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CA²⁺-ACTIVATED ADENYLYL CYCLASE 1 INTRODUCES CA²⁺-DEPENDENCE TO BETA-ADRENERGIC STIMULATION OF HCN2 CURRENT

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Introduction

The pacemaker current, I_f , plays an important role in determining diastolic depolarization in sinoatrial node cells (SAN) and in modulating rhythm in response to regulatory inputs. I_f is generated by the Hyperpolarization-activated Cyclic Nucleotide-gated channels (HCN; isoforms 1, 2 and 4) and carried by sodium and potassium ions. Two important properties of this channel are activation upon hyperpolarization and direct interaction with cyclic nucleotides (especially, cAMP) via a Cyclic Nucleotide Binding Domain (CNBD).

I_f sensitivity to internal Ca^{2+} was first described by Hagiwara and Irisawa [1]. In rabbit SAN cells, reduction of intracellular Ca^{2+} from 100 nM to 0.1 nM induced downregulation of I_f and shifted activation to more negative voltages. Then, Zaza et al [2], using inside-out macro patches, demonstrated that Ca^{2+} did not directly modulate pacemaker channels. Further, Rigg et al. [3] found that I_f in guinea pig SAN is regulated by Ca^{2+} and calmodulin, but via a mechanism independent of CAMKII activation.

In guinea pig SAN myocytes Ca^{2+} -chelation with BAPTA inhibits I_f [3,4]. In rabbit SAN, sarcoplasmic reticulum Ca^{2+} depletion by ryanodine does not affect basal I_f but reduces the shift in I_f activation induced by isoproterenol [5]. However, ryanodine does not affect the ability of I_f to be stimulated when the adrenergic cascade is bypassed by direct stimulation of adenylyl cyclase with forskolin [4] or by the introduction of permeable cAMP [5]. A similar observation was reported in thalamocortical cells, where Ca^{2+} -mediated upregulation of I_h required adenylyl cyclase activity [6]. Disruption of normal Ca^{2+} -homeostasis with ryanodine also influences sympathetic modulation of SAN cell rate in the same manner as it does I_f [5].

Nine adenylyl cyclase isoforms have been identified (AC 1–9), the features and tissue distribution of which differ significantly [7]. Some (e.g. AC5/6) are ubiquitously distributed throughout the body while others are largely limited to certain organs or tissues (AC1 in

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neuronal tissue, AC3 in olfactory epithelium, etc.). Three isoforms (AC1, AC3, AC8) are Ca^{2+} -activated in the sub-micromolar range, and of these, AC1 and AC8 are calmodulin- (but not CAMKII-) dependent [7]. In contrast, AC5/6 is inhibited in the presence of micromolar Ca^{2+} concentrations and highly regulated by PKA and PKC phosphorylation [8]. The predominant cardiac AC isoforms are AC5/6 [9]. However, the cardiac pacemaker region also expresses AC1, AC2 and AC8 [4,10]. Thus, Ca^{2+} -activated AC1 and AC8 in SAN may be responsible for the Ca^{2+} -dependence of β -adrenergic modulation of I_f [4].

Although the data cited above are consistent with the hypothesis that the Ca^{2+} modulation of pacemaker current depends on the presence of Ca^{2+} -stimulated AC isoforms, technical limitations such as the lack of reliable isoform-specific AC inhibitors complicate experiments to demonstrate a causal link within the SAN. Therefore we chose to over-express recombinant AC1 in neonatal rat ventricular myocytes (which do not have that isoform) and test whether this perturbation alters the Ca^{2+} -dependence of β -adrenergic effects on HCN current.

Methods

Cell culture and infection

Neonatal rat ventricular myocytes (NRVM) were isolated as previously described [11]. Briefly, 1–2 days old Wistar rats were euthanized by decapitation according to protocols approved by the Institutional Animal Care and Use Committee of Columbia University. This protocol conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Hearts were quickly removed and ventricles were dissociated by a standard trypsinization procedure. After pre-plating for one hour to minimize fibroblast contamination, myocytes were harvested and plated in protamine-sulfate coated dishes or onto fibronectin-coated coverslips (for action potential recordings). Cells were incubated in MEM (CellGro) containing 10% fetal bovine serum for 24 hr, then switched to serum free medium (SFM; [12]). Adenoviral infection was performed on culture day 1, at multiplicity of infection (moi) 0.5 for each construct. Cells were exposed to the infectious mix for 2 hrs, rinsed twice and then incubated in SFM at 37°C, 5% CO_2 for 3–4 days. For studies of pacemaker current, the cell monolayer was resuspended by brief exposure to 0.1% trypsin and the cells re-plated onto fibronectin-coated coverslips, then studied 2–6 hrs later.

