ROLE OF ENDOTHELIN-1 IN THE REGULATION OF THE SWELLING-ACTIVATED CI- CURRENT IN ATRIAL MYOCYTES

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ROLE OF ENDOTHELIN-1 IN THE REGULATION OF THE SWELLING-ACTIVATED Cl- CURRENT IN ATRIAL MYOCYTES

by

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<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
<td></td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
<td></td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-aminoethyl) benzenesulfonyl fluoride</td>
<td></td>
</tr>
<tr>
<td>AF</td>
<td>atrial fibrillation</td>
<td></td>
</tr>
<tr>
<td>AngII</td>
<td>angiotensin II</td>
<td></td>
</tr>
<tr>
<td>AP</td>
<td>action potential</td>
<td></td>
</tr>
<tr>
<td>APD</td>
<td>action potential duration</td>
<td></td>
</tr>
<tr>
<td>AT1R</td>
<td>AngII receptor type 1</td>
<td></td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
<td></td>
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<tr>
<td>C-H2DCFDA-AM</td>
<td>6-carboxy-2',7'-dichlorodihydrofluorescein diacetate-di(acetoxy-methyl ester)</td>
<td></td>
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<tr>
<td>cPKCα</td>
<td>conventional protein kinase C-α</td>
<td></td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
<td></td>
</tr>
<tr>
<td>DCPiB</td>
<td>4-(2-butyl-7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid</td>
<td></td>
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<tr>
<td>DIDS</td>
<td>4,4'-diisothiocyanostilbene-2, 2'-disulfonic acids</td>
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<tr>
<td>DPI</td>
<td>diphenyleneiodonium</td>
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EAD ................................................................. early afterdepolarization
E-C ................................................................. excitation-contraction
ECE .............................................................. endothelin converting enzyme
E_{Cl} ............................................................. chloride equilibrium potential
ECM .............................................................. extracellular matrix
EGF .............................................................. epidermal growth factor
EGFR ........................................................... epidermal growth factor receptor
ER ................................................................. endoplasmic reticulum
ERK ............................................................... extracellular signal regulated kinase
ERP ............................................................... effective refractory period
ET ................................................................. endothelins
ET_{A} ............................................................ endothelin receptor subtype A
ET_{B} ............................................................ endothelin receptor subtype B
FAK ............................................................... focal adhesion kinase
FRNK ........................................................... FAK-related non-kinase
GEF ............................................................. guanine-nucleotide exchange factor
GPCR .......................................................... G protein coupled receptor
HB-EGF ....................................................... heparin-binding EGF
I_{Ca,L} ........................................................ L-type Ca^{2+} current
I_{Ca,T} ........................................................ T-type Ca^{2+} channel
I_{Cl,Ca} ........................................................ calcium activated Cl^{-} current
I_{Cl,CAMP} .................................................. cAMP-activated Cl^{-} current
ICl,swell .......................................................... swelling activated Cl⁻ current
Iᵥ ........................................................... hyperpolarization-activated inward current
i.m. .......................................................... intramuscular
IMAC ........................................................ inner membrane anion channel
INa .......................................................... Na⁺ current
INaCa .................................................. Na⁺/Ca²⁺ exchanger (NCX)-generated current
IK-ACh .................................................. muscarinic K⁺ current
IK1 ........................................................ inwards rectifier K⁺ current
IKr ......................................................... rapid delayed rectifier K⁺ current
IKs ........................................................ slow delayed rectifier K⁺ current
IKur ...................................................... ultra-rapid delayed rectifier K⁺ current
IP₃R ................................................. phosphatidylinositol 3', 4', 5'-trisphosphate receptors
Iss ...................................................... steady-state background K⁺ current
I–V ........................................................ current-voltage
JNK .......................................................... c-jun N terminal kinase
MAPK .................................................. mitogen-activated protein kinase
MEK .................................................. mitogen-activated protein kinase kinase
MitoK_ATP ................................................ mitochondrial ATP-sensitive K⁺ channel
MMP ........................................................ matrix metalloproteinases
MPT ........................................................ mitochondrial permeability transition
NADP ................................................ nicotinamide adenine dinucleotide phosphate
NADPH ................................................ reduced form of nicotinamide adenine dinucleotide phosphate
NCX ................................................................. Na+/Ca\(^{2+}\) exchanger
NHE1 .............................................................................. Na\(^+\)/H\(^+\) exchanger 1
NMDG ........................................................................ N-methyl-D-glucamine
NOX ........................................................................... NADPH oxidase
nPKCd .............................................................. novel protein kinase C-\(\delta\)
nPKC\(\varepsilon\) .............................................................. novel protein kinase C\(\varepsilon\)
PDK ................................................................. PtdIns(3, 4, 5)\(P_3\) dependent kinase
PI-3K ........................................................... phosphoinositide-3 kinase
PLC\(\beta\) ............................................................... phospholipase C\(\beta\)
PKC ................................................................. protein kinase C
ppET-1 ............................................................. preproET-1
proHB-EGF .................................................. pro-heparin-binding EGF-like growth factor
PtdIns (4, 5)\(P_2\) ................................................ phosphatidylinositol 4', 5'-bisphosphate
PtdIns(1, 4, 5)\(P_3\) ............................................... inositol 1', 4', 5'-trisphosphate
PTK .............................................................. receptor protein tyrosine kinase
PYK2 ........................................................ Pro-rich Tyr-kinase 2
RIRR .......................................................... ROS-induced ROS release
SAN .............................................................. Sinoatrial Node
SHR ........................................................ spontaneously hypertensive rats
SOCE .......................................................... store-operated calcium entry
\(V_{\text{max}}\) ........................................................ maximum upstroke velocity,
VSMCs....................................................................................vascular smooth muscle cells
Swelling-activated Cl⁻ current (I_{Cl,swell}) is an outwardly rectifying Cl⁻ current that influences cardiac electric activities and acts as a potential effector of mechanoelectrical feedback that antagonizes the effects of stretch-activated cation channels. Persistent activation of I_{Cl,swell} has been observed in multiple models of cardiovascular diseases. Previously we showed that angiotensin II (AngII) signaling and reactive oxygen species (ROS) produced by NADPH oxidase (NOX) are involved in the activation of I_{Cl,swell} by both β1-integrin stretch and osmotic swelling. Because endothelin-1 (ET-1) is a potential downstream mediator of AngII and ETA receptor blockade abrogates AngII-induced ROS generation, we studied how ET-1 signaling regulates I_{Cl,swell} and the relationship between
AngII and ET-1 signaling. Under isosmotic conditions, ET-1 elicited an outwardly rectifying Cl− current that was fully blocked by the highly selective I_{Cl,swell} inhibitor DCPIB and by osmotic shrinkage. Selective ET_A blockade (BQ123), but not ET_B blockade (BQ788), fully suppressed the ET-1-induced current. ET-1-induced I_{Cl,swell} was abolished by blockade of EGFR kinase (AG1478) and PI-3K inhibitors (LY294002 and wortmannin), which also suppress β1-integrin stretch- and swelling-induced I_{Cl,swell}.

ET-1-induced I_{Cl,swell} was abrogated by ebselen, a membrane-permeant glutathione peroxidase mimetic that dismutates H₂O₂ to H₂O, suggesting that ROS were required intermediates in ET-1-induced activation of I_{Cl,swell}. Both NOX and mitochondria are important sources of ROS in cardiomyocytes. Blocking NOX with apocynin or mitochondrial complex I with rotenone both completely suppressed ET-1-induced ROS generation and activation of I_{Cl,swell}, indicating that ROS from both NOX and mitochondria were required to activate I_{Cl,swell}, and complete block by inhibitors of either ROS source suggests mitochondrial and NOX must act in series rather than in parallel.

I_{Cl,swell} elicited by antimycin A, which stimulates superoxide production by mitochondrial complex III, was insensitive to NOX inhibitor apocynin and the NOX fusion peptide inhibitor gp91ds-tat. Activation of I_{Cl,swell} induced by diazoxide, which stimulates mitochondrial ROS production by opening mitochondrial K_{ATP} channels, was not affected by gp91ds-tat. These data suggests that mitochondrial ROS is downstream from NOX in the regulation of I_{Cl,swell}. Mitochondrial ROS production that is enhanced by NOX ROS is likely to be responsible for the activation of I_{Cl,swell} by ET-1.
In order to determine the role of ERK in the proposed signaling pathway that regulates $I_{\text{Cl,swell}}$, we examined the effect of ERK inhibitors (PD 98059 and U0126) on the activation of $I_{\text{Cl,swell}}$ elicited by ET-1, EGF, and $H_2O_2$. ERK inhibitors partially blocked ET-1-induced $I_{\text{Cl,swell}}$ but fully inhibited activation of $I_{\text{Cl,swell}}$ in response to EGF. However, ERK inhibitors did not affect $I_{\text{Cl,swell}}$ elicited by exogenous $H_2O_2$.

We also established the relationship of ET-1 to AngII and osmotic swelling in the regulation of ET-1 $I_{\text{Cl,swell}}$. ET$_A$ blockade abolished $I_{\text{Cl,swell}}$ elicited by both AngII and osmotic swelling, whereas AT$_1$ blockade did not effect ET-1-induced $I_{\text{Cl,swell}}$, suggesting that ET-1 signaling is downstream from AngII and osmotic swelling.

HL-1 cell is a murine atrial cell line that retain phenotypic characteristics of adult cardiomyocytes. We showed that osmotic swelling and ET-1 turned on DCPIB-sensitive outwardly rectifying Cl$^-$ current in HL-1 cells with both physiological and symmetrical Cl$^-$ gradients. The swelling-induced current was suppressed by gp91ds-tat and rotenone but insensitive to apocynin. Blockade of ET$_A$ receptor (BQ123) and NOX (gp91ds-tat) completely inhibited ET-1-induced $I_{\text{Cl,swell}}$ in HL-1 cells. These data indicate that $I_{\text{Cl,swell}}$ is present in HL-1 cell and regulated by similar mechanisms as in native cells.

Finally, we confirmed the production of ROS by ET-1 signaling by flow cytometry of HL-1 cells using the nominally $H_2O_2$-selective fluorescent probe C-H$_2$DCFDA-AM. Exposure to ET-1 increased ROS production, as did $H_2O_2$, a positive control. ET-1-induced ROS production was fully suppressed by both gp91ds-tat and rotenone. HL-1 cell ROS production also was stimulated by the mitochondrial complex
III inhibitor antimycin A, and antimycin A-induced ROS production was blocked by rotenone but not by gp91ds-tat.

These data suggest that ET-1 ETₐ receptor signaling elicits I_{Cl,swell} by sequentially stimulating ROS production by NOX and mitochondria. ETₐ receptor signaling is downstream from AngII in the osmotic swelling-induced activation of I_{Cl,swell} and is upstream from EGFR kinase and PI-3K. Endothelin signaling is likely to be an important means of activating ROS production and I_{Cl,swell} in a variety of cardiovascular diseases.
Endothelins (ETs) are a family of 21-amino-acid peptides that consists of three identified members: ET-1, ET-2 and ET-3. ET-1 is the isoform predominantly responsible for cardiovascular effects (Kedzierski & Yanagisawa, 2001). ET-1 plays a critical role in the regulation of physiological cardiovascular function, but excessive levels of ET-1 have been linked to various cardiovascular pathologies.

ET-1 interacts with two G protein coupled receptor (GPCR) subtypes, ETA and ETB, which are widely and heterogeneously distributed throughout the heart (Russell & Molenaar, 2000). Recently reactive oxygen species (ROS) have been identified as crucial mediators in ET-1 signaling. ET-1-induced ROS generation plays an important role in certain physiological and pathophysiological effects, and is well positioned to be a potential regulator of redox sensitive ion channels and transporters, among which is the swelling-activated Cl⁻ current (I_{Cl,swell}).

I_{Cl,swell} is a cell volume and mechanosensitive anion current that has profound implications in cellular volume regulation, apoptosis, and cardiac electrical activity (for
Persistent activation of $I_{\text{Cl,swell}}$ has been observed in multiple cardiovascular disease models (Clemo et al., 1998; Clemo et al., 1999b; Clemo et al., 2000; Clemo et al., 2001; Patel et al., 2003). Previously we have shown that angiotensin II (AngII) signaling and ROS produced by NADPH oxidase (NOX) are responsible for the activation of $I_{\text{Cl,swell}}$ by both $\beta_1$-integrin stretch and osmotic swelling (Browe & Baumgarten, 2004; Browe & Baumgarten, 2006; Ren et al., 2008). Besides NOX, another important source of ROS in cardiomyocytes is mitochondria, and we have shown that mitochondrial ROS is also involved in the regulation of $I_{\text{Cl,swell}}$ (Browe & Baumgarten, 2007; Deng et al., 2009; Raucci, Jr. & Baumgarten, 2009). The localization of NOX in the sarcolemmal membrane and the high density of subsarcolemmal mitochondria in cardiomyocytes raise the feasibility of their crosstalk, and crosstalk between NOX and mitochondria has emerged as a new concept of cellular signaling (Kimura et al., 2005a; Kimura et al., 2005b; Caldiz et al., 2007).

The synthesis and release of ET-1 can be induced by AngII, and accumulating evidence indicates that ET-1 might act as a downstream mediator of AngII in multiple physiological and pathophysiological processes including ROS production and arrhythmogenic effects. It has been reported that ET-1 is a potential modulator of $I_{\text{Cl,swell}}$ (Du & Sorota, 2000) and anion efflux (Tilly et al., 1996a) in cardiac myocytes. However, the downstream signaling pathways and the relationship between AngII and ET-1 signaling in the regulation of $I_{\text{Cl,swell}}$ remain unknown.
1.1. ET-1 in Cardiac Physiology and Pathology

ET-1 was first identified in 1988 as a vasoconstrictor peptide produced by vascular endothelial cells (Yanagisawa et al., 1988). ET-1 is also produced by other cell types including leukocytes (Sessa et al., 1991), macrophages (Ehrenreich et al., 1990), smooth muscle cells (Hahn et al., 1990), and cardiomyocytes (Ito et al., 1993). Vascular endothelial cells secrete ET-1 preferentially towards the abluminal side, i.e. smooth muscle side, rather than the luminal side (Yoshimoto et al., 1991; Wagner et al., 1992). Under physiological conditions, the plasma concentration of ET-1 is lower than the pharmacological threshold. Therefore, ET-1 is generally regarded as an autocrine/paracrine factor rather than a circulating hormone.

Physiologically, ET-1 is essential for embryonic development of cardiovascular system, and endogenously generated ET-1 plays an important role in maintenance of vascular tone and blood pressure (Haynes & Webb, 1994; Veniant et al., 1994). ET-1 elicits a positive inotropic effect in cardiac muscle of most species investigated, and the extent of the positive inotropic effect of ET-1 on human heart appears to be chamber-specific and depends on the pathological state of the heart (Moravec et al., 1989; Endoh & Takanashi, 1991; Wang et al., 1991; Kasai et al., 1994; Dhein et al., 2000; Nagasaka et al., 2003; Mollmann et al., 2007). The positive inotropic effect of ET-1 recently has been largely attributed to intracellular ROS production and ROS-mediated stimulation of Na+/H+ exchanger 1 (NHE1) (Cingolani et al., 2006; Cingolani & Ennis, 2007; De Giusti et al., 2008). ET-1 is also implicated in the slow phase of stretch-induced inotropic response, which involves a sequential Ang II – ET release and subsequent increase of
Ca\textsuperscript{2+} entry (Cingolani et al., 2001; Cingolani et al., 2003; Ennis et al., 2005; Cingolani et al., 2005). It is widely accepted that the inotropic effect of ET-1 is mediated by ET\textsubscript{A} receptors (Ishikawa et al., 1988; Kelso et al., 2000), whereas the role of ET\textsubscript{B} receptor is contentious (Leite-Moreira & Bras-Silva, 2004).

The ET system is over-activated in multiple cardiovascular disorders including congestive heart failure (Moe et al., 2003), atrial fibrillation (Tuinenburg et al., 1998; Brundel et al., 2001; Masson et al., 2006), essential hypertension (Touyz & Schiffrin, 2003), salt sensitive hypertension (Feldstein & Romero, 2007), pulmonary hypertension (Jasmin et al., 2003), pre-eclampsia (Florijn et al., 1991), atherosclerosis (Bousette & Giaid, 2003), stable coronary artery disease (Pernow, 2004), acute myocardial infarction (Cernacek et al., 2003), ischemia/reperfusion injury (Pernow & Wang, 1997), myocarditis (Ono et al., 1999), sepsis induced myocardial dysfunction (Chopra & Sharma, 2007), and diabetic cardiovascular complications (Chakrabarti et al., 2002).

Furthermore, ET-1 participates in the structural remodeling of heart as a potent mitogenic (Shubeita et al., 1990; Ito et al., 1991), pro-fibrotic (Teder & Noble, 2000; Clozel & Salloukh, 2005), and pro-inflammatory factor (Hayasaki et al., 1996; Yang et al., 2004).

1.2. The Effect ET-1 on Cardiac Electrical Activity and Ion Channels

1.2.1. Sinoatrial Node (SAN)

In isolated rabbit SAN preparations, ET-1 exhibits a negative chronotropic effect via ET\textsubscript{A} receptors, which reflects prolongation of action potential duration (APD), a positive shift of take-off potential, and a decrease in the slope of the pacemaker potential.
This negative chronotropic effect was ascribed to the effects of ET-1 on various currents: inhibition of the L-type Ca\(^{2+}\) current (\(I_{\text{Ca,L}}\)), the T-type Ca\(^{2+}\) current (\(I_{\text{Ca,T}}\)), the hyperpolarization-activated inward current (\(I_f\)), and an increase of background K\(^+\) current (Tanaka et al., 1997; Tanaka et al., 1998; Ono et al., 2001). Modulation of Cl\(^-\) channels apparently was not considered. However, the effects of ET-1 on SAN may differ between species. Under exactly the same experimental conditions, ET-1 exerted a positive chronotropic effect on guinea-pig SAN (Ono et al., 2001).

