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The Functional Role of Chloride Channels in Cardiac Pacemaker Activity

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1. Introduction

Chloride (Cl^-) channels are transmembrane proteins in biological membranes, which form functional pores and allow the diffusion of negatively-charged Cl^- ions along the electrochemical gradients. These channels can also conduct other anions including I^-, Br^-, NO_3^-, aspartate, glutamate, etc. They are conventionally called Cl^- channels because Cl^- is the most abundant and physiological anions in organisms.

While cation (K^+, Na^+, and Ca^{2+}) channels have received most of attentions in the past 50 years, the role of Cl^- channels in the pacemaker activity of the heart has been largely ignored and understudied (Duan, 2009). The possible contribution of Cl^- currents to spontaneous cardiac action potentials was first described in multicellular preparations of Purkinje fibers by Carmeliet (Carmeliet, 1961) and Hutter & Noble in 1961 (Hutter & Noble, 1961). Later, investigators from several laboratories observed the Cl- dependence of the diastolic depolarization and action potential duration. They characterized a hyperpolarization-activated Cl^- current in multicellular preparations of rabbit sinoatrial node (SAN) (De Mello, 1963;Noma & Irisawa, 1976;Seyama, 1979). Unfortunately, studies in the late 1970s raised serious doubts about the existence and functional role of any type of Cl^- channels in the heart. The potential functional role of the hyperpolarization-activated Cl^- currents in cardiac pacemaker activity has therefore been completely disputed (Hume *et al.*, 2000;Duan *et al.*, 2005).

Recent studies have characterized the functional and molecular expression of several Cl^- channels in cardiac SAN cells from different species (Duan, 2009). These include 1) a hyperpolarization- and cell swelling-activated inwardly rectifying Cl^- current ($I_{Cl,ir}$)(Duan *et al.*, 2000), which may be encoded by ClC-2, a member of the ClC voltage-gated Cl^- channel gene superfamily (Thiemann *et al.*, 1992;Britton *et al.*, 2000;Duan *et al.*, 2000;Britton *et al.*, 2005;Huang *et al.*, 2009); 2) a volume-regulated outwardly-rectifying Cl^- current ($I_{Cl,vol}$)(Hagiwara *et al.*, 1992), which may be encoded by another member of the ClC superfamily, ClC-3 (Duan *et al.*, 1997b;Duan *et al.*, 1999a;Britton *et al.*, 2000); and 3) a Ca^{2+}-activated Cl^- current ($I_{Cl,Ca}$) (Verkerk *et al.*, 2002), which may be encoded by TEME16A (or Ano1) (Schroeder *et al.*, 2008;Caputo *et al.*, 2008;Yang *et al.*, 2008;Hartzell *et al.*, 2009). Therefore, experimental evidence are merging to support the potential role of Cl^- channels in

the regulation of cardiac pacemaker activity and these anion channels may represent novel therapeutic targets for arrhythmias. Several recent review articles have summarized the biophysical, pharmacological and molecular properties and the potential functional role of Cl^- channels in the heart (Hiraoka *et al.*, 1998;Hume *et al.*, 2000;Baumgarten & Clemo, 2003;Duan *et al.*, 2005;Duan, 2009). In this chapter, we will highlight the major findings and recent advances in the studies of Cl^- channels in the regulation of cardiac pacemaker activity.

2. Cl^- channels in the heart

The electrophysiological examination of Cl^- channels in the heart can be dated back 50 years ago to the original work of Hutter & Noble (Hutter & Noble, 1961) and Carmeliet (Carmeliet, 1961). However, the existence of any Cl^- channels in cardiovascular system was disputed by a series of studies in the late 1970s (Kenyon & Gibbons, 1979a;Kenyon & Gibbons, 1979b). The discovery of a β-adrenergic receptor activated Cl^- current in rabbit ventricular myocytes (Harvey & Hume, 1989) and a cAMP-activated Cl^- current ($I_{Cl,cAMP}$) in guinea pig heart by Gadsby and colleagues (Bahinski *et al.*, 1989) in 1989 inaugurated the new era of Cl^- channel research in the heart. Patch-clamp studies have now identified at least eight types of Cl^- currents in cardiac cells from different regions of the heart and in different species. The biophysical, pharmacological and molecular properties of these Cl^- channels have been well characterized and are summarized in Table 1 (Hiraoka *et al.*, 1998;Hume *et al.*, 2000;Baumgarten & Clemo, 2003;Duan *et al.*, 2005;Duan, 2009). Several of these Cl^- channels have been found to be expressed in SAN of different species (Table 1).

Current	Activation	Single Channel (pS)	I-V ($[Cl^-]_o \neq [Cl^-]_i$)	I-V ($[Cl^-]_o = [Cl^-]_i$)	Ion selectivity	SAN	Gene
$I_{Cl,PKA}$	Gs-AC-cAMP-PKA	7~13	Outward Rectifying	linear	$Br^- > Cl^- > I^-$	?	CFTR
$I_{Cl,PKC}$	PKC	7~13	Outward Rectifying	linear	$Br^- > Cl^- > I^-$	?	CFTR
$I_{Cl,ATP}$	ATP_o (P_2-receptor)	~12	Outward Rectifying	linear	$Br^- > Cl^- > I^-$	?	CFTR
$I_{Cl,Ca}$	$[Ca^{2+}]_i$	1~2	Outward Rectifying	linear	$I^- > Br^- > Cl^-$	Yes	TMEM 16?
$I_{Cl,swell}$	Cell swelling, membrane stretch	30~60	Outward Rectifying	Outward Rectifying	$I^- > Br^- > Cl^-$	Yes	ClC-3
$I_{Cl,b}$	Basally active	30~60	Outward Rectifying	Outward Rectifying	$I^- > Br^- > Cl^-$	?	ClC-3
$I_{Cl,ir}$	Basally active cell swelling, $[H^+]_o$	1~4	Inward Rectifying	Inward Rectifying	$I^- = Br^- > Cl^-$	Yes	ClC-2
$I_{Cl,acid}$	$[H^+]_o$	?	Outward Rectifying	Outward Rectifying	$Br^- > I^- > Cl^-$	?	?

