

# Stretch of $\beta 1$ Integrin Activates an Outwardly Rectifying Chloride Current via FAK and Src in Rabbit Ventricular Myocytes

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**ABSTRACT** Osmotic swelling of cardiac myocytes and other types of cells activates an outwardly rectifying, tamoxifen-sensitive  $\text{Cl}^-$  current,  $I_{\text{Cl,swell}}$ , but it is unclear whether  $\text{Cl}^-$  currents also are activated by direct mechanical stretch. We tested whether specific stretch of  $\beta 1$ -integrin activates a  $\text{Cl}^-$  current in rabbit left ventricular myocytes. Paramagnetic beads (4.5- $\mu\text{m}$  diameter) coated with mAb to  $\beta 1$ -integrin were applied to the surface of myocytes and pulled upward with an electromagnet while recording whole-cell current. In solutions designed to isolate anion currents,  $\beta 1$ -integrin stretch elicited an outwardly rectifying  $\text{Cl}^-$  current with biophysical and pharmacological properties similar to those of  $I_{\text{Cl,swell}}$ . Stretch-activated  $\text{Cl}^-$  current activated slowly ( $t_{1/2} = 3.5 \pm 0.1$  min), partially inactivated at positive voltages, reversed near  $E_{\text{Cl}}$ , and was blocked by 10  $\mu\text{M}$  tamoxifen. When stretch was terminated,  $64 \pm 8\%$  of the stretch-induced current reversed within 10 min. Mechanotransduction involved protein tyrosine kinase. Genistein (100  $\mu\text{M}$ ), a protein tyrosine kinase inhibitor previously shown to suppress  $I_{\text{Cl,swell}}$  in myocytes, inhibited stretch-activated  $\text{Cl}^-$  current by  $62 \pm 6\%$  during continued stretch. Because focal adhesion kinase and Src are known to be activated by cell swelling, mechanical stretch, and clustering of integrins, we tested whether these tyrosine kinases mediated the response to  $\beta 1$ -integrin stretch. PP2 (10  $\mu\text{M}$ ), a selective blocker of focal adhesion kinase and Src, fully inhibited the stretch-activated  $\text{Cl}^-$  current as well as part of the background  $\text{Cl}^-$  current, whereas its inactive analogue PP3 (10  $\mu\text{M}$ ) had no significant effect. In addition to activating  $\text{Cl}^-$  current, stretch of  $\beta 1$ -integrin also appeared to activate a nonselective cation current and to suppress  $I_{\text{K1}}$ . Integrins are the primary mechanical link between the extracellular matrix and cytoskeleton. The present results suggest that integrin stretch may contribute to mechano-electric feedback in heart, modulate electrical activity, and influence the propensity for arrhythmogenesis.

**KEY WORDS:** stretch-activated channels • swelling-activated channels • mechano-electric feedback • protein tyrosine kinase • arrhythmia

## INTRODUCTION

A volume-sensitive  $\text{Cl}^-$  current,  $I_{\text{Cl,swell}}$ , is broadly distributed throughout the heart (Sorota, 1999; Hume et al., 2000; Baumgarten and Clemo, 2003) and other tissues (Nilius et al., 1994).  $I_{\text{Cl,swell}}$  in cardiac cells is activated by osmotic swelling (Sorota, 1992; Tseng, 1992) or hydrostatic pressure-induced cell inflation (Hagiwara et al., 1992). The current is time independent over most of the physiological voltage range, partially inactivates at positive potentials, and exhibits outward rectification with physiologic or symmetric  $\text{Cl}^-$  gradients. Activation of  $I_{\text{Cl,swell}}$  in both cardiac and noncardiac cells occurs over several minutes and is regulated by multiple signaling molecules, including PKC (Duan et al., 1995; Clemo and Baumgarten, 1998), protein tyrosine kinase (PTK) (Sorota, 1995), phosphatidylinositol-3-kinase (PI-3K) (Shi et al., 2002), small GTP binding proteins such as Rho (Tilly et al., 1996; Nilius et al.,

1999), and protein phosphatases (Clemo et al., 1999a; Duan et al., 1999).

Questions have been raised as to whether  $I_{\text{Cl,swell}}$  is truly a mechanosensitive current (Hu and Sachs, 1997; Cazorla et al., 1999). Cell swelling is a nonspecific stimulus. Besides its mechanical effects, it reduces ionic strength, dilutes cytoplasmic ions and macromolecules, and relieves macromolecular crowding, thereby activating signaling molecules. These perturbations, rather than mechanical stretch, may be responsible, at least in part, for the activation of  $I_{\text{Cl,swell}}$  upon cell swelling. In support of this idea, directly stretching cardiac myocytes generally activates cation currents but not  $I_{\text{Cl,swell}}$  (Hu and Sachs, 1997; Kamkin et al., 2003).

Ideally, studies of cardiac stretch-activated channels would make use of the structures that normally transmit forces to myocytes within the intact heart. Mechanical forces are transmitted by integrins (Wang et al.,

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*Abbreviations used in this paper:* APD, action potential duration; ECM, extracellular matrix; FAK, focal adhesion kinase; G, Gauss; PI-3K, phosphatidylinositol-3-kinase; PTK, protein tyrosine kinase; SAC, stretch-activated current.

1993), a family of heterodimeric ( $\alpha\beta$ ), noncovalently associated, transmembrane glycoproteins that act as extracellular matrix (ECM) receptors and physically link the ECM and cytoskeleton (Ross, 2002). The  $\beta 1D$ -splice variant is the principal  $\beta$  isoform expressed in adult heart (Zhidkova et al., 1995). In myocytes, integrins are a component of costameres, macromolecular complexes in register with Z-lines that are specialized for force transmission (Danowski et al., 1992; Borg et al., 2000). The cytoskeletal proteins paxillin, talin, muscle integrin binding protein, and  $\alpha$ -actinin bind directly to the cytoplasmic tail of  $\beta 1$ -integrin, and additional cytoskeletal proteins, including vinculin, melusin, and desmin, are colocalized within the costamere (Ross and Borg, 2001).

Integrins also participate in cell signaling (Borg et al., 2000; Ross and Borg, 2001; Parsons, 2003). A variety of signaling molecules, adaptor proteins, and other cell surface receptors are colocalized with integrins and cytoskeletal proteins within costameres. Focal adhesion kinase (FAK) and members of the Src kinase family are the principal upstream PTKs activated by clustering of integrin receptors. FAK binds to the cytoplasmic domain of  $\beta 1$ -integrin, resulting in autophosphorylation of FAK at Y397. Src binds via its SH2 domain to Y397 of FAK. Once activated, FAK and Src recruit a number of other proteins, including PKC, PI-3K, protein phosphatases, small GTPases, and GTPase-activating proteins, and orchestrate a network of downstream signaling cascades (Parsons, 2003). It is striking that the signaling cascades initiated by integrins also are activated by both mechanical stretch and cell swelling (Sadoshima et al., 1996; Sadoshima and Izumo, 1997). Furthermore, some of these signaling molecules are known to regulate  $I_{Cl,swell}$ .

The aims of the present study were to determine whether  $Cl^-$  currents are activated by direct and specific mechanical stretch of integrins in cardiac myocytes and to explore the mechanism of mechanotransduction. Paramagnetic beads coated with mAb to  $\beta 1$ -integrin were employed to apply force. Stretch of integrins activated an outwardly rectifying, tamoxifen-sensitive  $Cl^-$  current resembling  $I_{Cl,swell}$ . The mechanotransduction pathway includes the PTK family members, FAK and/or Src. Preliminary reports appeared previously (Browe and Baumgarten, 2003a,b).

## MATERIALS AND METHODS

### Ventricular Myocyte Isolation

Left ventricular myocytes were freshly isolated from adult New Zealand white rabbits ( $\sim 3$  kg) of either gender by enzymatic dissociation. Hearts were excised and retrogradely perfused via the aorta with oxygenated, modified Tyrode solutions at 37°C. Successively, Ca-containing and Ca-free Tyrode solutions were run for 5 min each, and then, enzyme solution was run for 30 min. At

selected intervals, portions of the left ventricle were dissected, placed in test tubes, and gently agitated. After filtering through 210  $\mu m$  monofilament nylon mesh (Small Parts), myocytes were washed and stored in a modified KB solution. The yield of rod-shaped, quiescent myocytes typically was  $>70\%$ . All electrophysiological recordings were performed within 10 h of cell isolation. Single, rod-shaped myocytes that were quiescent displayed clear striations, and were free of membrane blebbing or other morphological irregularities were chosen for study.

Tyrode solution for cell isolation contained (mM): 130 NaCl, 5 KCl, 1.8 CaCl<sub>2</sub>, 0.4 KH<sub>2</sub>PO<sub>4</sub>, 3 MgCl<sub>2</sub>, 5 HEPES, 15 taurine, 5 creatine, 10 glucose, pH 7.25. For Ca-free Tyrode solution, CaCl<sub>2</sub> was replaced with 0.1 mM Na<sub>2</sub>EGTA. For enzyme solution, 1.5–1.75 mg/ml bovine serum albumin (Sigma-Aldrich), 0.5 mg/ml collagenase (type II; Worthington), and 0.05 mg/ml pronase (type XIV; Sigma-Aldrich) were added to Ca-free Tyrode. KB solution contained (mM): 120 K-glutamate, 10 KCl, 10 KH<sub>2</sub>PO<sub>4</sub>, 0.5 K<sub>2</sub>EGTA, 10 taurine, 1.8 MgSO<sub>4</sub>, 10 HEPES, 20 glucose, 10 mannitol, pH 7.2.

