HCN2 and HCN4 Isoforms Self-assemble and Co-assemble with Equal Preference to Form Functional Pacemaker Channels*

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Hyperpolarization-activated cyclic nucleotide-modulated (HCN) “pacemaker” channel subunits are integral membrane proteins that assemble as tetramers to form channels in cardiac conduction tissue and nerve cells. Previous studies have suggested that the HCN2 and HCN4 channel isoforms physically interact when overexpressed in mammalian cells, but whether they are able to co-assemble and form functional channels remains unclear. The extent to which co-assembly occurs over self-assembly and whether HCN2-HCN4 heteromeric channels are formed in native tissue are not known. In this study, we show co-assembly of HCN2 and HCN4 in live Chinese hamster ovary cells using bioluminescence resonance energy transfer (BRET), a novel approach for studying tetramerization of ion channel subunits. Together with results from electrophysiological and imaging approaches, the BRET data show that HCN2 and HCN4 subunits self-assemble and co-assemble with equal preference. We also demonstrate colocalization of HCN2 and HCN4 and a positive correlation of their intensities in the embryonic mouse heart using immunohistochemistry, as well as physical interactions between these isoforms in the rat thalamus by co-immunoprecipitation. Together, these data support the formation of HCN2-HCN4 heteromeric channels in native tissue.

Hyperpolarization-activated cyclic nucleotide-modulated (HCN)² channels, which underlie hyperpolarization-activated or funny currents (I_{h} or I_{f}) in excitable cells, are thought to be made up of subunits that assemble as tetramers to form functional channels (1). Four mammalian HCN isoforms (HCN1 to -4) (2–6) possess various overlapping patterns of expression in the heart and throughout the central nervous system, suggesting that they form heteromeric channels in these tissues (1, 7, 8). Previous studies suggest that the following combinations of HCN isoforms co-assemble and form functional channels in heterologous expression systems: HCN1 with HCN2 (9–12) and HCN1 with HCN4 (11). On the other hand, whether HCN2 and HCN4 isoforms co-assemble and form functional channels has not been shown and is an important objective of the present experiments.

The best evidence for co-assembly of HCN2 and HCN4 in native tissue comes from studies in the embryonic heart and adult thalamus. In the embryonic mouse heart, mRNA for HCN2 and both mRNA and protein for HCN4 have been found (13–16). Knock-out of HCN4 reduces, but does not abolish, I_{f} and speeds up rates of I_{f} activation in cardiomyocytes, consistent with the presence of other HCN isoforms in these cells (16). Immunohistochemical approaches in rats and mice have demonstrated HCN2 and HCN4 protein in thalamocortical relay nuclei (17, 18) and colocalization in cells of the ventrobasal complex and reticular nucleus of the thalamus (19). Knock-out of HCN2 reduces, I_{f} in thalamocortical neurons, consistent with the presence of other HCN isoforms in these cells (20).

Co-assembly of HCN2 and HCN4 is supported by evidence of interaction of the two isoforms in heterologous expression systems. When co-expressed in human embryonic kidney cells, HCN2 and HCN4 were found to colocalize and co-immunoprecipitate, and cell surface fluorescence of an HCN2 trafficking mutant channel was rescued when co-expressed with HCN4 (21). Reductions in HCN4 current density by co-expression with a nonfunctional HCN2 pore mutant in Chinese hamster ovary (CHO) cells (22) also suggests that HCN2 and HCN4 interact in a physical and/or functional way. Although these data could be explained by co-assembly of HCN2 and HCN4, they can also be readily explained by other interactions. For example, functional and physical associations between ion channel subunits have been reported between voltage-gated potassium channels that are distantly related in primary structure and do not co-assemble. One example is between the human-ether-a-go-go-related gene and KCNQ1, which exist as separate groups of homomeric channels within macromolecular complexes both in native tissue and when overexpressed in mammalian cells (23). Together, the data support interactions between HCN2 and HCN4 but do not demonstrate the formation of functional heteromeric channels.

Evidence in favor of functional co-assembly of HCN2 and HCN4 in live cells comes from single I_{f} channel recordings in...
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co-transfected CHO cells. These single channels possessed intermediate activation kinetics, in theory reflecting contributions from both subunits (24). However, whether the measured channels in that study truly represent HCN channels remains controversial (25–28).

Among the HCN isoforms that do co-assemble, whether they have a preference for self-assembly (homomerization) over co-assembly (heteromerization) is not known. Because the primary amino acid sequences of the four mammalian HCN isoforms are very similar, it is possible that their abilities to self-assemble or to co-assemble may also be similar. After exclusion of the nonconserved regions of the cytoplasmic N and C termini, the amino acid homology among the four mammalian isoforms is very high (≥94% conserved), and HCN2 and HCN4 isoforms bear the strongest conservation of primary sequence among the four isoforms (≥96% conserved) (29). Nevertheless, the differences between HCN2 and HCN4, both those within the conserved region and those within the cytoplasmic N and C termini, could modify the extent to which these isoforms co-assemble under different conditions.

In this study, we demonstrate that co-assembly of HCN2 and HCN4 in live CHO cells occurs with equal preference compared with self-assembly, using multiple approaches, including bioluminescence resonance energy transfer (BRET²), a novel approach to study the association of ion channel subunits. We also provide evidence in support of co-assembly of HCN2 and HCN4 in the rat thalamus and embryonic mouse heart.