Adenoviral vectors

AdGFP, AdmHCN2 and AdmHCN2R591E are described elsewhere [13]. Adenoviral constructs of FLAG-tagged AC1 and AC6 were a generous gift of Dr. Ross Feldman (University of West Ontario, London, Canada). All viruses were amplified in HEK 293 cells and titrated with anti-Adv Hexon antibodies. Final titers were 1×10^9 – 5×10^9 fluorescence forming units per ml (ffu/mL).

Immunoprecipitation and immunoblotting

72 hours after infection, cardiomyocytes were scraped into homogenization buffer (20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2 mM EGTA, 0.1 mM sodium orthovanadate, 0.042% β -mercaptoethanol, 1x protease inhibitor cocktail (Roche); 0.5mL per 100 mm dish), sonicated, and centrifuged at 185,000g for 1 hour at 4°C. The pellet was resuspended in solubilization buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% NP-40, 0.1 mM sodium orthovanadate and 0.1 mM sodium fluoride) and cleared of insoluble materials. A protein assay (Bio-Rad D_C Protein Assay) was performed on each set of samples, and all concentrations in the set were defined from the same calibration curve. Adenylyl cyclase was immunoprecipitated from 700 μg of particulate

fraction with anti-FLAG antibodies (F1804; Sigma) according to manufacturer's instructions. HCN2 was pulled down with 2 μ g of anti-HCN2 antibodies (APC-030; Alomone Labs, Israel) per 500 μ g of soluble materials. Proteins were separated by 7.5% SDS-PAGE and transferred to nitrocellulose for immunoblotting with anti-HCN2 or anti-FLAG antibodies. Samples for detection of adenylyl cyclases were run unheated. Loading and transfer efficacy were controlled by ponceau staining. Epitope specific binding was visualized by enhanced chemiluminescence (Pierce). Results were replicated in four different culture preparations. Isoform-specific antibodies (Santa Cruz) were used for detection of endogenous AC1 (sc-586) and AC5/6 (sc-590) in rat brain membrane (a gift from Dr. Lori Isom) and neonatal rat ventricular myocytes.

cAMP measurements

For cAMP measurements, besides indicated experimental treatments, all groups were pre-treated with 100 μ M IBMX (Sigma) to inhibit phosphodiesterase activity, then harvested and processed according to manufacturers' instructions. cAMP was determined using radioimmunoassay (Amersham Cyclic AMP (3H) Assay System, GE Healthcare, UK) or ELISA (Parameter™ Cyclic AMP Assay, R&D Systems, MN, USA).

Electrophysiology

Whole-cell HCN2 current and action potentials were recorded using patch electrodes. Cells were superfused with the following extracellular solution (mM): NaCl, 140; NaOH, 2.3; MgCl₂, 1; KCl, 5.4; CaCl₂, 1; HEPES, 5; glucose, 10; pH 7.4. Extracellular potassium was increased to 10mM and MnCl₂ (2 mM) and BaCl₂ (4 mM) (to eliminate I_{Ca} and I_{K1}) were added while recording HCN2 current. Pipettes were filled with solution containing (mM): aspartic acid, 130; KOH, 146; NaCl, 10; CaCl₂, 2; EGTA-KOH, 5; Mg-ATP, 2; HEPES-KOH, 10; pH 7.2. This formulation resulted in a calculated free intracellular Ca²⁺ concentration of ~100 nM (pCa~7). Calculations (MaxChelator) indicate that under these conditions, the addition of 5 μ M of the cell-permeable Ca²⁺- chelating agent BAPTA-AM (Invitrogen) extracellularly will not reduce diastolic Ca²⁺ but will prevent free Ca²⁺ from rising from this value.

Currents were recorded at 35°C. An Axopatch-200B amplifier and pClamp9.2 software (Axon Instruments) were used for data acquisition and analysis. The protocol included test voltage steps from -25 to -125 mV with increments of 10 mV (from the holding potential -20 mV), followed by a voltage step to a negative voltage (-100 to -115 mV), where the tail currents were recorded. To completely deactivate HCN current, a pulse to -5 mV was applied between sweeps. The normalized plot of tail current versus test voltages was fitted with a Boltzmann function. The voltage of half maximal activation and slope factor were then defined from that fitting. Time constants of activation were obtained by fitting the early part of current traces with a monoexponential function; the initial delay and slow activation phase were ignored. Data are not corrected for liquid junction potential (9.8mV); doing so would shift measured voltages negative.

Rate study

For rate determination, myocytes were plated into 24 well dishes, pre-coated with protamine sulfate, to form a continuous monolayer. Beating rate was determined by counting spontaneous beats in each well under the light microscope for 15 sec.

Statistics

All data are presented as mean \pm S.E.M. Statistical significance for multiple groups comparison was examined by One or Two Way ANOVA (followed by pairwise multiple

comparisons (Bonferroni)) as appropriate. In some cases unpaired *t* test was used for comparison of two means. Values of $p < 0.05$ were considered significant.