1.2.2. Atria

APD in cardiomyocytes is largely governed by the amplitude and kinetics of \(I_{\text{Ca,L}}\), rapid delayed rectifier K\(^+\) current (\(I_{\text{K,r}}\)) and slow delayed rectifier K\(^+\) current (\(I_{\text{K,s}}\)). Atria has intrinsically shorter APD than that of ventricle, a difference that is partially due to atrial-specific expression of the ultra-rapid delayed rectifier K\(^+\) current (\(I_{\text{Kur}}\)) and atrial-predominant expression of the muscarinic K\(^+\) current (\(I_{\text{K-ACh}}\)) (Schram et al., 2002). ET-1 hyperpolarizes the membrane and shortens the APD of guinea-pig and canine atrial myocytes (Yorikane et al., 1991; Ono et al., 1994). A recent study showed that ET-1 had no effect on the basal APD of human atrial cells but completely abolished the APD prolongation induced by isoproterenol (Redpath et al., 2006). As in SAN, the ionic basis for the atrial APD shortening effect of ET-1 appears to vary between species. In guinea-pig atria, ET-1 dose dependently inhibits \(I_{\text{Ca,L}}\) and activates \(I_{\text{K-ACh}}\) by stimulating a pertussis toxin-sensitive GTP-binding protein via \(ET_A\) receptor (Ono et al., 1994). The same group also demonstrated that ET-1 augments \(I_{\text{K,s}}\) in guinea-pig atrial myocytes (Ono, 2003). However, ET-1 was shown to inhibit \(I_{\text{K-ACh}}\) in rabbit and mouse atrial
myocytes (Spiers et al., 1996; Cho et al., 2005). ET-1 either reduces (Cheng et al., 1995) or has no effect on basal $I_{\text{Ca,L}}$ (Redpath et al., 2006) of human atrial cells, and dual effects, depending on the $I_{\text{Ca,L}}$ density before ET-1 treatment, were also reported (Boixel et al., 2001). Besides Ca$^{2+}$ and K$^{+}$ currents, ET-1 activates $I_{\text{Cl,swell}}$ in canine atrial myocytes (Du & Sorota, 2000). As an outwardly rectifying current that reverses near the Cl$^{-}$ equilibrium potential ($E_{\text{Cl}}$), activation of $I_{\text{Cl,swell}}$ shortens APD (Hiraoka et al., 1998). Another current that may contribute to shortening of APD is the Na$^{+}$/Ca$^{2+}$ exchanger (NCX)-generated current ($I_{\text{NaCa}}$) (Janvier et al., 1997). ET-1 activates NHE1 and increase [Na], which promotes NCX in reverse mode (Cingolani et al., 2001; Cingolani & Ennis, 2007). With a stoichiometry of 3Na$^{+}$:1Ca$^{2+}$, electrogenic NCX will generate a repolarizing outward current when operating in the reverse mode.

Besides the ion channels that have direct effects on APD, ET-1 also modulates other channels in atria. A recent study showed that ET-1 inhibits human atrial inward rectifier K$^{+}$ current ($I_{\text{K1}}$) via ET$_{A}$ receptors and a protein kinase C (PKC)-mediated pathway (Kiesecker et al., 2006). Potentially important for the present study, ET-1 inhibits the cAMP-activated Cl$^{-}$ current ($I_{\text{Cl,cAMP}}$), which results from CFTR (cystic fibrosis transmembrane conductance regulator), via ET$_{A}$ receptors in human atrial myocytes (Tsai et al., 2001). ET-1 also activates phosphatidylinositol 3′, 4′, 5′-trisphosphate receptors (IP$_{3}$R), which potentially plays a role in atrial excitation-contraction (E-C) coupling and contributes to arrhythmogenic spontaneous diastolic Ca$^{2+}$ transients (Lipp et al., 2000; Mackenzie et al., 2002; Zima & Blatter, 2004; Li et al., 2005). The effects of ET-1 on atrial Na$^{+}$ channels seem controversial. A stimulatory
effect was observed by Cheng et al. (1995), whereas ET-1 failed to significantly alter the maximum upstroke velocity, $V_{\text{max}}$, of the action potential, which is an indicator of the size of $I_{\text{Na}}$ (Geller et al., 2000; Magyar et al., 2000).

1.2.3. Ventricles

Most studies have shown that ET-1 prolongs APD in His bundle and ventricular myocardium (Szokodi et al., 1998; Geller et al., 1998a; Geller et al., 1998b; Merkely et al., 2002). APD in ventricular myocytes largely is determined by the amplitude and the kinetics of $I_{\text{Ca,L}}$, $I_{\text{Kr}}$ and $I_{\text{Ks}}$. The effect of ET-1 on ventricular $I_{\text{Ca,L}}$ is highly controversial: an increase (Lauer et al., 1992; Kelso et al., 1998; Woo & Lee, 1999; He et al., 2000), decrease (Tohse et al., 1990; Magyar et al., 2000), no change (Thomas et al., 1997; Watanabe & Endoh, 2000), or dual effects (Watanabe & Endoh, 1999) were all reported. The effects of ET-1 on ventricular $K^+$ channel appears to vary between the species, but overall, ET-1 exerts an inhibitory effect on $K^+$ channels. ET-1 was shown to inhibit the $I_{\text{Ks}}$ and partially inhibit $I_{\text{K-ACH}}$ in guinea pig ventricles (Kobayashi et al., 1996; Washizuka et al., 1997). In human ventricular myocytes, ET-1 inhibits $I_{\text{Kr}}$ (Magyar et al., 2000). In rat ventricular myocytes, however, the predominant effects of ET-1 is to inhibit a non-inactivating steady-state background $K^+$ current ($I_{\text{ss}}$) (James et al., 2001). Interestingly, ET-1 was reported to augment $I_{\text{Ks}}$ in guinea pig atrial myocytes, where it shorten APD (Ono, 2003), but inhibit $I_{\text{Ks}}$ in guinea pig ventricular myocytes, where it prolongs APD (Washizuka et al., 1997). The reason for the apparently differential effects is not known.

Previous studies have indicated that ET-1 regulates reappearance of T-type Ca$^{2+}$ channel ($I_{\text{Ca,T}}$) in failing hearts both in vivo and in vitro (Izumi et al., 2003). Reappearance
of $I_{\text{Ca},T}$ in hypertrophic and failing heart has been associated with $\text{Ca}^{2+}$ overload, triggered activity, and $\text{Ca}^{2+}$-dependent signaling that mediates cell apoptosis (Nuss & Houser, 1993; Martinez et al., 1999; Levine et al., 2000). Moreover, ET-1 induces spontaneous $\text{Ca}^{2+}$ transients and triggered activity in ventricular myocytes that are attributed to activation of $\text{IP}_3\text{R}$ (Proven et al., 2006).

### 1.3. ET-1 and Cardiac Arrhythmias

The majority of arrhythmias are attributed to three mechanisms: reentry, increased automaticity, and triggered activity. ET-1 not only induces or exacerbes ischemia-induced ventricular arrhythmias (Ezra et al., 1989; Salvati et al., 1991; Polontchouk et al., 2001), it also has a direct arrhythmogenic action that is not solely attributable to myocardial ischemia (Yorikane & Koike, 1990; Yorikane et al., 1990; Szabo et al., 2000). It has been indicated that prolongation of APD and generation of early afterdepolarization (EAD) are involved in the genesis of ET-1-induced ventricular arrhythmias (Szokodi et al., 1998; Geller et al., 1998a; Geller et al., 1998b; Merkely et al., 2000; Merkely et al., 2002). Reintroduction of $I_{\text{Ca},T}$ in ventricles and activation $\text{IP}_3\text{R}$ may contribute to ET-1-induced triggered activity (Levine et al., 2000; Proven et al., 2006). Moreover, ET-1 also causes regional heterogeneity and increase of action potential (AP) dispersion, which may favor a reentry mechanism (Geller et al., 1998a).

Although the arrhythmogenic effects of ET-1 were first identified in ventricles, ET-1 may participate in the genesis of atrial arrhythmias as well. It has been reported that plasma and tissue ET-1 are elevated in patients with atrial fibrillation (Tuinenburg et al., 1998; Brundel et al., 2001; Masson et al., 2006), and ET-1 was shown to induce
arrhythmic contractions in human right atrial tissues (Burrell et al., 2000). The pathophysiological basis underlying the arrhythmogenic effects of ET-1 on atria have not been fully understood. Nevertheless, shortening of effective refractory period (ERP), which is a consequence of APD abbreviation, is an important electrophysiological substrate that facilitates reentrant arrhythmias including atrial fibrillation (Shiroshita-Takeshita et al., 2005). In addition, activation of IP₃R by ET-1 is a potential mechanism for arrhythmogenic Ca²⁺ sparks and triggered activity in atria (Zima & Blatter, 2004; Li et al., 2005). ET-1 is also known to induce oxidative stress and inflammation, which have a considerable impact on the energetic, electrophysiological and mechanical properties of atria contributing to atrial remodeling and fibrillation (Carnes et al., 2001; Korantzopoulos et al., 2004; Engelmann & Svendsen, 2005; Korantzopoulos et al., 2005; Goldstein & Stambler, 2005; Kim et al., 2005; Dudley, Jr. et al., 2005; Wolin & Gupte, 2005; Boos et al., 2006).

1.4. ET-1-Induced Signaling

Both ETₐ and ETₐ receptor couple to the Gq family of heterotrimeric G proteins, and there is some evidence that they also couple to Gi/o (Hilal-Dandan et al., 1992; Hilal-Dandan et al., 1994; Hilal-Dandan et al., 1997). The best characterized ET-1 signaling pathway in cardiomyocyte are the diacylglycerol (DAG)/PKC cascades, the mitogen-activated protein kinase (MAPK) cascades, and the phosphoinositide-3 kinase (PI-3K)-dependent cascades. Recently, ROS signaling and epidermal growth factor receptor (EGFR)-mediated pathway also were identified.
1.4.1. DAG/PKC Signaling Cascades

ET-1 binding to ETA receptor stimulates exchange of GDP for GTP on Gαq and the heterotrimeric G protein dissociates into Gαq and βγ dimers. Both Gαq and βγ dimers are able to activate phospholipase Cβ (PLCβ) (Rhee, 2001; Fukami, 2002).

Following activation, PLCβ hydrolyzes the membrane phospholipid, phosphatidylinositol 4′, 5′-bisphosphate [PtdIns (4, 5)P2] to DAG and inositol 1′, 4′, 5′-trisphosphate [Ins(1, 4, 5)P3]. DAG is a physiological regulator of several PKC isoforms that translocate from the cytoplasm to the membrane fraction. Conventional PKC-α (cPKCα), novel PKC-δ (nPKCδ) and novel PKC-ε (nPKCε) are involved in ET-1 induced cellular response (Clerk & Sugden, 1997a; Clerk & Sugden, 1999; Zolk et al., 2004; Wolf et al., 2004; Takeishi et al., 2007). Moreover, PKC modulate multiple signaling pathways including MAPK and PI-3K cascade(Gomez et al., 1996; Chiloeches et al., 1999).

1.4.2. MAPK Cascades

Three MAPK cascades have been well characterized in the cardiomyocytes: the extracellular signal-regulated kinase (ERK) cascade, the c-jun N terminal kinase (JNK) cascade, and the P38-MAPK cascade. ERK1 and ERK2 are robustly activated by ET-1 in cardiac myocytes (Bogoyevitch et al., 1993; Bogoyevitch et al., 1994). The upstream of ERK1/2 are Raf (A-Raf, B-Raf, and c-Raf) and mitogen-activated protein kinase kinase (MEK1/MEK2). Several lines of evidence shows that the small G protein Ras is involved in Raf activation in cardiomyocytes (Avruch et al., 2001; Muslin, 2005), and PKC was shown to activate Ras in cardiac myocytes (Chiloeches et al., 1999). Another possible pathway for Ras activation is EGFR-mediated signaling, which results in the activation of
Sos, a guanine-nucleotide exchange factor (GEF) for Ras (Smith et al., 2004; Chan et al., 2006). Moreover, ET-1-induced ROS may also be involved in the activation of ERK1/2 because ERK signaling cascade has been shown to be redox sensitive (McCubrey et al., 2006).

ET-1 also evidently activates the JNK and p38-MAPK cascades in cardiac myocytes (Clerk & Sugden, 1997b; Clerk et al., 1998), but the upstream modulators have not been clearly defined. The involvement of nPKC-ε (Bayer et al., 2003), Rac (Clerk et al., 2001; Clerk et al., 2002), ERK1/2 (Clerk et al., 2002), and ROS (Sugden & Clerk, 2006) have been suggested.

1.4.3. PI-3K-Dependent Cascades

ET-1 activates the PI-3K/Akt pathway, although the activation is relatively weak in comparison to other agonists (Pham et al., 2001; Sugden, 2002). The PI-3K group of lipid kinases catalyze the formation of PtdIns(3, 4, 5)P3 from PtdIns(4, 5)P2. PtdIns(3, 4, 5)P3 remains in the membrane where it activates PtdIns(3, 4, 5)P3-dependent kinase (PDK), which in turn phosphorylate and activate Akt (Katso et al., 2001).

Possible upstream activators of PI-3K signaling include receptor protein tyrosine kinase (PTK) and βγ dimmers of heterotrimeric G-proteins (Hoyal et al., 2003; Heineke & Molkentin, 2006).

1.4.4. EGFR-Mediated Pathway

It has been demonstrated that ET-1 induces EGFR transactivation in cardiac myocytes, and this receptor transactivtion pathway is crucial for hypertrophy response.
induced by ET-1 and other GPCR agonist such as AngII (Pierce et al., 2001; Asakura et al., 2002; Kodama et al., 2002). The transactivation process involves activation of matrix metalloproteinases (MMP), which cleavages and allows shedding of extracellular surface heparin-binding EGF (Prenzel et al., 1999; Anderson et al., 2004). Upon EGF binding, EGFR kinase dimerizes, trans-autophosphorylates six specific tyrosine residues within its cytoplasmic tail, and then EGFR kinase is able to recruit and phosphorylate various adaptor and signaling molecules (Shah, 2002).

In heart, EGFR kinase is coupled to PI-3K (Oudit et al., 2004; Shah et al., 2006). Both PI-3K and EGFR kinase were shown to be involved in NOX activation (Seshiah et al., 2002; Shah & Catt, 2003; Shah & Catt, 2004).

**1.4.5. ROS Signaling**

An increasing body of evidence indicates that ROS are important second messengers that participate in multiple signaling pathways. ROS, such as H$_2$O$_2$ and O$_2^{-}$, can modify cysteine residues, heme groups, and iron-sulfur centers of signaling molecules, which may have a profound impact on the signaling pathways (Demple et al., 1999; Salmeen & Barford, 2005). The principal growth-promoting signaling pathways in cardiomyocytes, including ERK, JNK, P38 MAPK, and PI3K/Akt pathways, all are redox sensitive (for review, see Takimoto & Kass, 2007).

ET-1 induces ROS generation in cardiomyocyte, and ROS mediate the inotropic response to ET-1 (Cingolani et al., 2006; De, V et al., 2008; De Giusti et al., 2008) as well as multiple pathways associated with ET-1-induced cardiac hypertrophy (Cheng et
al., 1999; Tanaka et al., 2001; Cheng et al., 2005). In addition to redox-sensitive signaling molecules, ROS can modulate functions of ion channels and transporters, which may have profound impact on cardiac electric activities as well as other functions (for review, see Zima & Blatter, 2006).

1.4.6. Non-receptor PTKs and Other Signaling Pathways

The non-receptor PTKs present in cardiomyocytes including the Src family and the family consisting of focal adhesion kinase (FAK) and Pro-rich Tyr-kinase 2 (PYK2). ET-1 activates Src (Kovacic et al., 1998) and focal adhesion associated pathways that involves FAK, PYK2, and p130Cas (Eble et al., 2000). ET-1 also activates RhoA and Rac1, which are members of Rho family (Clerk et al., 2001). Moreover, ET-1 activates calcium/calcineurin pathways by modulating intracellular Ca^{2+} concentration and IP_3-induced Ca^{2+} release (Kakita et al., 2001; Iwai-Kanai & Hasegawa, 2004; Higazi et al., 2009)

1.5. ET-1 as a Potential Downstream Mediator of AngII

Ang II induces preproET-1(ppET-1) gene expression in multiple cell types including cardiomyocytes (Ito et al., 1993). The induction of ppET-1 gene was attributed to AngII receptor type 1 (AT_1R) and a PKC-dependent pathway (Sung et al., 1994), and the AP-1 binding sites in ppET-1 promoter is crucial (Imai et al., 1992; Hong et al., 2004). Besides transcriptional regulation, AngII also can trigger the release of preformed ET-1, which involves degranulation of ET-1-containing storage granules on the cell surface (Villa-Abrille et al., 2006).
Accumulating evidence indicates that ET-1 acts as a downstream mediator of AngII in multiple AngII-induced effects, including hemodynamic response, ROS production, inotropic effect, mitogenic effect, fibrosis, inflammation, and arrhythmogenesis. *In vivo* studies demonstrated that pretreatment with the nonselective ET receptor antagonist bosentan blunted the increase in blood pressure, the decrease in cardiac output, and the decrease in total peripheral conductance induced by acute Ang II infusion both in normotensive and spontaneously hypertensive rats (SHR) (Balakrishnan *et al.*, 1996). Chronic infusion of Ang II to Sprague-Dawley rats markedly enhanced ET-1 levels in vascular tissues and plasma, and ET$_A$-selective antagonist pretreatment abolished the rise in blood pressure as well as AngII-induced vascular hyperresponsiveness to a variety of vasoconstrictors (Rajagopalan *et al.*, 1997). In healthy human subjects, ET$_A$-selective antagonist BQ123 substantially attenuated both systemic and renal hemodynamic effects of low-dose AngII infusion (Montanari *et al.*, 2003). The role of ET-1 in AngII-evoked hemodynamic response was also verified in transgenic animals. In transgenic hypertensive mice overexpressing both human renin and angiotensinogen genes (dTGR), expression of ET-1 was robustly enhanced in cardiac and vascular tissues, and ET receptor antagonist significantly lowered blood pressure (Maki *et al.*, 1998; Maki *et al.*, 2004).

There is some evidence indicates that ET-1 mediates AngII-induce ROS production in both vascular smooth muscle cells (VSMCs) and cardiomyocytes. In VSMCs, AngII elicits superoxide production in a bimodal fashion: a first peak within 10-15 min after treatment followed by a second peak after 4-6 h (Laplante *et al.*, 2005).
Nonselective and ETA-selective receptor antagonists did not prevent the early peak but efficiently prevented the late peak, indicating that the late phase of the Ang II-induced superoxide production is ET-1 dependent whereas the early phase is independent of ET-1 (Laplante et al., 2005). Furthermore, blockade of ET_A receptors suppressed the AngII-induced expression of Nox1 and Nox4, two homologs of the p91phox (NOX2) subunit expressed in VSMCs (Laplante et al., 2005). Cingolani et al. (2006) reported that AngII-induced ROS production in cat ventricular myocytes was completely abolished by nonselective ET-1 receptor blocker TAK044 and the selective ET_A receptor blocker BQ-123, which argues for the crucial role of ET-1 in AngII-induced ROS production in cardiomyocytes.

AngII exerts a positive inotropic effect on cardiac muscle by increasing the intracellular Ca^{2+} transient (Petroff et al., 2000; Salas et al., 2001). ET receptor blockers abolished exogenous AngII-induced inotropic effects, indicating that the inotropic effect of AngII is mediated by ET-1 (Perez et al., 2003; Cingolani et al., 2006). The slow force response to myocardial stretch is ascribed to an autocrine/paracrine release of AngII and ET-1 (Cingolani et al., 1998; Cingolani et al., 2001). Blockade of ET_A receptors or AT_1 receptors canceled stretch-induced NHE-1 activation and inotropic effect. The direction of the cross-talk between AngII and ET-1 was demonstrated by the findings that ET_A receptor blockade blunted the activation of NHE-1 by exogenous Ang II, whereas AT_1 receptor blockade was unable to block the effect of ET-1, which suggests that AngII is upstream from ET-1 (Cingolani et al., 1998; Cingolani et al., 2001; Perez et al., 2003).
Both AngII and ET-1 exhibit potent growth-promoting effect on vasculature and cardiac tissue. Both in vitro and in vivo studies have suggested that ET-1 is involved in hypertrophic changes induced by AngII. Ito et al. (1993) and Xia et al. (2004) showed that AngII upregulated ppET-1 mRNA expression in cultured neonatal rat cardiomyocytes, and inhibition of ET-1 signaling by ET\textsubscript{A} receptor antagonist or antisense sequence against coding region of ppET-1 mRNA blunted hypertrophic response induced by AngII. Moreau et al. (1997) demonstrated that selective ET\textsubscript{A}-receptor antagonists prevented chronic AngII infusion-induced hypertrophic changes of vasculature. Ficai et al. (2001) showed that non-selective ET receptor antagonist bosentan prevented cardiac hypertrophy evoked by chronic Ang II infusion. Endogenous ET-1 also was shown to act as a downstream mediator for AngII in the development of cardiac hypertrophy in response to volume overload (Ishiye et al., 1995a; Ishiye et al., 1995b). The role of ET-1 in AngII-induced hypertrophy response was further supported by transgenic animal models. In transgenic rats harboring both human renin and human angiotensinogen genes (dTGR), the non-selective ET receptor antagonist SB 209670 significantly attenuated both cardiac hypertrophy and vascular wall thickening (Maki et al., 2004).

