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Invited review

Functional role of anion channels in cardiac diseases[1]

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Key words

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Abstract

In comparison to cation (K^+, Na^+, and Ca^{2+}) channels, much less is currently known about the functional role of anion (Cl^-) channels in cardiovascular physiology and pathophysiology. Over the past 15 years, various types of Cl^- currents have been recorded in cardiac cells from different species including humans. All cardiac Cl^- channels described to date may be encoded by five different Cl^- channel genes: the PKA- and PKC-activated cystic fibrosis tansmembrane conductance regulator (*CFTR*), the volume-regulated *ClC-2* and *ClC-3*, and the Ca^{2+}-activated *CLCA* or *Bestrophin*. Recent studies using multiple approaches to examine the functional role of Cl^- channels in the context of health and disease have demonstrated that Cl^- channels might contribute to: 1) arrhythmogenesis in myocardial injury; 2) cardiac ischemic preconditioning; and 3) the adaptive remodeling of the heart during myocardial hypertrophy and heart failure. Therefore, anion channels represent very attractive novel targets for therapeutic approaches to the treatment of heart diseases. Recent evidence suggests that Cl^- channels, like cation channels, might function as a multiprotein complex or functional module. In the post-genome era, the emergence of functional proteomics has necessitated a new paradigm shift to the structural and functional assessment of integrated Cl^- channel multiprotein complexes in the heart, which could provide new insight into our understanding of the underlying mechanisms responsible for heart disease and protection.

Introduction

Abnormalities of cardiac ion channels have been linked to a variety of inherited and acquired cardiac diseases including myocardial ischemia, hypertrophy, heart failure, and arrhythmias[1–5]. In addition, ion channels may also be mediators of the cardioprotective effects of ischemic preconditioning (IPC)[6,7]. While cation (K^+, Na^+, and Ca^{2+}) channels have received the most attention in the past four decades, the role of anion channels in the cardiovascular system has been largely ignored. Within the last 15 years, a re-surgence of interest in Cl^- channels in the cardiovascular system has led to the discovery of at least seven different types of Cl^- currents in cardiac cells from different regions of the heart and in different species[8]. Intensive efforts have been given to characterize the properties of these anion channels at the cellular and molecular levels. More details about the biophysical, pharmacological, and molecular properties of Cl^- channels in the heart can be found in several recent excellent review articles[8–11]. It has also been demonstrated in recent studies that Cl^- channels may be involved in the regulation of a large repertoire of cellular functions, including cellular excitability, cell volume homeostasis, intracellular organelles acidification, cell migration, proliferation and differentiation, and apoptosis[8,9,12]. With the recent identification of molecular entities responsible for cardiac Cl^- channels[8] and the genes mapped to specific human chromosomal locations[13], gene targeting and transgenic techniques have been used to delineate the functional role of Cl^- channels in the context of health and disease. It has been reported that Cl^- channels could contribute to: 1) arrhythmogenesis in myo-

cardial injury; 2) the adaptive remodeling of the heart during myocardial hypertrophy and heart failure; and 3) IPC. Therefore, anion channels represent very attractive novel targets for therapeutic approaches to the treatment of heart diseases. In this review, we will briefly summarize the major findings and recent advances in the study of functional role of anion channels in the heart.

Anion channels in the heart

Since the independent discovery of a cAMP-activated Cl^- current in the guinea pig heart by Bainski *et al*, Harvey and Hume in 1989[14,15], intensive efforts have been made to characterize Cl^- channels in the cardiovascular system at both the cellular and molecular levels. These have been