EXPERIMENTAL PROCEDURES

Molecular Biology—The construction of an HCN2 C-terminal deletion (lacking the cyclic nucleotide binding domain and the remainder of the C terminus distal to it, HCN2ΔCNBD) and another mutant lacking the entire N terminus was described previously (12, 30). An extracellular HA epitope was inserted between the S3 and S4 transmembrane domain of HCN2ΔCNBD (Fig. 4A). This construct was made by digesting HCN2-HA (kind gift from M. Sanguinetti) at common HCN2 restriction sites, such that the HA tag was removed and placed in HCN2ΔCNBD. For BRET² constructs, Renilla luciferase (Rluc) or green fluorescent protein (GFP) tags were added to the N-terminal end of HCN2, HCN2ΔN, HCN4, or Kv1.5 cDNA. BRET² vectors (pRlucC and pGFPC; PerkinElmer Life Sciences) were digested at restriction sites complementary to those present on 5′ and 3′ ends of HCN2, HCN2ΔN, HCN4, or Kv1.5, such that the tags were expressed in frame with the channel cDNA when ligated. Resulting sequences were confirmed by automated DNA sequencing (DNA sequencing core facility, Vancouver, Canada). All tagged constructs, except those made with HCN2ΔN, which does not form functional channels, were tested by patch clamp electrophysiology and produced currents characteristic of their wild type counterparts (data not shown).

Cell Culture and Transfection—CHO-K1 cells (American Type Culture Collection, Manassas, VA) were maintained in Ham’s F-12 medium (Invitrogen) supplemented with 50 µg/ml penicillin/streptomycin (Invitrogen) and 10% fetal bovine serum (Invitrogen) and incubated at 37 °C with 5% CO₂. For electrophysiology and immunocytochemistry, cells were plated onto glass coverslips in 35-mm dishes. After 48 h, CHO cells were transiently transfected with mammalian expression vectors encoding wild type and/or mutant channels (2 µg/dish) using FuGene6 transfection reagent (Roche Applied Science). For electrophysiology, cells were also co-transfected with the GFP reporter plasmid for identification using fluorescent microscopy (0.5–0.7 µg/dish).

Whole Cell Patch Clamp Electrophysiology and Analysis—One to 2 days following transfection, a shard of coverslip plated with cells was transferred to a recording chamber (~200-µl volume) and continually perfused (0.5–1.0 ml/min) with a low K⁺ extracellular solution (5.4 mM KCl, 135 mM NaCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH to 7.4 with NaOH). Following rupture of the patch membrane, the solution was changed to a high K⁺ recording solution (135 mM KCl, 5.4 mM NaCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH to 7.4 with KOH) to maximize current amplitude. The patch pipettes were filled with a solution containing 130 mM potassium aspartate, 10 mM NaCl, 0.5 mM MgCl₂, 5 mM HEPES, and 1 mM EGTA and adjusted to a pH of 7.4 with KOH. In some experiments, this solution was supplemented with 2 mM camp (as noted). Whole cell hyperpolarization-activated currents (Iₜ) were measured using borosilicate glass electrodes (Sutter Instrument Co.), which had a resistance of 2.0–4.0 megaohms when filled with the intracellular solution. Currents were recorded using an Axopatch 200B amplifier and Clampex software (Axon Instruments Inc.). Data were filtered at 2 kHz and were analyzed using Clampfit (Molecular Devices) and Origin (Microcal) software. All experiments were conducted at room temperature (20–22 °C). Currents were not leak-subtracted. The voltage dependence of Iₜ activation was determined from tail currents at ~65 mV following 2-s test pulses ranging from −50 to −150 mV in 20-mV steps. Normalized tail current amplitudes were plotted as a function of test potential, and values were fit with a Boltzmann function as follows,

\[
 f(V) = \frac{I_{\text{max}}}{1 + e^{(V - V_{1/2})/k}}
\]  
(Eq. 1)

to determine the midpoint of activation (V₁/₂) and slope factor (k). Each test pulse was followed by a 200–500-ms pulse to +5 mV to ensure complete channel deactivation, and the resting current was allowed to return to its base-line value before subsequent voltage pulses. Time constants to assess rates of Iₜ activation were generated using a single exponential fitting procedure. An initial delay during Iₜ activation was not well described by a single exponential function and therefore was not used in our fits (17, 31).