Results

AC1 and AC6 over-expression and interaction with mHCN2

First, we investigated the presence of endogenous AC1 and AC5/6 in our cultures using western blot analysis. Supplemental figure 1 demonstrates no detectable AC1 expression in NRVM cultures (left panel). The positive control, rat brain membranes, shows an epitope-specific band of expected molecular weight for rat AC1 (108kDa). On the other hand, probing neonatal myocyte membrane extracts with anti-AC5/6 antibodies reveals abundant reactivity at the expected molecular weight (suppl. fig. 1, right panel). This confirmed previously reported data regarding AC1 and AC5/6 expression in mammalian ventricle and validated the rationale for using NRVM as a model system for introducing a neuronal AC isoform.

Cultures were exposed to AdmHCN2 along with AdGFP (control), AC1 or AC6. Over-expression of recombinant AC1 and AC6 was confirmed by western blot (figure 1A). Anti-FLAG antibodies detected bands of the expected molecular weight for both isoforms (left panel). No anti-FLAG specific signal was found in AdGFP-infected cultures. To explore the possibility of direct interaction with HCN2 channels, we immunoprecipitated AC with anti-FLAG antibodies, separated the obtained proteins by PAGE and probed with anti-HCN2 antibodies (figure 1A; middle panel). Both AC1 and AC6 pulled down HCN2 protein (figure 1A), suggesting possible interaction between these AC isoforms and HCN2. No protein was detected in the GFP lane. These results were replicated in four different culture preparations. To confirm specificity of the FLAG pull down, we also performed a reverse experiment, immunoprecipitating with anti-HCN2 antibodies and detecting FLAG-specific signal that was pulled down with the channel protein. As expected, a signal was found in AC1 and AC6 infected cultures, but not in GFP group (figure 1A; right panel). Further, both AC1 and AC6 isoforms also co-immunoprecipitated with endogenous HCN2 (figure 1B).

Effect of AC overexpression on cAMP level

To investigate the functional effect of each AC isoform on basal cAMP, we measured total intracellular cAMP level in cultures infected with GFP, AC1 or AC6 (Figure 2A). Only AC1 induced a significant increase in total intracellular cAMP level ($p < 0.05$ vs. GFP). In the absence of added exogenous agonist, the β -adrenoreceptor blocker propranolol (1 μ M) had no effect on cAMP accumulation induced by AC1-overexpression (suppl. fig. 2). In addition, the AC expression level was functionally evaluated by measuring forskolin-stimulated (10 μ M) cAMP production in AC1 or AC6 infected cultures (Figure 2B). There was no significance difference between the two groups.

The higher basal cAMP level with AC1 is reflected in a more positive position of the HCN2 activation relation

HCN2 current was recorded on days 3 or 4 post-infection with AdmHCN2 along with GFP, AC1 or AC6. No differences in cell capacitance were observed. The current density did not differ significantly between groups (88 \pm 18 pA/pF, 127 \pm 34 pA/pF and 81 \pm 17 pA/pF in GFP, AC1 and AC6 co-infected cultures respectively; $p > 0.05$). However, the current in AC1 infected myocytes activated at significantly more positive voltages than in the GFP and AC6 groups (Figure 3A, B; $p < 0.001$). This shift was accompanied by acceleration of kinetics and is consistent with the channel being activated by cAMP-binding [14]. Average midpoints of activation were -69 ± 1.8 mV in GFP, -58 ± 1.8 mV in AC1 and -65 ± 1.2 mV in AC6 groups.

Figure 3C shows corresponding values for slope factors (which did not differ) in the three groups and panel D provides data on mean activation kinetics.

To investigate whether the observed effects of AC1 expression on HCN2 biophysics were due to direct cAMP activation of the channel, we co-expressed AC1 with HCN2R593E (HCN2RE), a mutant with markedly reduced (>1000 fold) affinity for cAMP [15,13]. Previously, we reported that in NRVM HCN2RE current activation is shifted about 12 mV negative compared to HCN2 [13]. AC1 did not change the voltage dependence of HCN2RE (figure 4A). V_{50} values were: GFP -87 ± 2 mV; AC1: -87 ± 2 mV; AC6: -84 ± 2 mV ($p>0.05$); corresponding values for slope factors were: 8.9 ± 0.6 mV, 11.5 ± 0.5 mV and 11.3 ± 1.0 mV ($n=6-8$) ($p>0.05$). The current density in the AC1 group did not differ from the GFP group (94.9 ± 15.2 pA/pF vs 66.7 ± 19.6 pA/pF in GFP, $p>0.05$). Together, these data are consistent with the effects of AC1 expression on HCN2 biophysical properties resulting from an elevation in basal cAMP.

Acceleration of spontaneous beating rate in AC1-infected cultures

On day 4 after plating, neonatal rat ventricular myocytes in culture beat spontaneously with an average rate of 69 ± 13 bpm ($n=17$). Over-expression of HCN2 significantly increased the beating rate to 167 ± 8 bpm ($n=34$; $p<0.05$ vs. non infected by unpaired *t*-test). AC1 co-expression further accelerated beating rate, whereas cultures infected with AC6 (which do not have elevated cAMP) did not show increase in spontaneous activity ($p>0.05$). Average values are shown in figure 4B.