### Experimental Solutions and Drugs

Ventricular myocytes were dispersed over a poly-L-lysine-coated glass-bottomed chamber ( $\sim 0.3$  ml) mounted on an inverted microscope (Diaphot; Nikon) and were visualized with Hoffman modulation optics (40 $\times$ ; NA = 0.55) and a high-resolution TV camera (CCD72; Dage-MTI). Bath solution was superfused at 2–3 ml/min at room temperature (22–23°C). Recordings were made in physiological bath solution containing (mM): 140 NaCl, 5 KCl, 2.5 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 10 HEPES, 5 glucose, pH 7.4 with a pipette containing (mM): 104 K-aspartate, 10 KCl, 10 NaCl, 2.5 MgATP, 8 K<sub>2</sub>EGTA, 0.1 CaCl<sub>2</sub>, 10 HEPES, pH 7.1 (liquid junction potential,  $-8.3$  mV). To isolate anion currents, bath Na<sup>+</sup> and K<sup>+</sup> were replaced with N-methyl-D-glucamine, and Ca<sup>2+</sup> was replaced with Mg<sup>2+</sup>; in the pipette, Na<sup>+</sup> and K<sup>+</sup> were replaced with Cs<sup>+</sup> (liquid junction potential,  $-13.2$  mV). For one set of experiments, a high Cl<sup>-</sup> pipette solution was employed in which all aspartate was replaced with an equimolar Cl<sup>-</sup> (liquid junction potential,  $-5.1$  mV). Pipette-free Ca<sup>2+</sup> was  $\sim 35$  nM (WinMAXC ver 2.4; www.stanford.edu/~cpatton/maxc.html).

Tamoxifen (20 mM; Sigma-Aldrich), genistein (100 mM), PP2 (10 mM), and PP3 (10 mM; Calbiochem) were prepared as stock solutions in DMSO and kept frozen ( $-4^{\circ}C$ ) in small aliquots until use.

### Paramagnetic Bead Method

Mechanical force was applied specifically to  $\beta 1$ -integrins using mAb-coated paramagnetic beads and an electromagnet, a method derived from those of Wang et al. (1993) and Glogauer et al. (1995; Glogauer and Ferrier, 1998). Mouse anti-human IgG<sub>1</sub> mAb for the  $\beta 1$  subunit of integrin (MAB2250; Chemicon), which cross-reacts with rabbit  $\beta 1$ -integrin, was attached to the surface of uniform,  $4.5 \pm 0.2$   $\mu m$  diameter ( $\pm SD$ ), superparamagnetic, polystyrene beads containing iron oxides (Dynabeads M-450 Pan Mouse IgG, 110.22; Dynal Biotech). These beads are supplied covalently bound to a mAb (human IgG<sub>4</sub>) for mouse IgG Fc that facilitates attachment of primary mAb to the beads. After resuspending the Dynabeads ( $4.0 \times 10^8$  beads/ml), 200  $\mu l$  of the suspension was transferred to a sterile 0.5-ml plastic vial, washed three times, and resuspended in PBS. Then, 8  $\mu l$  of MAB2250 (1 mg/ml) was added to the vial, and the vial was mounted on the drum of a rotary tumbler (Lortone) and slowly rotated for 30 min at 4°C. After washing three times to remove unbound MAB2250, the MAB2250-coated beads ( $\sim 0.1$  pg MAB2250/bead) were resuspended in PBS to a final volume of 200  $\mu l$  and stored at 4°C.

For control experiments, mouse anti-human IgG<sub>1</sub> mAb for transferrin receptors (CBL 137; Chemicon) was attached to the beads. The procedures for attaching antitransferrin receptor mAb to the beads, mAb dilutions, and storage conditions were identical to those used for the  $\beta$ 1-integrin mAb.

For each experiment, mAb-coated beads were resuspended, and a 20  $\mu$ l aliquot was diluted to 2 ml with bath solution to give  $\sim$ 4,000 beads/ $\mu$ l. Generally, 75–200  $\mu$ l of this final dilution of coated beads was added to myocytes in the chamber with the flow turned off and allowed to randomly settle on cells from above. After  $\sim$ 5 min, unbound beads were washed away by restoring superfusion. Typically 3–5 coated beads were firmly attached to myocytes selected for study, each presumably to multiple  $\beta$ 1 integrins.

The electromagnet consisted of a water-cooled coil connected to a constant current circuit (Fig. 1). The coil was made from 475 turns of 18 AWG copper magnet wire wrapped around a 1.1-mm thick conical copper form and was sealed with thermally conductive epoxy.

The electromagnet was placed directly on top of the bath, and patch pipettes passed through an ellipsoidal opening (23  $\times$  12 mm) at its base. Measurements with a Gauss meter (5080; F.W. Bell) indicated that the magnetic flux density ( $B$ ) was 35 Gauss (G) and was uniform in the x-y plane occupied by the myocytes, 4 mm below the face of the electromagnet. In contrast,  $B$  varied along the z-axis. Based on a plot of  $B$  versus distance, the magnetic flux density gradient,  $\partial B_z / \partial z$ , was 2,400 G/m at the level of the myocytes. This magnetic field induced a magnetic dipole moment within the Dynabeads that was parallel to the z-axis of the coil. As a result, the force vector exerted on the  $\beta$ 1-integrin-bound beads was oriented upward toward the coil and perpendicular to the long axis of the myocyte. The force per bead ( $F$ ) was estimated as 1.2 pN based on the equation:

$$F = \delta V_b M_s \partial B_z / \partial z,$$

where  $\delta$  is the fraction of bead volume,  $V_b$ , occupied by iron oxide,  $M_s$  is the saturated magnetization of iron oxide, and  $\partial B_z / \partial z$  is as defined above.  $\delta$  was taken as 20% (according to manufacturer's data),  $V_b$  was calculated from the nominal bead diameter,  $M_s$  was 4.8  $\times 10^5$  A/m (Glogauer and Ferrier, 1998), and  $\partial B_z / \partial z$  was measured.

### Electrophysiological Recordings

Pipettes were pulled using thin-walled borosilicate glass capillary tubing and then fire polished. The final pipette tip diameter was 3–4  $\mu$ m, and the pipette resistance in bath solution was 2–3 M $\Omega$ . A 150 mM KCl agar bridge was used as the ground.

Membrane currents were recorded with an EPC-7 amplifier (List-Medical) using the whole-cell configuration of the patch clamp technique. Seal resistances of 5–30 G $\Omega$  were typically achieved, and the membrane patch was ruptured by application of negative pressure or by a brief, 500-mV zapping pulse. 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, Inc.). 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. Voltages were corrected for the measured liquid junction potential before forming a seal. Membrane currents were low-pass filtered at 2 kHz (8-pole Bessel 902; Frequency Devices) and digitized at 10 kHz. For presentation, selected records were filtered at 50 Hz. Under conditions designed to isolate anion currents, the integrin stretch-induced current exhibited strong voltage-dependent inactivation, and I-V curves

were constructed using the average current at 20–35 ms. In experiments with physiologic bath Na<sup>+</sup>, steady-state I-V curves were plotted using the current at the end of the 500-ms pulse.

### Statistics

Data are reported as mean  $\pm$  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 one-way repeated measures ANOVA or a two-way ANOVA was performed, as appropriate, and the Student-Newman-Keuls test was used to compare groups. For simple comparisons of two groups, a one-tailed paired Student's  $t$  test was administered. Statistical analyses were done using SigmaStat 2.03 (SPSS), and  $P < 0.05$  was considered significant.

## RESULTS

### $\beta$ 1-Integrin Stretch Activates a Cl<sup>-</sup> Current

The paramagnetic bead method was used to test whether specific stretch of  $\beta$ 1-integrins activates a Cl<sup>-</sup> current (Cl<sup>-</sup> SAC) in rabbit left ventricular myocytes. Fig. 2 illustrates the effect of  $\beta$ 1-integrin stretch on membrane current under conditions designed to isolate Cl<sup>-</sup> currents. With anti- $\beta$ 1-integrin IgG<sub>1</sub> mAb-coated paramagnetic beads attached to the myocyte but no stretch stimulus (control), a small background current that partially inactivated at potentials positive to  $+10$  mV was observed (Fig. 2 A). The I-V relationship