Both AngII and ET-1 are important pro-fibrotic and pro-inflammatory factors. It has been shown that AngII induces ppET-1 gene expression in both vascular (An et al., 2007) and cardiac fibroblast (Cheng et al., 2003). In dTGR mice that overexpress renin and angiotensinogen, endothelin converting enzyme (ECE) inhibitor treatment significantly reduced fibrosis in the vessel wall and cardiac tissue (Muller et al., 2002). Another study using this transgenic model showed that non-selective ET receptor
antagonist bosentan inhibits NFκB and AP-1 activation as well as inflammatory gene expression regulated by these two transcription factors, suggesting that ET-1 is involved in Ang II-induced inflammation (Muller et al., 2000).

It has been well recognized that AngII is related to the genesis of ventricular arrhythmias under multiple pathological conditions, including hypertension, heart failure, ischemia, and acute myocardial infarction (for review, see Garg et al., 2006). Brunner and Kukovetz (1996) showed that the inhibition of reperfusion arrhythmias by Angiotension converting enzyme (ACE) inhibitors might be attributed to suppression of ET-1 secretion and action. ET\textsubscript{A} receptor antagonist ESB 209670 alone was equally anti-arrhythmic as ACE inhibitors, and they both almost completely suppressed reperfusion arrhythmias. I\textsubscript{Ca,T} reappears in hypertrophic ventricular cells, which may contribute to Ca\textsuperscript{2+} overload and triggered arrhythmias (Levine et al., 2000). AngII was shown to increase I\textsubscript{Ca,T} density and Ca\textsubscript{V}3.1 mRNA in cultured myocytes, and these effects are inhibited by non-selective ET receptor antagonist bosentan, suggesting that ET-1 may mediate AngII-induced I\textsubscript{Ca,T} upregulation (Ferron et al., 2003).

Numerous studies also have illuminated the crucial role of AngII in pathogenesis of atrial fibrillation (for review, see Healey et al., 2005). Although evidence indicating the interaction of AngII and ET-1 in genesis of atrial arrhythmias seems lacking right now, ET-1 is potentially a downstream mediator of AngII in the genesis of atrial fibrillation (AF). Presumably, the fast and irregular beating seen in AF would stretch atrial myocytes and trigger the autocrine/paracrine loop leading to sequential Ang II and ET-1 release, as discussed previously. ET-1 is elevated in plasma and tissue samples of
patients with AF (Tuinenburg et al., 1998; Brundel et al., 2001; Masson et al., 2006), and ET-1 has been shown to shorten APD in atrial cells (Yorikane et al., 1991; Ono et al., 1994), which contributes to electrical remodeling of atria that facilitates reentry (Nakashima et al., 2000). In addition to electrical remodeling, the arrhythmogenic properties of Ang II in AF are also attributed to atrial structural remodeling (Okazaki et al., 2006; Everett & Olgin, 2007), oxidative stress (Korantzopoulos et al., 2007), and inflammation (Boos et al., 2006; Wachtell et al., 2007). ET-1 may act as a downstream effector for AngII in all of these processes.

1.6. Cardiac I_{Cl,swell} and its Regulation

I_{Cl,swell} is a chloride current expressed in the heart and other cell types in many species including human (Hume et al., 2000). As suggested by the name, I_{Cl,swell} is activated by osmotic increase of cell volume. I_{Cl,swell} can also be activated by multiple stimuli under isosmotic conditions, including cell inflation (Hagiwara et al., 1992), anionic amphipaths (Tseng, 1992), \( \beta_1 \)-integrins stretch (Browe & Baumgarten, 2003), AngII (Browe & Baumgarten, 2004), EGF (Browe & Baumgarten, 2006), H$_2$O$_2$ (Browe & Baumgarten, 2004), and ET-1 (Du & Sorota, 2000). I_{Cl,swell} was classified as a mechanosensitive channel because its open probability can be modulated by deforming membrane patch with pipette pressure (Sato & Koumi, 1998). However, aspects of the single channel currents identified by these authors are different than those reported by Duan and Nattel (1994). Although the biophysical characteristics, pharmacology, as well as the regulation pathways of cardiac I_{Cl,swell} have been intensively investigated, the molecular identity of this channel remains elusive.
Several biophysical characteristics can be used to identify $I_{\text{Cl,swell}}$. $I_{\text{Cl,swell}}$ is outwardly rectifying and reverses near $E_{\text{Cl}}$ with either a physiological or symmetrical Cl$^-$ gradient, and the current is time-independent over the physiologic voltage range but partially inactivates at strongly positive potentials (Duan et al., 1995; Shuba et al., 1996). On the other hand, the time-dependent component can be blocked (Ren & Baumgarten, 2005). $I_{\text{Cl,swell}}$ is independent of cytoplasm Ca$^{2+}$ (Lemonnier et al., 2002; Zholos et al., 2005), and this channel is also permeant to other anions with the permeability sequence: $\Gamma^- > \text{NO}_3^- > \text{Br}^- > \text{Cl}^- > \text{Asp}^-$ (Hagiwara et al., 1992; Vandenberg et al., 1994).

Early studies of the pharmacology of cardiac $I_{\text{Cl,swell}}$ were made by Sorota et al. (1994). $I_{\text{Cl,swell}}$ is insensitive to Gd$^{3+}$ (Clemo & Baumgarten, 1997), which is a blocker of cation mechanosensitive channels. Tamoxifen and DIDS (4,4′-diisothiocyanostilbene-2,2′-disulfonic acids) emerged as useful tools to identify $I_{\text{Cl,swell}}$ under conditions that isolate anion currents, but they also inhibit multiple cation channels and transporters (for review, see Baumgarten & Clemo, 2003). DCPIB [4-(2-butyl-6,7-dichloro-2-cyclopentyl-1-on-5-yl) oxobutyric acid], which only recently became commercially available, is a highly selective $I_{\text{Cl,swell}}$ blocker. It does not inhibit either CFTR or Ca$^{2+}$-activated Cl$^-$ channels($I_{\text{Cl,Ca}}$) and also has no effect on several K$^+$, Ca$^+$, Na$^+$ channels (Decher et al., 2001).

$I_{\text{Cl,swell}}$ influences cardiac electric activities and acts as a potential effector of mechanoelectrical feedback that antagonizes the effects of stretch-activated cation channels (Baumgarten & Clemo, 2003). As $I_{\text{Cl,swell}}$ reverses between the resting and
plateau potentials of cardiomyocytes, activation of $I_{\text{Cl,swell}}$ depolarizes membrane potential ($E_m$) and shorten APD (Hiraoka et al., 1998; Decher et al., 2001).

Activation of $I_{\text{Cl,swell}}$ has been observed in multiple cardiovascular abnormalities, including dilated cardiomyopathy induced by rapid pacing (Clemo et al., 1999b) or aortic regurgitation (Clemo & Baumgarten, 1998; Clemo et al., 1999a), myocardial infarction (Clemo et al., 2001), and in human atrial myocytes from patients with atrial enlargement or elevated ventricular end-diastolic pressure (Patel et al., 2003). Based on its biophysical properties, $I_{\text{Cl,swell}}$ has profound implication in arrhythmogenesis. APD abbreviation resulting from $I_{\text{Cl,swell}}$ activation would dampen afterdepolarizations and protect against triggered arrhythmias, on the other hand, it favors reentry arrhythmias such as AF.

In addition to the effects on cardiac electric activities, $I_{\text{Cl,swell}}$ is closely related to multiple physiological and pathophysiological processes, including cell volume regulation, proliferation, differentiation, migration, apoptosis, ischemia-reperfusion-induced death of cardiomyocytes (for review, see Okada et al., 2009).

Previous studies indicated that H$_2$O$_2$, the longer lived, membrane permeable ROS, appears to be a regulator of $I_{\text{Cl,swell}}$. Exogenous H$_2$O$_2$ elicits a current with exactly same properties as $I_{\text{Cl,swell}}$ and scavenging H$_2$O$_2$ with catalase precludes activation of $I_{\text{Cl,swell}}$ (Browe & Baumgarten, 2004; Ren et al., 2008). Studies over the last decade have indicated that the NOX family of enzymes is the major source of ROS in the cardiovascular system (for review, see Griendling, 2006). Activation of $I_{\text{Cl,swell}}$ in response to integrin stretch and osmotic swelling was blocked by inhibitors of NOX (Browe & Baumgarten, 2004; Ren et al., 2008). Besides NOX, mitochondria are also
important source of ROS in cardiomyocytes and recently the crosstalk between NOX and mitochondria has emerged as a new concept of cellular signaling (Kimura et al., 2005a; Kimura et al., 2005b; Caldiz et al., 2007).

1.6.1. NOX-Derived ROS and Upstream Mediators

1.6.1.1. Integrin, FAK, and Src

Since $I_{\text{Cl,swell}}$ is a mechanosensitive current, how the mechanical forces are sensed and transmitted would be of importance. As the physical links between the extracellular matrix (ECM) and cytoskeleton, integrins are the best candidates for the mechanosensors (Force et al., 2002; Manso et al., 2006). Integrins colocalize with a variety of signaling molecules, adaptor proteins, and other cell surface receptors within costameres, among which are focal adhesion kinase (FAK) and members of the Src kinase family (Ross, 2002; Shai et al., 2002; Ross, 2004). FAK and Src are well positioned to be the principal upstream PTKs mediating the integrin-induced mechanotransduction (Brancaccio et al., 2006; Lal et al., 2007). FAK binds to the cytoplasmic domain of $\beta_1$-integrin and autophosphorylates at Y397. Src binds to Y397 of FAK via its SH2 domain. Activated FAK and Src recruit a number of signaling molecules, including PKC, PI-3K, protein phosphatases, small GTPases and trigger a network of downstream signaling cascades (Pham et al., 2000; Parsons, 2003).

The principle integrin isoform in heart is $\beta_1$D (Ross et al., 1998). The roles of $\beta_1$-integrins and its downstream FAK and Src in the regulation of $I_{\text{Cl,swell}}$ are evidenced by the findings that $\beta_1$-integrin stretch elicited $I_{\text{Cl,swell}}$ under isosmotic conditions (Browe &
Both the non-specific PTK inhibitor genistein and selective FAK/Src blocker PP2 fully inhibited β1-integrin stretch-induced I_{Cl,swell} (Browe & Baumgarten, 2003).

The role of Src in the regulation of I_{Cl,swell} apparently differs between stimuli. In contrast to its inhibition of the response to integrin stretch, the Src family PTK inhibitor PP2 substantially augment I_{Cl,swell} induced by osmotic swelling in atrial and ventricular myocytes (Du et al., 2004; Ren & Baumgarten, 2005). This was confirmed by Walsh and Zhang (Walsh & Zhang, 2005), who reported that both PP2 and expression of FAK-related non-kinase (FRNK), which acts as a FAK inhibitor, both enhanced the activation of I_{Cl,swell} upon osmotic swelling of cultured neonatal rat ventricular myocytes. The mechanisms underlying the differential regulation of I_{Cl,swell} by FAK and Src inhibitors are not understood.

### 1.6.1.2. AngII and AngII Receptor

A well-recognized effect of mechanical stretch is releasing paracrine/autocrine factors including AngII and ET-1, which interact with their receptors on cardiomyocytes and orchestrate various downstream signaling pathways (Sadoshima et al., 1993; van Wamel et al., 2000; van Wamel et al., 2001). Meanwhile, mechanical stretch can activate AngII receptor type 1 (AT_{1}R) without AngII binding, suggesting that AngII release may not be mandatory for AT_{1}R activation (Zou et al., 2004; Akazawa et al., 2006).

The role of AngII and AT_{1}R in the regulation of I_{Cl,swell} is supported by observations that exogenous AngII elicits I_{Cl,swell} under isosmotic conditions and that
AT_{1}R competitive antagonists, losartan and eprosartan blocked β1 integrin stretch- and osmotic swelling- induced I_{Cl,swell} (Browe & Baumgarten, 2006; Ren et al., 2008).

1.6.1.3. EGFR Kinase

Previous studies have established that EGFR is a common element in the signaling pathways activated by cell volume changes (Lezama et al., 2005), integrins (Moro et al., 2002), AT_{1} receptors (Shah & Catt, 2003), and ET_{A} receptors (Prenzel et al., 1999; Kodama et al., 2002; Anderson et al., 2004). Upon activation, EGFR translocates from caveolae to costameres, recruits and phosphorylates various downstream signaling molecules (Shah, 2002).

EGFR also are implicated in the regulation of I_{Cl,swell} (Du et al., 2004; Browe & Baumgarten, 2006; Ren et al., 2008). EGFR kinase blockers inhibit activation of I_{Cl,swell} by osmotic swelling, β1 integrin stretch, and AngII, while exogenous EGF elicits I_{Cl,swell} under isosmotic conditions (Browe & Baumgarten, 2006; Ren et al., 2008).

1.6.1.4 PI-3K

PI-3K are heterodimers composed of a p110 catalytic subunit and a regulatory subunit. The predominant PI-3K isoforms expressed in the cardiac myocytes are p110-p85 and p110-p101, which are activated by PTK and β subunits of G proteins, respectively (Kessler et al., 2001; Crackower et al., 2002). It has been demonstrated that PI-3K is activated by myocardial stretch, osmotic swelling, and integrin clustering (Bewick et al., 1999; Petroff et al., 2001; Torsoni et al., 2003). In cardiomyocytes, PI-3K
couples to EGFR and mediates the downstream signaling of EGFR and AT1 receptors (Seshiah et al., 2002; Oudit et al., 2004).

PI-3K inhibitors including wortmannin and LY294002 suppress EGF-, β1 integrin stretch-, and osmotic swelling-induced I_{Cl,swell}, suggesting that PI-3K lies downstream from EGFR in the signaling cascade leading to I_{Cl,swell} activation (Browe & Baumgarten, 2006; Ren et al., 2008).

1.6.1.5. NOX

The predominant NOX isoform expressed in cardiac myocytes is NOX2, and the expression of NOX4 is also reported (Byrne et al., 2003). NOX2 is composed of several components: a transmembrane flavocytochrome b_{558} complex consisting of a large gp91^{phox} (Nox2) and a smaller p22^{phox} subunit, cytosolic p47^{phox} and p67^{phox} subunits, and the small GTP-binding protein Rac (Vignais, 2002; Murdoch et al., 2006; Sirker et al., 2007). To make NOX2 functional, the cytosolic subunits and Rac need to translocate from cytosol to the membrane and assemble with gp91^{phox} and p22^{phox}. This process involves multiple signaling molecules, including PKC, Src and other PTKs, and PI-3K (Vignais, 2002; Murdoch et al., 2006; Sirker et al., 2007). The fully assembled NOX2 is able to transfer electron across the membranes, i.e. from intracellular NADPH or NADH as electron donors to extracellular molecular oxygen, to form O_2^•−, which is rapidly converted to H_2O_2 both spontaneously and by superoxide dismutase (SOD) (Vignais, 2002).
PI-3K has been shown to participate in the activation of NOX and the generation of ROS in response to diverse stimuli in several tissues (Hawkins et al., 2007). NOX is essential for activation of $I_{\text{Cl,swell}}$ in response to $\beta_1$ integrin stretch, AngII, EGF, and osmotic swelling. Blocking NOX with diphenyleneiodonium (DPI), 4-(2-aminoethyl) benzenesulfonfyl fluoride (AEBSF), apocynin or a membrane-permeant fusion peptide inhibitor of NOX assembly (gp91ds-tat) rapidly and completely blocked $I_{\text{Cl,swell}}$ induced by $\beta_1$ integrin stretch, AngII, EGF, and osmotic swelling (Browe & Baumgarten, 2006; Ren et al., 2008).

1.6.2 Mitochondria-Derived ROS and Upstream Mediators

Besides NOX, mitochondria are another source of ROS in cardiomyocytes. Excessive mitochondrial ROS production is observed in cardiomyocytes from experimental models of heart failure (Ide et al., 1999; Ide et al., 2000), myocardial infarction (Ide et al., 2001), and ischemia/reperfusion (Ambrosio et al., 1993). In addition to these pathological processes, recent studies indicate that mitochondrial-derived ROS are involved in the slow force response to myocardial stretch (Caldiz et al., 2007) as well as in the positive inotropic effects of AngII and ET-1 (Cingolani et al., 2006; De Giusti et al., 2008).

Acetylcholine (ACh) was shown to stimulate mitochondrial ROS production in ventricular myocytes via muscarinic receptors (Yao et al., 1999; Oldenburg et al., 2003). ACh-induced mitochondria ROS production is dependent on PI-3K, Src, mitochondrial ATP-sensitive $K^+$ ($\text{Mito}K_{\text{ATP}}$) channel opening (Oldenburg et al., 2003) and is mediated
by transactivation of EGFR through matrix metalloprotease-dependent release of HB-EGF (Krieg et al., 2004). Therefore, ACh and AngII activate essentially the same signaling cascade leading to ROS generation, except that the sources of ROS are thought to be different (mitochondria vs. NOX). The role of mitochondrial ROS in the regulation of I_{Cl, swell} was indicated by the preliminary data showing that exogenous ACh elicits I_{Cl, swell} that is blocked by mitochondria K_{ATP} channel blocker and mitochondrial complex I inhibitor rotenone (Browe & Baumgarten, 2007). Additionally, we also showed that I_{Cl, swell} could be activated by mitochondrial ROS without the involvement of NOX (Deng et al., 2009; Raucci, Jr. & Baumgarten, 2009).

1.6.3. Crosstalk between NOX and Mitochondria

The localization of NOX and the subsarcolemmal mitochondria in the cardiomyocytes raise the feasibility of their crosstalk, and there is some evidence indicating the interaction between NOX and mitochondria in ROS generation and ROS-dependent effects. AngII and ET-1 induce both NOX and mitochondrial ROS production (Seshiah et al., 2002; Kimura et al., 2005a; De Giusti et al., 2008). Inhibition of NOX or blockade of mitoK_{ATP} channel abrogated exogenous AngII-induced ROS production, activation of MAPK pathway, inotropic response, preconditioning, lipid peroxidation and apoptosis of cardiomyocytes (Kimura et al., 2005b; Zhang et al., 2007; Caldiz et al., 2007). A recent study also showed that blockade of either NOX or mitoK_{ATP} channel abolished ET-1-induced ROS production and inotropic effect (De Giusti et al., 2008). Complete abrogation by blockade of either source of ROS indicates that NOX and mitochondria act in serial rather than in parallel.
The underlying mechanism by which NOX interacts with mitochondria has not been illuminated. One possibility is that ROS produced by NOX directly modify and open mitochondria K\textsubscript{ATP} channels (Zhang \textit{et al.}, 2001). Another possibility is ROS-induced ROS release (Zorov \textit{et al.}, 2000).