Surprisingly, the HCN2RE mutant was as effective in accelerating spontaneous beating rate in NRVM cultures in the absence or presence of the AC1 isoform as the wild type channel despite its more negative V_{50} (160 ± 15 bpm; $n=12$). The observation that AC1 co-expression increases rate equally well with HCN2 or HCN2RE suggests additional cAMP dependent factors, besides the positive activation of expressed HCN2, contribute to the observed rate increase (see Discussion).

Representative spontaneous action potential recordings from cultures co-expressing WT HCN2 with AC1 or AC6 are shown in figure 5. Summary data of relevant action potential parameters are shown in Table 1 and indicate significant rate acceleration in the AC1 group and differences in the maximum diastolic potential (MDP) between the two groups. Maximum diastolic potential (MDP) was significantly more positive in AC1 cultures. In contrast, the rate of early diastolic depolarization (EDD), take off potential (TOP) and overshoot did not differ between AC1 and AC6 (figure 5; Table 1).

AC1 but not AC6 over-expression introduces Ca^{2+} -sensitivity to the β -AR response of HCN2

To stimulate β -adrenoreceptors we used a well known β -agonist isoproterenol (ISO) at a $1\mu\text{M}$ concentration, which produces a maximum effect in NRVM [16]. In GFP-expressing cultures, ISO induced the expected positive shift in the HCN2 activation relation (from -69.4 ± 1.8 mV to -59.8 ± 2.2 mV) (figure 6, $p<0.05$ by *t*-test). Compared to the GFP group, the HCN2 midpoint in the AC1 group was -58.7 ± 1.5 mV. The introduction of $5\mu\text{M}$ BAPTA-AM did not significantly shift this value, consistent with the calculation that this concentration of BAPTA-AM would minimally affect basal $[Ca^{2+}]_i$ and therefore basal cAMP level near the channels, unless more stringent chelating conditions were used (suppl. fig. 3). In the absence of BAPTA-AM, $1\mu\text{M}$ isoproterenol (ISO) induced a positive shift (to -52 ± 1.4 , $n=5$, $p<0.05$ vs. AC1 control), indicating that HCN2 was not fully saturated by cAMP under basal conditions. Importantly, this ISO effect was lost when cells were pre-incubated in BAPTA (-59 ± 1.7 mV, $n=8$; figure 6), consistent with BAPTA preventing an

ISO-induced increase in mean $[Ca^{2+}]_i$. In AC6-expressing myocytes, ISO shifted the HCN2 activation relation to a similar extent in the absence and presence of BAPTA-AM (-59 ± 1.5 mV and -58 ± 1.3 mV respectively; figure 6), confirming a lack of Ca^{2+} -sensitivity in this setting. Taken together, these data suggest that the presence of AC1 can account for the Ca^{2+} -sensitivity of HCN2 β -AR responsiveness.

To complement the HCN2 current data, we evaluated ISO-induced intracellular cAMP production in cultures with or without BAPTA-AM pre-treatment. In the AC1 group, three out of four experiments showed a noticeable decrease in cAMP level after BAPTA, while in one experiment it was slightly higher after BAPTA-AM. In contrast, in the AC6 group, all four preparations had an increased level of cAMP after pretreatment compare to untreated. However, none of those changes were statistically significant.

Discussion

This study tested the hypothesis that the presence of a Ca^{2+} -stimulated adenylyl cyclase isoform, as found in SAN [4,10], accounts for the reported Ca-dependence of the effect of adrenergic agonists to shift the I_f activation relation in SAN [5]. While this hypothesis might be addressed through methods such as siRNA to suppress AC1 in SAN, we chose a more direct approach to explore the impact of the presence of AC1, making use of NRVM cultures, which have certain similarities to, and advantages over, SAN cells in this context. Like SAN cells, NRVM cultures consist of small, spontaneously active cells lacking T-tubules. Both preparations appear to have a high basal cAMP level relative to adult ventricle [17,13] and automaticity that is decreased by I_f inhibition [18–20]. However, NRVM cells do not express AC1, providing a convenient model for testing the effect of adding a Ca^{2+} -stimulated cyclase isoform. Further, such exogenous expression can readily be accomplished with high efficiency via adenovirus [14] within the typical 7 day period during which these cells are maintained in culture.

However, inference from studies in NRVM on the ionic basis of automaticity in SAN should be made with caution, since there are important differences between these two cell types. For example, native I_f is small in NRVM cells, which is why we co-expressed HCN2 in these studies. In addition, while both show some Ca^{2+} -dependence to automaticity, SAN cells appear strongly dependent on the Na/Ca exchanger [21] while NRVM appear largely affected by altering Ca^{2+} influx [22]. Further, the NRVM action potential in culture has a Na dependent upstroke with maximal velocities on the order of 100 V/s [23], which is markedly higher than that of adult SAN cells [24]. It is because of these differences that we largely focused on the Ca^{2+} -dependence of HCN current and its adrenergic responsiveness rather than rate.