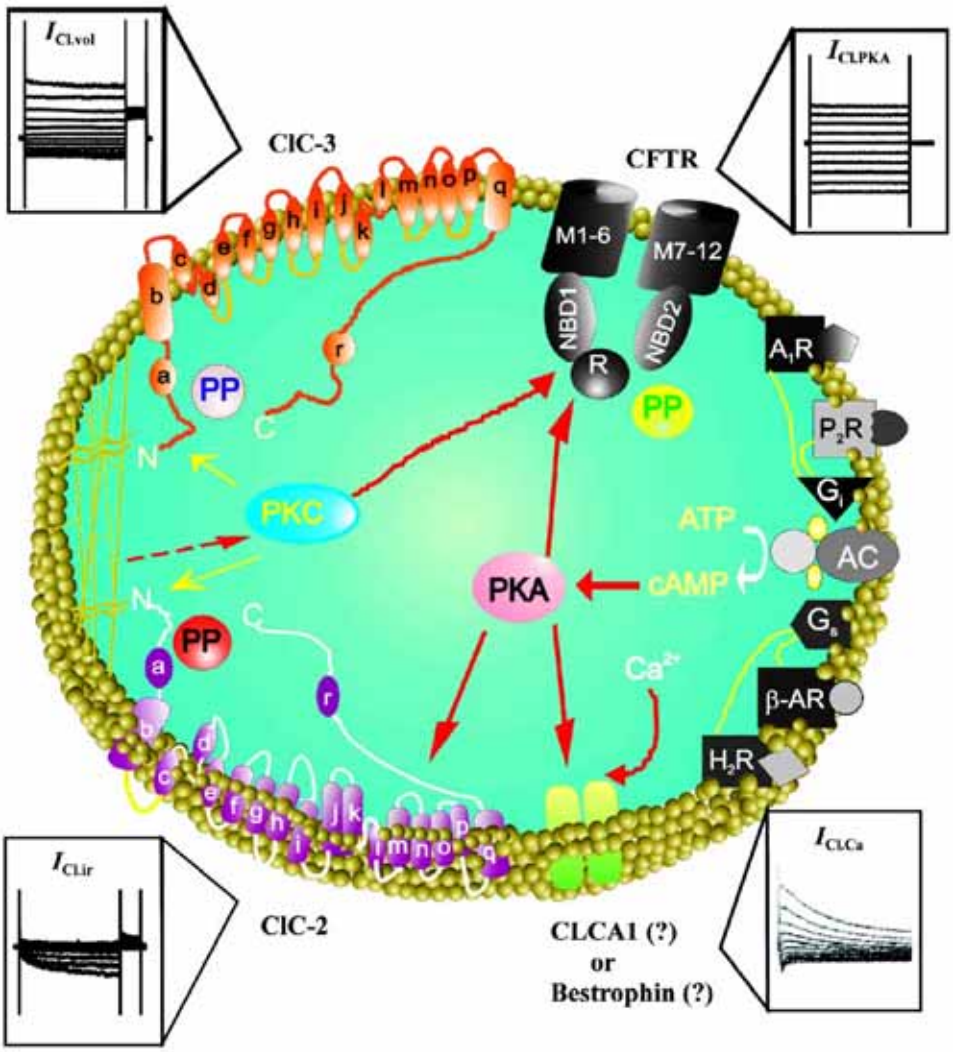

Figure 1. Schematic representation of anion channels in cardiac myocytes. Anion channels and their corresponding molecular entities or candidates are indicated in parentheses. I_{CLPKA}, Cl^- current regulated by adenylyl cyclasecAMP-protein kinase A pathway; CFTR, cystic fibrosis transmembrane conductance regulator; M1–6, CFTR transmembrane spanning segments 1–6; M7–12, CFTR transmembrane spanning segments 7–12; NBD1, nucleotide binding domain 1; NBD1, nucleotide binding domain 2; R, regulatory subunit; P, phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC); PP, serine-threonine protein phosphatases; Gi, heterodimeric inhibitory G protein; A_1R, adenosine type I receptor; M_2R, muscarinic type II receptor; AC, adenylyl cyclase; H_2R, histamine type II receptor; Gs, heterodimeric stimulatory G protein; β-AR, β-adrenergic receptor; P_2R, purinergic type 2 receptor; I_{Clvol}, Cl^- current regulated by cell volume; ClC-3, member of voltage-gated ClC Cl^- channel family; $I_{Cl\,Ca}$, Cl^- current regulated by intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$); CLCA1, member of a Ca^{2+}-sensitive Cl^- channel family (CLCA); *Bestrophin*: the *Bestrophin* gene family; I_{Clir}, inward rectifying Cl^- current; ClC-2, member of voltage-gated ClC Cl^- channel family. Membrane topology models (α-helices A-X) for ClC-2 and ClC-3 are modified from Dutzler *et al*[125].

recently reviewed and thoroughly described elsewhere[8–11] and will not be repeated in this review. Briefly, at the molecular level, all cardiac Cl^- channels described so far may fall into the following Cl^- channel gene families (Figure 1): 1) the cystic fibrosis transmembrane conductance regulator (CFTR), which is a member of the adenosine triphosphate-binding cassette (ABC) superfamily and may be responsible for the Cl^- currents activated by protein kinase A (PKA) (I_{ClPKA})[14–16], protein kinase C (PKC) (I_{ClPKC})[17,18], and extracellular ATP (I_{CLATP}) in the heart[19–21]; 2) ClC voltage-gated Cl^- channel superfamily: a) *ClC-2*, which is responsible for the hyperpolarization- and cell swelling-activated inwardly rectifying Cl^-current (I_{Clir})[22–24]; b) *ClC-3*, which is responsible for the volume regulated outwardly rectifying Cl^- current (I_{Clvol}), including the basally-activated (I_{Clb})[25] and swelling-activated ($I_{Clswell}$) components[25–34]; 3) *CLCA-1*, which was thought to be responsible for the Ca^{2+}-activated Cl^- current (I_{ClCa})[35–38]; and 4) *Bestrophin*, a new candidate for I_{ClCa}[39–42]. Further studies on the molecular and functional properties of these Cl^- channel genes are necessary to define the structure of the channel proteins and to elucidate the physiological and clinical significance of these channels.

Functional role of Cl^- channels in cardiac diseases

Theoretically, Cl^- channels could be involved in the regulation of cellular excitability, cell volume homeostasis, intracellular organelles acidification, cell proliferation and differentiation, and apoptosis[12]. Thus, they may have important physiological and pathological significance in cardiac function under normal and pathological (hypoxia, ischemia, myocardial infarction, hypertrophy, and heart failure) conditions. Mutations in several Cl^- channels have been known to result in human inherited diseases[13]. But the exact role of Cl^- channels in human cardiovascular physiology and pathophysiology is still unclear[8]. The ability to examine the exact role of Cl^- channels in human cardiovascular physiology and pathology has been hampered by the lack of specific pharmacological and molecular tools. With the recent identification of the molecular entities responsible for Cl^- channels in the heart[8] and the genes mapped to specific human chromosomal locations[13], it is now possible to overcome these obstacles by use of gene targeting and transgenic animals. We have been using a multitude of approaches from traditional methodologies including biophysics, biochemistry, electrophysiology, and pharmacology to state-of-the-art technologies including telemetry system, echocardiography, genomics, and proteomics to effectively and accurately define the role of each Cl^- channel in heart function in the context of health and disease.

Functional role in electrophysiology and arrhythmogenesis

Estimates of intracellular Cl^- activity (a^i_{Cl}) in cardiac myocytes from ion-selective microelectrode studies indicate the equilibrium potential for Cl^- (E_{Cl}) be more positive than the resting membrane potential under normal physiological conditions with an extracellular Cl^- concentration ($[Cl^-]_o$) of 145 mmol/L and an intracellular Cl^- concentration ($[Cl^-]_i$) of 10 to 20 mmol/L[43–46]. Because the E_{Cl} is within a membrane potential range (usually –65 to –40 mV) that can be either negative or positive to the actual membrane potential during the normal cardiac cycle, activation of cardiac Cl^- channels can generate both inward and outward currents (Figure 2). Thus, compared with cationic channels, Cl^- channels have the unique ability to cause both depolarization as well as repolarization during the action potential and produce significant effects on cardiac pacemaker activity and action potential characteristics.

The degree to which activation of Cl^- currents depolarizes the resting membrane or accelerates the repolarization of action potential depends critically on the actual value of E_{Cl} and the magnitude of the Cl^- conductance relative to the total membrane conductance. Under physiological conditions the transmembrane Cl^- gradient is asymmetrical. Thus, the activation of CFTR and ClC-3 Cl^- channels in the heart will result in outwardly rectifying currents. This will have more significant effects at positive potentials to accelerate repolarization and shortening of the action potential duration compared with smaller depolarizing effects at negative potentials near the resting membrane potential (Figure 2). The ability of Cl^- current activation to depolarize cardiac cells is also opposed by the presence of a large background K^+ conductance that normally controls the resting membrane potential. Both abbreviation of APD and depolarization of E_m upon activation of Cl^- channels may play a role in rhythm disturbance and likely contribute to arrhythmogenesis under pathological conditions.