Immunocytochemistry, Fluorescence Microscopy with Structured Illumination, and Determination of Pearson Correlation Coefficients—Forty-eight hours after transfection, cells on coverslips were washed briefly with PBS and fixed with 2% paraformaldehyde in PBS for 5 min. Fixed cells were washed twice with PBS, some were permeabilized for 10 min using 0.2% Triton X-100, and all were blocked with 10% normal goat serum (NGS) and 10% bovine serum albumin for 10 min. After one wash with PBS containing 1% NGS, cells were incubated with primary antibodies for 1 h at room temperature. Anti-HA (Sigma) or anti-Rluc (Chemicon International Inc.) mouse monoclonal antibodies were used as needed at a dilution of
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1.5% in 1% NGS, PBS. Cells were subsequently washed with PBS three times and incubated with Alexa-555-tagged goat anti-mouse secondary antibodies (Molecular Probes, Inc., Ontario, Canada) at a dilution of 1:1500 in PBS with 1% NGS plus bovine serum albumin for 1 h at room temperature in the dark. After washing three times in PBS, coverslips were rinsed in double-distilled H2O and mounted on slides using Gel Mount (Sigma). Cells were visualized using a Zeiss Axiovert 200 fluorescence microscopy with an Apotome structured illumination module and with a ×63 oil immersion objective lens. Results reported represent a minimum of four transfections for each set of the imaging experiments described. To correlate intensities of fluorescence for each pair of proteins, Pearson correlation coefficients were calculated from individual cells co-transfected with different combinations of Kv1.5 and/or HCN isoforms, tagged with GFP or Rluc. Calculation of the Pearson correlation coefficient from captured images of individual cells was determined by the following equation (Axiovision User Guide),

$$\Sigma((GVC1 - MVC1) \times (GVC2 - MVC2)) \over \sqrt{\Sigma(GVC1 - MVC1)^2 \times \Sigma(GVC2 - MVC2)^2}$$  \hspace{1cm} (Eq. 2)

where GV represents Gray value, MV is mean value, and C is channel.

The values range from -1 to +1, representing an increasing correlation of the intensities measured in two channels. In other words, the Pearson correlation coefficient describes the interdependence of varying intensities of fluorescence between two proteins of interest throughout a cell.

Bioluminescence Resonance Energy Transfer (BRET²) Analysis—BRET² experimental methods were based on a recent study examining the dimerization of G-protein-coupled receptors (32). CHO cells were transiently transfected with a constant amount (0.5 μg) of Rluc-tagged and varying amounts of GFP-tagged constructs (0.5–2.0 μg) in order to measure optimal expression ratios for BRET² experiments. Forty-eight hours after transfection, cells were washed twice with Dulbecco’s PBS (Invitrogen), detached with 0.05% trypsin-EDTA —Mouse day 18 embryos were obtained from CD1 mice. Isolated tissue was cryosectioned into 10-μm sections, mounted on poly-L-lysine-treated slides (Wax-it histology services; UBC), and stored at -80°C. Sections were fixed in 4% paraformaldehyde (PerkinElmer Life Sciences). Using a Victor 3V plate reader (PerkinElmer Life Sciences), expression of GFP-tagged constructs was assessed by directly exciting GFP with a 400–410-nm excitation filter. Expression of Rluc-tagged constructs was assessed using luminescence values obtained in the BRET² assay. -Fold level over background of emission was determined for both Rluc- and GFP-tagged constructs by comparing luminescence and fluorescence values with background values in untransfected cells. Although we varied the amount of cDNA of the GFP-tagged constructs, the resulting expression levels did not vary greatly, and therefore all samples were used in subsequent BRET² experiments. For BRET² measurements, DeepBlueC substrate (PerkinElmer Life Sciences) was added to cells (final concentration of 5 μM), and Rluc emission was measured through a 370–450-nm filter. Resulting GFP emission was in turn measured through a 500–530-nm filter. BRET² ratios

were calculated by the ratio of GFP emission to Rluc emission. Ratios were corrected for background by subtracting emission ratios from untransfected cells after the addition of DeepBlueC substrate. BRET² values are expressed as the mean ± S.D. for three or six independent experiments (as noted), performed in duplicate, using different Rluc-tagged/GFP-tagged transfection ratios in each experiment. Graphing and statistical analysis were done using Prism 4 software (GraphPad Software).

Immunoprecipitation and Western Blotting of Rat Thalamus—Rat thalami were obtained as previously described (33). Tissue was processed in radioimmune precipitation lysis buffer (50 mM Tris at pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 2 mM each Na2VO4 and NaF, and 10 μg/ml each aprotinin, pepstatin, and leupeptin) by multiple passes through 16-gauge and then 23-gauge syringes and incubated on ice for 30 min, followed by centrifugation to remove cell debris. Lysates were precleared with normal rabbit IgG and protein-A beads (Sigma) for 2 h at 4°C and then incubated with primary antibodies (rabbit anti-HCN2 or rabbit anti-HCN4; Alomone Laboratories) and protein A beads overnight at 4°C. For negative controls, primary antibodies were preincubated with supplied antigens for 1 h prior to incubation with precleared lysates. Beads were collected by centrifugation and washed three times in ice-cold PBS and then boiled in sample buffer with 2-mercaptoethanol for 10 min and loaded into 8% SDS-polyacrylamide gels. Gels were transferred to polyvinylidene difluoride membranes, and blots were washed twice in TBS-T and then blocked with 5% nonfat dry milk in TBS-T for 1 h. Blots were incubated with primary antibody overnight at 4°C (guinea pig anti-HCN4 (1:500) or rabbit anti-HCN2 (1:400)) (18) in 5% nonfat milk with 3% bovine serum albumin. After three washes with TBS-T, blots were incubated with horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:3000 in 5% nonfat milk for 1 h at room temperature. After three washes with TBS-T, signals were obtained with ECL detection reagents (GE Healthcare). All IP experiments were carried out three times (n = 3 rats).