Our key finding is that introducing AC1 into NRVM cells in culture results in the response of HCN2 to the adrenergic agonist isoproterenol becoming sensitive to disruption of intracellular Ca^{2+} homeostasis (figure 6). In conditions of normal intracellular Ca^{2+} , ISO causes a comparable shift of HCN2 activation in the absence of added cyclase, or when either AC1 or AC6 is expressed. However, after BAPTA-AM pre-treatment, an ISO response persists in AC6 expressing cells but not in AC1 expressing cells. Prior studies by us and others reported that in SAN cells disruption of Ca^{2+} homeostasis results in loss of I_f responsiveness to ISO [5] and that SAN expresses Ca^{2+} -stimulated cyclase isoforms AC1 and AC8 [4,10]. Our results, when considered along with these SAN studies, suggest that the presence of Ca^{2+} -stimulated cyclase isoforms in the SAN accounts for the Ca^{2+} -dependence of I_f adrenergic responsiveness in that tissue. The presence of Ca^{2+} -dependent cyclase may also explain previous observations that I_f is modified by Ca^{2+} and calmodulin,

but not CamKII [3], because AC1 and AC8 are Ca²⁺/calmodulin but not CaMKII dependent [7].

Several additional observations are of note in the present study. First, AC1 but not AC6 over-expression results in increased basal cAMP levels and a corresponding positive shift in the basal position of the HCN2 activation relation (figures 2 and 3), which is reversed by lowering basal intracellular Ca²⁺ (suppl. fig. 3). While the shift in basal HCN2 activation is consistent with a greater fraction of channels being cAMP-bound, this did not prevent ISO from causing a further shift in activation (under normal intracellular Ca²⁺) that was comparable to that with AC6 overexpression (5 mV vs 7 mV shift for AC1 vs AC6).

Virally delivered AC1 and AC6 were well expressed in this study and at functionally equivalent levels (figure 2B). This suggests the different effects of these two enzymes on cardiac myocytes might arise from distinct regulation [8] and/or ability to interact with other proteins [25]. For example, AC6 activity might be self limiting due to inhibition by PKA phosphorylation or elevation of intracellular Ca²⁺ [26]. Both AC1 and AC5/6 are known to reside in lipid rafts [27,10], but they interact with different anchoring proteins (Yotiao vs. AKAP79/150). Therefore, they may coordinate formation of different protein complexes [25] and lead to activation of different signaling pathways [28]. While our co-IP results (figure 1) indicate that HCN2 and AC1 are co-localized, whether they are in specialized membrane compartments requires further investigation.

Another interesting observation of this study relates to experiments on spontaneous rate. Our earlier action potentials measurements suggest that HCN2 expression in NRVM results in depolarization of the cells, with MDP settling near current activation threshold [14]. That is, HCN current limits the extent of membrane polarization. The more positive position of HCN2 is expected to result in even more depolarized MDP, and AC1-expressing NRVM support this hypotheses. However, other AP parameters such as EDD, take off potential and overshoot remain unaffected by AC1. Under such conditions (the less polarized MDP; identical EDD and TOP) the time to get from MDP to take-off potential is shortened, and so rate becomes faster. It is not surprising that EDD does not change since, for both AC6 and AC1 cells MDP is comparably near HCN2 threshold and thus the fraction of available current during diastole is similar.

Interestingly, HCN2RE, the mutated HCN2 with low cAMP affinity, resulted in a comparable spontaneous rate as HCN2 in the absence of added cyclase expression. This occurred despite the HCN2RE activation relation being more negative than that of HCN2, suggesting HCN2RE was still able to contribute significant depolarizing current during early diastole. The most likely explanation is our previous observation that in NRVM HCN2RE exhibits faster activation kinetics than HCN2 [13], which would result in more current earlier in diastole. We previously demonstrated with computer modeling that speeding HCN activation kinetics could result in faster spontaneous rates [29]. However, the fact that with AC1 co-expression there is an additional and comparable rate increase in HCN2 and HCN2RE cultures (figure 4B) despite the fact that HCN2RE voltage dependence is not affected by AC1 (figure 4A), suggests that AC1 may be increasing rate at least in part independently of any effect on the V₅₀ of expressed pacemaker channels. It is unlikely this is occurring through endogenous HCN channels, since we do not observe any effect of AC1 expression on total pacemaker current in the HCN2RE cultures (figure 4A). Therefore, the effect of AC1 (but not AC6) to further increase rate in HCN2 or HCN2RE cultures likely reflects effects of the increased basal cAMP on other targets relevant to pacing. This could include L-type Ca²⁺ channels and other components of Ca²⁺ homeostasis. In preliminary studies, we find that AC1 expression increases peak L-type current density ~50%. Further, a

separate preliminary study in a small set of cells found that AC1 expression alone (without HCN2 co-expression) increases rate ~40%.