CFTR and arrythmogenesis CFTR channels are closed under basal conditions but can be open under conditions where intracellular PKA- and PKC-dependent phosphorylation activity is increased. A major physiological role of activation of CFTR channels may be to minimize (oppose) the significant action potential prolongation associated with β-adrenergic stimulation of I_{Ca}. This is expected to contribute to action potential shortening during strong adrenergic stimu-

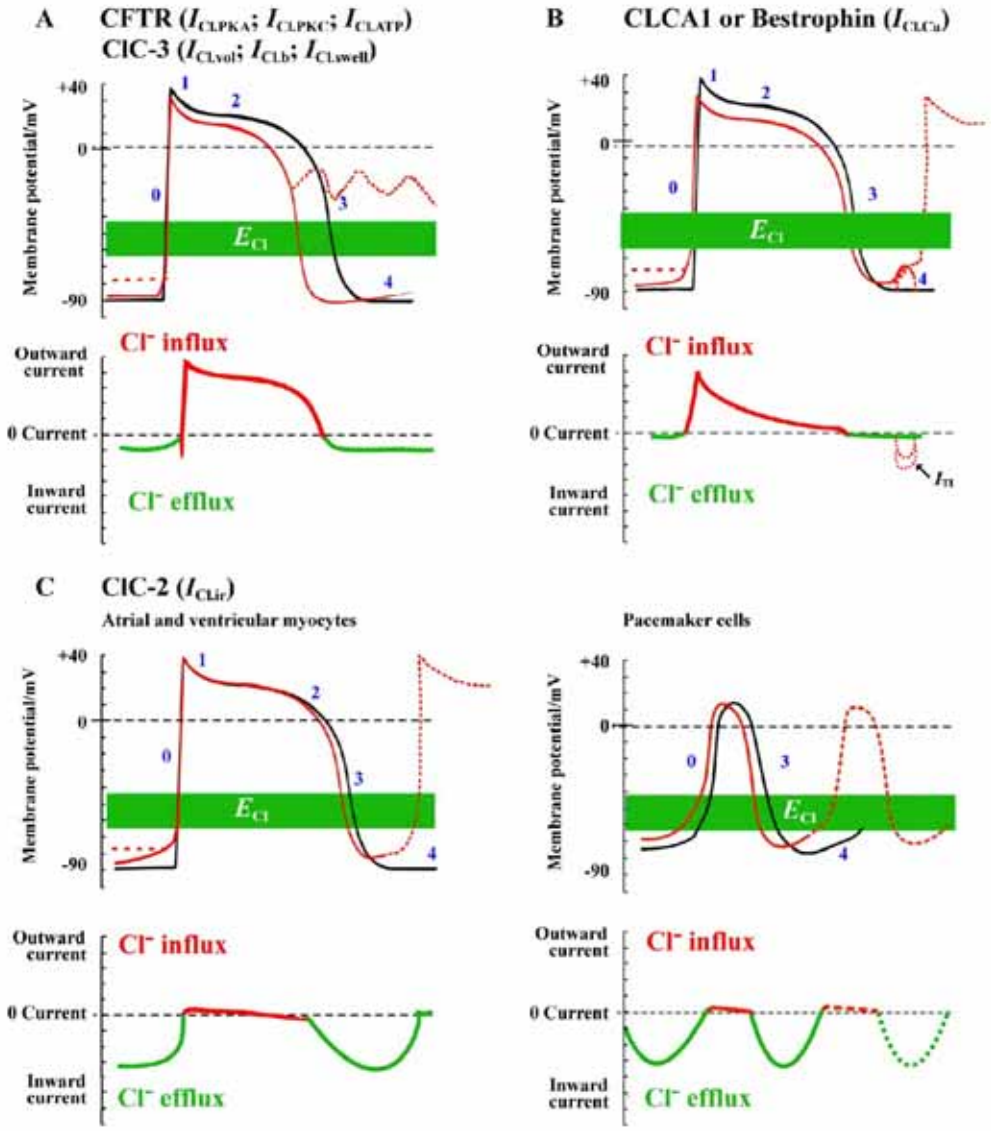

Figure 2. Modulation of cardiac electrical activity by sarcolemmal anion channels in cardiac myocytes. Changes in action potentials (top) and membrane currents (bottom) caused by activation of cystic fibrosis transmembrane conductance regulator (CFTR) and volume-regulated ClC-3 Cl^- channels (A), Ca^{2+}-activated Cl^- channels (B), ClC-2 channels (C) are depicted. $I_{Cl,CFTR}$ can be activated by stimulation of protein kinase A (PKA), protein kinase C (PKC), or purinergic receptors; $I_{Cl,vol}$ is activated by cell swelling induced by exposure to hypotonic extracellular solutions or possibly membrane stretch; $I_{Cl,Ca}$ is activated by elevation of localized $[Ca^{2+}]_i$. $I_{Cl,ir}$ is activated by hyperpolarization, cell swelling, acidosis. Range of estimates for normal physiological values for Cl^- equilibrium potential (E_{Cl}) is indicated in green in top panels in A–C; range of zero-current values corresponding to E_{Cl} is shown in green in bottom panels. Numbers in top panels of A–C illustrate conventional phases of a prototype ventricular action potential under control conditions (black) and after activation of I_{Cl} (red). In A, activation of I_{Cl} induces larger membrane depolarization and induction of early afterdepolarizations (EAD) under conditions where resting K^+ conductance is reduced (dashed red lines); in B, activation of $I_{Cl,Ca}$ during $[Ca^{2+}]_i$ overload results in oscillatory transient inward current (I_{Tl}) and induction of delayed afterdepolarization (DAD); in C, activation of $I_{Cl,ir}$ during hyperpolarization causes acceleration of phase 4 depolarization and automaticity (dashed red lines, left panel) and shortening of action potential duration, membrane depolarization and/or phase 4 depolarization and induction of abnormal electrical impulse (trigger activity) and automaticity (right panel).

lation and faster heart rates. Therefore, activation of CFTR channels may prevent excessive prolongation of APD and protect the heart against the development of early after depolarizations (EAD) and triggered activity caused by activation of Ca^{2+} channels in the presence of β-adrenergic stimulation. EAD arising from phase 2 and 3 underlie focal triggered tachyarrhythmias and repolarization abnormalities, which contribute to cardiac sudden death[47]. It is well-

established that APD prolongation favors EAD by allowing recovery of inward currents and, conversely, shortening of APD makes it more difficult to induce EAD. Therefore, activation of CFTR channels should protect against focal triggered arrhythmias. However, when background K^+ conductance is reduced in the case of myocardial hypokalemia, activation of CFTR channels will cause significant membrane depolarization and induce abnormal automaticity leading to the development of, EAD (dotted red lines in Figure 2A). These predicted effects of CFTR channel activation on APD and automaticity have been verified experimentally by manipulations of the Cl^- gradient or the use of Cl^- channel blockers[11,48–51]. Histamine was found to activate CFTR channels in ventricular myocytes and induce oscillatory activity and abnormal impulses in the heart, although the contribution of CFTR channels to these arrhythmogenic activities has not been further explored. It has been shown that activation of CFTR channels contributes to hypoxia-induced shortening in APD[52]. Activation of CFTR channels may accelerate the development of reentry because of the shortening of APD and refractoriness and a decrease in conduction velocity caused by a slight depolarization of diastolic potential leading to Na^+ channel inactivation.