Immunohistochemistry of Mouse Tissue Sections—Mouse day 18 embryos were obtained from CD1 mice. Isolated tissue was cryosectioned into 10-μm sections, mounted on poly-l-lysine-treated slides (Wax-it histology services; UBC), and stored at -80°C. Sections were fixed in 4% paraformaldehyde for 10 min and then washed twice with PBS. Sections were then permeabilized with 0.2% Triton X-100 for 15 min, washed three times in PBS, and then blocked with 10% normal donkey serum in PBS for 1 h at room temperature. Sections were then incubated with primary antibody overnight at 4°C. Primary antibody dilutions were as follows: guinea pig anti-HCN2 and anti-HCN4 (18), 1:500; goat anti-HCN4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:50; and rabbit anti-HCN2 (Alomone Laboratories), 1:200. For negative controls, tissue sections were incubated with either goat or guinea pig IgG or with rabbit anti-HCN2 preincubated with its supplied antigen (3:1 antigen/antibody ratio). Tissue sections were washed three times in PBS, followed by incubation with Alexa 488 or 555-tagged secondary antibodies raised in donkeys (1:1000; Molecular Probes). After three washes in PBS, 4’6-diamidino-2-phenylindole (1:50,000; Molecular Probes) was added to tissue for 5
that exhibit different stoichiometries, but they can report on the sum of all channels possessing both subunits.

We utilized the voltage-gated channel subunit Kv1.5 as a negative control. Kv1.5 is found in the same superfamily as the HCN channel family and has a similar overall structure but is not expected to co-assemble with HCN subunits. However, when co-expressed in CHO cells, Kv1.5 is highly colocalized with both HCN2 and HCN4, as determined by measuring colocalization coefficients in the present imaging studies (see below). Thus, Kv1.5 provided a critical negative control for potential interactions among subunits that did not involve co-assembly. These types of interactions include both specific interactions (e.g. between adjacent but separate tetrameric channels) and nonspecific interactions possibly due to overexpression of protein in intracellular compartments. Co-transfection of differentially tagged HCN2 and HCN4 with themselves provided positive controls for co-assembly.

**Homomeric and Heteromeric Combinations of HCN2 and HCN4 Produce Equally High Pearson Correlation Coefficients in CHO Cells**—We first acquired immunofluorescent images of CHO cells co-expressing constructs tagged with GFP or Rluc (Fig. 2A) and determined the extents to which the intensities of their fluorescence correlated. To quantify this, we utilized a Pearson correlation coefficient (see “Experimental Procedures”). For two subunits that co-assemble to form a tetrameric ion channel, their varying levels of expression throughout a cell would be expected to be interdependent. Therefore, the variation of their intensities of fluorescence would correlate to a greater extent than for two subunits that associate in another way (e.g. two subunits that form separate channels which are localized to a similar region) or that do not associate at all.

We co-transfected CHO cells with HCN2-GFP or HCN4-GFP with HCN2-Rluc, HCN4-Rluc, or Kv1.5-Rluc and correlated the intensities of fluorescence for each combination of two isoforms. A strength of this approach is that the same molecules (GFP and Rluc) were used for all correlations; thus, there is no variability in our measurements due to the use of different antibodies. We hypothesized that HCN2 or HCN4, when co-transfected with themselves or with each other, would yield Pearson correlation coefficients that were significantly higher than those produced by co-transfection of either HCN2 or HCN4 with Kv1.5, if they formed homomeric or heteromeric channels.

We found that Pearson correlation coefficients were significantly larger in cells co-transfected with HCN2-GFP and HCN2-Rluc, HCN2-GFP and HCN4-Rluc, HCN4-GFP and HCN4-Rluc, or HCN4-GFP and HCN2-Rluc than those determined from cells that were co-transfected with either HCN2-GFP or HCN4-GFP and Kv1.5-Rluc (Fig. 2, B and C). Since HCN isoforms do not co-assemble with Kv1.5, the larger Pearson correlation coefficients determined for the different combinations of HCN subunits suggest co-assembly among them. This conclusion is strengthened by our finding that coefficients determined from cells co-transfected with HCN2-GFP and HCN2-Rluc or with HCN4-GFP and HCN4-Rluc (which are expected to self-assemble) were not significantly different from those determined from cells co-transfected with HCN2-GFP and HCN4-Rluc, respectively.

**RESULTS**

**Tetrameric Assembly of HCN2 and HCN4 Isoforms**—Because of their similarity to voltage-gated potassium channels, HCN channels are probably tetrameric, and their co-assembly can be described by a binomial distribution. This distribution is determined from the proportion of subunits present to the $n^{th}$ power ($p^n$), where $n$ is equal to the number of different subunit isoforms in a given channel (34). When co-expressed, two isoforms with equal preference for homomeric and heteromeric assembly will co-assemble according to a binomial distribution, whereas any preference toward homomeric or heteromeric assembly would alter this distribution pattern.