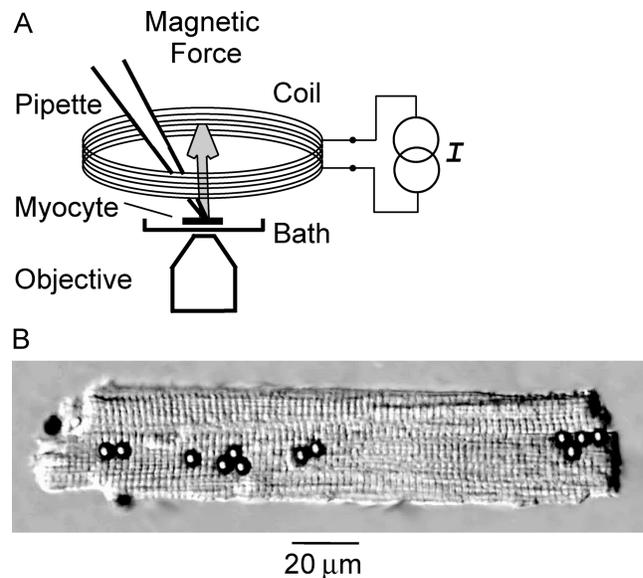
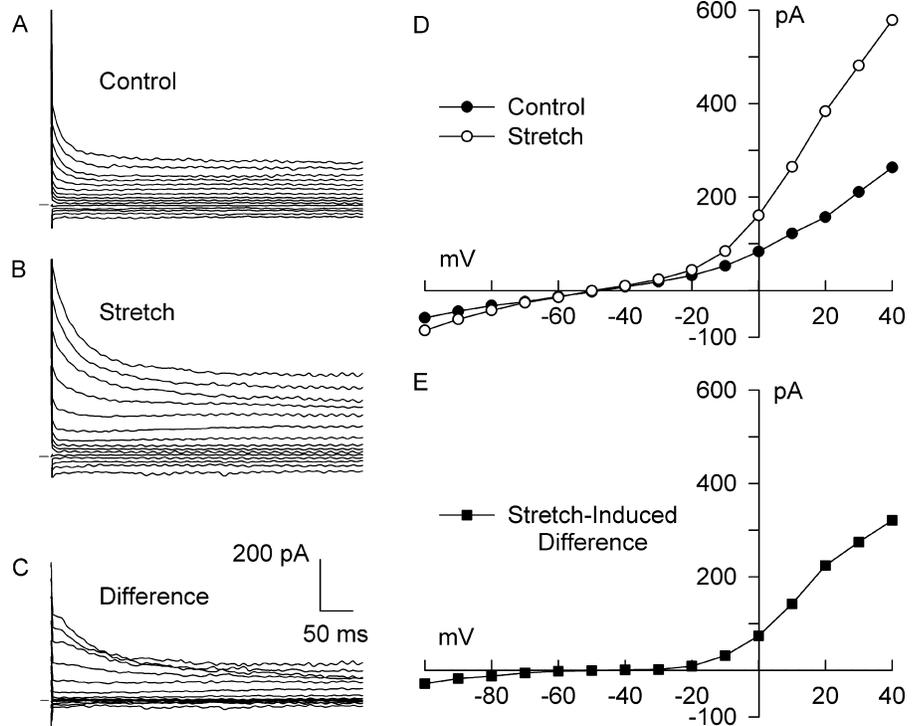


FIGURE 1. Paramagnetic bead method for stretching  $\beta$ 1-integrins. (A) Schematic diagram of the apparatus. A water-cooled electromagnet coil was placed directly on top of the bath. Energizing the coil with a constant current source,  $I$ , generated a magnetic flux density gradient of 2,400 G/m. The resulting force vector for each bead pointed upward toward the plane of the coil and perpendicular to the long axis of the myocyte. (B) Ventricular myocyte with 4.5- $\mu$ m diameter beads coated with anti- $\beta$ 1 integrin mAb attached to the upper surface. Out-of-focus beads are near chamber floor.

FIGURE 2. Stretch of  $\beta 1$ -integrins activated a  $\text{Cl}^-$  current ( $\text{Cl}^-$  SAC) in bath and pipette solutions designed to isolate anion currents. Membrane potential was stepped from  $-60$  mV to between  $-100$  and  $+40$  mV for 500 ms. Families of currents before stretch (A, Control), after 5 min of integrin stretch (B, Stretch), and the stretch-induced difference current (C, Difference) obtained by digital subtraction. Horizontal bar denotes 0 current. I-V relationships for membrane current (D) before ( $\bullet$ ) and after ( $\circ$ ) integrin stretch, and (E) for the stretch-induced difference current ( $\blacksquare$ ). The background current in the absence of stretch was outwardly rectifying, reversed near  $E_{\text{Cl}}$  ( $-52$  mV), and underwent partial inactivation at positive potentials. Stretch markedly augmented outward currents, and the resulting  $\text{Cl}^-$  SAC exhibited much stronger outward rectification, reversed near  $E_{\text{Cl}}$ , and partially inactivated at positive potentials.  $\text{Cl}^-$  SAC elicited by  $\beta 1$ -integrin stretch was  $1.13 \pm 0.10$  pA/pF at  $+40$  mV ( $n = 34$ ).



for the control current exhibited mild outward rectification and a reversal potential of about  $-50$  mV (Fig. 2 D), and its magnitude was not correlated with the number of beads on the myocyte. An outwardly rectifying background  $\text{Cl}^-$  current has been reported previously under similar experimental conditions and was attributed to  $I_{\text{Cl,swell}}$  (Sorota, 1992; Duan et al., 1995, 1997).

Static stretch of bead-attached  $\beta 1$ -integrins achieved by applying a magnetic flux density gradient of 2,400 G/m progressively increased the  $\text{Cl}^-$  current.  $\text{Cl}^-$  current after 5 min of static stretch partially inactivated at potentials positive to  $+10$  mV (Fig. 2 B), as previously shown for the control current, and the I-V relationship exhibited strong outward rectification (Fig. 2 D). The reversal potential was unchanged at  $-50$  mV, however. To more clearly define the current activated by integrin stretch, difference currents were calculated by subtracting the control current from the corresponding current recorded after stretch (Fig. 2 C). The stretch-induced difference currents partially inactivated at positive potentials, and their I-V relationship (Fig. 2 E) displayed strong outward rectification and reversed at  $-50$  mV, close to the calculated  $E_{\text{Cl}}$  of  $-52$  mV. The difference currents emphasize that stretch caused a much more prominent increase in outward than inward current.

A stretch-induced, outwardly rectifying  $\text{Cl}^-$  current was observed in 75% of myocytes tested (34 out of 45). In these cells, stretch increased outward current by  $1.13 \pm 0.10$  pA/pF at  $+40$  mV ( $n = 34$ ,  $P < 0.0005$ ), from  $2.89 \pm 0.77$  to  $4.01 \pm 0.85$  pA/pF. In contrast, in-

ward current increased only  $-0.23 \pm 0.06$  pA/pF at  $-100$  mV ( $n = 34$ ,  $P < 0.0005$ ), from  $-1.98 \pm 0.88$  to  $-2.21 \pm 0.93$  pA/pF. The reversal potential was  $-47 \pm 2$  mV ( $n = 4$ ). A small subset of these myocytes displayed a larger increase of inward current ( $-1.0 \pm 0.3$  pA/pF,  $n = 4$ ) that began after a delay of 4–6 min after the onset of  $\beta 1$ -integrin stretch.

When pipette solution was switched to high  $\text{Cl}^-$ , the reversal potential shifted to  $-19 \pm 1$  mV ( $n = 4$ ), 28 mV more positive than with the 20.2 mM  $\text{Cl}^-$  pipette solution, and the stretch-induced currents still exhibited strong outward rectification. The shift in reversal potential gives a  $P_{\text{Asp}}/P_{\text{Cl}}$  ratio of 0.2, similar to the value of 0.1 reported previously for  $I_{\text{Cl,swell}}$  (Vandenberg et al., 1994).

$\text{Cl}^-$  SAC activation required both anti- $\beta 1$  integrin mAb and the application of stretch. Current was unaffected by application of a magnetic field alone without beads ( $n = 4$ , ns) and by untreated anti-IgG beads that adhered to myocytes in a nonspecific manner ( $n = 4$ , ns). Furthermore,  $\text{Cl}^-$  SAC also was not elicited by specific stretch of cardiac transferrin receptors (Parkes et al., 2000; Upton et al., 2003) using antitransferrin receptor IgG<sub>1</sub> mAb-coated paramagnetic beads attached to the myocyte ( $n = 5$ , ns).

Activation of  $I_{\text{Cl,swell}}$  by osmotic swelling or pressure-induced inflation occurs over 3–5 min (Hagiwara et al., 1992; Tseng, 1992). Activation of  $\text{Cl}^-$  SAC also occurred slowly, and the time course at  $+40$  mV is illustrated in Fig. 3. The time course of activation was sig-

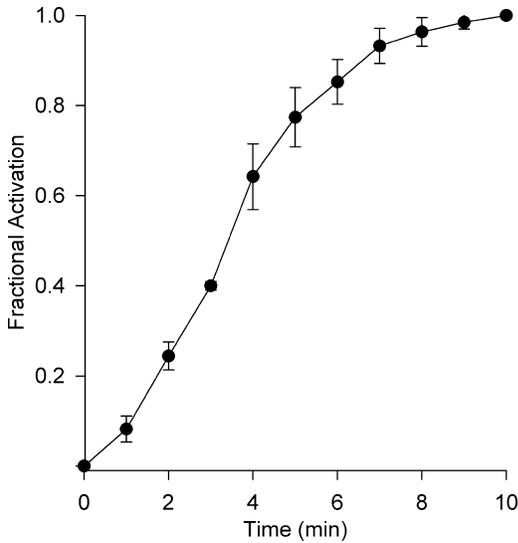


FIGURE 3. Time course of  $\text{Cl}^-$  SAC activation by  $\beta 1$ -integrin stretch. Stretch-induced currents at  $+40$  mV were recorded at 1-min intervals and normalized by the current at 10 min to obtain fractional activation. Activation of  $\text{Cl}^-$  SAC followed a sigmoidal time course with a half time of  $3.5 \pm 0.1$  min ( $n = 5$ ).

moidal with a half-time of  $3.5 \pm 0.1$  min ( $n = 5$ ), and steady-state was reached in  $\sim 10$  min.