Table 1. Characteristics of functionally identified Cl^- channels in heart*

* *I-V*, current-voltage relationships; SAN, sino-atrial node; Gs, G-protien; AC, adenylyl cyclase; PKA, protein kinase A; PKC, protein kinase C; CFTR, cystic fibrosis transmembrane conductance regulator; subscripts i and o, intracellular and extracellular, respectively

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) transporter superfamily and may be responsible for $I_{Cl,cAMP}$, including the Cl^- currents activated by protein kinase A (PKA) ($I_{Cl,PKA}$) (Harvey & Hume, 1989;Bahinski *et al.*, 1989;Nagel *et al.*, 1992), protein kinase C (PKC) ($I_{Cl,PKC}$)(Walsh & Long, 1994;Collier & Hume, 1995), and extracellular ATP ($I_{Cl,ATP}$) (Levesque & Hume, 1995;Duan *et al.*, 1999b;Yamamoto-Mizuma *et al.*, 2004a); 2) *ClC-2,* which may be responsible for $I_{Cl,ir}$(Duan *et al.*, 2000); 3) *ClC-3*, which may be responsible for $I_{Cl,vol}$, including the basally-activated ($I_{Cl,b}$) (Duan *et al.*, 1992;Duan & Nattel, 1994) and swelling activated ($I_{Cl,swell}$) components (Duan & Nattel, 1994;Duan *et al.*, 1995;Duan *et al.*, 1997a;Duan *et al.*, 1997b;Duan *et al.*, 1999a;Duan *et al.*, 2001;Wang *et al.*, 2003;Yamamoto-Mizuma *et al.*, 2004b;Duan, 2010); 4) *CLCA-1*, which was thought to be responsible for the Ca^{2+}-activated Cl^- current ($I_{Cl,Ca}$)(Collier *et al.*, 1996;Xu *et al.*, 2002;Britton *et al.*, 2002); 5) *Bestrophin,* a candidate also for $I_{Cl,Ca}$(Hartzell, 2008); and 6) TMEM16, a novel candidate for $I_{Cl,Ca}$ (Schroeder *et al.*, 2008;Caputo *et al.*, 2008;Yang *et al.*, 2008;Hartzell *et al.*, 2009). A novel Cl^- current activated by extracellular acidosis ($I_{Cl.acid}$) has also been observed in cardiac myocytes but the molecular identity for $I_{Cl.acid}$ is currently not known (Yamamoto & Ehara, 2006).

3. Cl^- channels in cardiac pacemaker activity

The diastolic depolarization of cardiac pacemaker cells in the SAN initiates spontaneous action potentials that propagate to the whole cardiac muscle through specific pathways and drives normal rhythmic contraction of the heart (Liang *et al.*, 2010;DiFrancesco, 2010). The ionic mechanisms of the pacemaker activity have been one of the central research subjects in cardiac electrophysiology (DiFrancesco, 2010). It is generally agreed now that multiple mechanisms such as the hyperpolarization-activated non-selective cationic "funny" current (I_f)(DiFrancesco, 2010),the L-type ($I_{Ca,L}$)(Kurata *et al.*, 2003) and T-type Ca^{2+} currents ($I_{Ca,T}$)(Ono & Iijima, 2010), Na^+/Ca^{2+} exchanger (I_{NCX})(Dobrzynski *et al.*, 2007), and a sustained inward current (I_{st})(Shinagawa *et al.*, 2000), may be involved in the generation and regulation of spontaneous pacemaker activity, although the relative contribution of these individual ionic currents are still under debate.

Early studies using ion-substitution strategies in multicellualr Purkinje fibers and SAN tissues provided initial experimental evidence for the potential physiological role of Cl^- and other anions in the regulation of membrane potentials, the diastolic depolarization and action potential duration of cardiac cells (Carmeliet, 1961;Hutter & Noble, 1961;De Mello, 1963;Noma & Irisawa, 1976). In 1961 Hutter & Noble (Hutter & Noble, 1961) reported that upon the substitution of extracellular Cl^- by impermeant anions the heart rate transiently increased and then eventually decreased to between 40 and 90 % of that found in Cl^- solution. The replacement of Cl^- by permeant anions caused either an arrest or an initial slowing followed by acceleration of the rhythm. These changes were explained by the passive movement of anions during the action potential, assuming that the E_{Cl} was -50 mV (Hutter & Noble, 1961). Later, de Mello (De Mello, 1963) and Noma & Irisawa (Noma & Irisawa, 1976) independently reported the Cl^--dependence of the diastolic depolarization in multicellular preparations of rabbit SAN tissue. Seyama (Seyama, 1977) also reported that 9 % of the total membrane conductance of the resting potential is carried by Cl^-. In 1979,

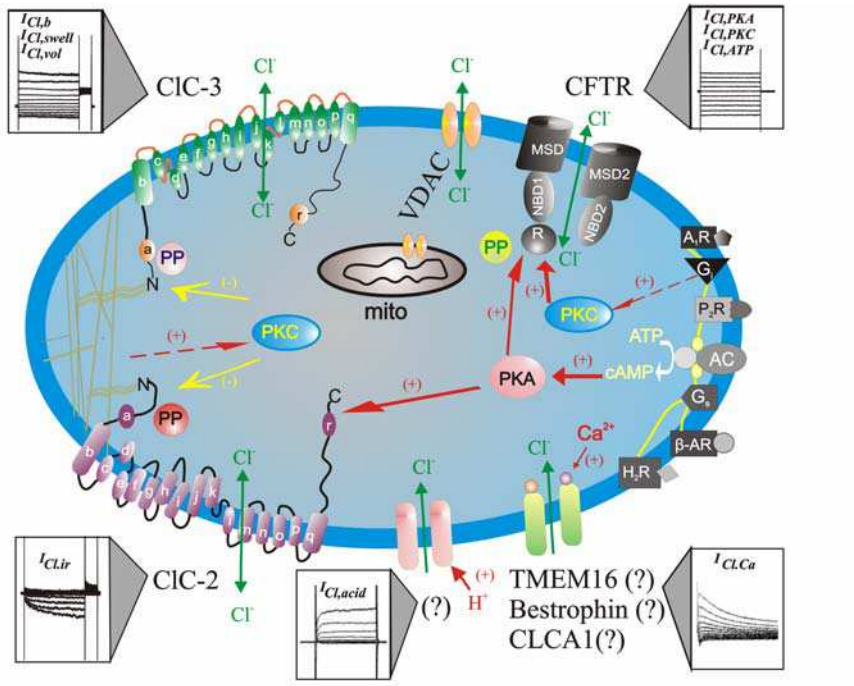

Fig. 1. Schematic representation of Cl- channels in cardiac myocytes. Cl- channels and their corresponding molecular entities or candidates are indicated. ClC-3, a member of voltage-gated ClC Cl- channel family, encodes Cl- channels that are volume-regulated ($I_{Cl,vol}$) and can be activated by cell swelling ($I_{Cl,swell}$) induced by exposure to hypotonic extracellular solutions or possibly membrane stretch. $I_{Cl,b}$ is a basally-activated ClC-3 Cl- current. ClC-2, a member of voltage-gated ClC Cl- channel family, is responsible for a volume-regulated and hyperpolarization-activated inward rectifying Cl- current ($I_{Cl,ir}$). Membrane topology models (α-helices a-r) for ClC-3 and ClC-2 are modified from Dutzler *et al* (Dutzler *et al*., 2002). $I_{Cl,acid}$ is a Cl- current regulated by extracellular pH and the molecular entity for $I_{Cl,acid}$ is currently unknown. $I_{Cl,Ca}$ is a Cl- current activated by increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$); Molecular candidates for $I_{Cl,Ca}$ include CLCA1, a member of a Ca^{2+} -sensitive Cl- channel family (CLCA), bestrophin-2, a member of the Bestrophin gene family, and TMEM16, transmembrane protein 16. CFTR, cystic fibrosis transmembrane conductance regulator, encodes Cl- channels activated by stimulation of cAMP-protein kinase A (PKA) pathway ($I_{Cl,PKA}$), protein kinase C (PKC) ($I_{Cl,PKC}$), or extracellular ATP through purinergic receptors ($I_{Cl,ATP}$). CFTR is composed by two membrane spanning domains (MSD1 and MSD2), two nucleotide binding domains (NBD1 and NBD2) and a regulatory subunit (R). P, phosphorylation sites for PKA and PKC; PP, serine-threonine protein phosphatases; Gi, heterodimeric inhibitory G protein; A_1R, adenosine type 1 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; proposed intracellular signaling pathway for purinergic activation of CFTR. VDAC, voltage-dependent anion channels (porin); mito, mitochondrion. (Duan, 2009)