The role of Ca^{2+} -stimulated adenylyl cyclase in the SAN is not entirely clear. Younes et al, [10] proposed that AC1 and 8 are responsible for basal AC activity, while other isoforms (AC5/6) respond to agonist stimulation, a concept consistent with the above reasoning. However, their proposal was based on changes in total cAMP level in response to β -adrenergic stimulation of SAN cells under control and Ca^{2+} -buffering conditions, and total cAMP might not reflect levels in submembrane domains. In our experiment, while having a clear BAPTA effect on ISO-stimulated current in AC1 cultures, we failed to observe a significant effect of BAPTA inhibition on total cAMP production. It has been reported that adrenergic stimulation of NRVM cultures results in localized cAMP elevations in regions on the order of 1 micron [30]. Thus, cAMP concentrations in the environment of I_f or other transmembrane channels may differ markedly from bulk measurements. The ability of ryanodine to interfere with the effect of β -adrenergic stimulation of I_f in SAN suggests Ca^{2+} -stimulated adenylyl cyclases are among the effectors of β -adrenergic activation and contribute to alteration of at least sub-membrane intracellular cAMP content in response to sympathetic inputs [5].

In conclusion, Ca^{2+} -activated adenylyl cyclases have emerged as new players in pacemaker function of the heart. They provide an important link between activity-dependent changes in intracellular Ca^{2+} content and production of cAMP, and thus a potential mechanism in SAN for coupling “membrane” and “Ca clock” mechanisms of automaticity [31]. How the presence of these isoforms in SAN impacts cardiac rhythm, including basal rate, stability of rhythm and response to autonomic stimulation, remains to be elucidated. Transgene expression in NRVM cultures provides a novel approach for dissecting these complex and interdependent mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Ca-stimulated Adenylyl Cyclase 1 (AC1) or AC6 was expressed with HCN2 in cardiac myocytes.
- AC1, but not AC6 increased $[cAMP]_i$, accelerated beating rate and shifted HCN2 activation positive.
- In both AC1 and AC6 groups, isoproterenol induced a positive shift of HCN2 activation relation.
- BAPTA-AM prevented the isoproterenol effect in the AC1 but not AC6 group.
- AC1 plays a key role in sensitivity of β -adrenergic stimulation of HCN to $[Ca]_i$.

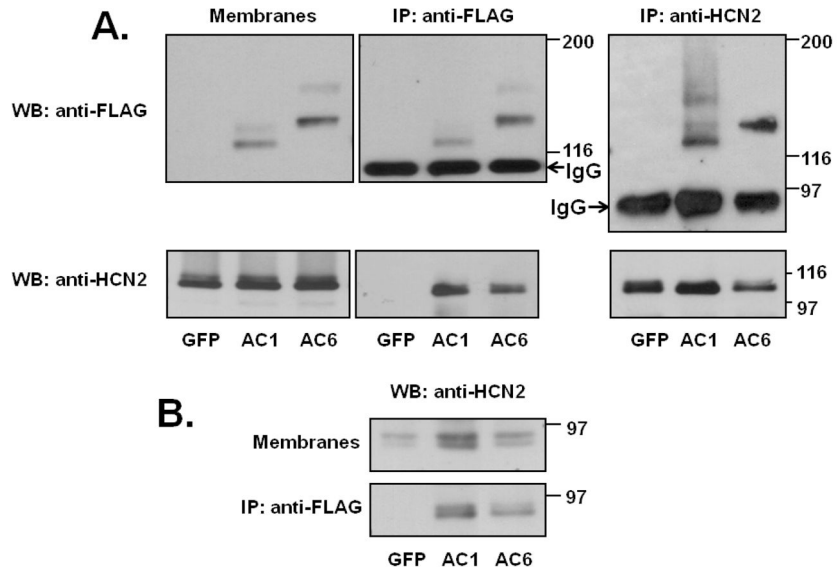


Figure 1.

A. Expression of recombinant adenylyl cyclase in NRVM. Cultures were co-infected with AdmHCN2 and one of the following: AdGFP, FLAG-tagged AdAC1 or FLAG-tagged AdAC6. 72 hours later cells were harvested, and the soluble membrane fraction was isolated. Soluble membranes from cultures infected with AdGFP, AdAC1 or AdAC6 were incubated with anti-FLAG or anti-HCN2 antibodies, separated by PAGE and probed with appropriate antibodies. **B.** Expression of endogenous HCN2 in NRVM and its co-immunoprecipitation with overexpressed adenylyl cyclases. The top panel shows detection of HCN2 in membrane fraction and the bottom panel – in anti-FLAG-immunoprecipitates.