With the ruptured patch technique,  $I_{\text{Cl,swell}}$  elicited by hypoosmotic swelling or positive pipette pressure only partially turns off after return to control conditions (Hagiwara et al., 1992; Sorota, 1992; Tseng, 1992). Sim-

ilarly, activation of  $\text{Cl}^-$  SAC was only partially reversed after termination of integrin stretch. Fig. 4 shows the background (Fig. 4 A) and outwardly rectifying  $\text{Cl}^-$  current induced by 6 min of stretch (Fig. 4 B) and their I-V relationships (Fig. 4 D). When the magnetic field was turned off, current slowly decreased in amplitude, but recovery was incomplete at 6 min (Fig. 4, C and D) and remained incomplete even after 10 min. Comparable behavior was observed in each myocyte in which reversibility was tested. On average, stretch significantly increased  $\text{Cl}^-$  current at  $+40$  mV from  $1.56 \pm 0.22$  to  $4.48 \pm 1.01$  pA/pF ( $P < 0.05$ ), and the current significantly decreased to  $2.61 \pm 0.35$  pA/pF after termination of stretch ( $P < 0.05$ ), a recovery of  $64 \pm 8\%$ . Although recovery appeared to be incomplete, the control and postrecovery currents were not significantly different. These data suggest that activation of  $\text{Cl}^-$  SAC is due to a reversible process rather than to mechanical damage of the membrane or to a nonspecific change in leak conductance. Recovery of inward  $\text{Cl}^-$  current at  $-100$  mV tended to behave similarly to recovery of outward current. Inward current increased from  $-0.36 \pm 0.19$  to  $-0.58 \pm 0.28$  pA/pF after stretch and recovered to  $-0.46 \pm 0.24$  pA/pF after termination of stretch. The currents at  $-100$  mV were not significantly different from each other, however.

Both the background  $\text{Cl}^-$  current and the  $\text{Cl}^-$  current recorded after stretch underwent partial inactivation at positive potentials in  $\sim 60\%$  (20 of 34) of myocytes. Fig. 5 compares the extent of inactivation of the currents be-

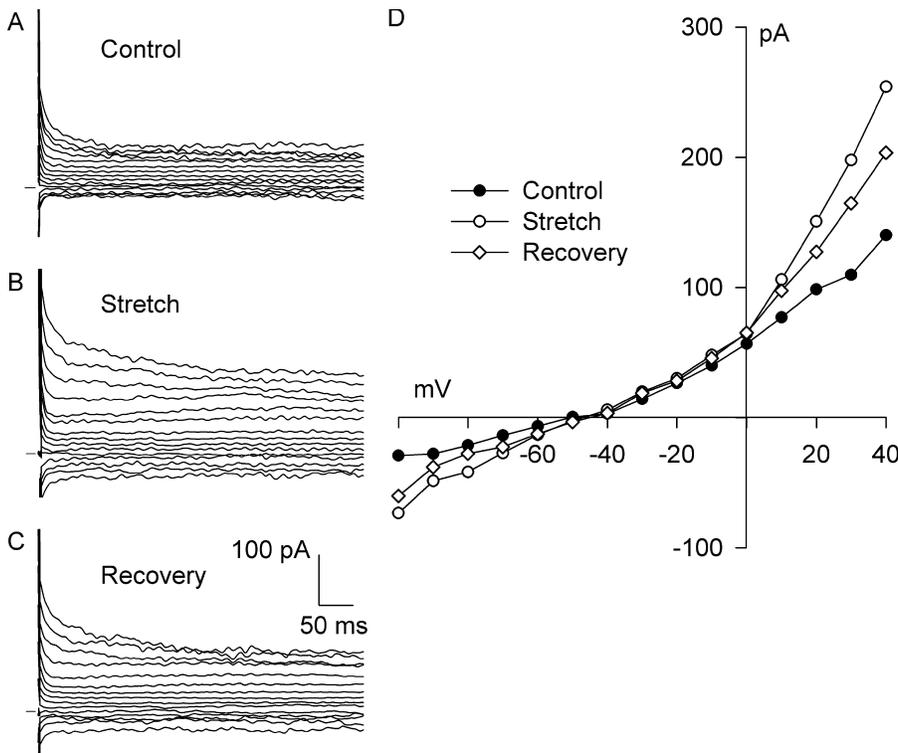
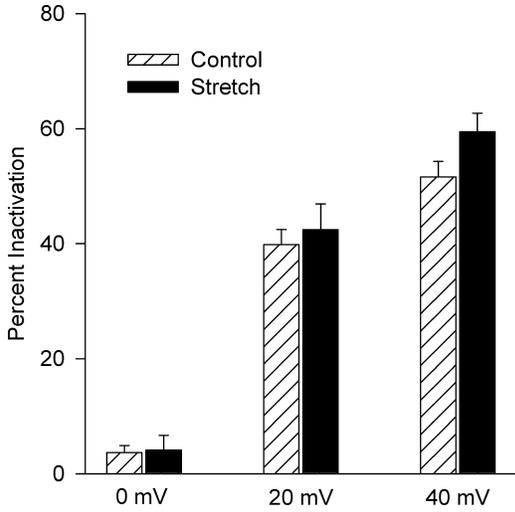


FIGURE 4.  $\text{Cl}^-$  SAC activation partially reversed upon termination of integrin stretch. Families of currents before (A, Control) and after (B, Stretch) activation of  $\text{Cl}^-$  SAC by 6 min of integrin stretch and after a recovery period of 10 min with the magnet turned off (C, Recovery). (D) I-V relationships for control ( $\bullet$ ), stretch ( $\circ$ ), and recovery ( $\diamond$ ). At  $+40$  mV,  $\text{Cl}^-$  SAC decreased by  $64 \pm 8\%$  ( $n = 4$ ) during the recovery period.



**FIGURE 5.** Stretch did not alter the voltage dependence of steady-state inactivation. The percentage of current undergoing inactivation was determined at 0, +20, and +40 mV both before and after 5–8 min of integrin stretch ( $n = 20$ ). Steady-state inactivation was significantly dependent on voltage ( $P < 0.001$ ) but was not significantly affected by stretch ( $P = 0.137$ ). Because the overall effect of stretch was not significant (2-way ANOVA), comparison of stretch and control at individual test potentials was precluded. The  $\text{Cl}^-$  SAC did not undergo inactivation at negative potentials before or after stretch.

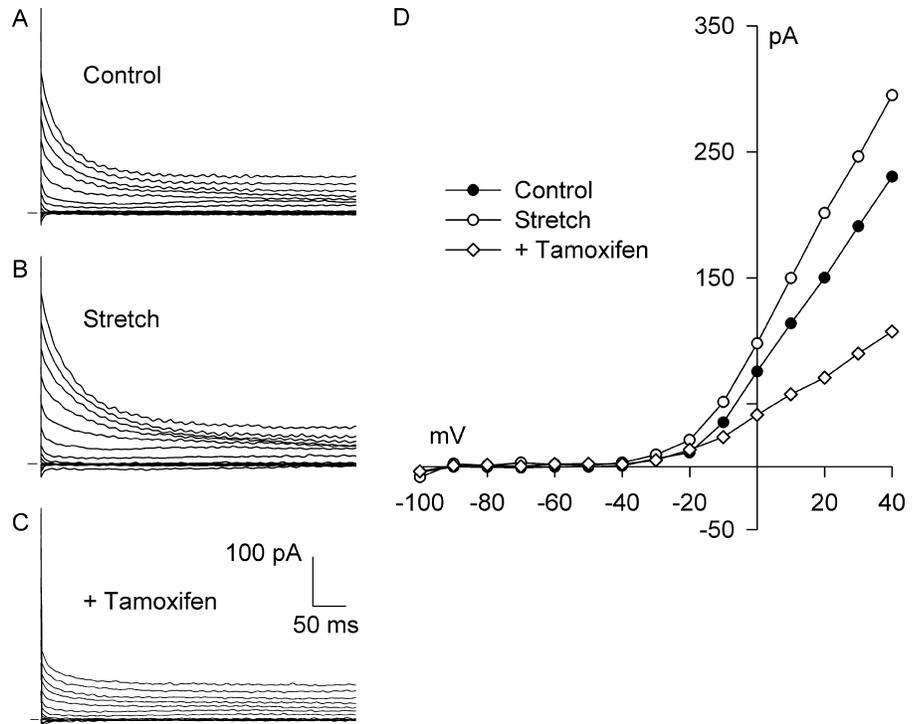
fore and after stretch and their voltage dependencies. Inactivation significantly increased at positive potentials. At +40 mV, control current inactivated by  $52 \pm 3\%$  ( $n = 20$ ) and after stretch by  $59 \pm 3\%$  ( $n = 20$ ). In contrast, at 0 mV inactivation was only  $4 \pm 1\%$  ( $n = 20$ ) and

$4 \pm 3\%$  ( $n = 20$ ), respectively. A two-way ANOVA confirmed a significant voltage dependence ( $P < 0.001$ ), but stretch did not significantly alter inactivation. In the remaining myocytes (14 of 34), neither the background current nor the  $\text{Cl}^-$  SAC exhibited prominent inactivation at positive potentials. The similarity of the extent of inactivation and whether or not it occurs suggests that the channels responsible for the background  $\text{Cl}^-$  current also are responsible for the  $\text{Cl}^-$  SAC.

*Tamoxifen Blocks  $\text{Cl}^-$  SAC*

Under conditions that isolate  $\text{Cl}^-$  currents, tamoxifen is regarded as a selective and potent inhibitor of  $I_{\text{Cl,swell}}$  and has been used to distinguish this current from the other major  $\text{Cl}^-$  currents (Hume et al., 2000; Baumgarten and Clemo, 2003). Tamoxifen also blocks several cation currents and gap junctions, however (for references see, Baumgarten and Clemo, 2003). Fig. 6 shows that  $10 \mu\text{M}$  tamoxifen blocks both  $\text{Cl}^-$  SAC and the background  $\text{Cl}^-$  current present before stretch. In the experiment illustrated, a relatively large, outwardly rectifying, background  $\text{Cl}^-$  current was recorded before application of stretch (Fig. 6, A and D), and 6 min of integrin stretch elicited additional outward current (Fig. 6, B and D). Fig. 6, C and D, illustrates the effect of tamoxifen ( $10 \mu\text{M}$ , 6 min) in the presence of continued stretch. Tamoxifen inhibited both the  $\text{Cl}^-$  SAC and a large fraction of the background  $\text{Cl}^-$  current.