using the voltage-clamp technique Seyama (Seyama, 1979) identified a time- and voltage-dependent inwardly rectifying Cl^- current in SAN cells of rabbit heart. This inwardly rectifying Cl^- current was only activated by membrane potentials more negative than -60 mV and might contribute to the diastolic depolarization. Eliminating this current component by a replacement of Cl^- with less permeable acetate caused a reduction in frequency of SAN rhythm and an increase in the amplitude of the action potential (Seyama, 1979). Later a similar Cl^- current was reported in frog sinus venosus (Brown *et al.*, 1977). Unfortunately, the biophysical and molecular properties of the hyperpolarization-activated inwardly rectifying Cl^- channels have never been further characterized. Instead, a later study found that substitution of extracellular Cl^- with larger anions including isethionate, glutamate, acetate, and aspartate, reduced the amplitude of I_f without changing the reversal potential and substitution with small anions such as iodide or nitrate supported an intact I_f (Frace *et al.*, 1992). Therefore, the possible important role of the hyperpolarization-activated Cl^- current described in the 1960s and 1970s has been either disputed (Frace *et al.*, 1992) or ignored (Hume *et al.*, 2000;Duan *et al.*, 2005). Very little is known about Cl^- channels and their potential functional role in the heart although recent efforts in the last twenty years have characterized the properties of several Cl^- channels in the heart at the cellular and molecular levels (Duan *et al.*, 2005;Duan, 2009).

3.1 $I_{Cl,ir}$ and ClC-2 Cl^- channels in cardiac pacemaker activity

ClC-2 was cloned originally from rat heart and brain (Thiemann *et al.*, 1992). Later several alternatively spliced forms were cloned from several other tissues and species, including human (Furukawa *et al.*, 1995;Malinowska *et al.*, 1995;Cid *et al.*, 1995;Chu *et al.*, 1996;Chu & Zeitlin, 1997;Furukawa *et al.*, 1998;Cid *et al.*, 2000;Loewen *et al.*, 2000;Furukawa *et al.*, 2002). Expression of ClC-2 cDNA in *Xenopus* oocytes or mammalian cells resulted in hyperpolarization-activated inward-rectifying Cl^- currents which are sensitive to changes in cell volume and extracellular pH (Jordt & Jentsch, 1997;Furukawa *et al.*, 1998;Park *et al.*, 1998;Stroffekova *et al.*, 1998;Cid *et al.*, 2000;Park *et al.*, 2001;Furukawa *et al.*, 2002).

The endogenous $I_{Cl.ir}$ in native cardiac myocytes was identified in guinea pig and mouse hearts for the first time in 2000 (Duan *et al.*, 2000). Under conditions in which cationic inward rectifier channels were blocked, $I_{Cl.ir}$ was activated by membrane hyperpolarization (-40 to -140 mV). Under isotonic conditions, the current activated slowly with a biexponential time course (time constants averaging τ_1=179.76±3.4 and τ_2=2073.66±87.6 ms at -120 mV). Hypotonic cell swelling accelerated the activation (τ_1=97.5±8.5 ms and τ_2=656.4±113.6 ms at -120 mV) and increased the current amplitude whereas hypertonic cell shrinkage inhibited the current (Figure 2). The inwardly rectifying current was carried by Cl^- and had an anion permeability sequence of Cl^->I^->>aspartate. $I_{Cl.ir}$ was blocked by 9-anthracene-carboxylic acid and cadmium (Cd^{2+}) but not by stilbene disulfonates and tamoxifen (Duan *et al.*, 2000). Subsequently, similar $I_{Cl.ir}$ was found in rat atrial and subepicardial and subendocardial ventricular myocytes (Komukai *et al.*, 2002a;Komukai *et al.*, 2002b). Acidosis (extracellular pH decreased from 7.4 to 6.5) increased $I_{Cl.ir}$, which may underlie the acidosis-induced depolarization of the resting membrane potential (Komukai *et al.*, 2002a;Komukai *et al.*, 2002b). The properties of $I_{Cl.ir}$ in guinea-pig and mouse cardiac myocytes are consistent with currents generated by expression of ClC-2 Cl^- channels.

RT-PCR and Northern blot analysis confirmed transcriptional expression of ClC-2 in both atrial and ventricular tissues and isolated myocytes from mouse and guinea pig hearts