\subsection*{1.6.4. PKC, Rho/Rho Kinase, and ERK1/2}

Besides ROS and its known upstream mediators, regulation of I\textsubscript{Cl,swell} by PKC, Rho/Rho Kinase and ERK1/2 were also reported. The regulation of I\textsubscript{Cl,swell} by PKC was attributed to the channel itself, i.e. the open probability of the channel was thought to be regulated by the phosphorylation state of the channel protein at a PKC consensus site (Hume \textit{et al.}, 2000). Rho and Rho kinase were shown to exert a permissive effect on the activation of I\textsubscript{Cl,swell} (Tilly \textit{et al.}, 1996b; Nilius \textit{et al.}, 1999). Previous studies also indicate that MAPK pathway plays a role in the regulation of I\textsubscript{Cl,swell}. Selective blocker of ERK1/2 inhibited I\textsubscript{Cl,swell} in ventricular myocytes from failing canine hearts (Clemo & Baumgarten, 1999) and the potentiation of I\textsubscript{Cl,swell} by ET-2 (Du & Sorota, 2000).

\subsection*{1.7. Potential Role of ET-1 in the Regulation of I\textsubscript{Cl,swell}}

ET-1 potentially is a downstream mediator of \(\beta\)1 integrin and AngII AT\(_1\) receptor signaling in the regulation of I\textsubscript{Cl,swell}. ET-1 is released by myocardial stretch and plays an critical role in stretch-induced cardiac hypertrophy (van Wamel \textit{et al.}, 2000; van Wamel \textit{et al.}, 2001; Cingolani \textit{et al.}, 2008) and stretch-induced inotropic effect (Alvarez \textit{et al.}, 1999; Cingolani \textit{et al.}, 2001; Aiello \textit{et al.}, 2002; Cingolani \textit{et al.}, 2003; Cingolani \textit{et al.}, 2005). Both synthesize and release of ET-1 can be stimulated by AngII, and there is
evidence that ET-1 mediates AngII-induced ROS production and inotropic response in cardiomyocytes (Cingolani et al., 2006; Villa-Abrille et al., 2006)

Although stimulatory effects of ET-1 and ET-2 on cardiac I_{Cl,swell} have been reported (Du & Sorota, 2000), the signaling cascades that links ET to I_{Cl,swell} has have not been determined. ET-1 induces NOX activation and ROS generation in various cell types including cardiomyocytes (Cheng et al., 1999; Duerrschmidt et al., 2000; Callera et al., 2003; Li et al., 2003; Amiri et al., 2004; Cingolani et al., 2006), and ET-1 also activates EGFR kinase and PI-3K (Pierce et al., 2001; Pham et al., 2001; Kodama et al., 2002; Sugden, 2002), which were shown to be upstream regulators of NOX in the activation of I_{Cl,swell} by β1 integrin stretch, AngII, and osmotic swelling (Browe & Baumgarten, 2006; Ren et al., 2008). We therefore propose that ET-1 elicits I_{Cl,swell} via a signaling cascade that involves EGFR kinase, PI-3K, NOX, and ROS.

ET-1 activates ERK1 and ERK2 in cardiomyocytes (Bogoyevitch et al., 1993; Bogoyevitch et al., 1994). ERK1/2 blockade inhibited the persistent activation of I_{Cl,swell} in the failing heart (Clemo & Baumgarten, 1999) and ET-2- induced potentiation of I_{Cl,swell} (Du & Sorota, 2000). NOX activation in response to AngII and other stimuli was shown to be dependent on ERK1/2 in non-cardiac cells (Laplante et al., 2003; Hazan-Halevy et al., 2005; Lo et al., 2005). Whether ERK is involved in the activation of NOX by ET-1 in cardiomyocytes and how ERK is involved in the pathway that regulates I_{Cl,swell} remain to be determined.

Besides eliciting ROS generation by NOX, ET-1 also induced mitochondrial ROS production, and mitochondrial source of ROS has been implicated in the inotropic effect
of ET-1 (Touyz et al., 2004; De Giusti et al., 2008). Blocking either NOX or mitoKATP channel cancelled ET-1-induced ROS production and positive inotropic effect, suggesting that NOX and mitochondria act in serial rather than parallel pathways (De Giusti et al., 2008). Furthermore, our preliminary data show that mitochondrial ROS is involved in the regulation of I_{Cl,swell} (Browe & Baumgarten, 2007; Deng et al., 2009; Raucci, Jr. & Baumgarten, 2009). Nevertheless, the role of mitochondrial ROS in the activation of I_{Cl,swell} by ET-1 and the potential interaction between NOX and mitochondria in this process have not been elucidated.

1.8. HL-1 Cells as a Potential Tool to Investigate I_{Cl,swell}

HL-1 cell are an immortalized mouse atrial muscle cell line derived from a primary culture of SV40 large T antegen-induced atrial tumors. This cell line can be repeatedly passaged, while maintaining their ability to contract and retaining differentiated cardiac morphological, biochemical, and electrophysiological properties (Claycomb et al., 1998). HL-1 cells have been used to study cellular signaling, calcium handling, and electrical properties of cardiomyocytes (McWhinney et al., 2000; Sartiani et al., 2002; Akhavan et al., 2003; George et al., 2003; Walker et al., 2007). They have also been used in various pathological models, including hypoxia (Cormier-Regard et al., 1998; Nguyen & Claycomb, 1999), apoptosis (Kitta et al., 2001a; Kitta et al., 2001b; Carlson et al., 2002; Kim et al., 2003), ischemia-reperfusion (Ruiz-Meana et al., 2003; Suzuki et al., 2004; Mirabet et al., 2005; Andersen et al., 2009), and electrical remodeling induced by rapid pacing (Yang et al., 2005).
Previous studies showed that renin-angiotensin system component are expressed in the HL-1 cells (Tsai et al., 2008) and that AngII induces expression of L-type calcium channels via a NOX-ROS dependent pathway (Tsai et al., 2007). It also has been demonstrated that ET-1 induces activation of the cardiac transcription factor GATA-4 and expression of connective tissue factor, suggesting that functional ET receptor-mediated signaling is maintained (Kitta et al., 2001a; Recchia et al., 2009).

No information is available regarding Cl- channels in HL-1 cells. If I_{Cl,swell} is present in HL-1 cells and is regulated by similar mechanism as in native cells, this immortalized cardiac cell line would be a valuable tool for understanding I_{Cl,swell} at the molecular level.

1.9. Aims of the Present Study

There were four principle aims for the present study. The first aim was to determine the signaling cascade by which ET-1 activates I_{Cl,swell}. The roles of ET receptor subtypes, EGFR kinase, PI-3K, ERK, NOX, and ROS were evaluated. The second aim was to examine the interaction between NOX and mitochondria in the ET-1-induced activation of I_{Cl,swell} and to determine if these sources of ROS acted in parallel or in series. The third aim was to determine whether ET-1 signaling cascade that regulates I_{Cl,swell} is downstream from AngII and osmotic swelling or whether it represents a parallel but independent pathway. The fourth aim was to determine whether I_{Cl,swell} is present in HL-1 cells and whether I_{Cl,swell} is regulated by the same mechanism as in native cells.
Chapter 2

MATERIALS and METHODS

2.1. Rabbits and Atrial Myocyte Isolation

Atrial myocytes were freshly isolated from adult New Zealand white rabbits (2.8 – 3.1 kg) of either gender by a pronase-collagenase enzymatic dissociation method. Rabbits were anesthetized with dual intramuscular (i.m.) injections; first a sedative dose of 0.2 ml xylazine HCl (100 mg/ml) administered in combination with 1.0 ml acepromazine maleate (10 mg/ml), followed by an anesthetic dose of 2.5 ml ketamine HCl (100 mg/ml). Hearts were excised, immediately tied via the aorta to the end of a Langendorff column, and retrogradely perfused for 5 minutes with Tyrode solutions that were oxygenated and maintained at 37°C, followed by 5 minutes with a “Ca²⁺-free” Tyrode solution, and then, the heart was perfused with enzyme solution for ~20 minutes. Atria were excised, minced, and placed in fresh enzyme solution. The tissues were bubbled with O₂ and gently shaken for two 15-minute cycles in a 37°C shaker bath. At the end of each cycle the supernatant was collected and replaced with fresh enzyme solution. The supernatants were filtered through 200 µm nylon mesh (Small Parts, Miami Lakes, FL), and isolated cells were pelleted by gentle centrifugation. Isolated myocytes
were washed twice and stored in a modified Kraft-Brühe solution (pH 7.2) before using. Rod-shaped quiescent cells with clear striations and no membrane blebs or other morphological irregularities were studied within 10 hr of isolation.

Tyrode solution for myocyte isolation contained (mM): 130 NaCl, 5 KCl, 1.8 mM CaCl₂, 0.4 KH₂PO₄, 3 MgCl₂, 5 HEPES, 15 taurine, 5 creatine, 10 glucose, pH 7.25 (adjusted with NaOH). For “Ca²⁺-free” Tyrode solution, CaCl₂ was replaced with 0.1 mM Na₂EGTA. For making enzyme solution, the “Ca²⁺-free” Tyrode solution was supplemented with 0.45 mg/ml collagenase (Cls 4; Worthington Biochemical, Lakewood, NJ), and 0.015 mg/ml pronase (Type XIV; Sigma-Aldrich, St. Louis, MO). The modified KB myocyte storage solution contained (mM): 120 K-glutamate, 10 KCl, 10 KH₂PO₄, 0.5 K₂EGTA, 10 taurine, 1.8 MgSO₄, 10 HEPES, 20 glucose, 10 mannitol, pH 7.2 (adjust with KOH).

2.2. Culture of HL-1 cells

HL-1 cardiac myocytes (passage 3 to 17), an immortalized mouse atrial cell line (Claycomb et al., 1998), were also studied. Tissue culture flasks first were coated with gelatin (0.02%)/fibronectin (0.5%) (2 ml in T25 or 6 ml in T75 flask) and incubated at 37°C overnight. HL-1 cells were cultured in pre-coated flasks using Claycomb medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 μg/ml penicillin/streptomycin, 0.1 mM norepinephrine (Sigma-Aldrich) and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). The cells were kept at 37°C in an atmosphere of 5% CO₂ and 95% air at a relative humidity of approximately 95%. Once the HL-1 cells reached confluence, the cells were passaged by splitting 1 to 2. To split the
cells, the cells were rinsed briefly with DPBS and then incubated with 0.05% trypsin/EDTA (3 ml for T25, 6 ml for T75 flask) at 37°C for 3 – 5 min until the cells were dislodged. Equal amount of soybean trysin inhibitor (25 mg/100 ml PBS) was added directly into the cells and the cells were transferred into a 15 ml centrifuge tube and centrifuged at 1100 rpm for 5 min. The supernatant was removed by aspiration and the pellet was gently suspended in 3 ml of supplemented Claycomb medium. The cells were transfer into gelatin/fibronectin-coated flasks for culturing or 15 ml centrifuge tube for electrophysiological studies.

2.3. Experimental Solutions and Drugs

Anion currents were recorded in Na⁺, K⁺, and Ca²⁺-free bath solution with equimolar replacement of Na⁺ and K⁺ with N-methyl-D-glucamine (NMDG⁺), while CaCl₂ was replaced with MgCl₂. In the Na⁺ and K⁺-free pipette solution used for recording anion currents, Na⁺ and K⁺ were replaced with equimolar amounts of Cs⁺. Bath solution contained (mM): 90 N-methyl-D-glucamine (NMDG)-Cl, 3 MgCl₂, 10 HEPES, 10 glucose, 5 CsCl, 0.5 CdCl₂ pH 7.4 (adjusted with CsOH), 70 mannitol and was isosmotic (300 mOsm/L; 1T). Mannitol was omitted in hyposmotic solutions to reduce osmolarity to 0.7-times (0.7T) that in isosmotic solutions, reduced to 35 mM to make 0.85T solution, and increased to 220 mM to make 1.5T hyperosmotic solution. By varying the mannitol concentration, solution osmolarity was adjusted at a constant ionic strength and avoided unintended alteration of ion activities. Pipette solution contained (mM): 110 Cs-aspartate, 20 CsCl, 2.5 Mg-ATP, 8 Cs₂-EGTA, 0.1 CaCl₂, 10 HEPES, pH 7.1 (adjusted with CsOH; liquid junction potential, −15 mV). The pipette free-Ca²⁺ was
~35 nM (WinMAXC ver 2.4; www.stanford.edu/~cpatton/maxc.html). $I_{\text{Cl,swell}}$ is essentially time-independent under these conditions (Ren & Baumgarten, 2005). Pipette and bath solution osmolarities were verified by freezing-point depression.

Endothelin-1 (200 µM, Calbiochem/EMD, Gibbstown, NJ) was prepared as stock solutions in 5% acetic acid and kept frozen (~20°C) in small aliquots until use. Angiotensin II (5 µM, Calbiochem) and BQ123 (1 mM) were dissolved in H$_2$O and kept frozen (~20°C). Stock solutions of BQ788 (100 µM, Calbiochem), AG1478 (1 mM, Calbiochem), wortmannin (1 mM, Calbiochem), LY294002 (50 mM, Calbiochem), PD98059 (10 mM, Calbiochem), U0216 (1 mM, Biomol International/Enzo Life Sciences, Plymouth Meeting, PA), ebselen (15 mM, Calbiochem), rotenone (20 mM, Sigma-Aldrich), diazoxide (4.33 mM, Sigma-Aldrich), and antimycin A$^1$ (20 mM, Sigma-Aldrich) were prepared in DMSO and frozen (~20°C) in small aliquots until use. EGF (3.3 µM, Calbiochem) and losartan (5 µM, Merck) were dissolved directly in bath solution and kept frozen (~20°C). The NOX inhibitor gp91ds-tat was synthesized by the Tufts University Core Facility, and the peptide stocks (1.2 mg/ml) were made in 150 mM NaCl plus 10 mM acetic acid and frozen (~20°C) in aliquots until use. DCPIB (4-[2-buty1-6,7-dicholo-2 cyclopentyl-1,2,3 -dihydro-1-oxo-1H-inden-5-yl) oxy] butanoic acid; 20 mM; Tocris Bioscience, Ellisville, MO) was prepared as a stock solution in ethanol.

$^1$ Antimycin A contains four closely related components, antimycin A1 (538.6 Da), A2 (534.6 Da), A3 (522.6 Da), and A4 (510.6 Da). Assays provided by Sigma-Aldrich indicated that the percentage of each component varied in the lots used: A1, 33 – 75%; A2, 19 – 20%; A3, 5 – 32%; A4, 10 – 18%. Therefore, solutions were prepared using the weighted average molecular weight.
and kept refrigerated (−4°C). A stock solution of apocynin (500 mM, Sigma-Aldrich) was prepared in DMSO and kept at room temperature until use.

2.4. Whole Cell Patch Clamp and Electrophysiological Recordings

Pipettes were manufactured from 7740 thin-walled borosilicate glass capillary tubing (1.5 mm o.d., 1.12 mm i.d., filament; Sutter Instrument, Novato, CA) using a P-97 micropipette puller (Sutter Instrument) and then fire polished. The final pipette tip diameter was 2 – 3 µm, and the corresponding pipette resistance in bath solution was 2 – 4 MΩ. Junction potentials were corrected, and a 3-M KCl-agar bridge served as the ground electrode. Freshly isolated atrial myocytes and HL-1 cells were dispersed over a glass bottomed cell chamber (~0.3 ml) mounted onto the stage of an inverted microscope (Diaphot; Nikon, Inc., Garden City, NY), and visualized with Hofmann modulation optics (40X; NA = 0.55) and a high resolution TV camera (CCD72; Dage-MTI; Michigan City, IN) that projected images onto a video monitor. A fluid flow system was attached to the cell chamber and bath solution was superfused at a rate of 2 – 3 ml/min. Typical seal resistances were 5 – 30 GΩ, and myocytes were dialyzed for at least 10 min before data were collected. Successive 500-ms steps were made from −60 mV to test potentials between −100 and +60 mV in +10 mV increments, and current-voltage (I–V) relationships were plotted from quasi steady-state currents. Currents were recorded with an Axoclamp 200B and Digidata 1322A under pClamp 9 (MDS Analytical Technologies, Sunnyvale, CA); they were digitized (5 kHz) after low-pass filtering (Bessel, 2 kHz) and were refiltered (Bessel, 500 Hz) in PClamp for presentation.
2.5. ROS Detection by Flow Cytometry

ROS production was assessed in HL-1 cells rather than freshly isolated myocytes, which unavoidably include damaged myocytes in the preparation. ROS was detected with C-H2DCFDA-AM [6-carboxy-2',7'-dichlorodihydro-fluorescein diacetate di(acetoxy-methyl ester)] (Invitrogen), which is converted into a non-fluorescent derivative (carboxy-H2DCF) by intracellular esterases. Carboxy-H2DCF is retained in the cytoplasm and is oxidized to fluorescent carboxy-DCF by intracellular ROS. Confluent cells were incubated with C-H2DCFDA-AM (5 µM) for 30 min at 37 °C, washed twice with DPBS, and then isolated using the splitting procedure. Single myocytes suspensions in DPBS were analyzed using an EPICS XL cytometer (Beckman Coulter). The geometric means of gated fluorescence distributions (excitation: 485nm; emission: 520 nm) were calculated using the EXPO32 software (Beckman Coulter). After gating, 30,000 to 40,000 cells contributed to each fluorescence distribution. Fluorescence histograms (1024 bins) were exported to Excel (Microsoft) and plotted in SigmaPlot (Systat) after filtering (5 point running average, replotted in 512 bins); this gave plots that were comparable to those displayed with the commonly used smoothing function in EXPO32.

2.6. 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. One-way repeated-measures ANOVA were performed, and pairwise comparisons were made by the Holm-Sidak method with an overall significance level of
$P < 0.05$ in SigmaStat 3.11 (Systat). For clarity, percent block ($\pm$SEM) of the drug-activated current is reported for several interventions and was calculated using each myocyte as its own control. Non-linear fitting of current activation was done in SigmaPlot10.1 (Systat). Some of the fluorescence histograms data were not normally distributed and were analyzed using Kruskal-Wallis One Way Analysis of Variance on Ranks followed by pairwise comparisons by Dunn's method in SigmaStat. These data are plotted using a box plot to denote the $25\%$, $50\%$, and $75\%$ percentiles and error bars to denote the $10\%$ and $90\%$ percentiles.
3.1. ET-1 Activates an Outwardly Rectifying Cl\(^-\) current with Properties of \(I_{Cl,swell}\)

Exposure to 10 nM ET-1 for 10 min elicited an outwardly rectifying Cl\(^-\) current that reversed near \(E_{Cl}\) (-43 mV). Families of membrane currents (Fig. 1A) and the corresponding I–V relationships (Fig. 1B) from typical recordings are shown. ET-1-induced currents were abolished by DCPIB (Fig.1 A, B), a highly selective \(I_{Cl,swell}\) inhibitor (Decher et al., 2001). Fig. 1C presents summary data from cells exposed to DCPIB in continued presence of ET-1. ET-1 (10 nM, 10 min) increased the outward Cl\(^-\) current at +60 mV from 0.54 ± 0.04 to 1.69 ± 0.09 pA/pF (\(n = 57, P < 0.001\)). Addition of DCPIB (10 \(\mu\)M, 6–12 min) with continued presence of ET-1 inhibited 99 ± 4% (\(n = 5, P < 0.001\)) of ET-1-induced current at +60 mV, and the current after DCPIB application was not significantly different from the control currents (\(n = 5, P = 0.84\)).