Here, we sought to determine whether HCN2 and HCN4 subunits co-assemble to form heteromeric channels and whether they exhibit a preference for homomeric versus heteromeric assembly. This was accomplished using two approaches in which HCN2 and HCN4 were tagged to Rluc or GFP epitopes. Co-transfection allowed us to obtain measurements from channels made up of a mixture of subunits possessing each tag (Fig. 1, bracketed section). Thus, both self-assembly and co-assembly could be determined independently and compared. These assays cannot measure channels made up of subunits containing the same tag or differentiate among channels
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A. GFP Rluc Merge

HCN2-GFP+Kv1.5-Rluc

HCN4-GFP+Kv1.5-Rluc

HCN2-GFP+HCN2-Rluc

HCN4-GFP+HCN4-Rluc

HCN2-GFP+HCN4-Rluc

HCN4-GFP+HCN2-Rluc

B. 

C. 

FIGURE 2. Correlation of fluorescent intensities in co-transfected CHO cells supports similar levels of self-assembly and co-assembly of HCN2 and HCN4. A, images of CHO cells co-transfected with combinations of HCN2, HCN4, and Kv1.5 constructs. Rluc-tagged constructs are shown in red, and GFP-tagged constructs are shown in green, with areas of colocalization depicted in yellow. Scale bars, 10 μm. B, colocalization scatterplots of representative images in A of cells co-transfected with HCN2-GFP and Kv1.5-Rluc, HCN2-Rluc, or HCN4-Rluc. Intensity of GFP fluorescence is represented on the x axis, and intensity of Alexa555 (or Rluc) fluorescence is shown on the y axis. The frequency of pixel overlap at a given relative fluorescence intensity is plotted with greater frequency of overlap in red (toward the origin of axes) and lower in blue. Pearson correlation coefficients determined from the values of intensities in each graph are shown. C, bar graph of Pearson correlation coefficients determined from cells co-transfected with different construct combinations. Values represent means ± S.D. for each set of constructs, and n values depict number of cells used to determine mean Pearson correlation coefficients over a minimum of four independent transfections. Statistical comparisons were carried out using a one-way analysis of variance followed by Bonferroni’s multiple comparison post-test comparing all pairs. The asterisks indicate significant differences from the correlation coefficients produced by co-transfection of Kv1.5-Rluc and HCN2-GFP or HCN4-GFP. All combinations of HCN construct co-expression show Pearson correlation coefficients that are not significantly different from each other (p > 0.05) but are significantly greater than those determined from combinations of Kv1.5Rluc with the HCN constructs (p < 0.001).

tively. These similarities also suggest that HCN2 and HCN4 isoforms form homomeric channels and heteromeric channels with equal preference.

It should be noted that Pearson correlation coefficients give more information about the nature of interaction between two proteins than simply quantifying the extent of colocalization. For example, the coefficients were significantly lower for combinations of Kv1.5 and HCN isoforms, but the colocalization coefficient (which measures pixel overlap between two channels) was high (r > 0.85) for all combinations, including those with Kv1.5 and HCN isoforms. This suggests that although Kv1.5 does not co-assemble with HCN2 or HCN4 isoforms, they do colocalize to similar regions within the cell. This is not unexpected, because separate homomeric channels may share similar pathways of biogenesis and trafficking, and thus they may be localized to similar areas of cells, especially when overexpressed.

Homomeric and Heteromeric Combinations of HCN2 and HCN4 Produce Equally High BRET2 Ratios in CHO Cells—In order to study co-assembly of HCN2 and HCN4 channels in live cells, we utilized BRET2 technology. This approach, which has been widely used to examine and demonstrate receptor-protein interactions and receptor dimerization (35), offers several advantages for the analysis of protein interactions. BRET2 measures two proteins that are located within 10 nm of each other. Like its methodological cousin, fluorescence energy transfer, BRET2 allows for measurements in live cells and avoids a number of issues associated with more invasive approaches. Unlike fluorescence energy transfer, BRET2 does not require an initial light source and thus avoids photobleaching. An added and powerful advantage is that BRET2 measurements are taken from a large population of live cells rather than from individual cells (36). Thus, BRET2 seems well suited for the determination of tetrameric ion channel assembly.

Once both Rluc- and GFP-tagged constructs are expressed in CHO cells, the addition of DeepBlueC substrate to live, intact cells is oxidized by Rluc, causing emission at 380 nm. Emission at this wavelength excites GFP if the two proteins are within 10 nm of each other (Fig. 3, A and B). To test the ability of our system to detect BRET2, we carried out experiments using CHO cells co-transfected with β1-adrenergic receptors tagged with GFP or Rluc. We were able to reproduce previously published results showing high BRET2 ratios with β1-adrenergic receptors (data not shown), which supports the formation of homodimers (32). We hypothesized that HCN2 or HCN4, when co-transfected with themselves or with each other, would yield BRET2 values that were significantly higher than those produced by co-transfection of either HCN2 or HCN4 with Kv1.5.