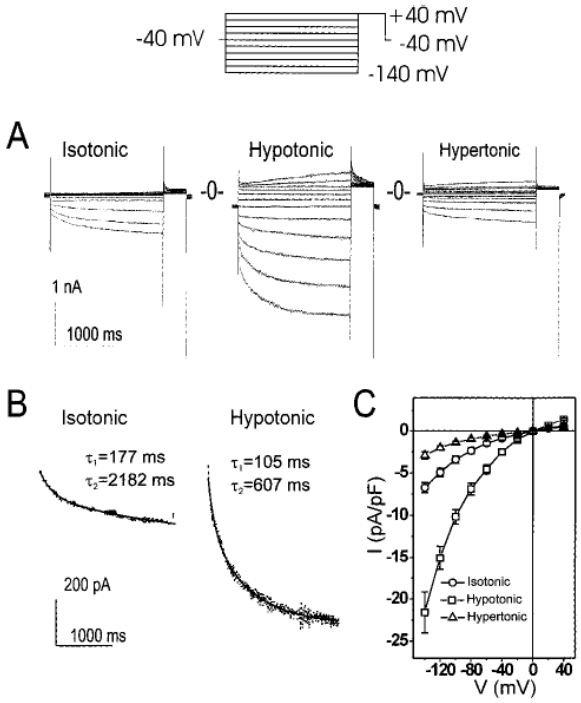

Fig. 2. Hyperpolarization-activated anion current and its sensitivity to cell volume in mouse ventricular myocytes. Currents were recorded using voltage-clamp protocols shown on top. Cells were held at -40 mV, and test potentials were applied from -140 to +40 mV in +20-mV increments for 2 seconds and then to +40 mV for 400 ms before return to the holding potential. The test potentials were applied at an interval of 10 seconds (insert on the top). **A,** Hyperpolarization voltage pulses activated inward currents under isotonic conditions (left panel). Subsequent exposure of the same cell to hypotonic solution (0.79T) caused a further increase in current amplitudes (middle panel). Further exposure of the cell to hypertonic solution caused a decrease in current amplitudes (right panel). **B,** Effects of hypotonic cell swelling on the time course of activation of the inward rectifying current. Representative current recordings from one cell after hyperpolarization to -120 mV under isotonic (left panel) and hypotonic (right panel) conditions, respectively, are shown. The points represent current activation data, and the solid lines are least-square curve fits obtained with the curve fitting program Clampfit (Axon Instruments). The activation process was best fit to a biexponential function with a fast time constant (τ_1) of 177 ms and a slow time constant (τ_2) of 2182 ms under isotonic conditions. Hypotonic cell swelling increased the amplitude of the current and also accelerated the activation kinetics (τ_1=105 ms, τ_2=607 ms). **C,** Mean *I*-V relationship from 5 different cells under isotonic, hypotonic, and hypertonic conditions. Currents were measured at the end of each 2-second test pulse. Mean reversal potentials (E_{rev}) of the currents under isotonic, hypotonic, and hypertonic conditions were 2.0±3.8 mV, 3.3±3.4 mV, and -1.7±3.3 mV (n=5, *P*=NS), respectively, which were very close to the predicted equilibrium potential of Cl^- (E_{Cl}=0 mV) with a symmetrical Cl^- gradient ($[Cl^-]_o$/ $[Cl^-]_i$=118/118 mmol/L).(Duan *et al.*, 2000)

(Duan *et al.*, 2000). The expression of ClC-2 in the heart was further characterized by immunohistochemistry and western blot from several species (Britton *et al.*, 2000). Later studies provided compelling evidence that $I_{Cl,ir}$ is encoded by ClC-2 and its alternatively spliced isoforms in the heart (Britton *et al.*, 2005;Huang *et al.*, 2009).

ClC-2 channels are activated by hyperpolarization, cell swelling, and acidosis and have an inwardly rectifying *I-V* relationship (Duan *et al.*, 2000;Komukai *et al.*, 2002a;Komukai *et al.*, 2002b;Britton *et al.*, 2005;Huang *et al.*, 2009). During the cardiac action potential, therefore, activation of the ClC-2 channels will conduct mainly an 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 the equilibrium potential for Cl^- (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) (DiFrancesco, 1984;DiFrancesco, 2006), the hyperpolarization-activated $I_{Cl,ir}$ through ClC-2 channels may normally play a much more prominent role in the sino-atrial (SA) or atrial-ventricular (AV) nodal regions of the heart.

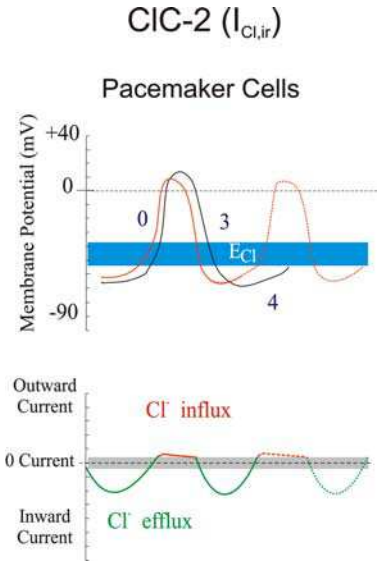

Fig. 3. Modulation of cardiac electrical activity by activation of ClC-2 channels in cardiac pacemaker cells. Changes in action potentials (*top panels*) and membrane currents (*bottom panels*) of cardiac pacemaker cells due to activation of ClC-2 channels are depicted. $I_{Cl,ir}$ is activated by hyperpolarization, cell swelling, and acidosis. *Top panel:* Numbers illustrate conventional phases of a prototype pacemaker action potential under control conditions (black) and after activation of I_{Cl} (red). Range of estimates for normal physiological values for Cl^- equilibrium potential (E_{Cl}) is indicated in blue. *Bottom panel:* Range of zero-current values corresponding to E_{Cl} is shown in grey. Activation of $I_{Cl,ir}$ in pacemaker cells during hyperpolarization causes acceleration of phase 4 depolarization and automaticity, shortening of action potential duration, and decrease in cycle length and action potential amplitude (dashed red line in *top panel*). (Adapted from Duan (Duan, 2009)).

$I_{Cl,ir}$ under basal or isotonic conditions is small, but can be further activated by hypotonic cell swelling (Duan *et al.*, 2000) and acidosis (Komukai *et al.*, 2002a;Komukai *et al.*, 2002b). 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 pathological 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). 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 (Hiraoka *et al.*, 1998;Carmeliet, 1999). 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 resting membrane potential and it is suggested that the acidosis-induced increase in $I_{Cl,ir}$ might underlie the depolarization of the resting membrane potential during acidosis or hypoxia (Komukai *et al.*, 2002a;Komukai *et al.*, 2002b).

Our recent study found that $I_{Cl,ir}$ was indeed functionally expressed in guinea-pig SAN cells (Huang *et al.*, 2009). $I_{Cl,ir}$ in guinea-pig SAN cells activated upon cell membrane hyperpolarization and hypotonic challenge has a strong inward rectification under symmetrical Cl^- conditions with a reversal potential close to the predicted value of E_{Cl}, and is inhibited by Cd^{2+} (Figure 4). All these properties are identical to those of $I_{Cl,ir}$ in atrial and ventricular myocytes of several species, including guinea-pig (Britton *et al.*, 2005), rat (Duan *et al.*, 2000;Komukai *et al.*, 2002a;Komukai *et al.*, 2002b;Britton *et al.*, 2005), and mouse (Duan *et al.*, 2000). $I_{Cl,ir}$ is neither a part of the I_f nor a result of Cl^- regulation of the I_f activity (Frace *et al.*, 1992) in SAN cells because a) $I_{Cl,ir}$ can be recorded in the presence of a strong I_f blocker (20 mM Cs^+) and in the absence of permeable cations; b) the reversal potential of $I_{Cl,ir}$ is close to the predicted E_{Cl}, suggesting the inward current is carried by Cl^-, not by cations; c) $I_{Cl,ir}$ but not I_f can be inhibited by Cd^{2+}; and d) $I_{Cl,ir}$ but not I_f is specifically inhibited by anti-*ClC-2* Ab. Our data from RT-PCR and immunohistochemistry provided direct evidence for the expression of *ClC-2* in SAN cells. In addition, dialysis of anti-*ClC-2* Ab but not the inactivated pre-absorbed Ab caused an inhibition of $I_{Cl,ir}$ but not I_f, $I_{Ca,L}$, I_{Ks} and $I_{Cl.vol}$. These results further support that *ClC-2* is the gene responsible for the endogenous Cl,ir channels not only in atrial and ventricular myocytes (Britton *et al.*, 2000;Britton *et al.*, 2005) but also in the SAN cells and that the Cl,ir in SAN cells may contribute to the regulation of pacemaker activity of the heart. Interestingly, the prevalence of functional endogenous $I_{Cl,ir}$ in the SAN cells (28/35, 80%) is apparently higher than that in the atrial or ventricular myocytes (Duan *et al.*, 2000). Whether this difference is due to the higher molecular expression or the different activation mechanisms is a legitimate question which may be very difficult to get a clear answer. Immunohistochemistry data reveals no significant difference between the SAN and atrial tissues. Theoretically, a Western blot of membrane fractions would help to quantitatively analyze the differences in the protein expression of ClC-2 in the SAN cells and the atrial myocytes. But, practically, in order to carry out the Western blot analysis on the isolated membrane fractions from SAN cells it would need to collect enough SAN cells from the guinea-pig heart, which means not only a requirement for a pool of tens of hearts but also an isolation and selection of true SAN cells without contamination from the

adjacent atrial cells. This is an extremely difficult task to accomplish. Although the confocal images of the SAN cells and atrail or ventricular myocytes would provide information on the subcellular distribution of ClC-2 channels in these cells it would not be able to give quantitative information for a decisive conclusion on the dynamic distribution of the ClC-2 channel protein and the relationship between the distribution and the function of the channels.

