000; Vignais, 2002), where ecSOD is positioned to convert O\(_{2}\)\(^{\bullet}\) to H\(_{2}\)O\(_2\) (Brahmajothi and Campbell, 1999). Third, direct application of exogenous H\(_{2}\)O\(_2\) in the absence of integrin stretch promptly activated a tamoxifen-sensitive Cl\(^{-}\) current with biophysical characteristics similar to those of Cl\(^{-}\) SAC; the ED\(_{50}\) was <10 μM. Although we refer to this as an H\(_{2}\)O\(_2\)-induced Cl\(^{-}\) current, the present data do not exclude the possibility that other reactive species participate in its regulation.

Activation of Cl\(^{-}\) current by H\(_{2}\)O\(_2\) was more rapid than the activation of Cl\(^{-}\) current by integrin stretch, as expected if H\(_{2}\)O\(_2\) is an intermediate in stretch-induced signaling. Nevertheless, H\(_{2}\)O\(_2\) and ROS activate a variety of signaling processes (Allen and Tresini, 2000). We cannot rigorously exclude the possibility that exogenous H\(_{2}\)O\(_2\) regulates Cl\(^{-}\) current, at least in part, by signaling cascades that are unaffected by integrin stretch.
Both gp91<sup>phox</sup> and Nox4, a homologue of gp91<sup>phox</sup>, are found in ventricle (Byrne et al., 2003). Knockout of gp91<sup>phox</sup> abrogates AngII-induced O<sub>2</sub>− production and ventricular hypertrophy, suggesting gp91<sup>phox</sup> underlies the stretch-induced, NADPH oxidase-dependent responses studied here. It is clear that AngII activates NADPH oxidase in vascular smooth muscle by a process that involves AT1 receptors, FAK and Src, and transactivation of EGF receptors (Seshiah et al., 2002). This may explain why block of FAK and Src inhibits Cl<sup>−</sup> transactivation of EGF receptors (Seshiah et al., 2002).

The stretch-induced, NADPH oxidase-dependent re-
gp91<sup>phox</sup> found in ventricle (Byrne et al., 2003). Knockout of the primary Cl<sup>−</sup>/H<sub>11001</sub> current (Browe and Baumgarten, 2003b). Moreover, in preliminary experiments, we found that Cl<sup>−</sup> SAC activated by integrin stretch was blocked by the EGF receptor inhibitor AG1478 (unpublished data).

Stress of cultured rat ventricular myocytes previ-
ously was shown to generate O<sub>2</sub>−· by a NADPH-dependent mechanism (Pimentel et al., 2001). Mechanical stimuli activate NADPH oxidase in isolated endothelial cells (Howard et al., 1997; De Keulenaer et al., 1998) and coronary artery denuded of endothelium (Oecler et al., 2003). NADPH oxidase also is activated by integrin clustering. In eosinophils, CR3 (CD11b/CD18) integrin-mediated adhesion activates NADPH oxidase by a pathway that includes Src, PKC, and PI-3K (Lynch et al., 1999). Application of particles coated with Ab for the α-integrin subunit of LFA-1, CR3, or CR4 (CD11a, CD11b, or CD11c, respectively) or the β2-integrin subunit of CR3 (CD18) stimulates NADPH oxidase in neutrophils by a process that requires cytoskele-
tal rearrangements but not phagocytosis (Serrander et al., 1999), and activation by anti-β2 integrin Ab de-
pends on PTK (Löfgren et al., 1999).

NADPH oxidase also appeared to be required to sup-
port the background Cl<sup>−</sup> current. The NADPH oxidase inhibitors DPI and AEBSF not only blocked Cl<sup>−</sup> SAC, but also suppressed the outwardly rectifying component of Cl<sup>−</sup> current present before integrin stretch. On the other hand, block of the AT1 receptor by losartan did not affect the Cl<sup>−</sup> current in the absence of stretch. Both gp91<sup>phox</sup> (Nox2) and Nox4 contribute to basal O<sub>2</sub>−· and H<sub>2</sub>O<sub>2</sub> production in the unstimulated heart (Bendall et al., 2002; Heymes et al., 2003; Byrne et al., 2003). There-
fore, background Cl<sup>−</sup> current seems to be regulated by NADPH oxidase independent of AT1 receptor activity. Others have attributed the background Cl<sup>−</sup> current in heart to I<sub>Cl/swell</sub> (Sorota, 1992; Duan et al., 1995, 1997).

