Online Supplement (Expanded Materials and Methods)

Part I: Methods

SA Node Cell Preparations and Electrophysiological Recordings

Single, spindle-shaped, spontaneously beating SANC, isolated from the rabbit hearts, using protocols approved by the Animal Care and Use Committee of our institution, as previously described (11, 13), were studied in Tyrode's solution at 35±0.1°C with a perfusion rate of 1-1.5 ml/min (chamber volume 300 μl). The perforated patch-clamp technique with 50 μmol/L β-escin4 (Sigma) added to the pipette solution was used to record spontaneous APs with an Axopatch-1D patch-clamp amplifier (Axon Instruments). The bath solution contained (in mmol/L): NaCl 140; KCl 5.4; $MgCl_2$ 1; HEPES 5; $CaCl_2$ 1.8; and glucose 5.5; pH 7.4. The pipette solution contained (in mmol/L) K-gluconate 120, NaCl 10, MgATP 5, HEPES 5, and KCl 20; pH 7.2. The AP rate was measured prior to, and in response to specific CCh concentrations (10 to 1000 nmol/l), followed by the washout in the presence or absence of PTX in the absence or presence of inhibition of I_f (CsCl) or $I_{K_{ACh}}$ inhibition (TQ) or during dual inhibition of both $I_{K_{ACh}}$ and I_f.

Voltage clamp recordings of I_f, I_{KACh} or $I_{Ca,L}$ currents

The whole-cell I_f or CCh induced currents were detected with an Axopatch-100D amplifier (Axon Instruments, Inc., Union City, CA) at 35 °C, low pass-filtered at 1 kHz, and sampled at 5 kHz with pCLAMP, version 6.0 software. Patch clamp pipette resistance was 3–5 megohms. Pipette solution contained (in mmol/L): 120 K-gluconate, 5

NaCl, 5 MgATP; 5 HEPES; 20 KCl;. 5 phosphocreatine, 0.2 NaGTP, pH 7. The bath solution contained (in mmol/L): NaCl 140; KCl 5.4; MgCl2 1; HEPES 5; $CaCl_2$ 1.8; and glucose 5.5; pH 7.4. 1 μM carbachol (CCh; Calbiochem) was added to the bath solution to induce the CCh sensitive current. Bath solutions containing drugs were applied rapidly via a 1.5 ml/min perfusion rate into an experimental chamber of 300 μl total volume. CCh-induced currents were measured in the voltage-clamp mode by applying voltage pulses of 500 ms, within the voltage range of -110 mV to + 40 mV in 10 mV increments starting from a holding potential -40 mV. To reduce interference between I_f and I_{KACh} currents, CCh induced current was measured in the presence of 2 mM CsCl added to the bath solution. To block CCh induced current we used an additional set of cells pretreated for 3 hours in KB solution at +4 °C with 1 μM tertiapin-Q, a specific I_{KACh} inhibitor (TQ, Sigma).

The ruptured patch-clamp technique was used to record L-type Ca^{2+} current, $I_{Ca,L}$, using an Axopatch -200D patch-clamp amplifier (Axon Instruments, Foster City, CA). Only spontaneous, regularly beating spindle-shaped SANC were chosen for $I_{Ca,L}$ recordings. The depolarizing voltage clamp pulses (300 ms) were applied from a holding potential of -50 mV with increments of 10 mV in the range -40 to +40 mV. The bath solution contained the following (in mmol/L): NaCl, 117; TEA-Cl, 20; CsCl, 5.4; $MgCl_2$, 1; HEPES, 5; $CaCl_2$, 1.8; 4-AP, 4; pH = 7.4; 10 μmol/L tetrodotoxin and 4 mmol/L 4-aminopyridine were added to block interfering currents. The pipette solution contained the following (in mmol/L): NaCl, 10; TEA-Cl, 20; CsCl, 110; EGTA, 10; MgATP, 5; HEPES, 10; pH, 7.2. To minimize the interference from rundown, $I_{Ca,L}$ was measured

every 11 seconds (step to 0 mV); the effect of CCh was measured after 2 min-CCh superfusion and compared to the amplitude of $I_{Ca,L}$ immediately before CCh addition.

Confocal imaging of Ca releases

SANC were placed on the stage of a Zeiss LSM-410 inverted confocal microscope (Carl Zeiss, Inc., Germany) and loaded with fluo-3 AM (Molecular Probes, Eugene, OR). All images were recorded in the linescan mode, with the scan line oriented along the long axis of the cell, close to sarcolemmal membrane; images were processed with IDL software (5.4, Research Systems, Boulder, CO) as described previously (2).