Volume sensitivity is one of the important characteristics of \(I_{Cl,swell}\). ET-1-induced current was abrogated by osmotic shrinkage with 1.5- times isomotic (1.5T) solution (Fig.1 D, E, F). Application of 1.5T solution containing ET-1 decreased the ET-1-induced Cl\(^-\) current at +60 mV from 1.79 ± 0.39 to 0.74 ± 0.22 pA/pF, an inhibition of 96 ± 6% (\(n = 4, P < 0.001\)).
**Figure 1:** ET-1 activated $I_{\text{Cl,swell}}$ in atrial myocytes. ET-1-induced Cl$^{-}$ current was outwardly rectifying and suppressed by DCPIB, a highly selective $I_{\text{Cl,swell}}$ blocker, and by osmotic shrinkage in 1.5-times isosmotic (1.5T) bath solution.  

(A, D) Families of currents before and after exposure to ET-1 (10 nM, 10 min) and after addition of DCPIB (10 μM, 5-10 min; A) or osmotic shrinkage in 1.5T (5-10 min, D) bath in the continued presence of ET-1. (B, E) I-V relationships for A and D. Kinetics of fractional activation of ET-1-induced current (B, Inset). Data for each experiment were individually fitted to sigmoidal relationship, $Y = 1/(1+\exp(-(x-x_0)/b))$, which gave $5.05 \pm 0.46$ for half-time for activation ($x_0$) and $1.51 \pm 0.15$ for slope factor (b). At +60 mV, ET-1-induced current was $1.2 \pm 0.1 \text{ pA/pF}$ ($n = 57$).  

(C, F) Normalized current from cells exposed to DCPIB and 1.5T solution in continuous presence of ET-1. DCPIB blocked $99.4 \pm 3.6\%$ ($n = 5$, $P < 0.01$) and osmotic shrinkage $95.4 \pm 5.7\%$ ($n = 4$, $P < 0.01$) of current elicited by ET-1.
3.2. ET-1 Activated $I_{Cl,swell}$ via ET$_A$ but not ET$_B$ Receptors

ET-1-induced Cl$^-$ current was suppressed by selective ET$_A$ blocker BQ123 but not by ET$_B$ blocker BQ 788 (Fig. 2). Addition of BQ 123 (10 µM, 10 min) with continued presence of ET-1 reduced the current at +60 mV from $1.37 \pm 0.22$ pA/pF to $0.57 \pm 0.08$ pA/pF, a value that was not significantly different from the control ($n = 4$, $P = 0.65$). At +60 mV, BQ123 inhibited ET-1 induced currents by $90 \pm 3\%$ ($n = 4$, $P < 0.01$), indicating that ET-1-induced current required activation of ET$_A$ receptors. In contrast, the selective ET$_B$ blocker BQ 788 (100 nM, 15 – 20 min) had no effect on the ET-1-induced current ($n = 4$, $P = 0.84$).

3.3. EGFR Kinase Regulates ET-1-induced $I_{Cl,swell}$

Based on evidence that EGFR kinase is activated by ET-1 in cardiomyocytes (Kodama et al., 2002) and its role in the activation of $I_{Cl,swell}$ (Browe & Baumgarten, 2006; Ren et al., 2008), we hypothesized that ET-1 elicited $I_{Cl,swell}$ via EGFR kinase. We tested the role of EGFR kinase in ET-1-induced activation of $I_{Cl,swell}$ using the selective EGFR kinase inhibitor AG1478. Fig.3 shows the normalized current of the cells that were exposed to ET-1 (10 nM, 10 min) and after addition of AG1478 (10 µM, 5 – 10 min) in the presence of ET-1. AG1478 (10 µM, 10 – 15 min) inhibited the ET-1-induced Cl$^-$ current by $88 \pm 3\%$ ($n = 5$, $P < 0.01$), reducing outward Cl$^-$ current at +60 mV from to $0.50 \pm 0.16$ to $1.83 \pm 0.47$ pA/pF, and the current after application of AG1478 (0.64 ± 0.17 pA/pF) was not significantly different from the control ($n = 5$, $P = 0.63$).

3.4. PI-3K is Involved in the Activation of $I_{Cl,swell}$ Induced by ET-1

There is evidence that EGFR kinase couples to PI3K (Shah et al., 2006) and PI-3K is involved in the regulation of $I_{Cl,swell}$ by β1-integrin stretch and osmotic swelling
Figure 2: ET-1 activated $I_{\text{Cl,swell}}$ via $\text{ET}_A$ but not $\text{ET}_B$ receptors. (A, D) Family of currents before and after exposure to ET-1 (10 nM, 10 min) and after addition of selective $\text{ET}_A$ blocker BQ123 (10 μM, 5-10 min; A) or $\text{ET}_B$ blocker BQ788 (100 nM, 15 – 20 min, D) in the presence of ET-1. (B, E) I-V relationships for A and D. (C, F) Current densities at +60 mV. $\text{ET}_A$ blocker BQ123 suppressed 90.5 ± 2.5% ($n = 4, P < 0.01$) of ET-1-induced $I_{\text{Cl,swell}}$, but $\text{ET}_B$ blocker BQ788 was ineffective ($n = 4, P = 0.84$). Signaling cascade is summarized in insets. The cascade denoted by $\times$ was excluded.
Figure 3: EGFR kinase and PI-3K were downstream from ET-1 in the signaling cascade leading to $I_{Cl, swell}$. After $I_{Cl, swell}$ was activated by ET-1 (10 nM, 10 min), the EGFR kinase blocker AG1478 (10 µM, 10 – 15 min) or the PI-3K blockers LY294002 (20 µM, 10 – 15 min) or wortmannin (500 nM, 8 – 15 min) were added in the continued presence of ET-1. AG1478 reduced the current at +60 mV from 1.83 ± 0.47 pA/pF to 0.64 ± 0.17 pA/pF (88.0 ± 3.0%; $n = 5$, $P < 0.01$), LY294002 from 1.63 ± 0.37 pA/pF to 0.45 ± 0.07 pA/pF (101.9 ± 7.0%; $n = 4$, $P < 0.01$) and wortmannin from 1.62 ± 0.18 pA/pF to 0.56 ± 0.05 pA/pF (91.7 ± 2.9%; $n = 4$, $P < 0.01$). Signaling cascade is summarized in insert. Previous studies (Browe & Baumgarten, 2006; Ren et al., 2008) demonstrated that EGFR is upstream from PI-3K and that PI-3K is upstream from $I_{Cl, swell}$. 
To evaluate signaling molecules downstream from EGFR kinase, we tested whether PI-3K participates in the activation of $I_{\text{Cl,swell}}$ in response to ET-1. LY 294002 and wortmannin are reversible and irreversible blockers of PI-3K, respectively. As shown in Fig. 3, both LY 294002 (10 µM, 10 – 15 min) and wortmannin (500 nM, 8 – 15 min) fully suppressed ET-1-induced activation of $I_{\text{Cl,swell}}$. Exposure to LY 294002 (10 µM) and wortmannin (500 nM) in the continued presence of ET-1 decreased the outward current at +60 mV by 102 ± 7% ($n = 4$, $P = 0.01$) and 92 ± 3% ($n = 4$, $P < 0.001$), respectively, and the currents after LY 294002 and wortmannin were not significantly different from the control ($n = 4$, $P = 0.94$ and $n = 4$, $P = 0.58$, respectively).

3.5. ROS are Required Intermediates in the ET-1-Induced Activation of $I_{\text{Cl,swell}}$

Previously we showed that ROS are required intermediates in the regulation of $I_{\text{Cl,swell}}$ by stretch, osmotic swelling and several signaling molecules (Browe & Baumgarten, 2004; Browe & Baumgarten, 2006; Ren et al., 2008). We examined the role of ROS in the ET-1-induced activation of $I_{\text{Cl,swell}}$ using ebselen, a membrane-permeant glutathione peroxidase mimetic that dismutates $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$. Figure 4 A and B shows the family of currents and I-V curves before and after stimulation of $I_{\text{Cl,swell}}$ with ET-1 (10 nM, 10 min) and after adding ebselen (15 µM, 15 min) in presence of ET-1. Fig. 4C shows the normalized current. Ebselen reduced the outward current at +60 mV from 1.80 ± 0.36 pA/pF to 0.45 ± 0.15 pA/pF, a block of 112.6 ± 10.2% ($n = 4$, $P < 0.01$).

3.6. NOX is Critically Involved in the Activation of $I_{\text{Cl,swell}}$ by ET-1

NOX has been implicated as the source of ROS that activates $I_{\text{Cl,swell}}$ (Browe & Baumgarten, 2004; Browe & Baumgarten, 2006; Ren et al., 2008). To test whether ET-1
Figure 4: ROS were required intermediates in ET-1-induced activation of $I_{Cl,swell}$.  
(A) Families of currents before and after stimulation of $I_{Cl,swell}$ by ET-1 (10 nM, 10 min) and after adding ebselen (15 µM, 15 min) in presence of ET-1.  Ebselen is a membrane-permeant glutathione peroxidase mimetic that dismutates $H_2O_2$ to $H_2O$.  (B) IV curves for A.  (C) Normalized current at +60 mV from cells exposed to ebselen in continuous presence of ET-1.  Ebselen inhibited 112.6 ± 10.2% ($n = 4$, $P < 0.01$) of the ET-1-induced current.  Signaling cascade is summarized in inset.
signaling requires NOX activity, we used apocynin, a selective NOX inhibitor. Fig. 5 shows that apocynin suppressed the current elicited by ET-1. ET-1 (10 nM, 10 min) increased the outward current at +60 mV from $0.43 \pm 0.08$ pA/pF to $1.16 \pm 0.18$ pA/pF. Apocynin (500 µM, 10 – 15 min) applied in continued presence of ET-1 suppressed 85.8 ± 6.5% ($n = 5$, $P < 0.001$) of the ET-1-induced current, and currents after block were not significantly different from control ($n = 5$, $P = 0.356$).

3.7. Mitochondrial ROS are Required for the ET-1-Induced Activation of $I_{Cl,swell}$

Although NOX is a required source of ROS, it remains unclear whether it is the only source of ROS involved in the activation of $I_{Cl,swell}$. An important alternative is mitochondria, and Complex III plays a central role in mitochondrial ROS generation (Chen et al., 2003). The highly selective Complex I inhibitor rotenone suppresses $e^-$ flow to Complex III and inhibits ROS generation by intact mitochondria (Chen et al., 2003). As shown in Fig. 6, application of rotenone (10 µM, 25 – 45 min) in the continued presence of ET-1 reduced the outward current at +60 mV from $1.90 \pm 0.16$ pA/pF to $0.96 \pm 0.14$ pA/pF ($n = 4$, $P < 0.001$), which amounts to block of 84.5 ± 4.4%, and the current after application of rotenone was not significantly different from control ($n = 4$, $P = 0.14$).

3.8. Activation of $I_{Cl,swell}$ by Mitochondrial ROS is Downstream from NOX

ET-1-induced $I_{Cl,swell}$ was completely blocked by inhibitors of either ROS source, suggesting that NOX and mitochondria must act in series rather than in parallel. To verify that mitochondrial ROS can activate $I_{Cl,swell}$, we used the Complex III inhibitor antimycin A and the mitoK$_{ATP}$ agonist diazoxide, which are both known to stimulate mitochondrial ROS production (Chen et al., 2003; Busija et al., 2005; Drose et al., 2009).
Figure 5: NADPH oxidase (NOX) was critically involved in the activation of $I_{\text{Cl,swell}}$ by ET-1. (A) Families of currents before and after activation of $I_{\text{Cl,swell}}$ by ET-1 (10 nM, 10 min) and after application of highly selective NOX inhibitor apocynin (500 nM, 10-15 min) in presence of ET-1. (B) I-V relationships for (A). (C) Current densities at +60 mV for activation of $I_{\text{Cl,swell}}$ by ET-1 and its block by apocynin. Apocynin suppressed 85.8 ± 6.5% ($n = 5$, $P < 0.001$) of the ET-1-induced current, and currents after block were not significantly different than control ($n = 5$, $P = 0.356$). Signaling cascade is summarized in insets.
Figure 6: ROS from mitochondria were required for ET-1-induced activation of $I_{\text{Cl,swell}}$. (A) Families of currents before and after activation of $I_{\text{Cl,swell}}$ by ET-1 (10 nM, 10 min) and after application of the highly selective mitochondrial electron transport blocker rotenone (10 µM, 25-45 min) in presence of ET-1. (B) IV relationships for (A). (C) Current densities of the cells exposed to rotenone in the continued presence of ET-1. Rotenone inhibited 84.5 ± 4.4% ($n = 4$, $P < 0.001$) of the ET-1-induced current, and current after block was not significantly different than control ($n = 4$, $P = 0.14$). Signaling cascade is summarized in insets.
As expected, both antimycin A and diazoxide elicited $I_{\text{Cl,swell}}$ (Fig. 7A, B). Antimycin A (10 µM, 5 – 10 min) increase the outward current at +60 mV from $0.54 \pm 0.11 \, \text{pA/pF}$ to $1.40 \pm 0.14 \, \text{pA/pF}$ ($n = 9$, $P < 0.001$) and diazoxide (50 µM, 5 – 10 min) from $0.60 \pm 0.13 \, \text{pA/pF}$ to $1.78 \pm 0.47 \, \text{pA/pF}$ ($n = 5$, $P < 0.01$).

Next we tested whether production of ROS by NOX was downstream from mitochondrial ROS in the activation of $I_{\text{Cl,swell}}$. Contrary to this scheme, $I_{\text{Cl,swell}}$ elicited by antimycin A and diazoxide was insensitive to two selective NOX blockers, apocynin (500 µM, 10 – 15 min; $n = 5$, $P = 0.69$, Fig. 7A) and the fusion peptide gp91ds-tat (500 nM, 15 – 20 min; Fig. 7A: $n = 6$, $P = 0.14$; Fig. 7B: $n = 5$, $P = 0.92$). These data and the finding that inhibitors of both NOX and mitochondrial ROS production (Fig. 5 and 6) fully suppressed $I_{\text{Cl,swell}}$ suggest that NOX must be upstream of mitochondrial ROS production rather than downstream.

**3.9. $I_{\text{Cl,swell}}$ Induced by Mitochondrial ROS is Insensitive to Osmotic Shrinkage**

Previously we showed that osmotic shrinkage suppressed the $I_{\text{Cl,swell}}$ elicited by EGF but failed to inhibit activation of $I_{\text{Cl,swell}}$ in response to $\text{H}_2\text{O}_2$, which is the most distal regulator of $I_{\text{Cl,swell}}$ in the proposed signaling pathway (Ren et al., 2008). We examined whether osmotic shrinkage-induced signaling is upstream from mitochondrial ROS. Fig. 8 shows that $I_{\text{Cl,swell}}$ induced by the complex III inhibitor antimycin A was insensitive to osmotic shrinkage with 1.5T bath solution. Antimycin A (10 µM, 5-10 min) increased the outward current +60 mV from $0.53 \pm 0.11 \, \text{pA/pF}$ to $1.86 \pm 0.07 \, \text{pA/pF}$ ($n = 4$, $P < 0.001$), and the current after switching to hyperosmotic bath solution (1.5T, 15 – 25 min) containing antimycin A was $1.92 \pm 0.13 \, \text{pA/pF}$, which was not significantly different from the value before osmotic shrinkage ($n = 4$, $P = 0.75$).
Figure 7: Activation of $I_{\text{Cl,swell}}$ by mitochondrial ROS was downstream from NADPH oxidase (NOX). Antimycin A ($A$; 10 µM), an inhibitor of Complex III, and diazoxide ($B$; 50 µM), a mitoK$_{ATP}$ agonist, are known to stimulate mitochondrial ROS production, and both elicited $I_{\text{Cl,swell}}$. Mitochondrial ROS-induced $I_{\text{Cl,swell}}$ was insensitive to two selective NOX blockers, apocynin (500 µM, 10-15 min; $n = 5$, $P = 0.69$) and the fusion peptide gp91ds-tat (500 nM, 15-20 min; $A$: $n = 6$, $P = 0.14$; $B$: $n = 5$, $P = 0.92$). Failure of apocynin and gp91ds-tat to block $I_{\text{Cl,swell}}$ implied that mitochondrial ROS production must be downstream from NADPH oxidase in the signaling cascade. Diagrams of signaling cascades assuming mitochondria were upstream (right) or downstream (left) from NADPH oxidase are shown as inserts. The cascade denoted by $\times$ (left) was excluded.
Figure 8: $I_{Cl,swell}$ induced by mitochondrial ROS was insensitive to osmotic shrinkage. (A) Families of currents before and after activation of $I_{Cl,swell}$ by antimycin A (10 µM, 5-10min) and after application of 1.5 times osmotic (1.5T) solution. (B) IV relationships for A. (C) Current densities for cells exposed to 1.5T solution in the continued presence of antimycin A. Antimycin A (10 µM, 5-10min) increased the outward current +60 mV from $0.53 \pm 0.11 \text{ pA/pF}$ to $1.86 \pm 0.07 \text{ pA/pF}$ ($n = 4$, $P < 0.001$), and the current after switching to hyperosmotic bath solution (1.5T, 15-25 min) with antimycin A was $1.92 \pm 0.13 \text{ pA/pF}$, which was not significantly different from the value before osmotic shrinkage ($n = 4$, $P = 0.75$).
3.10. Role of ERK in the Activation of $I_{cl,swell}$ Induced by ET-1, EGF, and H$_2$O$_2$