Identity of the H<sub>2</sub>O<sub>2</sub>-induced Cl<sup>−</sup> Current

The primary Cl<sup>−</sup> currents in cardiac myocytes are a PKA-dependent current due to the cardiac isoform of CFTR (I<sub>CFTR cardiac</sub>), the calcium-dependent transient outward Cl<sup>−</sup> current (I<sub>Cl,ca</sub>), and the volume-sensitive Cl<sup>−</sup> current (I<sub>Cl/swell</sub>) (Hume et al., 2000), and we previously suggested that the Cl<sup>−</sup> SAC is due to I<sub>Cl/swell</sub> (Browe and Baumgarten, 2003b). Several of the bio-
physical and pharmacological properties of the H<sub>2</sub>O<sub>2</sub>-induced current are consistent with Cl<sup>−</sup> SAC rather than either I<sub>CFTR cardiac</sub> or I<sub>Cl,ca</sub>. Cl<sup>−</sup> SAC and the H<sub>2</sub>O<sub>2</sub>-induced current both exhibit strong outward rectification, similar kinetics and voltage-dependence of inactiva-
tion, and steady-state I–V curves that are superimpos-
able. I<sub>Cl,Ca</sub> is time independent at all voltages (Shuba et al., 1996; Hume et al., 2000), whereas H<sub>2</sub>O<sub>2</sub>-induced current partially inactivated at positive potentials. I<sub>Cl,swell</sub> is initiated by elevation of cytoplasmic Ca<sup>2+</sup> and exhibits both inactivation at positive potentials and a bell-shaped I–V relationship if Ca<sup>2+</sup> handling is un-
compromised (Zygmont and Gibbons, 1991). When cy-
toplasmic Ca<sup>2+</sup> is set at an elevated level, however, I<sub>Cl,swell</sub> is time independent with a linear I–V relationship (Zyg-
munt, 1994). In contrast, the H<sub>2</sub>O<sub>2</sub>-induced current in-
activated and displayed outward rectification in Ca<sup>2+</sup>-
free bathing media with strongly buffered pipette Ca<sup>2+</sup>, conditions that reduced cytoplasmic free-Ca<sup>2+</sup> to <~35 nM and minimized Ca<sup>2+</sup> transients. Moreover, tamoxi-
fen completely blocked the H<sub>2</sub>O<sub>2</sub>-induced current, but I<sub>CFTR cardiac</sub> (Vandenbergh et al., 1994) and I<sub>Cl,ca</sub> (Val-
verde et al., 1993) are insensitive to tamoxifen.

One argument against the H<sub>2</sub>O<sub>2</sub>-induced current be-
ing the same as Cl<sup>−</sup> SAC is the kinetics of block by 10 
μM tamoxifen. The time constant was 4.1 min for the H<sub>2</sub>O<sub>2</sub>-induced current and 2.1 min for the Cl<sup>−</sup> SAC, whereas identical kinetics were expected if tamoxifen blocked at the same site in both cases. The action of tamoxifen is more complex than classic channel block, however. Tamoxifen can act as an ROS scavenger (Cus-
todio et al., 1994). This suggests that the approximately twofold slowing of block could have arisen because approx-
imately half of the tamoxifen was converted to an inactive form by exposure to 500 μM H<sub>2</sub>O<sub>2</sub>. In addition, tamoxifen is reported to inhibit NADPH oxidase in uterine smooth muscle (Jain et al., 1999). Because both NADPH oxidase and ROS regulate Cl<sup>−</sup> SAC/ I<sub>Cl/swell</sub> these actions of tamoxifen are likely to contrib-
ute to its block of both Cl<sup>−</sup> SAC with integrin stretch and I<sub>Cl/swell</sub> with osmotic swelling.

H<sub>2</sub>O<sub>2</sub> modulates multiple Cl<sup>−</sup> conductances in other systems. It activates native I<sub>Cl(Ca)</sub> in Xenopus oocytes indirectly via Na<sup>+</sup>–Ca<sup>2+</sup> exchange (Schlief and Heine-
mann, 1995), but suppresses a chlorotoxin-sensitive, time-independent Cl<sup>−</sup> conductance in retinal pigmented epithelium (Weng et al., 2002), and a sarcoplasmic reticulum Cl<sup>−</sup> channel (Kourie, 1997). Because the present studies were done under Na<sup>+</sup>-free conditions, stimulation of I<sub>Cl(Ca)</sub> via Na<sup>+</sup>–Ca<sup>2+</sup> exchange can be excluded.

Is NADPH Oxidase Directly Coupled to Cl<sup>−</sup> Channels?