Western blot of phospholamban

The detection of site-specific PLB phosphorylation was performed in SANC as previously described (9). SANC suspensions were equally divided into 6-8 parts, and each part was individually treated: with selected concentrations of CCh (1 nmol/l to 10 μmol/l) or solvent as a control for 5 min at +35ºC. After treatment, samples were centrifuged; the pellet was fully solubilized in lysis buffer and then snap frozen in liquid nitrogen and kept for further investigation. For detection of PLB, the pellet was boiled at 100ºC to dissociate PLB into its monomeric form. Proteins were resolved by 7.5% urea/SDS-PAGE gel and transferred (~10 μg protein/lane) to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). To detect PLB phosphorylation, the phosphorylation site-specific P-Ser-16 polyclonal antibody (1:10000, Badrilla) and HRP-conjugated secondary anti-rabbit antibody (1:10000, Bio-Rad) were employed. Membranes were exposed to the chemiluminescence reaction (ECL, Amersham Pharmacia Biotech) and quantified with a video detection system (Bio-Rad). To detect total PLB the same membranes were stripped with stripping buffer (Pierce), and

incubated with a total anti-PLB monoclonal (1:10,000) phospholamban antibody (Badrilla).

Adenylyl and Guanylyl cyclase activities

SANC were exposed to CCh (100 to 1000 nmol/l) in the presence of non-specific PDE inhibitor IBMX (100 μmol/l); the reaction was stopped by adding 100 % cold ethanol to the cell suspension according to the assay instruction. All groups, i.e. those with or without IBMX were incubated for a total time of 25 min. In groups treated with IBMX, drug was added immediately at the beginning of incubation. In groups that received CCh, drug was introduced at the 20^{th} min of total incubation time for the remaining 5 min of incubation. At 25 min, cAMP/cGMP production was stopped by adding 100% freezing cold ethanol. Cells were homogenized and then centrifuged at 14 g. The supernatant was used for cAMP or cGMP estimation using a cyclic AMP or cyclic GMP (^{125}I) assay system (Amersham Bitotech, USA). Total protein concentration was determined with a Bio-Rad system (Hercules, CA). The amount of the cAMP was expressed as pmol/mg protein.

Curve fitting procedure to determine the best fit value of IC_{50} in normalized dose response curves

To determine the IC_{50} CCh of a standard dose-response curve fitting model (GraphPad Software, Inc. 2007) was applied to the average BRR dose response data for CCh alone, for CCh plus the I_{KACh} inhibitor tertiapin-Q (TQ), and to the average dose response curve for reduction of PLB phosphorylation by CCh. The standard dose-response model uses four specific parameters: the minimum plateau, the maximum plateau, the IC_{50}, and the slope factor (which is often constrained to a standard value) to

calculate the best fit normalized response curve. The "IC_{50}-(EC_{50})-shift" model within the Prism 5 software (GraphPad Software, Inc. 2007) was used to determine the IC_{50} shift for the CCh+TQ effect on BBR and PLB phosphorylation reduction, using the control CCh effect on BRR served as the reference curve and a standart slope factor.

Part II: Numerical modeling

Rationale for the modeling

Previous numerical models of ChR stimulation effects on BRR (3, 14) **have** formulated important CCh-induced changes of I_{KACh}, I_f, and I_{CaL}. The present study explored the integration of CCh effects on these classical surface membrane mechanisms and PKA-dependent effects of intracellular Ca^{2+} cycling via NCX current (I_{NCX}) in BRR in response to CCh stimulation at the IC_{50} CCh. We used the experimentally measured changes of LCR characteristics (LCR period and LCR signal mass) to numerically predict the respective I_{NCX} changes in response to CCh stimulation that cannot be measured experimentally during spontaneous AP firing.

The numerical model

We used a modification of the numerical SANC model of primary rabbit SANC (10) based on the Kurata et al. model (7) that we had previously developed. Our model integrates an ensemble of individual multiple LCRs with other factors known to modulate SANC automaticity, including the rate of acceleration via β-AR stimulation (10) and the fine DD structure in rabbit SANC (1).

Model formulations to simulate BRR produced by CCh (100 nmol/l)

Simulations of CCh-dependent ion currents.

We calculated changes in I_{CaL}, I_f, and I_{KACh} at 100 nM CCh using formulations suggested previously by Zhang et al. 2002 (14). In short, the fractional block *b* of I_{CaL} by CCh was calculated using the following Michaelis-Menton–type equation:

$$b = b_{\max}[CCh] \ /(K_{0.5,Ca} + [CCh] \ ) ,$$

where $b_{max} = 0.56$ and $K_{0.5,Ca} = 90$ nmol/l.

The shift *s* (in mV) of the I_f activation curve by CCh was also described by a Michaelis-Menton–type equation:

$$s = s_{\max}[CCh]^{n_f} \ /(K_{0.5,f}^{n_f} + [CCh]^{n_f}) ,$$

where $s_{max} = -7.2$ mV, $n_f = 0.69$, and $K_{0.5,f} = 12.6$ nmol/l.