We compared BRET2 ratios from cells co-transfected with the same combinations of tagged constructs used in the imaging experiments (above). In addition, we used CHO cells transfected with Kv1.5-Rluc to determine background levels of emission at 510 nm. We found that BRET2 ratios determined from cells co-transfected with HCN2-GFP and HCN2-Rluc, with
HCN2-GFP and HCN4-Rluc, with HCN4-GFP and HCN2-Rluc, or with HCN4-GFP and HCN2-Rluc were significantly larger than those obtained from cells co-transfected with either HCN2-GFP or HCN4-GFP and with Kv1.5-Rluc (Fig. 3, C and D). Furthermore, we found that the BRET2 ratios determined from cells co-transfected with HCN2-GFP or HCN4-GFP and Kv1.5-Rluc were not significantly different from those determined from cells transfected with only Kv1.5-Rluc, indicating that our negative control produced nearly background levels of BRET2. These data strongly suggest that HCN2 and HCN4 co-assemble to form functional channels in live CHO cells. In addition, the levels of BRET2 determined from cells co-transfected with HCN2-GFP and HCN4-Rluc or with HCN4-GFP and HCN2-Rluc were not significantly different from those determined from cells transfected with only Kv1.5-Rluc, indicating that our negative control produced nearly background levels of BRET2. These data strongly suggest that HCN2 and HCN4 co-assemble to form functional channels in live CHO cells. In addition, the levels of BRET2 determined from cells co-transfected with HCN2-GFP and HCN4-Rluc or with HCN4-GFP and HCN2-Rluc were not significantly different from those determined from cells transfected with only Kv1.5-Rluc, indicating that our negative control produced nearly background levels of BRET2. These data strongly suggest that HCN2 and HCN4 co-assemble to form functional channels in live CHO cells. In addition, the levels of BRET2 determined from cells co-transfected with HCN2-GFP and HCN4-Rluc or with HCN4-GFP and HCN2-Rluc were not significantly different from those determined from cells transfected with only Kv1.5-Rluc, indicating that our negative control produced nearly background levels of BRET2. These data strongly suggest that HCN2 and HCN4 co-assemble to form functional channels in live CHO cells.

Importantantly, BRET2 values were similar between cells co-transfected with HCN4-GFP and HCN4-Rluc and with HCN2-GFP and HCN2-Rluc (between cells containing homomeric HCN2 channels or HCN4 channels), suggesting that the distances between GFP and Rluc tags in both homomeric channels were similar. Levels of emission among GFP-tagged constructs and among Rluc-tagged constructs were similar, also suggesting that the overall levels of expression among constructs were similar (for both BRET2 and imaging experiments).

As an additional negative control, we co-transfected CHO cells with wild type HCN2 or HCN4 and an N-terminally truncated HCN2 channel (HCN2ΔN). We have shown previously that the N terminus is required for assembly and expression of functional HCN2 channels (12, 30). The BRET2 ratios for cells co-transfected with HCN2ΔN-Rluc and HCN2-GFP or HCN2ΔN-Rluc and HCN4-GFP were significantly lower than wild type but not significantly different from each other or from the Kv1.5-Rluc negative control (Fig. 3E). These data support the co-assembly of HCN2 and HCN4 isoforms.
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**Functional Evidence for Co-assembly of HCN2 and HCN4 in CHO Cells**—To obtain direct evidence for functional co-assembly of HCN2 and HCN4 channels in CHO cells, we used immunofluorescent imaging and the whole cell patch clamp approach to examine cell surface expression and functional rescue, respectively, of a mutant HCN2 by wild type HCN4. This mutant HCN2 channel lacks the cyclic nucleotide binding domain (CNBD) and the distal C terminus (HCN2ΔCNBD), and, when expressed alone, it does not produce functional channels in CHO cells (12) or human embryonic kidney cells (37). This has been attributed to increased intracellular retention (12). We hypothesized that HCN4 would rescue mutant channel function and that cell surface expression of mutant HCN2 protein would be increased in cells transfected with both constructs.

In order to determine whether HCN2ΔCNBD protein could be rescued to the cell surface by HCN4, we utilized wild type and mutant HCN2 constructs with an HA epitope on the extracellular S3-S4 linker. We examined the fluorescence intensity on the surface of nonpermeabilized CHO cells transfected with HCN2-HA or HCN2ΔCNBD-HA alone, or nonpermeabilized cells co-transfected with HCN2ΔCNBD-HA and HCN4-GFP. At an exposure time of 50 ms, HCN2ΔCNBD-HA fluorescence was only visible at the cell surface in cells that also expressed HCN4-GFP (Fig. 4A). At this exposure time, intracellular fluorescence was observed at similar intensities in permeabilized cells transfected with HCN2-HA or HCN2ΔCNBD-HA or permeabilized cells co-transfected with HCN2ΔCNBD-HA and HCN4 (12), This strongly suggests that the mutant channel was rescued to the cell surface when co-expressed with HCN4.

To support functional heteromerization between these isoforms, we next compared $I_r$ in CHO cells transfected with HCN2, HCN4, or HCN2ΔCNBD and HCN4. Because wild type HCN2 homomeric channels display faster activation kinetics and open at more positive potentials than homomeric HCN4 channels (Fig. 4D), we hypothesized that in cells transfected with HCN4, co-transfection with HCN2ΔCNBD would confer faster rates of $I_r$ activation and shift channel activation to more positive potentials. The rates of $I_r$ activation were significantly and considerably faster in cells co-transfected with both HCN4 and HCN2ΔCNBD compared with $I_r$ in cells transfected with wild type HCN4 alone (Fig. 4, B and C) and were nearly as fast as for the wild type HCN2 channel. This could be explained in at least three ways. First, it could mean that the mutant HCN2 subunit dominated the rate at which the mixed channels open. This is consistent with previous findings from HCN1 and HCN2 co-expression, where the faster HCN1 isoform largely dictated heteromeric channel activation kinetics (10). Second, it could be explained by the presence of a large proportion of channels containing predominantly the mutant HCN2 subunit. This seems less likely, given the fact that $I_r$ density increased dramatically (see below), suggesting that the channels also contained a large proportion of HCN4 subunits. Third, this could reflect an intrinsic ability of the mutant subunit to activate more quickly than even the wild type HCN2. Although HCN2ΔCNBD does not produce functional channels in mammalian cells, it does form functional channels in Xenopus oocytes, which activate more quickly than wild type HCN2.