Inhibition of Cl<sup>−</sup> SAC and background Cl<sup>−</sup> current by 60 μM DPI and 2 mM AEBSF was both complete and
rapid. The kinetics of block appears to place a limit on the complexity of the signaling pathway between NADPH oxidase and the Cl− channel. One possibility is that NADPH oxidase is a closely coupled Cl− channel regulator. That is to say, it may regulate Cl− SAC by a direct molecular interaction, as well as by production of ROS. Such a coupling might explain why exogenous H2O2 did not fully reactivate Cl− SAC in the presence of either DPI or AEBSF. Recently, studies demonstrated that knockout of CIC-3, which has been postulated to underlie cardiac IClswell (Duan et al., 1999), leads to suppression of Nox 2 activity in stimulated leukocytes (Moreland et al., 2004) but up-regulation of Nox 1 in vascular smooth muscle (Miller et al., 2004). In addition, β2 integrin cross-linking can trigger a Cl− efflux that regulates the generation of ROS in neutrophils (Menegazzi et al., 1999).

An alternative possibility is that NADPH oxidase blockers directly inhibit Cl− channels independent of their action on NADPH oxidase. This possibility seems unlikely because DPI and AEBSF are structurally distinct molecules. Apocynin, a third structurally distinct NADPH oxidase blocker, and DPI also inhibit swelling-induced IClswell in rabbit ventricular myocytes (Ren, Z., personal communication; unpublished data). Moreover, the hypothesis that NADPH oxidase blockers directly inhibit Cl− channels fails to explain why extracellular catalase abrogated the response to stretch or why exogenous H2O2 mimicked stretch by eliciting a Cl− current. Nevertheless, precise understanding of the relationship between NADPH oxidase and the Cl− channel and the action of NADPH oxidase blockers awaits further investigation.

NADPH Oxidase, Cl− Current, and Cardiac Pathophysiology

IClswell blockers reportedly abolish ischemia-, drug-, and hypoxic stress–induced preconditioning and exert protective effects in ischemia/reperfusion (for review see Baumgarten and Clemo, 2003). Mohazzab-H et al. (1997) demonstrated that NADPH oxidase is activated during ischemia/reperfusion, and it is well established that H2O2 and ROS are critically important in preconditioning (LeBuffe et al., 2003), myocardial injury (Li and Jackson, 2002), and apoptosis (von Harsdorf et al., 1999). As noted above, NADPH oxidase can be blocked and ROS can be scavenged by agents that also block IClswell such as tamoxifen (Jain et al., 1999) and DIDS (Schwingshackl et al., 2000). Indeed, block of NADPH oxidase or scavenging of ROS by such agents, rather than a direct block of Cl− SAC or IClswell, may contribute to their effects on preconditioning, oxidant injury and apoptosis. Alternatively, a functional coupling between NADPH oxidase and Cl− channels may lead to inhibition of NADPH oxidase when Cl− channels are blocked.

NADPH oxidase and IClswell are concurrently up-regulated by chronic cardiac disease in animal models and man. Hypertrophy and heart failure trigger increased NADPH-dependent, DPI-inhibitable O2− production due either to increased expression of NADPH oxidase subunits (Li et al., 2002) or increased translocation (Heymes et al., 2003), as well as chronic activation of IClswell (Clemo et al., 1999). Furthermore, increased expression of NADPH oxidase subunits (Fukui et al., 2001; Krijnen et al., 2003) and chronic activation of IClswell (Clemo et al., 2001) are found in the infarct and peri-infarct zones after acute myocardial infarction. The degree to which up-regulation of NADPH oxidase accounts for concurrent up-regulation of IClswell under these conditions is unclear, however.

NADPH Oxidase, ROS, and Cation Channels

The stretch-induced activation of NADPH oxidase and production of ROS also may help explain two other consequences of β1 integrin stretch in rabbit ventricular myocytes: activation of a nonselective cation current and inhibition of the inward rectifier, IK1 (Browe and Baumgarten, 2003b). ROS and oxidant stress activate a nonselective cation current (Matsuura and Shattock, 1991; Jabr and Cole, 1995) and inhibit IK1 (Matsuura and Shattock, 1991; Jabr and Cole, 1993) in ventricular myocytes. Other cation channels also are modulated by ROS (Kourie, 1998).

In summary, direct and specific stretch of β1 integrin elicits Cl− SAC in ventricular myocytes by a mechanism that involves release of AngII, engagement of AT1 receptors in an autocrine/paracrine loop, activation of NADPH oxidase, and production of ROS. AngII or H2O2 applied exogenously in the absence of stretch also activates Cl− SAC.

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