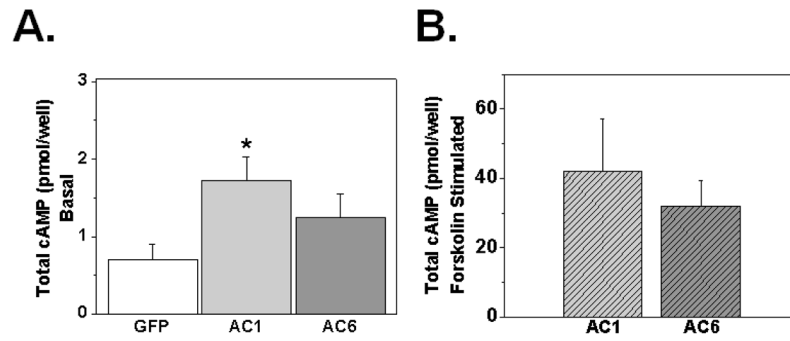


Figure 2.

Effect of AC1 and AC6 over-expression on basal and forskolin stimulated intracellular cAMP production (**A**). Cells were plated in 22-mm multiwell dishes, infected with AdGFP, AdAC1 or AdAC6 at moi 0.5 on day 1 and studied 72 hours later. n=6, *p<0.05 versus GFP by One Way ANOVA. **B**. Forskolin-stimulated (10 μ M) cAMP production was assessed by ELISA (n=4, p>0.05 by unpaired *t*-test).

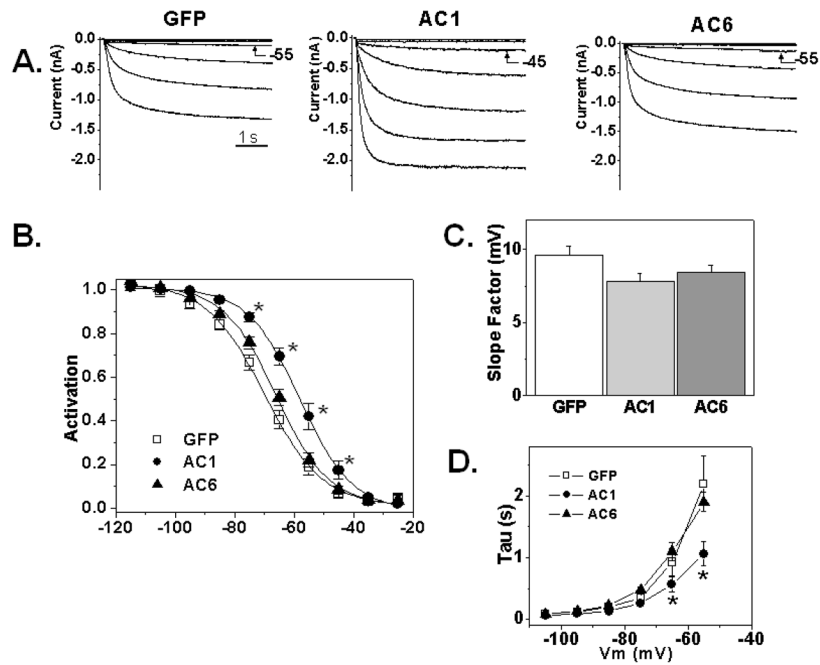


Figure 3.

Effect of over-expression of AC isoforms on HCN2 current. **A.** Original recordings of HCN2. The current was evoked by applying hyperpolarizing voltages from -25 to -85 mV for 5 seconds. **B.** Average fractional activation of measured HCN2 current. The solid lines are the fits to the Boltzmann function. AC1 activation relation differs from GFP and AC6 ($p < 0.001$) when tested by Two Way ANOVA with Bonferroni post test for pairwise comparison. (*- $p < 0.001$ vs GFP and AC6). **C.** Slope factor of activation in AdGFP, AdAC1 and AdAC6 groups. **D.** Kinetics of the HCN2 activation. AC1 curve differs significantly from GFP and AC6 by Two Way ANOVA ($p < 0.001$); the asterisks (*) indicate voltages that differ from GFP by pairwise multiple comparison (Bonferroni t-test; $p < 0.05$)