![FIGURE 4. HCN4 rescues an HCN2 trafficking mutant to the cell surface and forms functional heteromeric channels.](image)

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*FIGURE 4. HCN4 rescues an HCN2 trafficking mutant to the cell surface and forms functional heteromeric channels.*

**A.** Images of nonpermeabilized CHO cells transfected with HCN2ΔCNBD, containing an HA tag (above) or co-transfected with HCN4-GFP (below). Note increased fluorescence on the surface of the cell transfected with both proteins (as shown by arrows). This increase in cell surface fluorescence indicates the rescue of the HCN2-trafficking mutant by HCN4 to the cell surface. Scale bars, 10 μm. Images are representative of typical cells visualized from at least five transfections. **Top right, schematic depicting the location of the HA epitope within the S3-S4 extracellular loop of HCN2ΔCNBD.** B, representative current traces, normalized to a maximum value of 1, recorded from cells expressing HCN4, HCN2ΔCNBD with HCN4, or HCN2 in response to a test voltage step to −150 mV from a holding potential of −35 mV. C, time constants of $I_r$ activation ($τ_{act}$) versus test voltage determined from current traces recorded from CHO cells expressing HCN4 (443.7 ± 38.7 ms), HCN2ΔCNBD with HCN4 (293.7 ± 24.2 ms), or HCN2 (244.0 ± 27.3 ms) (right). Asterisks, significant differences from HCN4 (one-tailed t test, *p < 0.05*). D, activation curves from cells expressing HCN4, HCN2ΔCNBD with HCN4, or HCN2. V, and k values were −119.6 ± 1.5 mV and 13.8 ± 2.1, −110.7 ± 2.0 mV and 13.9 ± 1.2, and −107.7 ± 2.7 mV and 13.4 ± 1.2, respectively (n = 7 cells). E, bar graph depicting whole cell current densities (normalized to cell capacitance) of HCN4 (259.8 ± 31.9 pA/picoFarads) or HCN2ΔCNBD with HCN4 (658.8 ± 118.9 pA/picoFarads). *, a significant difference between HCN2ΔCNBD co-transfected with HCN4 and HCN4 (one-tailed t test, p < 0.05). For each bar graph, the number of cells in each group is shown in brackets above each bar. Values represent means ± S.E.
channels (38). Thus, when HCN2ΔCNBD is co-transfected with HCN4 as in our experiments, the resulting channels might be expected to activate more quickly than otherwise, even in channels that contain equal numbers of both subunits.

We found that $I_a$ activation curves were significantly shifted to more positive potentials (Fig. 4D). These data support the co-assembly of HCN2ΔCNBD and HCN4 to form functional channels. Additionally, the slope of the $I_a$ activation curve was unchanged in all cases. This is consistent with a predominantly heteromeric population of channels, since two independent populations of homomeric channels with different $V_1/2$ values would be expected to produce a single less steep activation curve (9).

Cyclic AMP binding to the CNBD allows HCN2 and HCN4 channels to open more easily in response to hyperpolarization (39), and maximum facilitation of opening by cAMP requires all four subunits (40). Because the mutant HCN2 subunits lack the binding site for cAMP, heteromeric channels made up of HCN2ΔCNBD and HCN4 isoforms would be expected to respond less strongly to cAMP. To determine the ability of the channels to respond to cAMP, we compared $I_a$ activation curves in the presence or absence of cAMP (2 mM cAMP in the pipette solution) from cells transfected with HCN4 or co-transfected with HCN4 and HCN2ΔCNBD. As expected, with cAMP present, the $V_1/2$ of activation significantly shifted ($t$ test, $p < 0.02$) to more positive potentials in cells expressing only HCN4 by $\sim$10 mV (without cAMP, $V_1/2 = -118.6 \pm 1.8$ mV, $n = 26$ cells; with cAMP, $V_1/2 = -109.1 \pm 3.4$ mV, $n = 7$ cells). However, when HCN4 was co-expressed with HCN2ΔCNBD, there was no significant difference ($t$ test, $p > 0.05$) in $V_1/2$ with or without cAMP present (without cAMP, $V_1/2 = -109.8 \pm 3.1$ mV, $n = 12$ cells; with cAMP, $V_1/2 = -115.2 \pm 2.4$ mV, $n = 5$ cells). The lack of cAMP action is consistent with a predominantly heteromeric population of channels in cells expressing HCN4 and HCN2ΔCNBD. Also, the position of the $I_a$ activation curve in cells expressing HCN4, in the presence of cAMP, and in cells expressing HCN4 and HCN2ΔCNBD were similar (\sim109 mV). This similarity is expected because cAMP binding removes the inhibitory effect of the CNBD on the transmembrane regions, as would deletion of the CNBD, such that the channels open at more positive voltages (38).