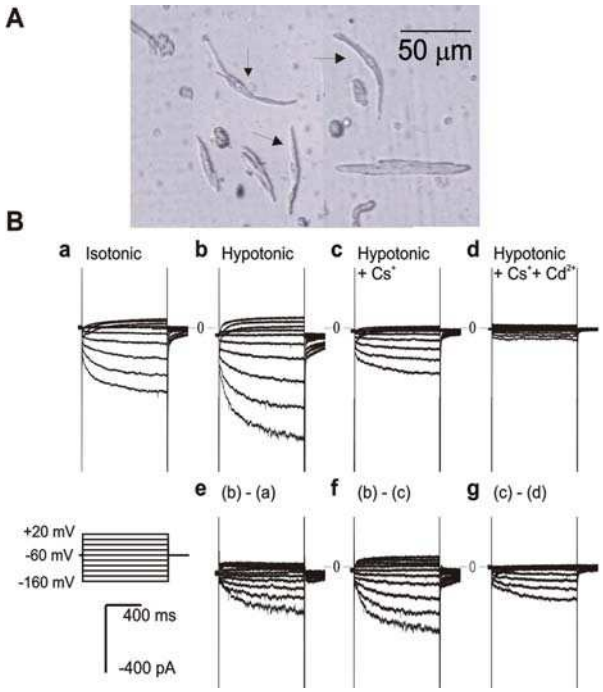

Fig. 4. Whole-cell currents recorded from SAN cells of guinea-pig heart. A. An example of single SAN cells (arrows) isolated from the SAN region of guinea pig heart by enzymatic dispersion. B. Whole-cell currents recorded from SAN cells. When cations (Na^+ and K^+) were included in the extracellular solutions, inward currents were slowly activated upon hyperpolarization under isotonic (a) conditions. Exposure of the same cell to hypotonic extracellular solution caused cell swelling and an increase in the inward current amplitude (b). The difference current caused by hypotonic cell swelling is shown in panel e. Subsequent replacement of 20 mmol/L of NaCl with CsCl caused a significant inhibition of the inward current (c). The Cs^+-sensitive current is shown in panel f. Subsequent addition of 0.2 mmol/L of Cd^{2+} to the hypotonic solution caused an inhibition of the inward current (d). The Cd^{2+}-sensitive currents are shown in panel g. (Huang *et al*., 2009)

Because the E_{Cl} in cardiac cells under physiological conditions ranges between -65 to -35 mV (Baumgarten & Fozzard, 1981;Vaughan-Jones, 1982), which is very close to the maximum diastolic potential (MDP) of SAN cells, the contribution of $I_{Cl,ir}$ to SAN cell action potential is unique and also more complicated than the activation of I_f and other cation currents (Figure 3). When the membrane potential is negative to E_{Cl}, opening of Cl,ir channel may conduct an

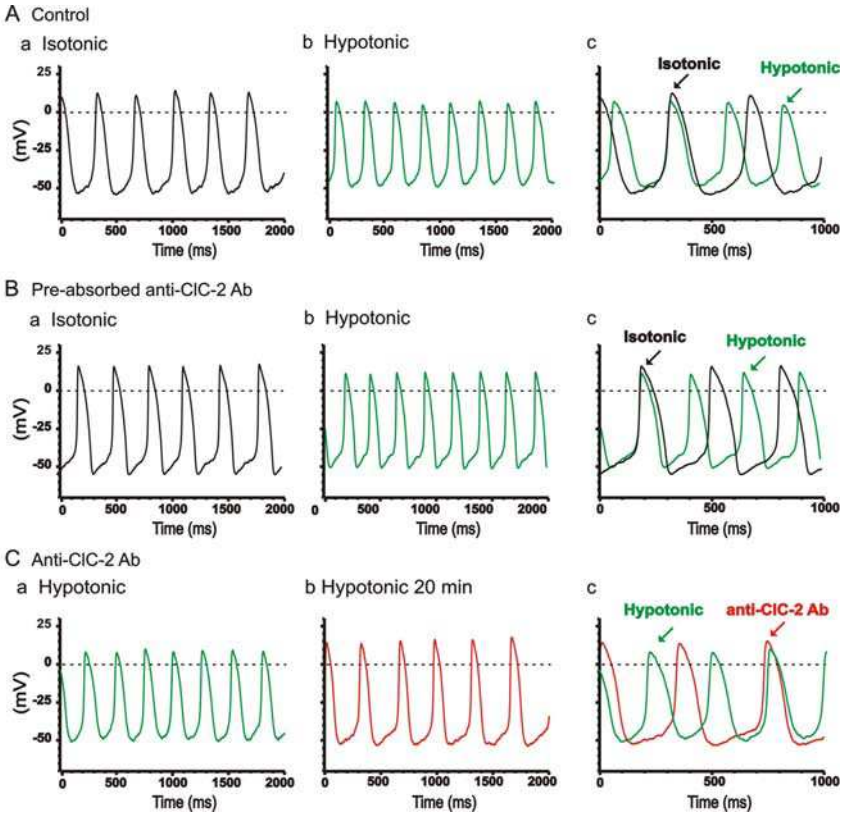

Fig. 5. Effects of Anti-*ClC-2* Ab on pacemaker action potential in SAN cells. A. Representative spontaneous action potentials recorded from an SAN cell by current-clamp (no current injection) with pipette solution containing no anti-ClC-2 Ab under isotonic (a) and hypotonic (b) conditions. For comparison, the action potentials recorded under these conditions were superimposed with an expanded time scale in panel c. The dotted lines indicate zero voltage. **B.** Spontaneous action potentials recorded from a SAN cell by current-clamp using a pipette solution containing pre-absorbed anti-ClC-2 Ab (control) and the cell was exposed to isotonic (a) and hypotonic (b) solutions. For comparison, the action potentials recorded under these conditions were superimposed with an expanded time scale in panel c. **C**. SAN cells were exposed to hypotonic solution for 20 min to fully activate $I_{Cl,ir}$ before whole-cell recordings. Action potentials were recorded immediately after membrane rupture (**a**) and after dialysis of anti-ClC-2 Ab for 20 min (**b**). Panel **c** shows the expanded and superimposed action potentials as shown in panel a and panel b.(Huang *et al.*, 2009)