ERK is involved in the activation of $I_{cl,swell}$ in failing heart (Clemo & Baumgarten, 1999) and potentiation of $I_{cl,swell}$ by ET-2 (Du & Sorota, 2000). In order to determine the role of ERK in the signaling pathway that regulates $I_{cl,swell}$, we examined the effect of ERK inhibitors on the current elicited by EGF, ET-1, and H$_2$O$_2$. As illustrated in Fig. 9A, EGF-induced $I_{cl,swell}$ was fully inhibited by the ERK1 blocker PD98059 and ERK1/2 blocker U0216. EGF (10 nM, 10 min) increased the outward current at +60 mV by $1.16 \pm 0.03$ pA/pF, from $0.57 \pm 0.13$ pA/pF to $1.73 \pm 0.16$ pA/pF ($n = 9$, $P < 0.001$). At +60 mV, PD98059 (10 µM, 10 min) reduced the EGF-induced current by $101.4 \pm 7.0\%$, from $1.81 \pm 0.22$ pA/pF to $0.56 \pm 0.13$ pA/pF ($n = 5$, $P < 0.01$) and U0126 (5 µM, 10 min) blocked $105.5 \pm 5.1\%$, reducing the current from $1.63 \pm 0.27$ pA/pF to $0.52 \pm 0.16$ pA/pF ($n = 4$, $P < 0.01$). In contrast, ET-1-induced $I_{cl,swell}$ (10 nM, 10 min) was partially inhibited by PD98059 and U0126 (Fig. 9B). PD98059 (10 µM, 15 min) blocked $69.9 \pm 4.0\%$ ($n = 5$, $P < 0.05$) and U0216 (5 µM, 15 min) inhibited $68.6 \pm 7.4\%$ ($n = 5$, $P < 0.01$) of ET-1-induced current. Although these concentrations of PD98059 and U0126 were sufficient to fully suppress $I_{cl,swell}$ elicited by EGF, it was important to verify their potency in response to ET-1. Increasing the concentration of PD98059 from 10 to 30 µM and U0216 from 5 to 10 µM in the same cells did not result in additional block (Fig. 9B). As shown in Fig. 9C, exogenous H$_2$O$_2$ (100 µM, 5 min) induced activation of $I_{cl,swell}$, and PD98059 (10 µM, 15 min) had no effect on the H$_2$O$_2$-induced current ($n = 4$, $P = 0.57$).
Figure 9: ERK-dependent and ERK-independent activation of $I_{\text{Cl,swell}}$ by ET-1, EGF and $H_2O_2$. (A) EGF-induced $I_{\text{Cl,swell}}$ (10 nM, 10 min) was fully inhibited by the ERK1 blocker PD98059 (10 µM, 15 min; 101.4 ± 7.0%, $n = 5$, $P < 0.01$) and the ERK1/2 blocker U0126 (5 µM, 10 min; 105.5 ± 5.1%, $n = 4$, $P < 0.01$). (B) By contrast, ET-1-induced $I_{\text{Cl,swell}}$ (10 nM, 10 min) was partially inhibited by ERK blockers. PD98059 (10 µM) blocked 69.9 ± 4.0% of ET-1-induced $I_{\text{Cl,swell}}$ ($n = 5$, $P < 0.02$); increasing PD98059 to 30 µM in 2 of these cells had no additional effect. Similarly, U0126 (5 µM) blocked 68.6 ± 7.4% ($n = 5$, $P < 0.02$), and increasing U0126 to 10 µM ($n = 5$) in the same cells had no additional effect. (C) $H_2O_2$ (100 µM, 5 min) elicits $I_{\text{Cl,swell}}$ at a site distal to ERK and was unaffected by PD98059 ($n = 4$, $P = 0.57$).
3.11. **AngII Elicited I_{Cl,swell} via ET\_A Receptors**

The results described so far indicate that activation of I_{Cl,swell} by ET-1 involve some of the same signaling molecules that are involved in the activation of I_{Cl,swell} by AngII (Browe & Baumgarten, 2004; Browe & Baumgarten, 2006; Ren et al., 2008). This raises a question: Are ET-1 and AngII part of a single signaling cascade or do they represent two independent signaling pathways that converge? If AngII and ET-1 act in series, block of one should suppress activation by the other peptide. Consistent with a series arrangement with ET-1 downstream from AngII, Fig.10 A shows that activation of I_{Cl,swell} by AngII (5 nM, 5 – 10 min) was completely abolished by ET\_A receptor blockade. Application of AngII (5 nM, 5 – 10 min) increased the outward current at +60mV from 0.40 ± 0.02 pA/pF to 1.38 ± 0.24 pA/pF and selective ET\_A receptor blocker BQ123 (1 µM, 5 – 10 min) blocked 92 ± 3% (n = 5, P < 0.01) of AngII-induced current. In contrast, the AT\_1R blocker losartan (5 µM, 15 min) did not alter ET-1-induced I_{Cl,swell} (n = 4, P = 0.92) (Fig.10 B).

3.12. **Osmotic Swelling Elicited I_{Cl,swell} via ET\_A Receptors**

Previously we showed that osmotic swelling activates I_{Cl,swell} via AngII AT\_1 signaling (Ren et al., 2008). This and our observation that ET-1 ET\_A receptor is downstream from AngII in the cascade regulating I_{Cl,swell} imply that the response to osmotic swelling also should be suppressed by an ET\_A inhibitor. Confirming this idea, Fig. 10 C shows that BQ123 inhibited the activation of I_{Cl,swell} in response to osmotic swelling. Osmotic swelling (0.7T, 10 min) induced the outward current at +60mV from 0.67 ± 0.16 pA/pF to 1.80 ± 0.42 pA/pF and application of the selective ET\_A receptor blocker BQ123 (1 µM, 5 – 10 min) in 0.7T solution blocked 91 ± 4% of osmotic swelling-induced current (n = 4, P <0.01).
**Figure 10: AngII and osmotic swelling (0.7T) elicited $I_{Cl,swell}$ via $EA$ receptors.** (A) Activation of $I_{Cl,swell}$ by AngII (5 nM, 5-10 min) was suppressed (92.0 ± 3.2%; $n = 5$, $P < 0.01$) by the $EA$ blocker BQ123 (1 µM, 5 – 10 min). (B) Confirming AT1 was upstream from EA, the AT1 blocker losartan (5 µM, 15 min) did not alter ET-1-induced $I_{Cl,swell}$ ($n = 4$, $P = 0.92$). (C) $I_{Cl,swell}$ elicited by osmotic swelling (0.7T, 5 min) was abolished (91 ± 4%; $n = 4$, $P < 0.01$) by BQ123 (1 µM, 5 – 10 min). Signaling schemes are shown in inserts. The cascade denoted by $\times$ (middle) was excluded.
Chapter 4

RESULTS IN CULTURED MOUSE HL-1 ATRIAL CELLS

Immortalized HL-1 murine atrial myocytes share a number of phenotypic properties with adult atrial myocytes and recently have emerged as a useful alternative for studying cardiac physiology and pathophysiology (Claycomb et al., 1998; White et al., 2004). Although several cation channels have been described in HL-1 cells, the presence and behavior of Cl⁻ channels has not been considered. This chapter documents the presence and regulation of I_{Cl,swell} in HL-1 cells and makes use of this cell line to study ET-1-induced ROS production by flow cytometry.

4.1. I_{Cl,swell} is Present in the Mouse Atrial HL-1 Cells

The effect of osmotic swelling in HL-1 myocytes was studied in 0.85T rather than 0.7T hypoosmotic bath solution because exposure to 0.7T rapidly caused all the cells in the chamber to rupture. Despite applying a more modest osmotic challenge than was used with adult myocytes, 0.85T (5 – 10 min) elicited a robust outwardly rectifying current in HL-1 cells. With a physiologic Cl⁻ gradient, osmotic swelling increased the outward current at +60 mV from 4.20 ± 0.93 pA/pF to 30.69 ± 2.75 pA/pF (n = 7, P <0.001), and
the selective $I_{\text{Cl,swell}}$ inhibitor DCPIB blocked 110.5 ± 11.2\% ($n = 6$, $P < 0.001$) of the swelling-induced current (Fig 11).

To verify that the outwardly rectifying current was $I_{\text{Cl,swell}}$, we also studied it with a symmetrical Cl$^-$ gradient. $I_{\text{Cl,swell}}$ undergoes outward rectification in both physiological and symmetrical Cl$^-$, whereas a number of other Cl$^-$ currents are linear under symmetrical conditions (Hume et al., 2000). As expected for $I_{\text{Cl,swell}}$, Fig. 12 shows that the current elicited by osmotic swelling in symmetrical Cl$^-$ remained outwardly rectifying. Swelling increased the current at +60 mV from 0.45 ± 0.18 pA/pF to 12.19 ± 0.58 pA/pF ($n = 4$, $P < 0.001$), and DCPIB blocked 100.9 ± 2.9\% ($n = 4$, $P < 0.001$).

4.2. Exogenous H$_2$O$_2$ Elicits $I_{\text{Cl,swell}}$ in HL-1 Cells

Another characteristic of $I_{\text{Cl,swell}}$ in adult myocytes is its activation by H$_2$O$_2$ (Browe & Baumgarten, 2004). Exogenous H$_2$O$_2$ also induced an outwardly rectifying Cl$^-$ current that was sensitive to DCPIB in HL-1 cells (Fig. 13). H$_2$O$_2$ (100µM, 5-10min) increased the outward current at +60 mV from 2.70 ± 1.23 to 24.18 ± 4.40 pA/pF ($n = 6$, $P < 0.001$), and DCIPB blocked 101.3 ± 12.6\% ($n = 4$, $P < 0.001$) of the H$_2$O$_2$-induced current.

4.3. NOX Regulates Swelling-Induced $I_{\text{Cl,swell}}$ in HL-1 Cells

Next we studied whether ROS were involved in the activation of $I_{\text{Cl,swell}}$ in HL-1 cells upon osmotic swelling and identified the source of ROS. Previous studies showed that the selective NOX inhibitor apocynin fully inhibited the osmotic swelling-induced $I_{\text{Cl,swell}}$ in rabbit ventricular cardiomyocytes (Ren et al., 2008), and we showed (Fig. 5)
Figure 11: Osmotic swelling activated a DCPIB-sensitive outwardly rectifying Cl\textsuperscript{−} current in HL-1 cells with physiological Cl\textsuperscript{−} gradient. (A) Families of currents before and after exposure to 0.85T solution (5-10 min) and after addition of DCPIB (10 μM, 5-10 min) in the presence of 0.85T solution. (B) I-V relationships for A. Cell capacitance was 24.8 pF. (C) Normalized current for cells exposed to DCPIB in 0.85 T solution. DCPIB blocked 110.5 ± 11.2% of swelling-induced current (n = 6, P <0.001).
Figure 12: Osmotic swelling induced an outwardly rectifying Cl\(^-\) current in HL-1 cells with symmetrical Cl\(^-\) gradient. (A) Families of currents before and after exposure to 0.85T bath solution (5-10 min) and after addition of DCPIB (10 \(\mu\)M, 5-10 min) in the 0.85T bath solution. (B) I-V relationships for A. Cell capacitance was 76.5 pF. (C) Normalized current for cells that were exposed to DCPIB in 0.85 T bath solution. DCPIB blocked 100.9 ± 2.9% of swelling-induced current \((n = 4, P < 0.001)\).
Figure 13: H₂O₂ activated I Clswell in HL-1 cells. H₂O₂ elicited an outwardly rectifying Cl⁻ current that was blocked by DCPIB. (A) Families of currents before and after exposure to H₂O₂ (100 μM, 5-10 min) and after addition of DCPIB (10 μM, 5-10 min) in the presence of 0.85T solution. (B) I-V relationships for A. Cell capacitance was 39.0 pF. (C) Normalized current for cells exposed to DCPIB in continued presence of H₂O₂. H₂O₂ (100μM, 5-10min) increased the outward current at +60 mV from 2.70 ± 1.23 to 24.18 ± 4.40 pA/pF (n = 6, P < 0.001), and DCIPB blocked 101.3 ± 12.6% (n = 4, P < 0.001) of H₂O₂-induced current.
that it also blocked $I_{Cl,swell}$ in rabbit atrial myocytes. However, as shown in Fig. 14, apocynin (500µM, 15min) failed to inhibit the activation of $I_{Cl,swell}$ in response to osmotic swelling in HL-1 cells ($n = 5, P = 0.82$). Apocynin is a pro-drug that must be activated by myeloperoxidase (Heumuller et al., 2008). This raised the possibility the insensitivity to apocynin reflected a failure to activate the pro-drug rather than a lack of involvement of NOX, and therefore, we tested another selective NOX inhibitor, the fusion peptide gp91ds-tat. By contrast to apocynin, gp91ds-tat completely inhibited osmotic swelling-induced $I_{Cl,swell}$ in HL-1 cells (Fig. 15). After swelling increased the current at +60 mV from $1.99 \pm 0.62$ pA/pF to $14.52 \pm 2.54$ pA/pF, addition of gp91ds-tat (500 nM, 10 – 15 min) in 0.85T solution blocked 103.1 ± 3.3% of the swelling-induced current ($n = 4, P <0.001$).

### 4.4. Mitochondrial ROS Regulate $I_{Cl,swell}$ in HL-1 Cells

Mitochondrial ROS is critically involved in the regulation of $I_{Cl,swell}$ in rabbit atrial myocytes (Fig. 6). We examined whether mitochondrial ROS also regulates $I_{Cl,swell}$ in HL-1 cells. Swelling-induced $I_{Cl,swell}$ in HL-1 cells was fully inhibited by the Complex I blocker rotenone (Fig.16). Application of rotenone (10 µM, 15 – 30 min) in 0.85T solution reduced the outward current at +60 mV from $15.95 \pm 3.3$ pA/pF to $0.97 \pm 0.18$ pA/pF. The osmotic swelling-induced current in HL-1 cells was inhibited $110.8 \pm 9.6\%$ ($n = 5, P <0.001$) by rotenone and the current after application of rotenone was not significantly different from control ($n = 5, P = 0.72$).
Figure 14: Apocynin failed to block $I_{\text{Cl,swell}}$ in HL-1 cells. (A) Families of currents before and after exposure to 0.85T solution (5-10 min) and after addition of apocynin (500µM, 15min) and DCPIB (10 µM, 5-10 min) in the presence of 0.85T solution. (B) I-V relationships for A. Cell capacitance was 39.9 pF. (C) Normalized current for cells exposed to apocynin and DCPIB in 0.85 T solution. Apocynin (500µM, 15min) had no effect on the activation of $I_{\text{Cl,swell}}$ in response to osmotic swelling in HL-1 cells ($n = 5$, $P = 0.82$). In contrast, DCPIB (10 µM, 5-10 min) blocked $106.0 \pm 4.4\%$ of swelling-induced current of the same cells ($n = 4$, $P < 0.001$).
Figure 15: Gp91ds-tat inhibited activation of I_{Cl\text{swell}} by osmotic swelling in HL-1 cells. (A) Families of currents before and after exposure to 0.85T solution (5-10 min) and after application of gp91ds-tat (500 nM, 10-15 min) in 0.85T solution. Gp91ds-tat is a fusion peptide that specifically blocks the docking site of p47^{phox}. (B) I-V relationships for A. Cell capacitance was 38.7 pF. (C) Normalized current from cells exposed to gp91ds-tat in 0.85 T solution. Gp91ds-tat (500 nM, 10-15 min) blocked 103.1 ± 3.3% of swelling-induced current (n = 4, P <0.001).
**Figure 16:** Rotenone inhibited swelling-induced $I_{Cl_{swell}}$ in HL-1 cells. 

(A) Families of currents before and after exposure to 0.85T solution (5-10 min) and after application of rotenone (10 µM, 15-30 min) in 0.85T solution. 

(B) I-V relationships for A. Cell capacitance was 38.0 pF. 

(C) Normalized current from cells exposed to rotenone in 0.85T solution. Rotenone (10 µM, 15-30 min) blocked 110.8 ± 9.6% ($n = 5, P < 0.001$) of swelling-induced current ($n = 5, P < 0.001$).
4.5 ET-1 Induces $I_{Cl,\text{swell}}$ in HL-1 Cells

Fig. 17 and 18 show that exposure to ET-1 induced an outwardly rectifying Cl\(^-\) current that is blocked by DCPIB in HL-1 cells in both physiological and symmetrical Cl\(^-\). ET-1 (10 nM, 5-10 min) increased the outward current at +60 mV from 0.99 ± 0.38 pA/pF to 27.79 ± 2.00 pA/pF ($n = 5$, $P < 0.001$) in physiological Cl\(^-\) and from 0.92 ± 0.40 pA/pF to 16.78 ± 4.01 pA/pF ($n = 4$, $P < 0.001$) in symmetrical Cl\(^-\). DCPIB blocked 100.2 ± 0.35% ($n = 4$, $P < 0.001$) and 100.4 ± 0.88% ($n = 4$, $P < 0.001$) of swelling-induced current in physiological and symmetrical Cl\(^-\), respectively.

4.6 ET-1 Induces $I_{Cl,\text{swell}}$ in HL-1 Cells via $\text{ET}_A$ Receptors

Selective $\text{ET}_A$ blocker BQ123 suppressed ET-1-induced $I_{Cl,\text{swell}}$ in HL-1 cells (Fig. 19). ET-1 (10 nM, 5-10 min) increased the current at +60 mV from 0.79 ± 0.28 to 36.62 ± 5.68 pA/pF, and application of BQ 123 (10 µM, 10 min) with continuous presence of ET-1 reduced the current to 0.55 ± 0.06 pA/pF, a block of 101.3 ± 1.4% ($n = 4$, $P < 0.001$). Current after application of BQ123 was not significantly different from the control ($n = 4$, $P = 0.96$).

4.7 NOX is Essential for ET-1-Induced $I_{Cl,\text{swell}}$ in HL-1 Cells

ET-1-induced $I_{Cl,\text{swell}}$ was blocked by the specific NOX inhibitor gp91ds-tat in HL-1 cells (Fig.20). At +60 mV, ET-1 increased the current by 36.53 ± 5.98 pA/pF, from 1.46 ± 0.82 to 37.09 ± 6.80 pA/pF, and gp91ds-tat (500 nM, 10-15 min) applied in continued presence of ET-1 decreased the outward current to 1.79 ± 0.88 pA/pF. The NOX blocker gp91ds-tat suppressed 98.6± 0.9% ($n = 4$, $P < 0.01$) of the ET-1-induced
Figure 17: ET-1 activated a DCPIB-sensitive outwardly rectifying Cl⁻ current in HL-1 cells with physiological Cl⁻ gradient. (A) Families of currents before and after exposure to ET-1 (10 nM, 10 min) and after addition of DCPIB (10 μM, 5-10 min) in the presence of ET-1. (B) I-V relationships for A. Cell capacitance was 47.2 pF. (C) Normalized current for cells exposed to DCPIB solution in continuous presence of ET-1. DCPIB blocked 100.2 ± 0.35% (n = 4, P < 0.001) of the current elicited by ET-1.
Figure 18: ET-1 induced an outwardly rectifying Cl⁻ current that was sensitive to DCPIB in HL-1 cells with symmetrical Cl⁻ gradient. (A) Families of currents before and after exposure to ET-1 (10 nM, 10 min) and after addition of DCPIB (10 μM, 5-10 min) in the presence of ET-1. (B) I-V relationships for A. Cell capacitance was 81.6 pF. (C) Normalized current from cells exposed to DCPIB solution in continuous presence of ET-1. DCPIB blocked and 100.4 ± 0.88% (n = 4, P < 0.001) of the ET-1-indued current.
Figure 19: ET-1 activated I_{Clswell} in HL-1 cells via ET\textsubscript{A} receptors. (A) Families of currents before and after exposure to ET-1 (10 nM, 10 min) and after addition of BQ 123 (10 µM, 10 min) in the presence of ET-1. (B) I-V relationships for A. Cell capacitance was 49.4 pF. (C) Normalized current for cells exposed to BQ123 in continuous presence of ET-1. ET-1 blocked 101.3 ± 1.4% (n = 4, P < 0.001) of the current elicited by ET-1.
**Figure 20:** NOX was involved in the activation of $I_{\text{Cl,swell}}$ by ET-1 in HL-1 cells. (A) Families of currents before and after exposure to ET-1 (10 nM, 10 min) and after addition of gp91ds-tat (500 nM, 10-15 min) in the presence of ET-1. (B) I-V relationships for A. Cell capacitance was 46.8 pF. (C) Normalized current for cells exposed to gp91ds-tat in continuous presence of ET-1. Gp91ds-tat suppressed $98.6 \pm 0.9\%$ ($n = 4$, $P < 0.01$) of the ET-1-induced current.
current, and currents after block were not significantly different from control ($n = 4, P = 0.96$).