Similar results were obtained in each myocyte tested using both physiologic and high  $\text{Cl}^-$  pipette solutions. With physiologic  $\text{Cl}^-$  pipette solution, stretch increased



**FIGURE 6.** Tamoxifen, an inhibitor of  $I_{\text{Cl,swell}}$ , blocks both  $\text{Cl}^-$  SAC and background  $\text{Cl}^-$  current. Families of currents before (A, Control) and after (B, Stretch) activation of  $\text{Cl}^-$  SAC by 6 min of integrin stretch and after application of  $10 \mu\text{M}$  tamoxifen for 6 min with continued stretch (C, +Tamoxifen). (D) I-V relationships for A-C. Each reversed near  $E_{\text{Cl}}$ . Tamoxifen blocked all of the  $\text{Cl}^-$  SAC and a large fraction of the background  $\text{Cl}^-$  current. At +40 mV, tamoxifen blocked  $116 \pm 5\%$  ( $n = 5$ ) of the stretch-induced current.

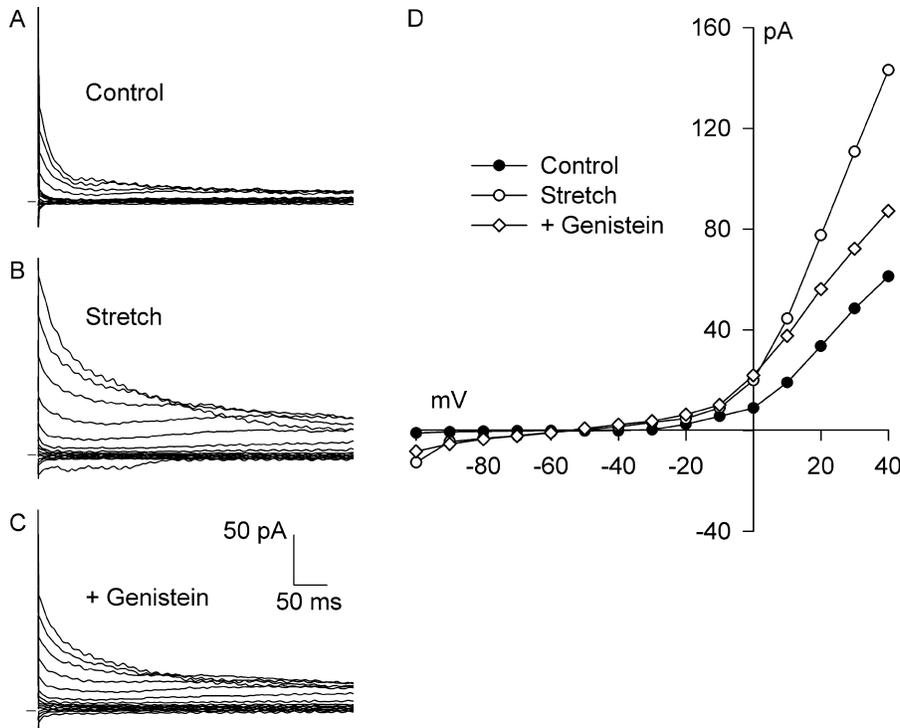


FIGURE 7. The PTK inhibitor genistein partially blocks Cl<sup>-</sup> SAC. Current records before (A, Control) and after (B, Stretch) activation of Cl<sup>-</sup> SAC by 6 min of integrin stretch and after application of 100  $\mu$ M genistein for 8 min with continued stretch (C, +Genistein). (D) I-V relationships for A–C. Each reversed near  $-50$  mV. At  $+40$  mV, genistein blocked  $62 \pm 6\%$  ( $n = 4$ ) of the stretch-induced current.

the Cl<sup>-</sup> current at  $+40$  mV from  $1.79 \pm 0.44$  to  $2.91 \pm 0.59$  pA/pF, and tamoxifen (10  $\mu$ M, 6–8 min) significantly decreased the current to  $1.63 \pm 0.48$  pA/pF. Thus, tamoxifen blocked  $116 \pm 5\%$  of the Cl<sup>-</sup> SAC at  $+40$  mV ( $n = 5$ ,  $P < 0.01$ ). At  $-100$  mV, the effects of both stretch and tamoxifen on Cl<sup>-</sup> current were small and not statistically significant. With high Cl<sup>-</sup> pipette solution, tamoxifen blocked  $150 \pm 19\%$  of the Cl<sup>-</sup> SAC at  $+40$  mV ( $n = 3$ ,  $P < 0.005$ ).

#### Protein Tyrosine Kinases Regulate Cl<sup>-</sup> SAC

Although the data indicate that mechanical stretch elicits a Cl<sup>-</sup> current, the time course of activation is slow enough to allow for participation of signaling molecules in the transduction process. Moreover, cell swelling (Sadoshima et al., 1996), mechanical stretch (Sadoshima and Izumo, 1997), and integrin clustering (Clark and Brugge, 1995; Giancotti and Ruoslahti, 1999) activate protein tyrosine kinases (PTKs), and PTKs have been implicated in the activation of  $I_{Cl,swell}$  in heart (Sorota, 1995) and other tissues (Voets et al., 1998; Shi et al., 2002). Therefore, we tested whether mechanical activation of Cl<sup>-</sup> SAC involved signaling by PTK.

Fig. 7 shows the effect of genistein, a broad-spectrum PTK inhibitor. A small, outwardly rectifying background current that partially inactivated at positive potentials was present before stretch (Fig. 7, A and D), and 6 min of integrin stretch significantly increased the Cl<sup>-</sup> current and the degree of rectification (Fig. 7, B and D). Superfusion of genistein (100  $\mu$ M, 8 min) partially in-

hibited the Cl<sup>-</sup> current (Fig. 7, C and D). At  $+40$  mV, genistein inhibited  $62 \pm 6\%$  of the stretch-induced current ( $n = 4$ ,  $P < 0.01$ ); stretch increased the Cl<sup>-</sup> current from  $2.25 \pm 1.00$  to  $3.21 \pm 1.14$  pA/pF, and genistein reduced the current to  $2.62 \pm 1.05$  pA/pF. The effect of genistein at  $-100$  mV was similar, but the currents were smaller and the changes were not statistically significant. These data suggest that PTK is involved in the activation of Cl<sup>-</sup> current by integrin stretch. On the other hand, genistein appeared to enhance the Cl<sup>-</sup> current in one additional myocyte studied.

FAK and Src are the two principal upstream PTKs activated by both mechanical stretch and integrin clustering (Sadoshima and Izumo, 1997; Parsons, 2003). PP2, a selective inhibitor of FAK and Src (Hanke et al., 1996), was used to test whether these PTKs regulate Cl<sup>-</sup> SAC. As previously shown, integrin stretch activated an outwardly rectifying Cl<sup>-</sup> current (Fig. 8, A, B, and D). Then, while maintaining stretch, the myocyte was superfused with 10  $\mu$ M PP2 for 10 min. PP2 inhibited both the Cl<sup>-</sup> SAC and a large fraction of the background Cl<sup>-</sup> current (Fig. 8, C and D).

At  $+40$  mV, PP2 (10  $\mu$ M, 8–10 min) blocked  $134 \pm 13\%$  of the Cl<sup>-</sup> SAC ( $n = 6$ ,  $P < 0.001$ ); stretch increased Cl<sup>-</sup> current from  $1.61 \pm 0.28$  to  $2.49 \pm 0.28$  pA/pF, and PP2 reduced the current to  $1.28 \pm 0.27$  pA/pF. The current after PP2 was significantly less than the control current ( $P < 0.05$ ). Similar behavior was observed at  $-100$  mV, but the currents and effect of PP2 were smaller, and the differences were not statistically significant.

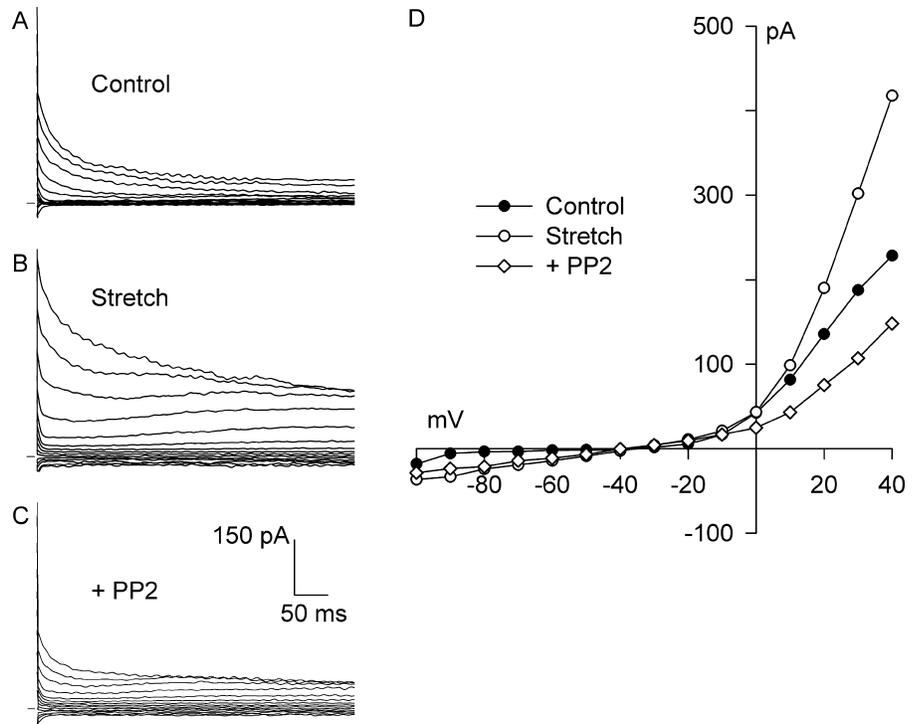


FIGURE 8. PP2, a selective inhibitor of Src and FAK, blocks both Cl<sup>-</sup> SAC and background Cl<sup>-</sup> current. Current records before (A, Control) and after (B, Stretch) activation of Cl<sup>-</sup> SAC by 5 min of integrin stretch and after application of 10  $\mu$ M PP2 for 10 min with continued stretch (C, +PP2). (D) I-V relationships for A–C. Each reversed near  $-50$  mV. At  $+40$  mV, PP2 blocked  $134 \pm 13\%$  ( $n = 6$ ) of the stretch-induced current.