ClC-3 and arrhythmogenesis The current through ClC-3 channels under basal or isotonic conditions is small, but can be further activated by stretching of the cell membrane by inflation and/or cell swelling induced by exposure to hypoosmotic solutions. Activation of ClC-3 channels is expected to induce a similar effect on cardiac action potentials as that of activation of CFTR channels (Figure 2A) because both Cl^- currents through both channels are relatively time- and voltage-independent over the physiological range of membrane potentials[53,54]. Activation of ClC-3 channels might produce more significant action potential shortening than CFTR channels because of its stronger outwardly rectifying property. Because myocardial cells swell during hypoxia and ischemia, and the washout of hyperosmotic extracellular fluid after reperfusion induces further cell swelling, activation of ClC-3 channels may also contribute to hypoxia, ischemia and reperfusion induced shortening in APD and arrhythmias[9,53,54]. Abbreviation of APD and, therefore, the effective refractory period reduces the length of the conducting pathway needed to sustain reentry (wavelength). In principle, this favors the development of atrial or ventricular fibrillation, which depends on the presence of multiple reentrant circuits or rotating spiral waves. $I_{Clswell}$ also may slow or enhance the conduction of early extrasystoles, depending on the timing. In the case of myocardial hypertrophy and heart failure, ionic remodeling is one of the major features of pathophysiological changes[55]. It has been found that the current densities and molecular expression of several major repolarizing K^+ channels (such as Kv4x) are significantly reduced, which may be responsible for the prolongation of APD and development of EAD[55]. However, under these conditions, I_{Clvol} is constitutively active[56]. The persistent activation of I_{Clvol} may limit the APD prolongation and make it more difficult to elicit EAD. Indeed, as shown in Figure 3, in myocytes from hearts in failure, block of I_{Clvol} by tamoxifen significantly prolonged APD and decreased the depolarizing current required to elicit EAD by about 50% (Figure 4B) and hyperosmotic cell shrinkage, which also inhibits I_{Clvol}, was almost equivalent to tamoxifen in causing EAD in these myocytes (Figure 4C)[9]. Therefore, the consequences of activation of I_{Clvol} are very complex. It may be detrimental, beneficial, or simultaneously both in different parts of the heart.

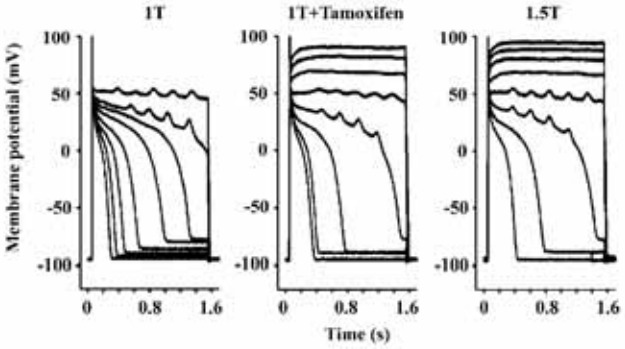

Figure 3. Activation of I_{Clvol} shortened APD and increased threshold for inducing early afterdepolarizations (EAD) in canine ventricular myocytes isolated from infarction and peri-infarction zones 30 days after left anterior descending artery ligation (MI). EAD were elicited with depolarizing pulses (20–200 pA) and current-voltage relationships were obtained from the same cells (not shown). Threshold for inducing EAD was 120 pA in a post-MI ventricular myocyte in 1T. Tamoxifen (10 μmol/L) reduced the threshold for induction of EAD in isotonic (1 T) solutions from 120 to 60 pA. Hyperosmotic shrinkage (in 1.5 T solutions), which also inhibits I_{Clvol}; reduced threshold for eliciting EAD to 40 pA. All panels from same cell under perforated patch conditions. (From Baumgarten and Clemo[9] with permission from Elsevier Science).

It has been shown that mechanical stretching or dilation of the atrial myocardium is able to cause arrhythmias. Since $I_{Clswell}$ was also found in sino-atrial (S-A) nodal cells, ClC-3 channels may serve as a mediator of mechanotransduction and play a significant role in the pacemaker function if they act as the stretch activated channels in these cells[9,57]. Baumgarten's laboratory has recently demonstrated that $I_{Clswell}$ in ventricular myocytes could be directly activated by

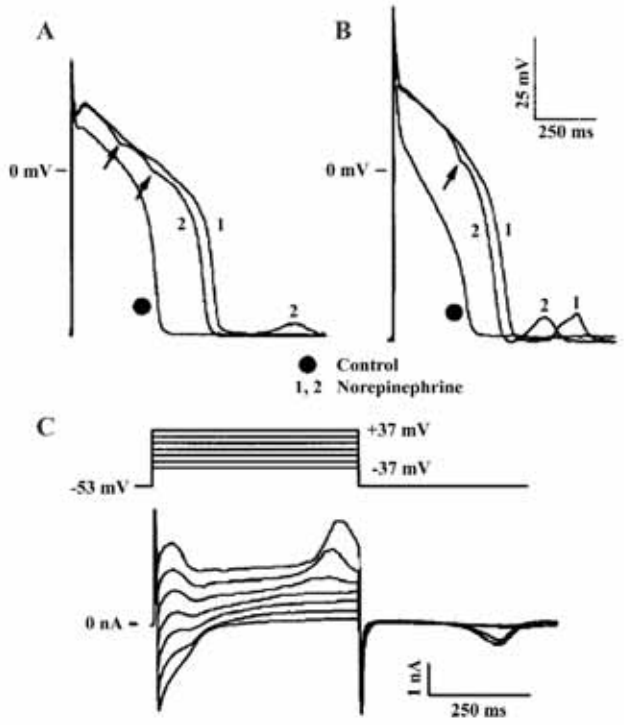

Figure 4. Contribution of I_{ClCa} to delayed afterdepolarization in ventricular and Purkinje myocytes. (A) Ventricular action potentials were recorded during control (•), 38 (1), and 45 (2) seconds after application of norepinephrine. Norepinephrine elevated the plateau and prolonged the action potential duration (1) and eventually caused delayed afterdepolarization (DAD) accompanied by transient repolarizations during the plateau (arrows). (B) Purkinje action potentials were recorded during control (•), 49 (1), and 52 (2) seconds after application of norepinephrine. Both action potentials recorded in the presence of norepinephrine were accompanied by DAD, but only the action potential recorded at (2) shows a transient repolarization (arrow) as well. (C) Membrane currents in ventricular myocyte activated by voltage steps from -53 mV to potentials between -23 and +37 mV in the presence of norepinephrine (1 mmol/L). Depolarization to -23 mV induced a large Ca^{2+} current which is superimposed with a transient outward current. This became more pronounced with stronger depolarization (From Verkerk *et al*[64] with permission from American Heart Association).