**4.8 Measurement of ROS Production by Flow Cytometry**

Fig. 21 shows the representative gated log fluorescence histograms from a typical experiment. ET-1 itself is not fluorescent, as no increase in fluorescence was observed after treatment of HL-1 cells with ET-1 (10 nM, 20 min) in the absence of C-H$_2$DCFDA-AM (Fig. 21 A). By contrast, ET-1 increased fluorescence above background levels in C-H$_2$DCFDA-AM-loaded cells, which indicates that ET-1 enhanced ROS production (Fig.21 B). Pretreatment with rotenone (10µM) or gp91ds-tat (500 nM) for 1 hr prevented ET-1-induced C-H$_2$DCFDA-AM fluorescence suggesting that both NOX and mitochondrial ROS production were involved (Fig.21 C). Summary data based on the geometric means of the fluorescence distributions are shown in Fig. 22 and confirmed the interpretation of the electrophysiologic data. ET-1 significantly increased ROS production as compared to that under background conditions, and ROS production was statistically unchanged from background after either rotenone or gp91ds-tat pretreatment.

The increase of C-H$_2$DCFDA-AM fluorescence by ET-1 followed an exponential time course, and the steady state was reached at ~20 min (Fig. 23). The time course data were fitted individually to an exponential function $f=a*(1-exp(-1/\tau*x))$ where $a$ is the amplitude of C-H$_2$DCFDA-AM fluorescence and $\tau$ is the time constant. The mean amplitude was 6.2 ± 0.7 and $\tau$ was 12.2 ± 1.7 min.
Figure 21: Gated log fluorescence (F) histograms from a typical experiment showing that ET-1-induced ROS production was abrogated by gp91ds-tat and rotenone. Intracellular ROS was detected with C-H2DCFDA-AM, which is converted into a non-fluorescent derivative (carboxy-H2DCF) by intracellular esterases. Carboxy-H2DCF is retained in the cytoplasm and is oxidized to fluorescent carboxy-DCF by intracellular ROS. The numbers represent the geometric mean of the fluorescence density. (A) Negative control (without loading the cells with C-H2DCFDA-AM) in the presence and absence of ET-1. ET-1 itself was not fluorescent. (B) Cells loaded with C-H2DCFDA-AM (2.5 µM) and treated with ET-1 (10 nM, 10 min or 40 min) or, as a positive control, H2O2 (100µM, 10 min). ET-1 and H2O2 increased fluorescence. (C) C-H2DCFDA-AM-loaded cells that were pretreated with rotenone (10 µM) or gp91ds-tat (500 nM) for 1 hr before stimulation with ET-1 (10 nM, 20 min). Consistent with the patch clamp data, both rotenone and gp91ds-tat fully suppressed ET-1-induced ROS production.
**Figure 22:** ET-1-induced the C-H$_2$DCFDA-AM fluorescence was abrogated by rotenone and gp91ds-tat. ET-1 (10 nM, 20 min) and H$_2$O$_2$ (100 µM, 10 min) significantly increased fluorescence above background, and after pretreatment (1 hr) with rotenone (10 µM) or gp91ds-tat (500 nM), the ET-1-induced fluorescence was not significantly different than control ($n = 4 – 10$, $P < 0.05$). The response to H$_2$O$_2$ served as a positive control. For each experiment, the geometric mean fluorescence after an experimental intervention was calculated as the fold-increase relative to background fluorescence (FL/FL$_0$). Because the summary data sets were not normally distributed, Kruskal-Wallis One Way ANOVA on Ranks was applied, and pairwise comparisons were made by Dunn's method; 25%, 50%, and 75% percentiles are denoted in box plots, and error bars represent 10% and 90% percentiles.
Figure 23: Time course of the C-H$_2$DCFDA-AM fluorescence induced by ET-1. ET-1 induced fluorescence is plotted as fold-increase relative to background fluorescence (FL/FL$_0$). The data were fitted individually to a sigmoidal function, $f = 1 + a/(1 + \exp \left(-(x-x_0)/b\right))$, where $a$ is the amplitude of C-H$_2$DCFDA-AM fluorescence, $x_0$ is the half time for activation, and $b$ is the slope factor. The mean amplitude of FL/FL$_0$ was 5.1 ± 0.6, $x_0$ was 9.5 ± 1.3, and $b$ was 7.1 ± 1.8 (solid line). **, significant differences from background at $t = 0$ ($n = 5$; $P < 0.01$). Time-course data were assessed with a repeated measures ANOVA and compared to control ($t = 0$) by the Holm-Sidak method. Symbols and error bars are mean ± SEM.
Flow cytometry also was used to confirm that antimycin A activated mitochondrial ROS production independent of effects on NADPH oxidase. Antimycin A (20 μM, 20 min) significantly increased ROS production 3.6-fold relative to background ($n = 3, P < 0.05$). Pretreatment with rotenone (10 μM, 20 min) reduced antimycin A-induced ROS production to 1.2-fold above background ($n = 2, ns$), whereas after pretreatment with gp91ds-tat (500 nM, 20 min) antimycin A-induced ROS production remained elevated by 3.5-fold ($n = 2, P < 0.05$).
5.1. **ET-1 Activates an Outwardly Rectifying Cl⁻ Current with Properties of I$_{Cl,swell}$ in Atrial Myocytes.**

The primary Cl⁻ channels expressed by atrial myocytes include I$_{Cl,cAMP}$, I$_{Cl,Ca}$, and I$_{Cl,swell}$ (for review, see Hume *et al.*, 2000). In the present study, we showed that ET-1 activated an outwardly rectifying Cl⁻ current with properties of I$_{Cl,swell}$ rather than either I$_{Cl,cAMP}$ or I$_{Cl,Ca}$. First, the ET-1-induced Cl⁻ current was blocked by highly selective I$_{Cl,swell}$ blocker DCPIB. Both I$_{Cl,cAMP}$ and I$_{Cl,Ca}$ are insensitive to DCPIB (Decher *et al.*, 2001). Second, the ET-1-induced was suppressed by osmotic shrinkage. Volume sensitivity is an important feature of I$_{Cl,swell}$ that distinguishes it from I$_{Cl,PKA}$ and I$_{Cl,Ca}$ (Hume *et al.*, 2000). Finally, the ET-1-induced Cl⁻ current is outward rectifying with symmetrical Cl⁻ gradient, which is a fundamental characteristic of I$_{Cl,swell}$ (Hume *et al.*, 2000; Baumgarten & Clemo, 2003). In contrast, I$_{Cl,PKA}$ and I$_{Cl,Ca}$ are linear in symmetrical Cl⁻ (Hume *et al.*, 2000).
Du and Sorota (2000) reported that ET-1 potentiates $I_{Cl,swell}$ after it is tuned on by hydrostatic positive-induced cell inflation. However, the effect of ET-1 on $I_{Cl,swell}$ without pre-activation by cell inflation or osmotic swelling was not investigated in the study. The present study showed that ET-1 elicits $I_{Cl,swell}$ under isomotic conditions without cell inflation, which argues for a role of ET-1 as an activator of $I_{Cl,swell}$.

5.2. ET-1 Activates $I_{Cl,swell}$ via $ETA$ but not $ETB$ Receptors

ET-1 interacts with two receptor subtypes, $ETA$ and $ETB$. Both $ETA$ and $ETB$ receptors are expressed in the atrial myocytes, although $ETA$ is the predominant isoform (Russell & Molenaar, 2000). ET-1-induced myocardial hypertrophy, fibrosis, inflammation, and arrhythmogenic effects are generally linked to $ETA$ receptors (Chen et al., 2001; Rothermund et al., 2002; Fraccarollo et al., 2002), but other studies suggest that $ETB$ receptors may also play a role (Hocher et al., 1999; Burrell et al., 2000; Cullen et al., 2001; Lee et al., 2004; Yang et al., 2004).

Previously we showed that ROS are required intermediates for the activation of $I_{Cl,swell}$ ventricular cardiomyocytes (Browe & Baumgarten, 2004; Ren et al., 2008). $ETA$ receptors were shown to be responsible for ET-1-induced ROS production in VSMCs (Laplante et al., 2005) and cardiomyocytes (Cingolani et al., 2006). However, in human umbilical vein endothelial cells ET-1-induced ROS generation was attributed to $ETB$ receptors (Dong et al., 2005). In agreement with previous studies, we showed that selective $ETA$ receptor blockade abolished the activation of $I_{Cl,swell}$ induced by ET-1 in atrial myocytes, whereas an $ETB$ receptor blocker had no effect. $ETB$ receptors are the predominant ET receptor subtypes expressed in the endothelial cells (Winkles et al.,
1993), and it is likely that there are tissue-specific differences in the linkage between ROS production and the ET receptor subtypes.

5.3. Transactivation of EGFR kinase and its Potential Role in the ET-1-Induced Activation of I_{Cl,swell}

EGFR kinase is an important member of the ErbB family of receptor tyrosine kinases. It dimerizes and trans-autophosphorylates six specific tyrosines within its cytoplasmic tail upon ligand binding (Prenzel et al., 1999; Anderson et al., 2004). We previously showed that EGFR kinase blockade fully inhibited the activation of I_{Cl,swell} by β1-integrin stretch and osmotic swelling, and exogenous EGF elicited I_{Cl,swell} under isosmotic conditions in ventricular myocytes (Browe & Baumgarten, 2006; Ren et al., 2008). EGF also has been shown to participate in the regulation of I_{Cl,swell} in other cell types including C127 mammary cells (Abdullaev et al., 2003) and liver-derived HTC cells (Varela et al., 2004). The present study provides the first evidence that EGFR kinase mediates the activation of I_{Cl,swell} by ET-1. First, ET-1 induced activation of I_{Cl,swell} was fully inhibited by selective EGFR kinase blocker AG1478, which inhibits ATP binding to EGFR kinase. Second, in agreement with the studies in the ventricular cardiomyocytes (Browe & Baumgarten, 2006), exogenous EGF elicited an outwardly rectifying Cl\(^{-}\) current with properties of I_{Cl,swell} under isosmotic condition in atrial cardiomyocytes.

Transactivation of EGFR kinase by ET-1 involves the cleavage of membrane-tethered pro-heparin-binding EGF-like growth factor (proHB-EGF) by matrix metalloproteinases (MMPs) and shedding of soluble heparin-binding EGF (HB-EGF), which binds to and activates EGFR kinase (Prenzel et al., 1999). We did not test the
importance of MMP dependent proHB-EGF cleavage in the activation of $I_{C_{L,swell}}$ by ET-1. One would predict, however, that MMP inhibitors would suppress $I_{C_{L,swell}}$, and that this may be an unexpected consequence of their application for other purposes.

5.4. PI-3K Regulates ET-1-Induced $I_{C_{L,swell}}$

PI-3K is activated by mechanical stretch (Petroff et al., 2001), integrin clustering (Franchini et al., 2000), osmotic swelling (Bewick et al., 1999), AngII (Rabkin et al., 1997), EGFR kinase (Krieg et al., 2004) and ET-1 (Foschi et al., 1997; Araki et al., 2000; Pham et al., 2001; Shi-Wen et al., 2004). Previous studies showed that inhibition of PI-3K suppressed the activation of $I_{C_{L,swell}}$ in response to $\beta_1$-integrin stretch (Browe & Baumgarten, 2006), osmotic swelling (Feranchak et al., 1999; Shi et al., 2002; Wang et al., 2004; Ren & Baumgarten, 2005), AngII (Ren et al., 2008), and EGF (Browe & Baumgarten, 2006). The role of PI-3K in the activation of $I_{C_{L,swell}}$ by ET-1 was supported by the observation that two structurally different PI-3K blockers, wortmannin and LY 294002, fully inhibited ET-1-induced $I_{C_{L,swell}}$.

PI-3K has been shown to participate in the activation of NOX and generation of ROS in response to diverse stimuli in various tissues (Hawkins et al., 2007). There are several mechanisms by which PI-3K regulates NOX assembly and activation. First, PI-3K leads to phosphorylation of the $\text{p}47^{\text{phox}}$ subunit and the PI-3K products, PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$, bind to the PX domains of $\text{p}47^{\text{phox}}$ and direct it to the plasma membrane (Zhan et al., 2002; Hoyal et al., 2003). Second, PI-3K and its products stimulate Rac, which also is essential for NOX assembly (Park et al., 2004). Moreover, H$_2$O$_2$ generated by NADPH oxidase feeds forward to potentiate RTK – PI-3K signaling.
and HB-EGF-induced EGFR kinase phosphorylation (Bae et al., 1997; Rhee et al., 2000; Frank & Eguchi, 2003; Rhee et al., 2003)

Wortmannin and LY294002 inhibited PI-3K by different mechanisms. Wortmannin covalently modifies the p110 catalytic subunit of PI-3K, whereas LY294002 competes with ATP for the p110 active site. Previously we showed that β1 integrin stretch- and osmotic-induced I_{Cl,swell} were completely inhibited by LY294002 but only partially suppressed by 500 nM wortmannin (Browe & Baumgarten, 2006; Ren et al., 2008). In present study, ET-1-induced I_{Cl,swell} was completely abolished by wortmannin. The reason for this discrepancy was not investigated. One possibility is that activation of I_{Cl,swell} by β1 integrin stretch and osmotic swelling involve monomeric class II PI-3K-C2α, which is more resistant to wortmannin (IC_{50} = 400 nM) than dimeric PI-3K (IC_{50} = 1–10 nM) (Oudit et al., 2004).

### 5.5. ROS are Essential for the ET-1-Induced Activation of I_{Cl,swell}

H_{2}O_{2} is a membrane permeant, longer-lived ROS that interacts with a large number of signaling molecules and ion channels (for review, see Lambeth, 2004). We and others showed that I_{Cl,swell} is elicited by exogenous H_{2}O_{2} in rabbit ventricular myocytes (Browe & Baumgarten, 2004), HeLa cells (Varela et al., 2004) and HTC cells (Shimizu et al., 2004). Decomposing H_{2}O_{2} with catalase inhibited the activation of I_{Cl,swell} in response to β1 integrin stretch and osmotic swelling (Browe & Baumgarten, 2004; Ren et al., 2008). Consistent with previous studies, scavenging H_{2}O_{2} using ebselen abrogated I_{Cl,swell} induced by ET-1. Besides H_{2}O_{2}, ebselen also reacts with peroxynitrite as well as hydroperoxides including membrane-bound phospholipid and cholesterylester
hydroperoxidase (Schewe, 1995). Although $I_{Cl,swell}$ was activated by exogenous H$_2$O$_2$, we cannot exclude the possibility that other ROS are also involved in the regulation of $I_{Cl,swell}$.

The underlying mechanism by which H$_2$O$_2$ regulates $I_{Cl,swell}$ remains to be understood. Our data indicates that H$_2$O$_2$ must act at a site distal in the signaling cascade because H$_2$O$_2$-induced activation is not reversed by osmotic shrinkage with 1.5T bath solution (Ren et al., 2008), whereas activation by ET-1 was abrogated by osmotic shrinkage (Fig. 1). H$_2$O$_2$ may directly modify ion channels and alter their activities or influence the redox-sensitive signaling pathways that regulate the ion channels (for review, see Zima & Blatter, 2006). A recent study indicated that activation of PLC$\gamma$ and subsequent intracellular Ca$^{2+}$ mobilization mediate H$_2$O$_2$-induced $I_{Cl,swell}$ in HTC cells, suggesting that H$_2$O$_2$ operates via redox-sensitive signaling pathways (Varela et al., 2007). However, the Ca$^{2+}$-dependent mechanism is difficult to explain the activation of $I_{Cl,swell}$ by H$_2$O$_2$ under present conditions, because the bathing solution was Ca$^{2+}$ free and cytoplasmic free Ca$^{2+}$ was clamped to ~35 nM with EGTA. Moreover, a number of studies found that $I_{Cl,swell}$ is independent of cytoplasmic Ca$^{2+}$ but is inhibited by store-operated calcium entry (SOCE) following endoplasmic reticulum (ER) calcium store depletion (Lemonnier et al., 2002; Zholos et al., 2005). We can not exclude the possibility that H$_2$O$_2$ regulates $I_{Cl,swell}$ via redox-sensitive signaling pathways in cardiomyocytes. Another possible mechanism is that H$_2$O$_2$ modified the channel itself. Because the molecular identity of the channel is still unclear, how the direct modification by ROS affects the properties of $I_{Cl,swell}$ remains to be further studied.
5.6. ROS derived from NOX are Required for the Activation of $I_{\text{Cl,swell}}$ by ET-1.

NOX transfers $e^-$ from intracellular NADPH to extracellular $O_2$ and produces $O_2^{-*}$, which readily undergoes dismutation to $H_2O_2$ spontaneously or by extracellular superoxide dismutase (SOD) (Bedard & Krause, 2007). Previous studies showed that blockade of NOX inhibited the activation of $I_{\text{Cl,swell}}$ by $\beta_1$ integrin stretch, osmotic swelling, and AngII in cardiomyocytes (Browe & Baumgarten, 2004; Ren et al., 2008). Additionally, expression of the dominant negative p47$^{5379A}$ NOX subunit suppresses osmotic swelling-induced $I_{\text{Cl,swell}}$ in HeLa cells (Varela et al., 2004), and the non-selective flavin-inhibitor DPI inhibited $I_{\text{Cl,swell}}$ in both HeLa and HTC cells (Varela et al., 2004; Shimizu et al., 2004).

ET-1 activates NOX in multiple cell types including cardiomyocytes (De Giusti et al., 2008), VSMCs (Fei et al., 2000; Li et al., 2003; Amiri et al., 2004) and endothelial cells (Duerrschmidt et al., 2000; Dong et al., 2005). In support of the role of NOX in the activation of $I_{\text{Cl,swell}}$ by ET-1, we showed that ET-1-induced $I_{\text{Cl,swell}}$ was blocked by selective NOX inhibitor apocynin, which prevents NOX assembly by conjugating thiol residues, and the specific NOX inhibitor gp91ds-tat blocked ET-1-induced ROS generation in HL-1 myocytes.

Both NOX2 (gp91$^{phox}$) and NOX4 are expressed in cardiac tissue (Cave et al., 2006). In the present study we did not distinguish which isoform is responsible for the activation of $I_{\text{Cl,swell}}$ by ET-1, but the involvement of NOX2 is favored. First, it has been shown that NOX2 rather than NOX4 mediates AngII-induced $O_2^{-*}$ production in
cardiomyocytes (Bendall et al., 2002), and AngII-induced ROS production was shown to be mediated by ET-1 ET_A signaling (Cingolani et al., 2006). Second, the selective NOX inhibitor gp91ds-tat, the fusion peptide that blocks the docking site of the p47^phox subunit of NOX, abrogate ET-1-induced ROS production in HL-1 cells. Because NOX4 activity does not depend on cytoplasmic subunits including p47^phox and its homologues (Bedard & Krause, 2007), the activity of NOX4 should not be suppressed by gp91ds-tat.