Finally, we found that current density was significantly greater (more than doubled) in cells expressing both HCN4 and HCN2ΔCNBD compared with cells expressing either HCN2 or HCN4 alone (Fig. 4E). Again, this is consistent with the rescue of a significant amount of HCN2ΔCNBD subunits to the cell surface by co-assembly with HCN4 and the formation of heteromeric channels.

**Evidence Supporting Co-assembly of HCN2 and HCN4 in Native Tissue**—Colocalization of HCN2 and HCN4 has been suggested in both the mouse and rat thalamus (17–20). To determine whether HCN2 and HCN4 physically interact in the rat thalamus, we used immunoprecipitation techniques. We found that when HCN4 was immunoprecipitated from thalamic tissue protein lysate, HCN2 was also detected in complex with HCN4 (Fig. 5A). Similarly, we were also able to immunoprecipitate HCN2 from tissue lysate, resulting in co-immunoprecipitation of HCN4 (Fig. 5B).

There is evidence that HCN2 and HCN4 mRNA and HCN4 protein are found in myocytes of the mouse embryonic heart (13–16). However, the presence and cellular distribution of HCN2 protein and whether HCN2 and HCN4 colocalize are unknown. Therefore, we analyzed the expression patterns of HCN2 and HCN4 in hearts from embryonic (embryonic day 18) mice, using specific antibodies and immunohistochemistry. In tissue sections of the atrium and ventricle, we found some areas where HCN2 and HCN4 colocalized (Fig. 6). Other areas were also identified that showed expression of either HCN2 or HCN4 or that did not express either isoform. Next, we measured the Pearson correlation coefficient in regions of HCN2 and HCN4 co-expression. To do this, we measured four regions of co-expression from different fields of view. We found positive correlations of $\sim$0.5 in these regions of co-expression (Fig. 6C).
Our results demonstrate that HCN2 and HCN4 isoforms co-assemble to form functional heteromeric channels in live CHO cells. Furthermore, they suggest an equal preference for co-assembly versus self-assembly between HCN2 and HCN4. These experiments complement and extend upon existing studies that demonstrate interactions, but not functional co-assembly, between HCN2 and HCN4 isoforms when expressed in mammalian cells (21, 22).

Our results are the first to suggest co-assembly of ion channel subunits of the voltage-gated cation channel family based on BRET². This approach offers a number of advantages over previous studies, which includes the determination of interactions in live populations of cells without resorting to more invasive approaches. It is possible that the higher BRET² values produced by co-transfected combinations of HCN2 and HCN4 are due to homomeric channels that are located within 10 nm of each other. The similarity of BRET² values between cells expressing either HCN2 or HCN4 (positive controls) and cells expressing both HCN2 and HCN4 suggests that adjacent and intersubunit interactions contribute equally to the measured values. Nevertheless, affinity of HCN2 and HCN4 homomeric channels for each other and physical interactions between them could explain the unusual clustering and gating cooperativity of Iₙ channels seen in single channel studies of sinoatrial myocytes and human embryonic kidney cells expressing HCN2 (28, 39).

In this study, we found novel evidence in support of co-assembly of HCN2 and HCN4 in native tissue. We show that HCN2 and HCN4 co-immunoprecipitate from the rat thalamus and that HCN2 and HCN4 colocalize and their intensities correlate positively in isolated regions of the embryonic mouse heart. Despite our inability to demonstrate co-immunoprecipitation of HCN2 and HCN4 from embryonic heart tissue, our functional data demonstrating a predominantly heteromeric population channels in CHO cells transfected with HCN2 and HCN4 suggest that they co-assemble in cardiomyocytes expressing both isoforms.

In summary, our results demonstrate that HCN2 and HCN4 heteromerize and homomerize with equal preference when co-expressed in CHO cells. Our data also support the co-assembly of HCN2 and HCN4 in the rat thalamus and mouse embryonic heart. The propensity of HCN2 and HCN4 to co-assemble when co-expressed suggest that other mechanisms may be actively invoked, if need be, to prevent this from happening. The extent of co-assembly may depend upon cell- or species-specific factors, such as HCN interactions with other proteins, varying affinities of different isoforms for each other, the total and relative amounts of each isoform that are synthesized, and the location of sites of synthesis within the cell. Individual cells may regulate each of these factors in their own way to control the location of sites of synthesis within the cell. Individual cells may regulate each of these factors in their own way to control the localization and stoichiometry of homomeric and heteromeric channel formation. The ability to monitor self-assembly, co-assembly, and stoichiometry of HCN channels in native tissue remains an important future goal, but adaptation of the

![FIGURE 6. HCN2 and HCN4 colocalize within certain regions of the embryonic mouse heart.](image)

To determine whether HCN2 and HCN4 physically interact in the embryonic heart, we again used immunoprecipitation techniques. We found that these isoforms could not be co-immunoprecipitated from this tissue. This negative result may be due to the distribution of HCN2 and HCN4 in regions where they do not colocalize or to the overall lower amounts of HCN protein. However, our functional data demonstrate a predominantly heteromeric population of channels in CHO cells transfected with HCN2 and HCN4, which supports their co-assembly in cardiomyocytes containing both isoforms.
approaches described in this paper for native tissue may be a useful starting point.

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