I_{KACh} was described as follows:

$$I_{KACh} = g_{KACh} \{[K]_e / (10 + [K]_e)\} \times \{ (V_m - E_K)/[1+\exp ((V_m - E_K - 140)F/2.5RT)]\},$$

where $[K]_e$ is the extracellular K^+ concentration, E_K is the K^+ equilibrium potential, and the conductance g_{KAch} is defined as follows:

$$g_{KACh} = g_{KACh,\max} jk[CCh]^{n_{KACh}} /(K_{0.5,KACh}^{n_{KACh}} + [CCh]^{n_{KACh}}) ,$$

where $g_{KAch,max} = 0.304615$ nS/pF (i.e. 0.0198 μS per 65 pF cell in) , $K_{0.5,KACh} = 280$ nmol/l , $n_{KACh} = 1.5$. The I_{KACh} inactivation variables *j* and *k* are defined below:

$$dj/dt = \alpha_j (1 - j) - \beta_j j$$

$$dk/dt = \alpha_k (1 - k) - \beta_k k$$

where α_j, β_j, α_k, β_k are rate constants ($\alpha_j = 73.1$ s^{-1}, $\alpha_k = 3.7$ s^{-1}). β_k and β_j are voltage-dependent:

$$\beta_j = 120/\{1+\exp[-(V_m + 50)/15]\}$$

$$\beta_k = 5.82/\{1+\exp[-(V_m + 50)/15]\}$$

Simulation of the LCR changes.

Individual LCRs are generated in the model as originally described in Vinogradova et al. (10). In short, the entire sarcolemma with adjacent junctional SR is virtually divided into segments of an average LCR size as measured by confocal microscopy. Each segment is described by a separate set of variables approximating the "local" Ca^{2+} cycling, I_{CaL} and I_{NCX}. The model algorithm makes some local SR segments permeable to Ca^{2+}, with the release rate being described by a sinusoid function. The number of LCRs (N_{LCR}), which fire in the model during each cycle is 42, i.e. the average number of LCRs evaluated from LCR line scan confocal images in rabbit SANC (1). The moments of the LCR firing in the model reflect the LCR periods: they are normally distributed, with the distribution center and the spread being close to that observed experimentally. Although, this kind of modeling does not provide a mechanism for the LCR initiation, it has been successfully used to evaluate the impact of the LCRs on the I_{NCX}, the fine DD structure, and ultimately AP firing rate.

We changed the following model parameters of LCRs and Ca^{2+} SR pumping in accordance with the experimental results obtained at 100 nmol/l [CCh]:

1) N_{LCR} is decreased to $N_{LCR,CCh}$= 42· 71.5/100 ~ 30, respectively in line with the reduction of the LCR rate in our confocal measurements to 71.5% of control.

2) The effective LCR size (L_{LCR}) was directly estimated from our confocal measurements in this study, and for the basal conditions L_{LCR}= 6.9 μm. L_{LCR} at 100 nmol/l CCh was

estimated from the reduction of the LCR size to 72.6% of control: $L_{LCR,CCh}$= 6.9 μm · 72.6/100 ~ 5 μm.

3) The LCR period after 3 min of 100 nmol/l [CCh] increased on average to 158.8% of control. Therefore, we increased t_{phase}, the phase of the LCR occurrence in our model (i.e. the center of the Gaussian distribution of the LCR periods) from t_{phaseh} = 260 ms to $t_{phase,CCh}$ = 260 ms· 158.8/100 ~ 413 ms.

4) We decreased the SR pumping rate (P_{up} = 0.012 M/s) by 40% to P_{up}_CCh = 0.0072 M/s to match an experimental estimate for the decrease in the index of PKA-dependent phosphorylation signaling (also by ~40%).

Model formulations to simulate BRR produced by CCh 100 nM and calyculin A

Simulations of CCh-dependent ion currents.

We changed only phosphorylation-independent I_{KCh} and I_f changes induced by 100 nM CCh as described above but I_{CaL} remained unchanged.

Simulation of the LCR changes.

We changed the following model parameters of LCRs and Ca^{2+} SR pumping in accordance with the experimental results obtained at 100 nmol/l [CCh] and ***calyculin A***:

1) N_{LCR} is decreased to $N_{LCR,CCh+CyA}$= 42· 85.4/100 ~ 36, respectively in line with the reduction of the LCR rate in our confocal measurements to 85.4% of control.

2) The effective LCR size (L_{LCR}) slightly increased to 107.7% of control: $L_{LCR,CCh+CyA}$ = 6.9 μm · 107.7/100 ~ 7.4 μm.