mechanical stretch through selectively stretching β1-integrins with mAb-coated magnetic bEAD[9,58,59]. Although it has been suggested that stretch and swelling activate the same anion channel in some non-cardiac cells, further study is needed to determine whether this is true in cardiac myocytes.

Ca^{2+}-activated Cl^- channel and arrhythmogenesis As illustrated in Figure 2B, the activation of I_{ClCa} will have considerably different effects on cardiac action potentials and resting membrane potential from those of CFTR and ClC-3 channels, even though I_{ClCa} is also expected to be outwardly rectifying under physiological conditions. This is because the kinetic behavior of I_{ClCa} is significantly determined by the time course of the $[Ca^{2+}]_i$ transient[60]. Normally, I_{ClCa} will have insignificant effects on the diastolic membrane potential, as resting $[Ca^{2+}]_i$ is low. When $[Ca^{2+}]_i$ is substantially increased above the physiological resting level, however, I_{ClCa} carries a significant amount of transient outward current. I_{ClCa} will be activated early during the action potential in response to an increase in $[Ca^{2+}]_i$ associated with Ca^{2+}-induced Ca^{2+} release (CICR). The time course of decline of the $[Ca^{2+}]_i$ transient will determine the extent to which I_{ClCa} contributes to early repolarization during phase 1 (Figure 2B). In the rabbit left ventricle, I_{ClCa} contributes to APD shortening in subendocardial myocytes but not in subepicardial myocytes. These differences in functional expression of I_{ClCa} may reduce the electrical heterogeneity in the left ventricle[61]. In Ca^{2+}-overloaded cardiac preparations, I_{ClCa} can contribute to the arrhythmogenic transient inward current (I_{Ti}, Figure 2B)[62]. I_{Ti} produces delayed after-depolarization (DAD)[63] and induces triggered activity (red dotted line in Figure 2B), which is an important mechanism for abnormal impulse formation. In sheep Purkinje and ventricular myocytes, activation of I_{ClCa} was found to induce DAD and plateau transient repolarization (Figure 4)[64]. Therefore, blockade of I_{ClCa} may be potentially antiarrhythmogenic by reducing DAD amplitude and triggered activity based on DAD. However, the role of I_{ClCa} in phase 1 repolarization and the generation of EAD and DAD of either normal or failing human heart seem very limited[65–67]. Therefore, the clinical relevance of I_{ClCa} blockers remains to be determined.

ClC-2 and arrhythmogenesis ClC-2 channels are activated by hyperpolarization, cell swelling, and acidosis and have an inwardly rectifying *I-V* relationship. During the cardiac action potential, therefore, the ClC-2 channel will conduct a mainly inward current as a result of Cl^- efflux at negative membrane potentials and cause a depolarization of the resting membrane potential of cardiac cells. At membrane potentials more positive than E_{Cl}, ClC-2 may conduct a small outward current as a result of Cl^- influx and may accelerate repolarization of the action potential. It is also possible that, in a manner analogous to the role and tissue distribution pattern of the cationic pacemaker channels (I_f), Cl.ir channels normally play a much more prominent role in the SA or AV nodal regions of the heart (Figure 2C). The hyperpolarization-activated inward rectifying Cl^- current (I_{Clir}) through ClC-2 channels under basal or isotonic conditions is small, but can be further activated by hypotonic cell swelling[22] and acidosis[23,24]. The volume-sensitivity of the channel also suggests its role in cell volume regulation. The sensitivity of ClC-2 to $[H^+]_o$ and cell volume may be of pathologi-

cal importance during hypoxia- or ischemia-induced acidosis or cell swelling. Therefore, it may be possible that the significance of $I_{Cl,ir}$ in the heart becomes more prominent under some pathological conditions (ischemia or hypoxia)[68]. As a matter of fact, ischemia and acidosis have consistently been shown to depolarize the resting membrane potential of cardiac myocytes, increase automaticity and cause lethal arrhythmias, although the mechanism has remained obscure[1,11]. It is reasonable to suggest that an increase in ClC-2 conductance could be responsible for these phenomena and be pro-arrhythmic. Drugs targeting ClC-2 channels could be anti-arrhythmic. Therefore, the ClC-2 channels could have important clinical significance for such cardiac diseases as arrhythmias, ischemia and reperfusion, and congestive heart failure. Activation of ClC-2 current should mainly cause a depolarization of the RMP and it is suggested that the acidosis-induced increase in $I_{Cl,ir}$ might underlay the depolarization of the resting membrane potential during acidosis or hypoxia[23,24].

It should be pointed out that prediction of the consequences of activation of Cl^- channels is complex. Most studies that have examined the contribution of Cl^- currents to the cardiac action potential and arrhythmias have relied on anion antagonist and substitution experiments. The pharmacological specificity of many of these anion channel antagonists can be problematic, and anion substitution, in addition to altering anion movement through channels, can have other unpredictable side effects on other transport proteins and signaling pathways[69,70]. With the recent identification of the molecular entities responsible for Cl^- channels in the heart, it is now possible to combine electrophysiological, molecular biological, and especially gene-targeting techniques in the study of cardiac Cl^- channels to effectively and accurately define the role of each Cl^- channel in heart function. However, as the distribution of various Cl^- channels in the heart varies among cell types and regions[8], activation of these channels may increase the dispersion of the electrophysiological properties and provide substrates for heart diseases involving cardiac arrhythmias and myocardial remodeling.