To verify that the profound inhibitory action of PP2 was not due to a nonspecific effect, PP3, an inactive analogue of PP2, also was tested. Although integrin stretch activated Cl<sup>-</sup> current (Fig. 9, A, B, and D), exposure to 10  $\mu$ M PP3 for 10 min while maintaining stretch did not significantly affect the current (Fig. 9, C and D). Similar results were obtained in all four myocytes examined, and block at  $+40$  mV was  $1 \pm 11\%$  ( $n = 4$ ); stretch increased Cl<sup>-</sup> current from  $1.31 \pm 0.21$  to  $2.36 \pm 0.14$  pA/pF, and after PP3, Cl<sup>-</sup> current was  $2.34 \pm 0.19$  pA/pF. Together, these results suggest that FAK and/or Src, members of the PTK family, play a key role in the activation of Cl<sup>-</sup> SAC by integrin stretch.

#### *$\beta$ 1-Integrin Stretch Modulates Cation Currents*

The effect of integrin stretch also was examined in physiological Tyrode solution in order to determine whether cation SAC is activated by integrin stretch. Steady-state I-V relationships are shown in Fig. 10 A. In physiological Tyrode solution, K<sup>+</sup> conductance dominates, and the steady-state I-V relationship reversed at  $-77$  mV. After 5 min of integrin stretch, the steady-state I-V relationship was more linear, reversed at  $-38$  mV, and appeared to be rotated counter-clockwise, except at very negative potentials. Whereas outward current positive to  $-20$  mV was enhanced, inward current at  $-100$  mV was inhibited. The stretch-induced difference current (Fig. 10 B) crossed the voltage axis twice, at  $-91$  and  $-23$  mV, indicating that stretch of integrins modulated more than one membrane current. On av-

erage, integrin stretch in physiological bathing media shifted the reversal potential positive by  $42.7 \pm 4.7$  mV ( $n = 3$ ,  $P < 0.01$ ), from  $-73.0 \pm 2.1$  to  $-30.3 \pm 6.2$  mV. Outward current at  $+40$  mV increased by  $5.30 \pm 1.56$  pA/pF ( $n = 3$ ,  $P < 0.05$ ), from  $4.02 \pm 2.87$  to  $9.33 \pm 2.45$  pA/pF. This enhancement of steady-state current was four to five times greater than the enhancement of peak current in solutions designed to isolate anion currents. At intermediate voltages,  $-40$  to  $-80$  mV, stretch shifted the current in an inward direction. At  $-60$  mV, for example, the stretch-induced current was  $-3.37 \pm 0.65$  pA/pF ( $n = 3$ ,  $P < 0.05$ ). In contrast, inward current at  $-100$  mV was inhibited by  $3.86 \pm 1.12$  pA/pF ( $n = 3$ ,  $P < 0.05$ ), decreasing from  $-18.51 \pm 0.51$  to  $-14.65 \pm 1.24$  pA/pF. This behavior is distinct from the small increase in inward anion current at  $-100$  mV noted previously and suggests I<sub>K1</sub> is inhibited by integrin stretch. A similar stretch-induced inhibition of I<sub>K1</sub> was reported recently by Isenberg et al. (2003) in guinea pig ventricular myocytes stretched with a glass stylus. In a fourth myocyte studied under physiological conditions, reversal potential did not shift, and changes in the I-V curve were similar to those seen in solutions designed to isolate anion currents. Based on these data, one can infer that integrin stretch in physiological bathing media modulates cation currents in  $\sim 75\%$  of myocytes.

Fig. 10 C compares the steady-state I-V relationship after stretch in physiological bath solution to that under conditions that isolate Cl<sup>-</sup> currents. Assuming independence, the contribution of cations to the stretch-

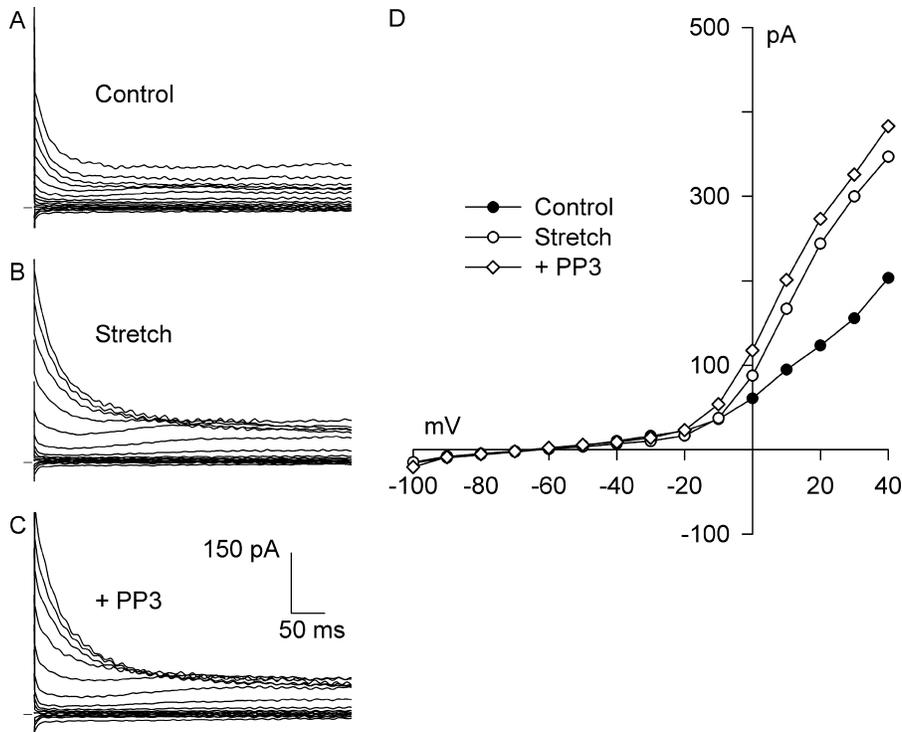


FIGURE 9. PP3, an inactive analogue of PP2, did not affect Cl<sup>-</sup> SAC or background Cl<sup>-</sup> current. Current records before (A, Control) and after (B, Stretch) activation of Cl<sup>-</sup> SAC by 5 min of integrin stretch and after application of 10  $\mu$ M PP3 for 10 min with continued stretch (C, +PP3). (D) I-V relationships for A–C. Each reversed near  $-50$  mV. At  $+40$  mV, PP3 did not significantly alter the stretch-induced current,  $-1 \pm 11\%$  ( $n = 4$ ).

activated current was obtained by subtracting the curves. Voltages between  $-60$  and  $+40$  mV were considered to minimize contributions from  $I_{K1}$  in physiological Tyrode solution. The difference I-V relationship (Fig. 10 D) was nearly linear and reversed at  $-10$  mV, suggesting integrin stretch activated a nonselective cation current. A nonselective stretch-activated cation current with a linear I-V relationship previously was recorded in cardiac myocytes in response to longitudinal stretch using two concentric pipettes or two patch electrodes, or by means of a glass stylus (Zeng et al., 2000; Zhang et al., 2000; Isenberg et al., 2003).

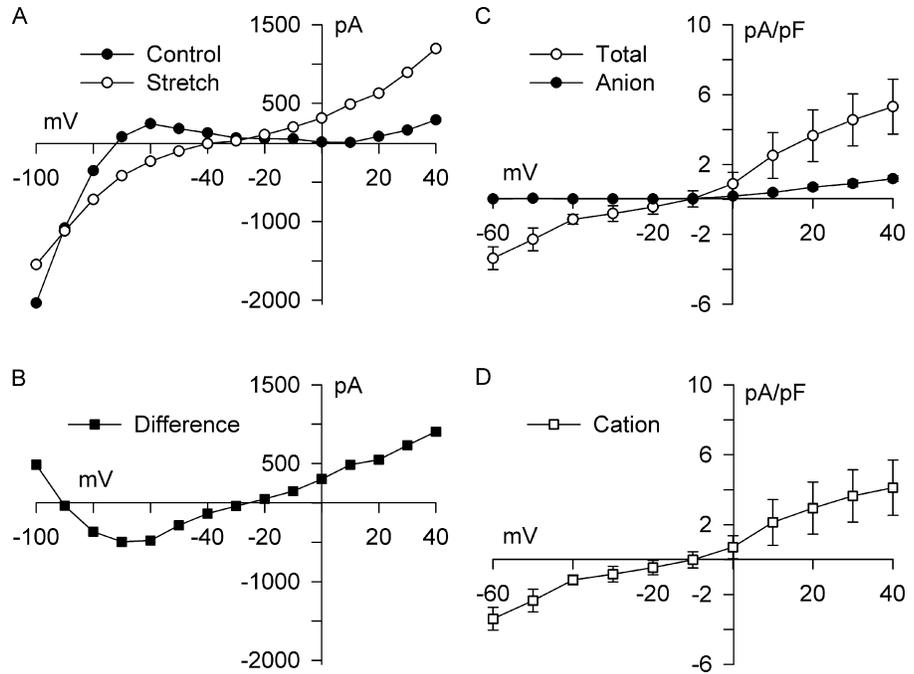
#### DISCUSSION

Contrary to previous claims (Hu and Sachs, 1997; Cazorla et al., 1999), cardiac Cl<sup>-</sup> channels can be activated by a mechanical stimulus that does not alter cell volume or dilute the cytoplasmic contents. In solutions designed to isolate anion currents, stretch of  $\beta 1$ -integrins with mAb-coated paramagnetic beads elicited a Cl<sup>-</sup> current in  $\sim 75\%$  of rabbit left ventricular myocytes. The current was outwardly rectifying, partially inactivated at positive voltages, and was blocked by tamoxifen; its reversal potential followed  $E_{Cl}$ . When stretch was terminated, the Cl<sup>-</sup> current largely returned to its control value. In contrast, application of a magnetic field either with beads that had not been coated with primary mAb or with beads coated with antitransferrin receptor mAb of the same class and subtype (mouse IgG<sub>1</sub>) as the anti- $\beta 1$  integrin mAb failed to