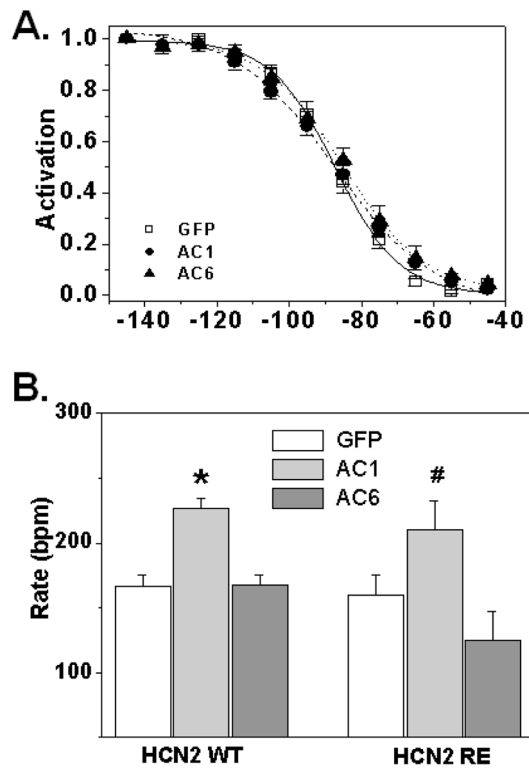


Figure 4. **A.** Effect of AC isoforms on HCN2RE fractional activation (n=6–8, $p > 0.05$ by Two Way ANOVA). **B.** Effect of AC1 or AC6 co-expression with mHCN2 or mHCN2RE on the spontaneous beating rate of 4-day old NRVM cultures. *,# $p < 0.05$ versus corresponding GFP-control (One Way ANOVA).

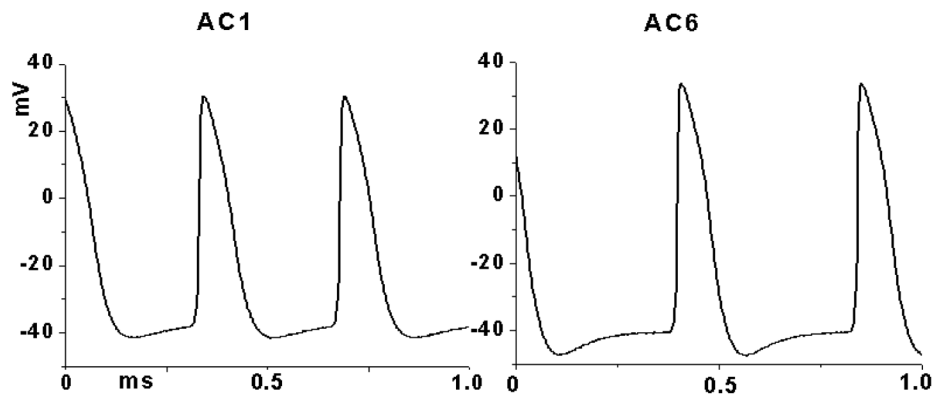


Figure 5. Sample traces of spontaneous action potentials recorded from NRVM monolayers co-expressing HCN2 WT with AC1 (left) or AC6 (right).

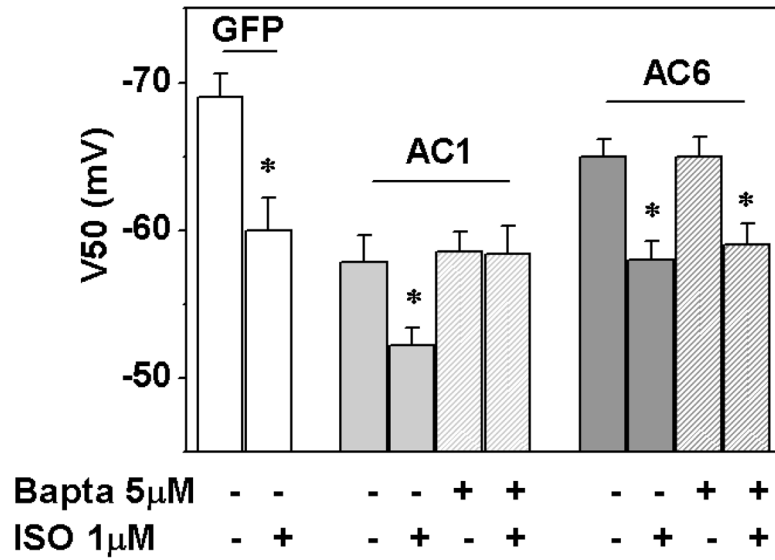


Figure 6.

Midpoint of activation (V_{50}) of mHCN2 and its responsiveness to β -adrenergic receptor agonist - isoproterenol (ISO, $1\mu\text{M}$). Cells were pre-treated with $5\mu\text{M}$ BAPTA-AM for 8 minutes; ISO perfusion was started 1 minute before recording. Two Way ANOVA was used for group comparison where ISO midpoint values were compared in relation to the presence/absence of BAPTA in AC1 or AC6 groups. * $p < 0.05$ versus corresponding no ISO; $n = 5-9$.

Table 1

Spontaneous action potential parameters from cultures co-expressing WT HCN2 with AC1 or AC6.

Parameters	HCN2+AC1	HCN2+AC6
Rate (Hz)	3.0±0.1 *	2.6±0.1
MDP (mV)	-44.4±1.8 *	-51.6±2.6
EDD (mV/ms)	0.042±0.005	0.043±0.01
TOP (mV)	-39.8±2.8	-45.1±2.6
Overshoot (mV)	30.4±1.2	35.4±2.3
n	7	5

* p<0.05 vs AC6 (defined with unpaired t-test)