Functional role of Cl^- channels in cardiac IPC

Ischemia causes myocardial damage and leads to infarction through apoptosis (programmed cell death) and necrosis. IPC is a phenomenon in which brief ischemic episodes elicit protection of the heart against sustained ischemia. It has been suggested that both sarcolemmal and mitochondrial ATP-sensitive potassium channels (sarc-K_{ATP} and mito-K_{ATP}, respectively) may serve as triggers or end-effectors. PKC may link cellular signal events during ischemia to the activation of end-effectors, which will somehow prevent or delay apoptosis and protect the cardiac myocytes. The precise mechanism of IPC, however, remains to be elucidated. Several recent studies have pointed to a potential role of Cl^- channels in IPC.

$I_{Clswell}$ and ClC-3 in IPC It has been reported that the block of $I_{Clswell}$ in rabbit cardiac myocytes inhibits preconditioning by brief ischemia, hypoosmotic stress[71,72] and adenosine receptor agonists[73]. These studies are solely based on the use of several Cl^- channel blockers, such as anthracene-9-carboxylic acid (9-AC) and 4-acetamide-4'-isothiocyanato-stilbene-2,2'-disulfonic acid (SITS). As mentioned above, these pharmacological tools lack specificity to a particular Cl^- channel in the heart and may also act on other ion channels or transporters. Therefore it has been very difficult to confirm the causal role of $I_{Clswell}$ in IPC[74]. The exact role of $I_{Clswell}$ in IPC needs to be further determined adequately using more specific approaches. To specifically test whether the volume-regulated Cl^- channels are indeed involved in IPC, we have recently established *in vitro* and *in vivo* IPC models in *ClC-3* knockout mice ($ClCn3^{-/-}$). Our preliminary results indicate that targeted inactivation of *ClC-3* gene prevented protective effects of late IPC but not of early IPC, suggesting that $I_{Clswell}$ may contribute differently to early and late IPC[75]. The underlying mechanisms for these differential effects are currently unknown. Recent reports, however, suggest that $I_{Clswell}$ and ClC-3 might play an important role in apoptosis. Cl^- channel blockers 4,4'-diisothiocyano-stilbene-2,2'-disulphonate (DIDS) and 5-nitro-2-(3-phenylpropyllamino)-benzoic acid (NPPB) were as potent as a broad-spectrum caspase inhibitor in preventing apoptosis and elevation of caspase-3 activity and improved cardiac contractile function after ischemia and *in vivo* reperfusion[76]. Transgenic mice overexpressing Bcl-2 in the heart had significantly smaller infarct size and reduced apoptosis of myocytes after ischemia and reperfusion[77]. It has been shown that Bcl-2 induces up-regulation of I_{Clvol} by enhancing ClC-3 expression in human prostate cancer epithelial cells[78]. Cell shrinkage is an integral part of apoptosis, suggesting that I_{Clvol} and ClC-3 might be intimately linked to apoptotic events through regulation of cell volume homeostasis[78,79].

CFTR channels and IPC Several lines of evidence suggest that CFTR channels could be involved in IPC including: (1) sarc-K_{ATP} blockers, such as glibenclamide, which suppress IPC protection, also block CFTR channels in noncardiac[80,81] and cardiac cells[19,82]; (2) PKC and PKA, two essential second messengers in IPC[83,84] can activate CFTR

channels[8,19,85]; and (3) triggers of IPC (nitric oxide, opioids, and adenosine *etc*) can all regulate CFTR channel function[8]. We have directly tested whether activation of CFTR channels is involved in IPC by studying hemodynamics and tissue injury of hearts isolated from WT and two strains of *CFTR* knockout ($CFTR^{-/-}$) mice subjected to ischemia and reperfusion. In isolated mouse heart perfused in the Langendorff or working heart mode, we have recently found that targeted inactivation of *CFTR* gene prevented protection on cardiac function and myocardium injury against sustained ischemia by ischemic preconditioning (Figure 5)[86]. Our *in vivo* studies using both wild type and *CFTR* knockout mice also demonstrated that CFTR was an important mediator in both early and late ischemic preconditioning in the heart[87]. Several mechanisms may be responsible for a functional role of CFTR channels in mouse heart IPC: (1) It has been demonstrated that cardiac CFTR plays a role in early action potential shortening during hypoxia and ischemia[52]. Activation of CFTR will also decrease resting membrane potential and action potential duration, thereby limiting intracellular Ca^{2+} overload and cell damage[8]; (2) The CFTR channel is an important transporter of sphingosine 1-phosphate (S-1-P)[88], which has recently emerged as an important lipid messenger involved in IPC[89]; (3) CFTR is permeable not only to Cl^-, but also to larger organic ions, as well as reduced and oxidized forms of glutathione (GSH)[90]. Therefore CFTR may contribute to the control of oxygen stress-induced apoptosis and the regulation of inflammation and the immune responses; (4) CFTR might decrease intracellular pH and modulate apoptosis[91]; (5) CFTR functions as a regulator of volume-dependent homeostatic cell mechanisms in cell proliferation and apoptosis[92]. We are currently in the