stimulate the current, and the magnetic field alone failed to stimulate the current. These data suggest that Cl<sup>-</sup> channels were activated by a specific stretch of  $\beta 1$  integrins rather than by some other nonspecific process or as a general response to myocyte stretch. Because mechanical stretch rather than volume changes serve as the stimulus, the current should be considered a Cl<sup>-</sup> SAC. The conclusion that certain cardiac Cl<sup>-</sup> channels are stretch activated is supported by observations that amphiphiles that cause membrane curvature without cell swelling (crenators) activate Cl<sup>-</sup> current (Tseng, 1992). The same amphiphiles are thought to activate the mechanosensitive 2-P K<sup>+</sup> channels, TRAAK and TREK, via membrane curvature (Patel et al., 2001). In addition, the open probability of cardiac Cl<sup>-</sup> channels is increased by applying negative pressure to inside-out and positive pressure to outside-out patches from human atrial cells (Sato and Koumi, 1998).

One important difference between this and previous attempts to provoke Cl<sup>-</sup> currents with mechanical perturbations is the means of applying force to myocytes. The paramagnetic bead method takes advantage of the specificity of mAb to apply force in a defined, reproducible, and quantifiable manner. Because integrins are an innate mechanical link between the ECM and cytoskeleton and function as mechanosensors in multiple cell types (Salter et al., 1997; Mobasheri et al., 2002; Shyy and Chien, 2002), it is appealing to think that stretch of  $\beta 1$  integrins represents a physiologically relevant means of mechanical stimulation. On the other hand, ECM normally interact simultaneously with both

FIGURE 10.  $\beta 1$ -integrin stretch activates a nonselective cation current and suppresses  $I_{K1}$ . Recordings were made using physiological bath and pipette solutions using the same voltage-clamp protocol as in previous figures. (A) I-V relationships for steady-state current before (Control, ●) and after (Stretch, ○) 5 min of integrin stretch, and (B) for the stretch-induced difference current (Difference, ■). Integrin stretch increased current over most of the voltage range, attenuated inward rectification, and shifted reversal potential positive. The difference current (B) reversed at  $-91$  and  $-23$  mV, suggesting that stretch modulated multiple currents. This is consistent with suppression of inwardly rectifying  $I_{K1}$  (appears inverted; difference current = stretch – control) and augmentation of a linear poorly selective current. (C) To characterize the stretch-activated cation component and minimize contributions from  $I_{K1}$ , steady-state I-V relationships were obtained between  $-60$  and  $+40$  mV in physiological solutions ( $n = 3$ ; Total, ○) and in solutions designed to isolate anion currents ( $n = 3$ ; Anion, ●). Stretch-activated total current was significantly greater than  $Cl^-$  SAC both at  $+40$  mV ( $5.30 \pm 1.56$  vs.  $1.19 \pm 0.17$  pA/pF;  $P < 0.05$ ) and  $-60$  mV ( $-3.37 \pm 0.65$  vs.  $-0.02 \pm 0.01$  pA/pF;  $P < 0.02$ ). (D) The stretch-activated cation current (Cation, □) was estimated as the difference between the total and anion currents in C assuming independence. The I-V relationship for the cation SAC was linear and reversed at  $-10$  mV.



the  $\alpha$  and  $\beta$  subunits of the integrin heterodimer (Clark and Brugge, 1995). Therefore, there may be differences in the information delivered and the responses evoked by stretching ECM in vivo or just  $\beta 1$  integrins. Furthermore, cardiac myocytes express other  $\beta$ -integrin isoforms, including  $\beta 3$  and  $\beta 5$  (Ross and Borg, 2001), and force also is transmitted from the ECM to the cytoskeleton by the dystrophin–dystroglycan complex (Petrof et al., 1993). These molecules and the attached cytoskeleton also are potential mechanotransducers for SAC. By coupling various mAb and ECM components to beads and applying either a static or time-dependent magnetic field, the paramagnetic bead method should prove useful in unraveling how external mechanical forces ultimately regulate cellular function.

#### Identity of the $Cl^-$ SAC

The main  $Cl^-$  channels identified in cardiac myocytes are the PKA-dependent, cardiac variant of the cystic fibrosis transmembrane conductance regulator ( $I_{CFTR,cardiac}$  or  $I_{Cl,PKA}$ ), the calcium-dependent transient outward  $Cl^-$  current ( $I_{Cl,Ca}$  or  $I_{to2}$ ), and the volume-sensitive  $Cl^-$  current ( $I_{Cl,swell}$  or  $I_{Cl,vol}$ ) (Sorota, 1999; Hume et al., 2000). All three of these currents are outwardly rectifying with a physiological  $Cl^-$  gradient, as was the  $Cl^-$  SAC. Nevertheless, based on biophysical and pharmacological characteristics, it seems unlikely that the  $Cl^-$  SAC re-

corded here is due to either  $I_{CFTR,cardiac}$  or  $I_{Cl,Ca}$ .  $Cl^-$  SAC partially inactivated at positive potentials, whereas  $I_{CFTR,cardiac}$  is time independent at all voltages (Shuba et al., 1996; Hume et al., 2000).  $I_{Cl,Ca}$  is triggered by elevation of cytoplasmic  $Ca^{2+}$  and displays both inactivation at positive potentials and a bell-shaped I-V relationship when  $Ca^{2+}$  handling is normal (Zygmunt and Gibbons, 1991). When cytoplasmic  $Ca^{2+}$  is clamped at an elevated level, however,  $I_{Cl,Ca}$  is time independent (Zygmunt, 1994). In contrast,  $Cl^-$  SAC inactivated and exhibited outward rectification in  $Ca^{2+}$ -free bathing media with 8 mM EGTA in the pipette solution, conditions that reduced cytoplasmic-free  $Ca^{2+}$  to  $\sim 35$  nM and minimized  $Ca^{2+}$  transients. Furthermore, 10  $\mu M$  tamoxifen blocked  $Cl^-$  SAC, but  $I_{CFTR,cardiac}$  (Vandenberg et al., 1994) and  $I_{Cl,Ca}$  (Valverde et al., 1993) are insensitive to tamoxifen. It also is unlikely that  $Cl^-$  SAC is due to  $ClC-2$ , which yields an inwardly rectifying  $Cl^-$  current in heart (Duan et al., 2000), or P-glycoprotein, which is insensitive to tamoxifen (Ehring et al., 1994).

Although activation of a novel  $Cl^-$  channel by integrin stretch rigorously cannot be ruled out, the characteristics of  $Cl^-$  SAC resemble those of  $I_{Cl,swell}$ . Like  $I_{Cl,swell}$  (Sorota, 1999; Hume et al., 2000; Baumgarten and Clemo, 2003), the  $Cl^-$  SAC activated slowly, was outwardly rectifying, partially inactivated at positive potentials, was blocked by tamoxifen, was poorly permeant to aspartate, and was not observed in every myocyte. On

the other hand, several quantitative differences should be noted. The current density for  $\text{Cl}^-$  SAC,  $1.13 \pm 0.10$  pA/pF at +40 mV, was less than that typically reported for  $I_{\text{Cl,swell}}$  in mammalian ventricle, 2 to 10 pA/pF (Vandenberg et al., 1996). This could be due to the fact that  $\text{Cl}^-$  SAC was activated by stretching only localized patches of integrin receptors, whereas  $I_{\text{Cl,swell}}$  arises from swelling of the entire cell. Second, in  $\sim 60\%$  of the myocytes  $\text{Cl}^-$  SAC inactivated to a greater degree than is typical for cardiac  $I_{\text{Cl,swell}}$  (Tseng, 1992; Duan et al., 1995; cf., Shuba et al., 1996). The reason why  $\text{Cl}^-$  SAC exhibited prominent inactivation in some cells but not in others is unclear. Nevertheless, both the extent of inactivation at positive potentials, if any, and its voltage dependence were identical for the background  $\text{Cl}^-$  current and  $\text{Cl}^-$  SAC in each cell studied. The similarity of inactivation is consistent with the idea that the  $\text{Cl}^-$  SAC and the background  $\text{Cl}^-$  current, which has been attributed to  $I_{\text{Cl,swell}}$  (Sorota, 1992; Duan et al., 1995, 1997), arise from the same channel. The possibility that  $\text{Cl}^-$  SAC is partially activated by binding of the anti- $\beta 1$ -integrin mAb-coated beads without stretch and contributes to the background current cannot be excluded, however. Finally, the outward rectification of  $\text{Cl}^-$  SAC was greater than for  $I_{\text{Cl,swell}}$  in rabbit ventricular myocytes (Clemo and Baumgarten, 1997) with both physiological and high pipette  $\text{Cl}^-$  concentrations. Perhaps the quantitative differences arise in part from the dilution of an intracellular regulator or blocking ion upon cell swelling but not after integrin stretch.