Inward current (Cl^- efflux), which will depolarize the MDP and increase the diastolic depolarization slope (DDs). At the beginning of diastolic depolarization, the impedance of the cell is very large and activation of a small current may contribute significantly to the depolarization of the action potential (Zhang *et al.*, 2002). Therefore, both the smaller instantaneous $I_{Cl,ir}$ activated at membrane potentials near the MDP and the larger time-dependent $I_{Cl,ir}$ activated at membrane potentials more negative than the MDP may

contribute pacemaker current to the phase 4 depolarization of the action potential of SAN cells. When the membrane potential is positive to E_{Cl}, opening of Cl,ir channel may conduct an outward current (Cl^- influx) and make the membrane potential (possibly including the MDP) more negative. But the inward rectification property of Cl,ir may limit the amplitude of the outward current and its contribution to repolarization and APD. Since the MDP may be determined normally by multiple mechanisms (Dobrzynski *et al*., 2007;Liu *et al*., 2007) such as I_f, I_{sus}, $I_{Ca,T}$, $I_{Ca,L}$, I_{NCX}, and possibly $I_{Cl,Ca}$ (Verkerk *et al*., 2002) and $I_{Cl,swell}$ (Hagiwara *et al*., 1992), the contribution of changes in $I_{Cl,ir}$ to the MDP during hypotonic stress may be further complicated by changes in other ionic currents which may also respond to hypotonic cell swelling such as $I_{Cl,swell}$ (Hagiwara *et al*., 1992;Duan *et al*., 2005;Duan, 2009) and I_{Ks} (Rees *et al*., 1995). In addition, activation of $I_{Cl,ir}$ may also cause a dynamic change in the E_{Cl} (Staley *et al*., 1996). The analysis of the relationship of the activation of $I_{Cl,ir}$ to the E_{Cl} and the consequent role of this relationship in determining the MDP has been limited by the lack of potent and specific $I_{Cl,ir}$ blocker. The identification of *ClC-2* as the gene responsible for Cl,ir channels in the heart and the availability of specific anti-*ClC-2* Ab provided us specific approach to effectively examine the functional role of Cl,ir in the heart.

In the isolated SAN cells, dialysis of anti-*ClC-2* Ab through the pipette solution for 20 min inhibited $I_{Cl,ir}$ (Figure 5B) and reversed the hypotonic stress-induced increase in DDs and decrease in MDP, APA, APD_{90}, and CL under hypotonic conditions, suggesting that $I_{Cl,ir}$ may play a role in the regulation of diastolic depolarization and the firing rate of spontaneous action potential of SAN cells under stressed conditions. Anti-*ClC-2* Ab, however, did not have significant effect on the hypotonicity-induced shortening of APD_{50} (Huang *et al*., 2009). This may suggest that the contribution of the $I_{Cl,ir}$ to the repolarization at positive potentials is rather small because of its inward rectification property. These data may provide new mechanistic insight into the tonicity regulation of spontaneous beating rate in guinea–pig SAN reported by Ohba in 1986 (Ohba, 1986). In that study it was found that decreasing the osmolarity by 30% increased the heart rate by 6% and increasing the osmolarity to 130, 150, and 170% decreased the heart rate to 94, 89, and 73%, respectively (Ohba, 1986).

As mentioned above it is possible that the observed anti-*ClC-2* Ab-induced reduction in pacemaker activity under hypotonic conditions may be due to a non-specific block of hypotonic activation of I_{Ks} (Rees *et al*., 1995) and $I_{Cl,swell}$ (Hagiwara *et al*., 1992;Duan *et al*., 2005;Duan, 2009). But we found that anti-*ClC-2* Ab failed to affect I_{Ks} and $I_{Cl,swell}$ under either isotonic or hypotonic conditions. These results are consistent with the observation that anti-*ClC-2* Ab has no effect on APD_{50} and strongly suggest that the activation of ClC-2 channels may play an important role in the diastolic depolarization and firing rate of SAN pacemaker activity but have very little impact on the repolarization at positive potentials during hypotonic stress. In addition, dialysis of pre-absorbed anti-ClC-2 Ab did not cause any changes in the response of the SAN pacemaker activity to hypotonic stress (Figure 5A and B) or in the hypotonic activation of $I_{Cl,ir}$. Therefore, it is highly unlikely that the anti-*ClC-2* Ab-induced reduction in the current amplitude of $I_{Cl,ir}$ and pacemaker activity in the SAN cells under hypotonic conditions are not due to the effects of anti-*ClC-2* Ab but the potential effects of dialysis with pipette solutions *per se*.

In agreement with our findings in the isolated cells, targeted inactivation of ClC-2 channels caused a decrease in HR, especially under exercise stress (Figure 6). The resting HR of the *Clcn2*$^{-/-}$ mice was slower but not significantly different from that of the *Clcn2*$^{+/+}$ and *Clcn2*$^{+/-}$ mice. This may be explained by the fact that $I_{Cl,ir}$ through ClC-2 channels under basal or