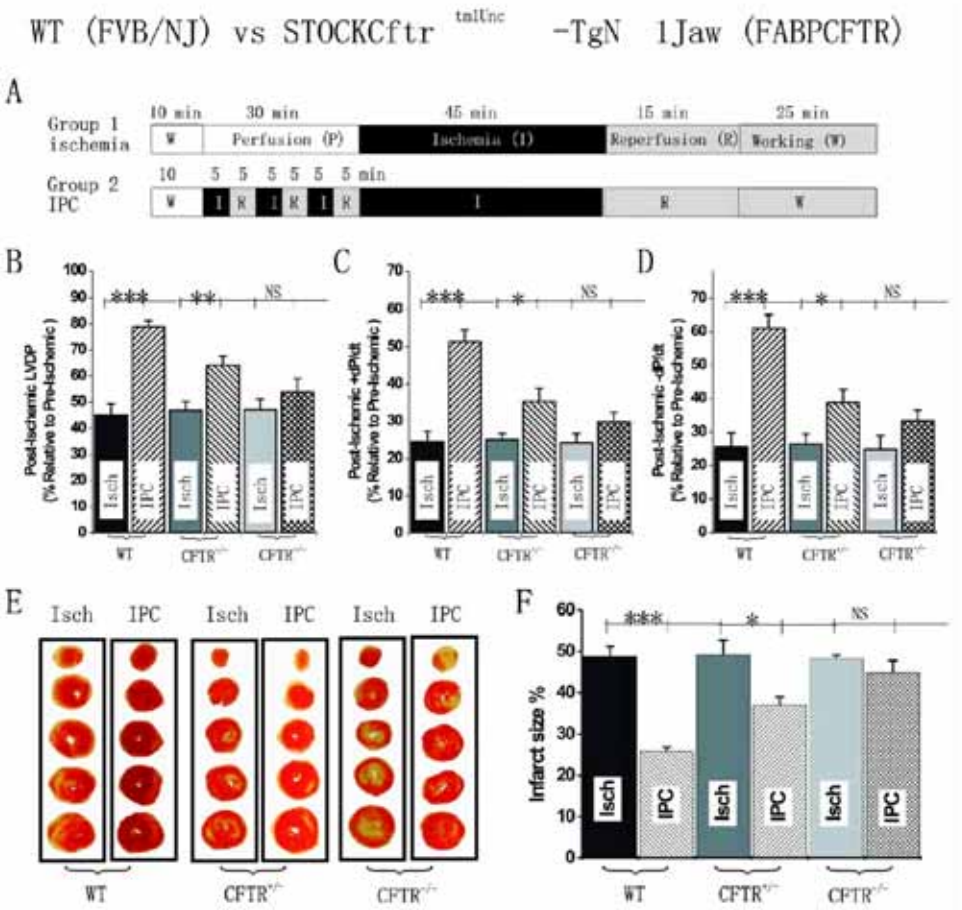

Figure 5. Effects of cystic fibrosis transmembrane conductance regulator (CFTR) gene knockout (FABPCFTR) on ischemic preconditioning (IPC) in isolated working mouse heart. (A) Experimental protocol. (B–D) Recovery of left ventricular contractile (B,C) and relaxation (D) function of WT (FVB/NJ), $CFTR^{-/+}$ and $CFTR^{-/-}$ (FABPCFTR) mice after 45 min of ischemia and 40 min of reperfusion. (E,F) IPC on infarct size of ventricles. (E) Representative ventricle transverse slices after ischemia (Isch) or IPC. (F) Mean infarct size measured from age-matched WT, $CFTR^{-/+}$, or $CFTR^{-/-}$ mouse heart after ischemia (Isch) or IPC (n=6 for each group). *P<0.05, **P<0.01, ***P<0.001. (From Chen *et al*[86] with permission from American Heart Association)

process of investigating these potential mechanisms and the relative role of CFTR in early and late preconditioning.

$I_{Cl,Ca}$ in IPC It has been well known that ischemia/reperfusion usually causes a cytosolic overload of Ca^{2+} in cardiac myocytes[93,94]. Therefore, it is very possible that $I_{Cl,Ca}$ may be activated during ischemia and reperfusion[38,62,64,95-98]. But, no information for the possible involvement of $I_{Cl,Ca}$ in IPC is currently available.

Functional role of Cl^- channels in myocardial hypertrophy and heart failure Myocardial hypertrophy and its progression to dilated cardiomyopathy or heart failure are characterized by not only structural remodeling, including hypertrophic growth of cardiac myocytes (changes in cell volume) and changes in the cytoskeleton and extracellular matrix (ECM)[99,100] but also ionic remodeling, that is, changes in expression and activity of many ion channels. It should be pointed out that ionic remodeling during the progression of hypertrophy to heart failure provides not only substrates for arrhythmias but also cellular mechanisms for structural remodeling. During the remodeling process, multiple neurohormonal and intracellular signaling cascades, including tyrosine kinases, PKA, PKC, protein phosphatases, MAP kinases, and endothelin, are activated[101]. These second messengers are well-known effective regulators of various ion channels. Indeed, it has been found that several cation channels, such as K^+ channels, Ca^{2+} channels, and stretch-activated non-selective channels, undergo significant changes. Recent evidence also supports possible involvement of anion channels in the remodeling process.

$I_{Clswell}$ and ClC-3 in myocardial hypertrophy and heart failure $I_{Clswell}$ is persistently activated in ventricular myocytes from a canine pacing-induced dilated cardiomyopathy model[102]. Using the perforated patch-clamp technique, Clemo *et al* found that, even in isotonic solutions, a large 9-AC-sensitive, outwardly rectifying Cl^- current was recorded in heart failure myocytes but not in normal myocytes. Graded hypotonic cell swelling (90%–60% hypotonic) failed to activate additional current while graded hypertonic cell shrinkage caused an inhibition of the "basal" Cl^- current in failure myocytes. Moreover, the maximum current density of the $I_{Clswell}$ in failure myocytes was about 40% greater than that in osmotically swollen normal myocytes. Constitutive activation of $I_{Clswell}$ is also observed in several other animal models of heart failure, such as a rabbit aortic regurgitation model of dilated cardiomyopathy[103,104] and a dog model of heart failure caused by myocardial infarction[105]. In human atrial myocytes obtained from patients with right atrial enlargement and/or elevated left ventricular end-diastolic pressure, a tamoxifen sensitive $I_{Clswell}$ was also found to be persistently activated[106]. It is not known at this time whether $I_{Clswell}$ is also persistently activated in hypertrophied non-failure (or non-dilated) myocytes in the above described models or in the human heart. In a rat aortic constriction model, however, a 9-AC-sensitive Cl^- current is present in hypertrophied ventricular myocytes but not in control myocytes, and this hypertrophy-activated Cl^- current seems to contribute to the shortening of APD in the hypertrophied cells[107]. It is not known, however, whether this hypertrophy-activated Cl^- current is the same as $I_{Clswell}$ because the volume-sensitivity of this Cl^- current was not assessed. Nevertheless, it is possible that persistent activation of $I_{Clswell}$ is a common response of cardiac myocytes to hypertrophy or heart failure-induced remodeling. The mechanism for this phenomenon is still not clear. Perhaps the cell volume increase caused by hypertrophy and cell membrane stretch caused by dilation, are both involved in the activation of $I_{Clswell}$. Alternatively, the persistent activation of $I_{Clswell}$ may be caused by signaling cascades activated during hypertrophy and heart failure independent of changes in cell length and volume, or both. $I_{Clswell}$ could be activated by direct stretching of β1-integrin through focal adhesion kinase (FAK) and/or Src[58]. Mechanical stretch of myocytes also releases angiotensin II (AngII), which binds to AT1 receptors (AT1R) and stimulates FAK and Src in an autocrine-paracrine loop. A recent study by Browe and Baumgarten suggests that the stretch of β1-integrin in cardiac myocytes activates $I_{Clswell}$ by activating AT1R and NADPH oxidase and, thereby, producing reactive oxygen species. In addition, NADPH oxidase may be intimately coupled to the channel responsible for $I_{Clswell}$, providing a second regulatory pathway for this channel through membrane stretch or oxidative stress[59]. This finding is very important for further understanding of the mechanism for hypertrophy activation of $I_{Clswell}$ and ClC-3 channels and their relationship to hypertrophy and heart failure as it is very well known that Ang II plays a crucial role in myocardial hypertrophy and heart failure[108].