Stretch and cell volume may regulate the same  $\text{Cl}^-$  channel in other cell types. Plating human neutrophils on surfaces coated with mAb for  $\beta 2$  integrin or the  $\alpha$  subunits of LFA-1, CR3, and gp150/95 integrins results in  $\text{Cl}^-$  efflux (Menegazzi et al., 1999) that was markedly inhibited by MK-447 and analogue A, which block  $I_{\text{Cl,swell}}$  in neutrophils (Simchowicz et al., 1993). Moreover, stretch and swelling activate the same anion channel in cultured opossum kidney cells (Ubl et al., 1988).

#### *Mechanism of Mechanotransduction*

The slow time course of activation of  $\text{Cl}^-$  SAC and the fact that localized stretch induced a significant whole-cell current indicates the involvement of a signaling cascade in the transduction process. In support of this idea, genistein, a broad-spectrum isoflavone PTK inhibitor, significantly but incompletely blocked  $\text{Cl}^-$  SAC in the presence of continued  $\beta 1$ -integrin stretch. Incomplete block may arise from nonspecific effects of this agent. Genistein activates  $I_{\text{CFTR,cardiac}}$  in a PTK-independent manner at the concentration used (Chiang et al., 1997), and it is likely that the observed incomplete block reflects the balance between block of  $\text{Cl}^-$  SAC and stimulation of  $I_{\text{CFTR,cardiac}}$ .

Stronger evidence for the involvement of PTK was obtained with PP2, a specific blocker of FAK and Src (Hanke et al., 1996) that competitively antagonizes the binding of ATP to the activated kinase domain (Zhu et al., 1999). PP2, but not its inactive analogue PP3, completely blocked  $\text{Cl}^-$  SAC as well as a significant fraction of the background  $\text{Cl}^-$  current. To our knowledge, this is the first evidence for the involvement of FAK and/or Src in the regulation of cardiac  $\text{Cl}^-$  currents.

The regulation of  $\text{Cl}^-$  SAC by PTK family members is consistent with the idea  $I_{\text{Cl,swell}}$  may be responsible for the  $\text{Cl}^-$  SAC. Genistein blocks  $I_{\text{Cl,swell}}$  in canine atrial myocytes (Sorota, 1995). Moreover, the Src family of PTK regulates  $I_{\text{Cl,swell}}$  in noncardiac cells. Src activator peptide, EPQ(pY)EEIPI, significantly enhances  $I_{\text{Cl,swell}}$  in cultured rabbit nonpigmented ciliary epithelial cells (Shi et al., 2002). In addition, p56<sup>lck</sup>, a Src kinase, rescues defective activation of  $I_{\text{Cl,swell}}$  in p56<sup>lck</sup> knockout human T lymphocytes (Lepple-Wienhues et al., 1998) and activates an outwardly rectifying  $\text{Cl}^-$  current independent of CFTR (Lepple-Wienhues et al., 2000). Finally, as previously noted, FAK and Src are upstream PTKs that orchestrate integrin-mediated signaling (Parsons, 2003), and integrins and PTK are activated by both mechanical stretch and swelling of myocytes (Sadoshima et al., 1996; Sadoshima and Izumo, 1997).

#### *Pathophysiological Activation of $\text{Cl}^-$ SAC*

Cardiac disease, including dilated cardiomyopathies, valvular disease, and myocardial infarction, results in chamber dilatation and wall motion abnormalities that ultimately stretch cardiac myocytes. We postulate that global or regional stretch of myocytes in situ by forces transmitted from ECM to integrins will lead to activation of  $\text{Cl}^-$  SAC, at least in part, via integrin signaling involving Src and/or FAK. Because  $\text{Cl}^-$  SAC is outwardly rectifying and reverses between the plateau and resting potential, its activation will tend to generate a small diastolic depolarization, reduce action potential duration (APD), and limit the overall prolongation of APD resulting from the contributions of other ionic currents that are typically altered or remodeled by disease processes. In the ventricle, the effect on APD may be protective by suppressing arrhythmias due to early afterdepolarizations. On the other hand, abbreviation of APD upon stretch would favor reentrant tachycardias.

Activation of FAK and Src in cardiac disease is well established. Acute pressure overload due to aortic constriction results in phosphorylation of FAK and Src (Franchini et al., 2000; Laser et al., 2000; Babbitt et al., 2002), and association of FAK with other components of costameres including Src, Grb2, PI-3K, and actin (Franchini et al., 2000). Similarly, acute volume overload produced by balloon inflation causes phosphoryla-

tion of FAK, association of FAK with Src, Grb2, and ERK (Domingos et al., 2002), and activation of Src (Takeishi et al., 2001). Chronic stretch triggers altered  $\beta 1$ -integrin expression (Babbitt et al., 2002), and the up-regulation and remodeling of ECM (Bendall et al., 2002; Cleutjens and Creemers, 2002), the natural integrin ligand. Furthermore,  $\beta 1$ -integrins (Ross et al., 1998), FAK (Pham et al., 2000; Taylor et al., 2000), and Src (Takeishi et al., 2001) have a role in compensatory hypertrophy, and  $\beta 1$ -integrins may also participate in the progression to decompensated heart failure (Goldsmith et al., 1999).

Activation of cardiac  $\text{Cl}^-$  channels in animal models of cardiovascular disease and in man also has been established.  $I_{\text{Cl,swell}}$  is persistently activated under isotonic conditions in ventricular myocytes from dilated cardiomyopathy induced by rapid pacing (Clemo et al., 1999b) or aortic regurgitation (Clemo and Baumgarten, 1998; Clemo et al., 1999a), in myocytes isolated 30 d after infarction from the infarct and peri-infarct zones (Clemo et al., 2001), and in human atrial myocytes from patients with atrial enlargement or elevated ventricular end-diastolic pressure (Patel et al., 2003). In addition, an outwardly rectifying  $\text{Cl}^-$  current is up-regulated in a pressure overload hypertrophy model (Bénitah et al., 1997), but the identity of the current was not established. It remains to be seen whether the regulation of  $\text{Cl}^-$  currents activated in cardiac disease is the same as for  $\text{Cl}^-$  SAC.

#### *Effect of Integrin Stretch on Cation Currents*

In addition to activating  $\text{Cl}^-$  SAC, stretch of  $\beta 1$ -integrins appeared to activate a poorly selective cation current with a linear I-V relationship that reversed near  $-10$  mV, and to suppress the inwardly rectifying potassium current,  $I_{\text{K1}}$ . The decrease in inward current at  $-100$  mV (Fig. 10) underestimates the suppression of  $I_{\text{K1}}$  because inward current is simultaneously augmented by the poorly selective cation current. Other means of cardiac myocyte stretch have been shown to activate nonselective cation currents (Zeng et al., 2000; Zhang et al., 2000; Isenberg et al., 2003) and recently to inhibit  $I_{\text{K1}}$  (Isenberg et al., 2003). Although these phenomena were not studied in detail, they are likely to be important because the effects of stretch on cation currents were larger in amplitude than those on  $\text{Cl}^-$  currents. On the other hand, stretch was applied locally. The relative efficiency of the resulting signaling cascades to propagate throughout the cell and activate anion and cation SAC and inhibit  $I_{\text{K1}}$  are unknown. Therefore, the magnitude of effects on anion and cation currents of mechanical stimuli that stretch integrins over the entire sarcolemma remains uncertain.

Integrin stretch previously has been shown to modulate cation currents in other cell types. For example,

stretching integrins with collagen-coated beads triggers  $\text{Ca}^{2+}$  transients in fibroblasts that were regulated by the actin cytoskeleton (Wu et al., 1999) and blocked by  $\text{Gd}^{3+}$  (Glogauer et al., 1995), a cation SAC channel blocker. Furthermore, integrin ligand-coated beads modulate  $\text{Ba}^{2+}$  current through L-type  $\text{Ca}^{2+}$  channels in rat smooth muscle cells (Wu et al., 1998) by a process requiring the activity of FAK and Src (Wu et al., 2001). We cannot exclude the possibility that stretch of integrins activates other currents in heart.

#### *Electrophysiological Consequences of Integrin Stretch*

Whereas  $\text{Cl}^-$  SAC alone is expected to shorten APD and cause slight diastolic depolarization, the overall effect of integrin stretch is likely to be more complex. Activation of a poorly selective cation SAC and inhibition of  $I_{\text{K1}}$  will result in significant diastolic depolarization, a more pronounced shortening of the plateau, and a slowing of the final portion of repolarization. Full analysis of the combined effects must await detailed measurements of anion and cation SAC with widespread rather than localized stretch of integrins, as might occur under physiologic and pathophysiologic conditions. Nevertheless, the predicted electrophysiologic consequences of integrin stretch are likely to contribute to arrhythmias associated, for example, with dilated cardiomyopathies and atrial enlargement.

In summary, this study shows for the first time that specific mechanical stretch of  $\beta 1$ -integrins in cardiac myocytes activates a  $\text{Cl}^-$  current with biophysical and pharmacological characteristics that resemble  $I_{\text{Cl,swell}}$ . Mechanotransduction involves PTK, particularly FAK and/or Src. Furthermore, stretch of  $\beta 1$ -integrin in cardiac myocytes also appears to activate a poorly selective cation current and inhibit  $I_{\text{K1}}$ .

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