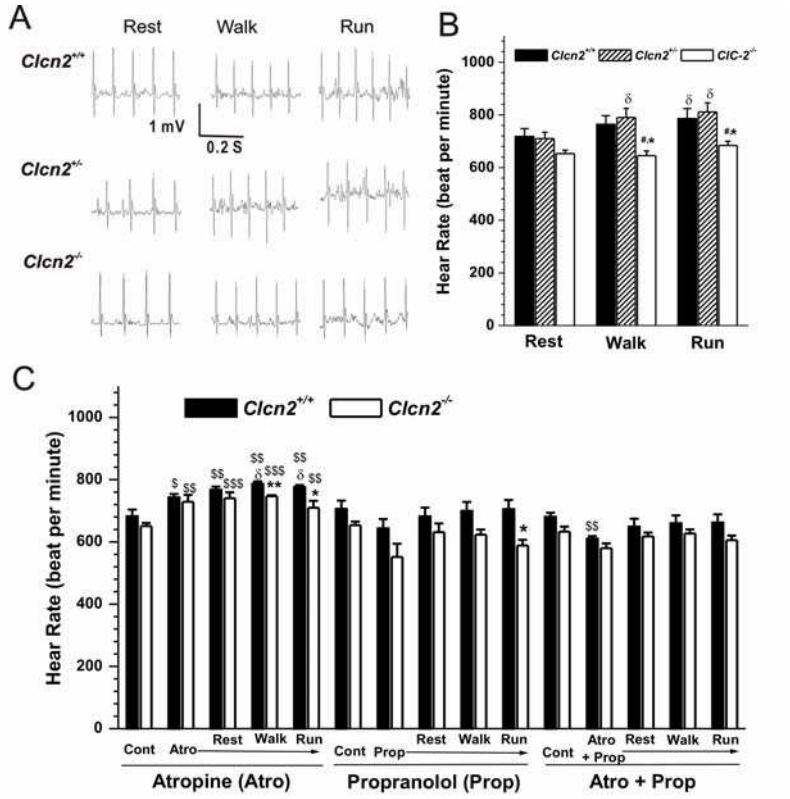

Fig. 6. Telemetry ECG recordings in *Clcn2*$^{-/-}$ mice and their *Clcn2*$^{+/+}$ and *Clcn2*$^{+/-}$ littermates during treadmill exercises. **A.** Representative ECG (Lead II) recordings in *Clcn2*$^{+/+}$, *Clcn2*$^{+/-}$, and *Clcn2*$^{-/-}$ mice while they were subjected to treadmill exercise at a) Rest period: acclimation at 0 m/min, incline 0 º for 5 min; b) Walk period: walking at 5m/min, incline 0 º for 5 min; c) Run period: running at 15 m/min, uphill incline 8 º for 5 min. **B**. Mean heart rate during the last minute of each treadmill exercise segment for the *Clcn2*$^{+/+}$ (n=6), *Clcn2*$^{+/-}$ (n=5), and *Clcn2*$^{-/-}$ (n=7) mice. **C**. Mean heart rate of the *Clcn2*$^{+/+}$ (n=5) and *Clcn2*$^{-/-}$ (n=4) mice before (Control, Cont) and after the intraperitoneal injection of atropine (Atro), propranolol (Prop), or atropine plus propranolol (Atro + Prop) during the last minute of each treadmill exercise segment (Rest, Walk, and Run). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs *Clcn2*$^{+/+}$, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs *Clcn2*$^{+/-}$; $ $P < 0.05$, $$ $P < 0.01$, $$$ $P < 0.001$ vs control (Cont); □ $P < 0.05$ vs Rest. (Huang *et al.*, 2009)

isotonic conditions is relatively small. It has been known that, however, $I_{Cl,ir}$ is activated in a larger scale by cell swelling (Duan *et al.*, 2000;Britton *et al.*, 2005), acidosis (Komukai *et al.*, 2002a;Komukai *et al.*, 2002b), and PKA phosphorylation (Kajita *et al.*, 2000a;Kajita *et al.*, 2000b;Cuppoletti *et al.*, 2004a;Cuppoletti *et al.*, 2004b;Huang *et al.*, 2008). Indeed, hypoxia, ischemia, and acidosis have consistently been shown to increase automaticity and cause lethal arrhythmias, although the mechanism has remained obscure (Carmeliet, 1999;Duan *et*

al., 2005). Activation of $I_{Cl,ir}$ may explain, at least in part, the increase in automaticity under these stressed or pathological conditions (Komukai *et al.*, 2002a;Komukai *et al.*, 2002b). We found that during acute exercise the maximal HR is lower in *Clcn2*$^{-/-}$ mice than in *Clcn2*$^{+/+}$ and *Clcn2*$^{+/-}$ mice, suggesting that activation of ClC-2 channels in the heart may contribute to the chronotropic response of the mouse to exercise stress. It is possible that activation of ClC-2 channels by β-stimulation induced PKA phosphorylation (Fritsch & Edelman, 1996;Sherry *et al.*, 1997;Kajita *et al.*, 2000a;Kajita *et al.*, 2000b;Cuppoletti *et al.*, 2004a;Cuppoletti *et al.*, 2004b;Huang *et al.*, 2008) may contribute to the regulation of HR during exercise. It has been known that several consensus PKA phosphorylation sites are well conserved in the ClC-2 sequences from different species (Cuppoletti *et al.*, 2004b). These results provide strong evidence for the molecular and functional expression of ClC-2 encoded endogenous Cl,ir channels in the SAN cells. The significance of $I_{Cl,ir}$ in the heart may become more prominent under stressed or pathological conditions. Our results may also shed new light on understanding mechanisms for arrhythmias such as sinus node dysfunction or sick sinus syndrome (Dobrzynski *et al.*, 2007). Cardiac ClC-2 channels may thus represent new therapeutic targets for arrhythmias, congestive heart failure, and ischemic heart diseases.

3.2 $I_{Cl,vol}$ and ClC-3 Cl^- channels in cardiac pacemaker activity

Activation of the volume-regulated outwardly rectifying Cl^- current ($I_{Cl,vol}$) is expected to produce depolarization of the resting membrane potential and significant APD shortening because of its strong outwardly rectifying property (Sorota, 1992;Duan & Nattel, 1994;Du & Sorota, 1997;Duan *et al.*, 1997b;Duan *et al.*, 1999a). Under basal or isotonic conditions $I_{Cl,vol}$ is small (Duan *et al.*, 1992;Sorota, 1992;Duan *et al.*, 1997b;Hume *et al.*, 2000), but can be further activated by stretching of the cell membrane by inflation (Hagiwara *et al.*, 1992;Du & Sorota, 1997) or direct mechanical stretch of membrane β_1-integrin (Browe & Baumgarten, 2003) and/or cell swelling induced by exposure to hypoosmotic solutions (Sorota, 1992;Du & Sorota, 1997;Duan *et al.*, 1997b;Duan *et al.*, 1999a). A strech of the cell membrane by applying positive pressure to inflate the cell activated a Cl^- current with characteristics similar to $I_{Cl,vol}$ in SAN cells isolated from rabbit heart (Hagiwara *et al.*, 1992). Later studies further confirmed the presence of both cell strech- and swelling-activated Cl- currents in SAN cells (Kohl *et al.*, 1994;Arai *et al.*, 1996;Lei & Kohl, 1998). 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. Baumgarten's laboratory has recently demonstrated that $I_{Cl,swell}$ in ventricular myocytes can be directly activated by mechanical stretch through selectively stretching β1-integrins with mAb-coated magnetic beads (Browe & Baumgarten, 2003;Baumgarten & Clemo, 2003;Browe & Baumgarten, 2004;Browe & Baumgarten, 2006). The stretch-activated Cl^- current may contribute to the pacemaker rhythm under physiological conditions, since the stimulus is almost continuously present in the intact heart (Hagiwara *et al.*, 1992). $I_{Cl,vol}$ may also serve as a mediator of mechanotransduction and volume regulation and play a significant role in the pacemaker function if they act as both the stretch activated channels and cell swelling activated channels in these cells (Hagiwara *et al.*, 1992;Kohl *et al.*, 1994;Arai *et al.*, 1996;Lei & Kohl, 1998;Baumgarten & Clemo, 2003).

The short isoform of ClC-3 (sClC-3), a member of the ClC superfamily of voltage-dependent Cl^- channels, has been proposed to be the molecular correlate of a key component of the

native $I_{Cl,vol}$ in cardiac myocytes. (Duan *et al.*, 1997b;Hume *et al.*, 2000;Duan, 2009). A series of recent independent studies from many laboratories further strongly corroborated this hypothesis (please see recent reviews by Duan (Duan, 2009;Duan, 2010) and Hume et al. (Hume *et al.*, 2010)). It has been demonstrated that ClC-3 is widely expressed in cardiac tissues (Britton *et al.*, 2000;Hume *et al.*, 2000). Further studies are needed to answer the questions whether ClC-3 channels are expressed in SAN cells and whether ClC-3 is responsible for both $I_{Cl,vol}$ and the stretch-activated Cl^- channels in these cells.