3) The LCR period after 3 min of 100 nmol/l [CCh] increased on average to 110.8% of control. Therefore, we increased t_{phase}, the phase of the LCR occurrence in our model (i.e.

the center of the Gaussian distribution of the LCR periods) from t_{phaseh} = 260 ms to $t_{phase,CCh+CyA}$ = 260 ms· 110.8/100 ~ 288 ms.

Other Ca^{2+} cycling parameters were identical in all simulations of basal and slowed AP firing (i.e. they remain the same as in our previous model (10)). Specifically, LCR period SD=30 ms, t_{width}=60 ms, $P_{rel,inst}$=0.5 ms^{-1}, $P_{rel,spont}$=4 ms^{-1} τ_{dif}=0.07 ms, L_{sub}=0.04 μm.

Other minor changes of the original model.

The model used in the present study, similar to the original Kurata et al. model (7) (version with I_{st}=0) but does not have background Ca^{2+} current and I_{st}. In fact, the non-selective steady current I_{st} has neither molecular identity, nor specific blockers. Furthermore, it exhibits many properties of I_{NCX} and I_{CaL}. Since Kurata et al. model (7) ("I_{st}=0" version) has a longer than usual APD_{50} value of ~120 ms, we slightly accelerated I_{CaL} inactivation to get more realistic APD_{50} values from 90 to 100 ms (Table 1 in (7)):

$$\tau_{fL} = 0.6\cdot (257.1 \exp (- (V+ 32.5)/13.9)^2 + 44.3)$$

To avoid possible problems with "degeneracy" (i.e. the existence of a continuum of equilibrium points) and slow ion concentration drifts (4-6) (review (12)), we fixed intracellular $[Na^+]$ and $[K^+]$ to 8 mmol/l and 140 mmol/l, respectively, as suggested in the most recent 2003 Kurata et al. model version (8). Specific ion conductances in the model were as follows (in nS/pF): g_{CaL} =0.29; g_{bNa}=0.003348; g_h =0.1125; g_{CaT}=0.2977; g_{Kr} =0.057474; g_{Ks}=0.0259; g_{to} =0.144; g_{sus} =0.016; $g_{KAch,max}$ = 0.304615. $I_{NaK,max}$ =0.9 pA/pF asnd k_{NaCa} =125 pA/pF.

207 **Online Supplement References**

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Online supplement Figure Legends

Fig.S-1. Representative examples of continuously recorded APs at varying concentrations of CCh.

Fig. S-2 A. Representative control total current-voltage relationship, left traces; total current-voltage relationship in the presence of CsCl, middle traces (same cell in left traces after 2 min perfusion with CsCl); total CCh induced current-voltage relationship in the presence of CsCl, right traces (same cell after 2 min perfusion with 1 μM CCh with solution containing 2 mM CsCl). B. Representative traces of total current-voltage relationship in cell perfused with I_{KACh} blocker TQ, left traces (a different cell pretreated and superfused with 1 μM terteapin-Q); total current-voltage relationship in a TQ treated cell superfused with CsCl, middle traces; total current-voltage relationship in TQ treated cell subsequently exposed to 1μM CCh in solution containing CsCl (right traces).

Fig. S-3. Representative continuous AP recordings from spontaneously beating SANC exposed to various [CCh] in cells in which I_{KACh} was disabled.

Fig. S-4. A. Suppression of L type Ca^{2+} current by10 μM CCh. A representative example of L type Ca^{2+} current records in response to voltage clamp to 0 mV from holding potential of -50 mV prior to and during CCh exposure. B. Average effects of CCh to suppress L type Ca^{2+} current (n=4; * - $p<0.05$).

Fig. S-5. Schematic illustration of the interplay of Ca^{2+}, basal Ca^{2+}-activated AC, cAMP, PDE activity and PKA activity, cast in the context of sarcoplasmic reticulum Ca^{2+} cycling, L type Ca^{2+} channels and other ion channels. Ca^{2+} activation of basal AC activity results in a feed forward "fail safe" system to generate cAMP and cAMP/PKA dependent phosphorylation. Ca^{2+} cycling protein phosphorylation (PLB, Ryanodine Receptors, L type Ca^{2+} channels, and crosstalk of these) generates local, spontaneous submembrane Ca^{2+} releases. Ca^{2+} activation of I_{NCX} during

DD initiates and initiates spontaneous SANC AP's, as reported previously (2-6, 7-10). Global increases in cytoplasmic Ca^{2+} triggered by APs activate ACs. Ca^{2+}, cAMP or PKA-dependant phosphorylation also modulates other ion channels. The dual basal Ca^{2+}/cAMP-PKA "feed-forward" regulation is kept in check by a high basal PDE activity, which prevents cAMP/PKA signaling to become excessive i.e., acts as negative feedback mechanism to prevent an excessive basal beating rate, and to insure reserve cAMP/PKA modulation of spontaneous beating via activation of β-ARs.