5.7. Mitochondrial ROS are Essential for the Activation of I_{Cl,swell} by ET-1.

Besides NOX, another important ROS generating system in cardiomyocytes is mitochondria, and our preliminary data suggest that mitochondrial ROS are involved in the regulation of I_{Cl,swell} by ACh, ceramide, and HIV protease inhibitors (Browe & Baumgarten, 2007; Deng et al., 2009; Raucci, Jr. & Baumgarten, 2009). Complex I and Complex III are two redox centers that leak single e^- to molecular O_2 to generate O_2^- (Chen et al., 2003; Turrens, 2003). O_2^- generated by Complex I is exclusively released into the matrix of intact mitochondria and degraded by the matrix anti-oxidant system (Muller et al., 2004). In contrast, Complex III releases O_2^- to both sides of the mitochondrial inner membrane and plays a central role in the production of ROS by mitochondria (Chen et al., 2003; Turrens, 2003; Muller et al., 2004). A mitochondrial source of ROS has been implicated ET-1 signaling in VSMCs (Touyz et al., 2004; Callera et al., 2006) and cardiomyocytes (Caldiz et al., 2007).

The role of mitochondrial ROS in the activation of I_{Cl,swell} by ET-1 was supported by the findings that selective Complex I inhibitor rotenone, which inhibits mitochondrial ROS generation by limiting the e^- to Complex III, completely blocked the ET-1-induced
Moreover, pretreatment by rotenone prevented the ET-1-induced ROS production in HL-1 cells.

5.8. Proposed Direction of the Crosstalk Between NOX and Mitochondria: Activation of \( I_{\text{Cl,swell}} \) by Mitochondrial ROS is Downstream from NOX

The crosstalk between NOX and mitochondria has been suggested in several recent studies. AngII, which is a well-known activator of NOX, induces mitochondrial ROS production in VSMCs (Kimura et al., 2005a). Either inhibition of NOX or blockade of mitochondrial ROS production by blocking mitoK\(_{\text{ATP}}\) channel abrogated multiple AngII-induced effects, including ROS production, the slow force response, preconditioning, lipid peroxidation, and apoptosis (Kimura et al., 2005b; Caldiz et al., 2007). A recent study also showed that NOX inhibitor or mitochondrial K\(_{\text{ATP}}\) channel inhibitors abolished ET-1-induced ROS production and inotropic effect (De Giusti et al., 2008). The present study suggests that ROS from both NOX and mitochondria are involved in the activation of \( I_{\text{Cl,swell}} \). This raises a question: How are these sources of ROS arranged? Because blockade of either NOX or mitochondrial source of ROS completely inhibited ET-1-induced ROS generation as well as the activation of \( I_{\text{Cl,swell}} \), the two ROS generating systems must work in serial rather than in parallel. We also showed that the activation \( I_{\text{Cl,swell}} \) induced by osmotic swelling, which was shown to be dependent on NOX, was blocked by rotenone (data not shown), and stimulation of \( I_{\text{Cl,swell}} \) by ACh, which was ascribed to mitochondrial ROS, was inhibited by NOX inhibitor apocynin (data not shown). Taken together, these data indicates serial crosstalk between NOX and mitochondria in the regulation of \( I_{\text{Cl,swell}} \).
Both NOX and mitochondrial ROS production can be stimulated by ROS. ROS stimulates the signaling pathway that leads to the activation of NOX (Cai, 2005), and local release of ROS may trigger self-sustained oscillations of mitochondrial membrane potential and ROS production that propagate from one mitochondrion to another throughout myocytes, which is referred to as mitochondrial ROS-induced ROS release (RIRR) (Zorov et al., 2000; Aon et al., 2003; Aon et al., 2008). Therefore, there are two possibilities for the direction of the crosstalk: mitochondria is upstream from NOX and induces ROS production by NOX. Alternatively, NOX is upstream from mitochondria and enhances mitochondrial ROS production.

Antimyosin A induces $\mathbf{O}_2^{-}$ production from the $Q_o$ site of Complex III by binding to a distal site near $Q_i$ and preventing $e^-$ transfer from cytochrome $b_{H}$ (Chen et al., 2003; Turrens, 2003). Diazoxide elicits mitochondrial ROS production by opening mitoK$_{ATP}$ channels (Pain et al., 2000; Oldenburg et al., 2003). Preliminary data shows that two stimulators of mitochondrial ROS, antimycin A and diazoxide, induce $I_{Cl,swell}$ in ventricular myocytes that is inhibited by 5-HD, a blocker of the MitoK$_{ATP}$ channel (Browe & Baumgarten, 2007).

In the present study, we showed that the activation of $I_{Cl,swell}$ in response to antimycin A and diazoxide was insensitive to the selective NOX inhibitor apocynin and the fusion peptide inhibitor gp91ds-tat. These results rule out the possibility that NADPH oxidase is downstream from mitochondrial ROS production. Rather, the data suggest that mitochondrial ROS is downstream from NOX in the signaling pathway regulating $I_{Cl,swell}$. We propose that low levels of ROS produced by NOX might trigger the production of a
greater amount of ROS by mitochondria, which is responsible for the activation of $I_{\text{Cl,swell}}$. Subsarcolemmal mitochondria are well-positioned for this role (Palmer et al., 1977); they are located adjacent to both NOX on surface membrane and to $I_{\text{Cl,swell}}$.

The underlying mechanism by which NOX interacts with mitochondria has not been illuminated. One possibility is that ROS produced by NOX directly modify and open mitoK$_{\text{ATP}}$ channels by oxidizing SH groups within the channel (Zhang et al., 2001). Another possibility is mitochondrial RIRRR, which is generated by circuits requiring mitochondrial membrane channels including the mitochondrial permeability transition (MPT) pore and the inner membrane anion channel (IMAC) (Zorov et al., 2006). The role of MPT and IMAC was not examined in the current study.

5.9. Osmotic Shrinkage-Induced Signaling is Proximal to Mitochondrial ROS in the Regulation of $I_{\text{Cl,swell}}$

The signaling induced by osmotic shrinkage is not been fully understood. Previous studies indicated that integrins (Sheikh-Hamad et al., 1997; Sheikh-Hamad et al., 2000; Pedersen et al., 2001; Haussinger et al., 2006), FAK (Lunn & Rozengurt, 2004; Lunn et al., 2007), Src family kinases (Lunn & Rozengurt, 2004; Reinehr et al., 2004), Rho family GTPases (Lunn & Rozengurt, 2004), and EGFR kinase (Rosette & Karin, 1996; Cheng et al., 2002; Reinehr et al., 2003) are involved in shrinkage-activated responses in non-cardiac cells. Osmotic shrinkage was shown to inhibit EGF receptor-mediated signaling in kidney cells (Copp et al., 2005) and suppress the PI-3K-Akt and MEK1/2-ERK1/2 pathways in NIH3T3-fibroblasts (Nielsen et al., 2008).
Osmotic shrinkage with hyperosmotic bathing media inhibited the activation of $I_{\text{Cl,swell}}$ induced by β1 integrin stretch and EGF (Browe & Baumgarten, 2006) but failed to inhibit H$_2$O$_2$-induced $I_{\text{Cl,swell}}$, suggesting that H$_2$O$_2$ acts at sites distal to those regulated by osmotic shrinkage (Ren et al., 2008). In the present study, we showed that the activation of $I_{\text{Cl,swell}}$ in response to antimycin A, which stimulates mitochondrial ROS directly, also was insensitive to osmotic shrinkage. These data indicates that mitochondrial ROS is distal from the sites that are regulated by osmotic shrinkage. Based on previous studies, we propose that osmotic shrinkage might regulate $I_{\text{Cl,swell}}$ at site(s) between EGFR kinase and mitochondrial ROS.

5.10. ERK-Dependent and ERK-Independent Activation of $I_{\text{Cl,swell}}$ by ET-1, EGF, and H$_2$O$_2$

ERK signaling plays a pivotal role in the cardiac hypertrophy and cardiomyocyte survival (Heineke & Molkentin, 2006; Muslin, 2008). ERK is activated by mechanical stretch (Caldiz et al., 2007; Lal et al., 2007), AngII (Fischer et al., 1998; Ruf et al., 2002; Booz et al., 2003), EGF (Kodama et al., 2002; Duquesnes et al., 2009), ET-1 (Bogoyevitch et al., 1993; Bogoyevitch et al., 1994), and H$_2$O$_2$ (Aikawa et al., 1997; Fukuzawa et al., 2002; Daou & Srivastava, 2004; Mehdi et al., 2005). The present and previous studies demonstrate that all of these signaling effectors are upstream regulators of $I_{\text{Cl,swell}}$ (Du & Sorota, 2000; Browe & Baumgarten, 2003; Browe & Baumgarten, 2004; Browe & Baumgarten, 2006).

ERK1/2 blockade was shown to inhibit the persistent activation of $I_{\text{Cl,swell}}$ in the failing heart (Clemo & Baumgarten, 1999) and the potentiation of $I_{\text{Cl,swell}}$ by ET-2 (Du &
Sorota, 2000). In the current study we showed that EGF-induced $I_{\text{Cl,swell}}$ was fully inhibited by blockade of ERK1/2 with PD98059 or ERK1 with U0216. However, both PD98059 and U0216 partially inhibited ET-1-induced $I_{\text{Cl,swell}}$. These data suggest that EGF activated $I_{\text{Cl,swell}}$ via an ERK-dependent pathway and there are both ERK-dependent and independent pathway involved in ET-1 signaling, but the pathway is likely to be complex. Although ERK is known to be activated by $H_2O_2$, $H_2O_2$ also appears to be distal from ERK signaling in the regulation of $I_{\text{Cl,swell}}$, as ERK1/2 inhibitor PD98059 had no effect on $H_2O_2$ elicited $I_{\text{Cl,swell}}$. Other aspects of the complicated ERK1/2 signaling cascade were not explored.

5.11. ETA Receptors Mediate the Activation of $I_{\text{Cl,swell}}$ by AngII and Osmotic Swelling

Although the primary goal of the present study was to understand regulation of $I_{\text{Cl,swell}}$ by ET-1, we also established the relationship of ET-1 to the effects of osmotic swelling and AngII. Both osmotic swelling and $\beta_1$ integrin stretch activate $I_{\text{Cl,swell}}$ via AngII AT$_1$ signaling that ultimately leads to ROS production (Browe & Baumgarten, 2004; Ren et al., 2008). AngII induces synthesis and release of ET-1 in cardiomyocytes, and there is evidence that ET-1 mediates AngII-induced ROS production as well as other ROS dependent effects (Ito et al., 1993; Cingolani et al., 2006; Villa-Abrille et al., 2006). We found that the activation of $I_{\text{Cl,swell}}$ induced by AngII and osmotic swelling was abolished by ET$_A$ receptor blockade whereas AT$_1$R blocker had no effect on activation of $I_{\text{Cl,swell}}$ induced by ET-1, suggesting that ET-1 ET$_A$ signaling is downstream from AngII and osmotic swelling. We did not measure ET-1 release in response to AngII and osmotic
swelling, but it is likely that the atrial myocyte under study was exposed to ET-1 released by the same myocyte as well as by other myocytes in the chamber.

We postulate that the acute effect of AngII and osmotic swelling is mediated by release to preformed ET-1 rather than the induction of ET-1 transcription and synthesis. Prolonged exposures to AngII or hyposmotic bath solution can not be accomplished in the present patch clamp studies that used each myocyte as its own control. On the other hand, it is likely that the upregulation of the ET-1 transcription is involved in the persistent activation of I_{Cl,swell} under the situations that RAAS is chronically overactive, such as congestive heart failure and atrial fibrillation.

The signaling by which AngII induces transcription and release of ET-1 in cardiomyocytes was not understood. ROS and ERK1/2 are involved in AngII-induced ET-1 transcription in multiple non-cardiac cell types, including vascular smooth muscle cells (Hong et al., 2004), endothelial cells (Hsu et al., 2004), and fibroblasts (Cheng et al., 2003; An et al., 2007). ET-1 was shown to directly modulates its own expression in human endothelial cells and mesangial cells (Iwasaki et al., 1995; Evans et al., 2003). It is plausible that ET-1-induced ROS production and activation of ERK feed forward to enhance its expression and contribute to the profound and long-lasting effects of ET-1.

5.12. Proposed Model for the Regulation of I_{Cl,swell} by ET-1

A scheme accounting for both our present and previous studies is shown in Fig. 24. ET-1-induced I_{Cl,swell} was abrogated by selective blockade of ET\(_A\) receptor, EGFR kinase and PI-3K, suggesting that ET-1 activates I_{Cl,swell} via a signaling cascade involving ET\(_A\) receptor, EGFR kinase and PI-3K. PI-3K is downstream from EGFR kinase because inhibition of PI-3K suppressed EGF-induced I_{Cl,swell} (Browe & Baumgarten, 2006). PI-
**Figure 24.** Simplified schematic diagram of ET-1 signaling responsible for the activation of $I_{Clswell}$. Dark Blue, signaling pathways; Green, stimulates ROS and $I_{Clswell}$; Red, blocks ROS and $I_{Clswell}$; Arrow, acts by stimulating pathway; T, acts by inhibiting pathway; X, fails to block pathway; Dashed Line, one or more steps unknown. Results with ERK blockers are omitted because the signaling pathway remains unclear. Effects of osmotic shrinkage are also omitted.
3K has been implicated in the assembly and activation of NOX. The $O_2^{-\bullet}$ produced by NOX undergoes rapidly dismutation to $H_2O_2$, which has been shown to be a distal downstream mediator of the activation of $I_{Cl,\text{swell}}$ by $\beta1$ integrin stretch and osmotic swelling (Browe & Baumgarten, 2004; Ren et al., 2008). Consistent with previous findings, ET-induced $I_{Cl,\text{swell}}$ was abolished by membrane-permeant glutathione peroxidase mimetic ebselen and selective NOX inhibitor apocynin. Moreover, pretreatment of specific NOX inhibitor gp91ds-tat abolished ET-1-induced ROS production. The low level of ROS produced by NOX might induce a larger ROS production by mitochondria, and this amplification of ROS production is likely to be responsible for the activation of $I_{Cl,\text{swell}}$. In agreement with this idea, Complex I inhibitor rotenone fully blocked the ET-1-induced ROS production as well as the activation of $I_{Cl,\text{swell}}$. Mitochondrial ROS is downstream from NOX because the $I_{Cl,\text{swell}}$ elicited by mitochondrial ROS stimulators antimycin A and diazoxide is insensitive to NOX inhibitors.

5.13. $I_{Cl,\text{swell}}$ is Regulated by ROS and ET-1 in HL-1 Cells

HL-1 atrial myocytes are a recently developed model for studying cardiac myocytes. This preparation is characterized as an immortalized mouse atrial muscle cell line that retains certain morphological, biochemical, and electrophysiological properties of adult cardiomyocytes (Claycomb et al., 1998). HL-1 cells offer several advantages over acutely isolated adult myocytes, including the ability to apply a variety of molecular methods that require the culture of the targeted cells. $Cl^-$ currents have not previously been studied in HL-1 atrial myocytes, and the present experiments were undertaken to
examine whether $I_{Cl,swell}$ is present in these cultured cells and whether its regulation was similar to that in adult cells.

$I_{Cl,swell}$ in HL-1 cells was identified by the sensitivity to DCPIB and outward rectification in both physiological and symmetrical $Cl^-$. $I_{Cl,swell}$ was present at a much higher current density than typically observed in adult myocytes. Moreover, using the same approaches employed in adult myocytes, we demonstrated that activation of $I_{Cl,swell}$ was controlled by ET1-1 and both NOX and mitochondrial ROS. Only one difference was noted. The NADPH oxidase inhibitor apocynin was ineffective in HL-1 cells, although $I_{Cl,swell}$ still was fully suppressed by gp91ds-tat, another selective NADPH oxidase inhibitor. Apocynin is a prodrug that must be converted to its active form, usually by peroxidases (Heumuller et al., 2008). This raises the possibility that insufficient peroxidases are present in HL-1 cells to effectively convert apocynin to its active form.

HL-1 myocytes also were very useful for flow cytometry. Acutely isolated preparations of adult cardiac myocytes contain damaged and dead cells that produce ROS. This creates a serious technical problem in measuring ROS production by flow cytometry. In contrast, HL-1 cell preparations contain far fewer damaged or dead cells, and the background ROS production and the background activation of $I_{Cl,swell}$ were much lower. The present studies suggest that HL-1 cells are likely to be useful for future studies of $I_{Cl,swell}$ as well as ROS generation by other stimuli.

5.14. Implication of the Present Study

Excessive levels of ET-1 have been identified in multiple cardiovascular diseases, including congestive heart failure (Moe et al., 2003), atrial fibrillation (Tuinenburg et al.,...
1998; Brundel et al., 2001; Masson et al., 2006), essential hypertension (Touyz & Schiffrin, 2003), salt sensitive hypertension (Feldstein & Romero, 2007), pulmonary hypertension (Jasmin et al., 2003), pre-eclampsia (Florijn et al., 1991), atherosclerosis (Bousette & Giaid, 2003), acute myocardial infarction (Cernacek et al., 2003), ischemia/reperfusion injury (Pernow & Wang, 1997), myocarditis (Ono et al., 1999), sepsis induced myocardial dysfunction (Chopra & Sharma, 2007), and diabetic cardiovascular complications (Chakrabarti et al., 2002). As shown in the present study, ET-1 activates $I_{\text{Cl,swell}}$ under isosmotic conditions, which implies that activation of $I_{\text{Cl,swell}}$ might be involved in the pathogenesis of cardiovascular diseases in which ET-1 is over-produced. Recent studies showed that $I_{\text{Cl,swell}}$ is involved in the apoptosis of cardiomyocytes and hippocampal neurons induced by ischaemia–reperfusion (Wang et al., 2005; Inoue et al., 2007). Besides the role in apoptosis, $I_{\text{Cl,swell}}$ also will affect cardiac electrical activity and may potentially participate in arrhythmogenesis. The APD shortening effect of $I_{\text{Cl,swell}}$ might protect against EAD and torsade de pointes. On the other hand, it favors reentrant arrhythmias such as atrial fibrillation. The pathophysiological role of $I_{\text{Cl,swell}}$ in atrial fibrillation and other cardiovascular diseases still awaits to be established by in vivo studies.

The finding that mitochondrial ROS is responsible for the activation of $I_{\text{Cl,swell}}$ indicates that this current also will be turned on in a variety of situations that mitochondrial ROS production is activated, including heart failure (Ide et al., 1999; Ide et al., 2000), myocardial infarction (Ide et al., 2001), and ischemia/reperfusion (Ambrosio et al., 1993). The crosstalk between NOX and mitochondria established by this study
might be a general scheme that is implicated in multiple signaling events including regulation of $I_{\text{Cl,swell}}$ by other stimuli than ET-1, the inotropic response, and ischemia/reperfusion injury.
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