3.3 Ca^{2+}-activated Cl^- channels and cardiac pacemaker activity

Ca^{2+}-activated chloride channels (CACCs) are widely distributed in cardiac tissues and play important roles in the regulation of cardiac excitability (Verkerk *et al.*, 2003a). Using perforated patch-clamp methodology Verkerk et al. identified a Ca^{2+}-activated Cl^- current ($I_{Cl,Ca}$) in one third of the spontaneously active rabbit SAN cells (Verkerk *et al.*, 2002). It is not known why $I_{Cl,Ca}$ is non-uniformly distributed among SAN cells. But it may be related to the inhomogeneity of function and structure of the SA node (Boyett *et al.*, 2000). $I_{Cl,Ca}$ is activated during the pacemaker cycle. The current is transient outward with a bell-shaped current–voltage relationship. Stimulation of adrenoceptors with noradrenaline doubled the $I_{Cl,Ca}$ density. Action potential clamp measurements demonstrate that $I_{Cl,Ca}$ is activated late during the action potential upstroke. Current clamp experiments show, both in the absence and presence of noradrenaline, that blockade of $I_{Cl,Ca}$ by DIDS (4,4,-diisothiocyanostilbene-2,2,-disulphonic acid) increases the action potential overshoot and duration, measured at 20 % repolarization, but has no effect on intrinsic interbeat interval, upstroke velocity, diastolic depolarization rate and the action potential duration measured at 50 and 90 % repolarization (Verkerk *et al.*, 2002). Therefore, it was suggested that $I_{Cl,Ca}$ may have a limited role in pacemaker synchronization or action potential conduction.

Even though $I_{Cl,Ca}$ is also expected to be outwardly rectifying under physiological conditions the activation of $I_{Cl,Ca}$ will have considerably different effects on cardiac action potential and resting membrane potential from those of ClC-3 channels. This is because the kinetic behavior of $I_{Cl,Ca}$ is significantly determined by the time course of the $[Ca^{2+}]_i$ transient (Zygmunt & Gibbons, 1991). Normally, $I_{Cl,Ca}$ 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, $I_{Cl,Ca}$ carries a significant amount of transient outward current. $I_{Cl,Ca}$ will activate 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 $I_{Cl,Ca}$ contributes to early phase 1 repolarization. In the rabbit left ventricle, $I_{Cl,Ca}$ contributes to APD shortening in subendocardial myocytes but not in subepicardial myocytes. These differences in functional expression of $I_{Cl,Ca}$ may reduce the electrical heterogeneity in the left ventricle (Verkerk *et al.*, 2004). In Ca^{2+}-overloaded cardiac preparations, $I_{Cl,Ca}$ can contribute to the arrhythmogenic transient inward current (I_{TI}) (Zygmunt, 1994). I_{TI} produces delayed afterdepolarization (DAD) (January & Fozzard, 1988) and induces triggered activity, which is an important mechanism for abnormal impulse formation. In sheep Purkinje and ventricular myocytes, activation of $I_{Cl,Ca}$ was found to induce DAD and plateau transient repolarization (Verkerk *et al.*, 2000). Therefore, blockade of $I_{Cl,Ca}$ may be potentially antiarrhythmogenic by reducing DAD amplitude and triggered activity based on DAD. However, the role of $I_{Cl,Ca}$ in phase 1

repolarization and the generation of EAD and DAD of either normal or failing human heart seem very limited (Verkerk *et al.*, 2003b). Therefore, the clinical relevance of $I_{Cl,Ca}$ blockers remain to be determined.

The molecular identity of CACCs in the heart remains to be determined. CLCA-1 and bestrophins were initially proposed as candidates for CACCs in cardiac tissues(Hartzell *et al.*, 2005;O'Driscoll *et al.*, 2008). It has been demonstrated that at least three members of the murine Bestrophin family, mBest1, mBest2 and mBest3, are expressed in mouse heart. Whole-cell patch clamp experiments with HEK cells transfected with cardiac mBest1 and mBest3 both elicited a calcium sensitive, time independent Cl^- current, suggesting mBest1 and mBest3 may function as pore-forming Cl^- channels that are activated by physiological levels of calcium(O'Driscoll *et al.*, 2008). Very recently, independent studies from three laboratories have identified a new gene, TMEM16 (or Ano1), as a candidate for CACCs (Schroeder *et al.*, 2008;Caputo *et al.*, 2008;Yang *et al.*, 2008). Whether TMEM16 forms the functional endogenous CACCs and how TMEME16 interacts with and the bestrophins in native cardiac myocytes and its functional role in pacemaker activity and electrophysiology remain to be explored.

3.4 Angiotensin II-activated Cl^- channels and cardiac pacemaker activity

An angiotensin-II-activated Cl^- current was found in rabbit SAN cells (Bescond *et al.*, 1994). The current is sensitive to Cl^- channel blockers anthracene-9-carboxylic acid and diphenylamine-2-carboxylic acid and can be totally inhibited by the competitive angiotensin II-receptor 1 (AT1) antagonist losartan and by the presence of intracellular protein kinase C inhibitor. It is suggested that in SAN cells there exist protein kinase-C-sensitive Cl^- channels which may be activated by angiotensin II via the stimulation of the AT1 receptors (Bescond *et al.*, 1994). The molecular mechanism and the exact functional role of these channels, however, have not been further explored.

4. Conclusion

Experimental evidence are merging to support the potential role of several Cl^- channels in the regulation of cardiac pacemaker activity and these anion channels may represent novel therapeutic targets for arrhythmias involving abnormal pacemaker activities.

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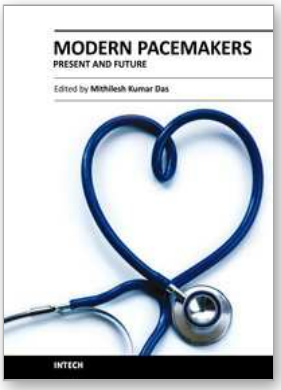

Modern Pacemakers - Present and Future

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The book focuses upon clinical as well as engineering aspects of modern cardiac pacemakers. Modern pacemaker functions, implant techniques, various complications related to implant and complications during follow-up are covered. The issue of interaction between magnetic resonance imaging and pacemakers are well discussed. Chapters are also included discussing the role of pacemakers in congenital and acquired conduction disease. Apart from pacing for bradycardia, the role of pacemakers in cardiac resynchronization therapy has been an important aspect of management of advanced heart failure. The book provides an excellent overview of implantation techniques as well as benefits and limitations of cardiac resynchronization therapy. Pacemaker follow-up with remote monitoring is getting more and more acceptance in clinical practice; therefore, chapters related to various aspects of remote monitoring are also incorporated in the book. The current aspect of cardiac pacemaker physiology and role of cardiac ion channels, as well as the present and future of biopacemakers are included to glimpse into the future management of conductions system diseases. We have also included chapters regarding gut pacemakers as well as pacemaker mechanisms of neural networks. Therefore, the book covers the entire spectrum of modern pacemaker therapy including implant techniques, device related complications, interactions, limitations, and benefits (including the role of pacing role in heart failure), as well as future prospects of cardiac pacing.

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