The functional and clinical significance of $I_{Clswell}$ in the hypertrophied and dilated heart is currently unknown. Using a mouse aortic banding model of myocardial hypertrophy, we have recently found that targeted disruption of ClC-3 gene ($ClCn3^{-/-}$) accelerated the development of myocardial hypertrophy and the discompensatory process[109], suggesting that activation of I_{Clvol} might be important in the adaptive remodeling of the heart during pressure overload. Further studies on the mechanism for the ClC-3 channels' effects on hypertrophy and heart failure are in progress in our laboratory. It is well accepted that in most cells activation of I_{Clvol} represents one important trigger to initiate regula-

tory volume decrease (RVD) when cells swell[12]. Cell volume homeostasis, therefore, could be an important function of $I_{Clswell}$ activation in the heart. Activation of Cl^- conductance causes significant changes in APD and intracellular Ca^{2+} concentration, and should also affect excitation-contraction (E-C) coupling, contractility, and other hemodynamic functions of the heart[8,11]. Recent studies suggest that $I_{Clswell}$ and ClC-3 channels play important roles in cell proliferation[110], differentiation[111], migration[112], and apoptosis[78,79]. All of these have been demonstrated as important cellular processes in myocardial remodeling during hypertrophy and heart failure[113].

CFTR in myocardial hypertrophy and heart failure Remodeling of CFTR channels has been observed in myocardial hypertrophy and heart failure. Using *in situ* mRNA hybridization in a combined pressure and volume overload model of heart failure in the rabbit, Wong *et al* found that the normal epicardial to endocardial gradient of *CFTR* mRNA expression is reversed due to a significant decrease in epicardial expression of CFTR mRNA in the rabbit left ventricle[114]. A post-translational change in the CFTR expression could be responsible for this phenomenon[115]. The loss of the normal transmural gradient of repolarising ion channels is likely to contribute to instability of repolarisation in the hypertrophied heart and hence increased risk of cardiac arrhythmias in patients with heart failure. The exact functional and clinical significance of the changes in CFTR expression during hypertrophy and heart failure is currently not clear and merits further study.

I_{ClCa} in myocardial hypertrophy and heart failure The critical role of Ca^{2+} in cardiac development, function, and disease is undisputable. Despite the heterogeneous etiology and overt manifestations of heart failure, abnormalities in Ca^{2+} handling are prominent, and alterations in Ca^{2+} homeostasis are a hallmark of myocardial hypertrophy and heart failure[116]. Ca^{2+} transients in failing cardiac myocytes, for example, are characterized by diminished amplitude, elevated diastolic Ca^{2+} levels, and prolonged decay of the Ca^{2+} transients. In non-cardiac cells, I_{ClCa} could be an important mediator of apoptosis[117]. But, information on the possible involvement of I_{ClCa} in heart failure is currently very limited. It is reported that I_{ClCa} may play little, if any, role in the electrical remodeling of human end-stage failing heart[66,67,118].

Conclusions and future directions

Although the field of anion channels in cardiac physiology and pathophysiology lags significantly behind that of cation channels, the gap can now be narrowed with the recent identification of molecular entities responsible for cardiac Cl^- channels[8], their genes mapped to specific human chromosomal locations[13] and the use of gene targeting and transgenic animals. Recent efforts not only at the cellular and molecular levels but also the isolated organ and whole animal levels have provided strong evidence that Cl^- channels may play an important role in cardiac diseases, including arrhythmias, myocardial ischemia, hypertrophy, and congestive heart failure. Anion channels in the heart, therefore, may represent important novel targets for therapeutic agents against heart diseases.

Despite these exciting developments, further investigations of the cellular and molecular mechanisms by which the Cl^- channel proteins function to impart a physiological or a pathophysiological phenotype may require a multitude of approaches for the assessment of the Cl^- channel functions in healthy and diseased hearts. Although global knockout mice are invaluable experimental models and functional genomics remains a powerful approach to understanding the function of cardiac Cl^- channels, several theoretical and practical problems should be considered. First, homologous recombination gene targeting is based on the assumption that targeting will result in specific loss of the gene's product and will not directly affect the expression of other genes. In reality, however, even though the loss of the gene's product can be verified, the upregulation of another gene in the vicinity of the targeting can occur[119] and may readily escape detection. Such upregulation could have an important effect on the observed phenotype. Second, a knockout may not always be a knockout[120] such as when the targeted gene is widely or ubiquitously expressed, when alternative splicing variants of the gene exist[121], and when functional channels are actually heteromultimeric and the structure might be associated with modulatory subunits, such as Barttin for ClC channels[122]. Accessory proteins may be involved in the determination of the stability of the channel complex in the membrane and in the modulation of biophysical, pharmaco-logical, and regulatory properties of the channel. Recent evidence suggests that Cl^- channels, like cation channels[52,123,124], may function as a multiprotein complex or functional module. A functional anion channel module may be a complex composed of the following: (a) pore forming subunit for ion transportation; (b) auxiliary subunits for modulating pore gating; and (c) proteins as second messengers tightly coupled to channel function. These proteins might be intimately linked to certain physiological functions and belong to the same subproteome. Manipulation of one gene in the subproteome may cause changes in other proteins of the same subproteome. Therefore, the functional consequences of disrupting the specific gene are very difficult to

predict unless the changes in the entire subproteome are examined. Similar phenotypes can be attained from alternative protein pathways within cellular networks, which are influenced by disease, environmental, internal, and biochemical stimuli. Therefore, caution should be taken when conventional global gene knockout animals are used in functional studies. Alternatively, tissue-specific conditional or inducible knockout or knockin animal models may be more valuable in the phenotypic studies of specific genes by limiting the effect of upregulation or developmental compensation on the phenotype of manipulated genes. Many phenotypic changes may actually be a result of posttranslational changes caused by protein modifications such as phosphorylation or dephosphorylation. Therefore, it is clear that conventional functional genomics may provide only limited information on the functional module of multiprotein complexes. We are now facing the challenge of a major paradigm shift in the study of integrated anion channel functions. In the postgenomic era, the recent advances in the genome resources including genome-wide microarray profiling together with advancement in the application of functional proteomics and bioinformatics will certainly facilitate our understanding of the functions of anion channels in the cardiovascular system. It is feasible that anion channels may become novel targets for therapeutic approaches to the treatment of cardiovascular diseases.

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