Role of Subunit Heteromerization and N-Linked Glycosylation in the Formation of Functional Hyperpolarization-activated Cyclic Nucleotide-gated Channels*

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The coassembly of homologous subunits to heteromeric complexes serves as an important mechanism in generating ion channel diversity. Here, we have studied heteromerization in the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel family. Using a combination of immunoprecipitation, electrophysiology, and coexpression systems, we found that upon coexpression in HEK293 cells almost all dimeric combinations of HCN channel subunits give rise to the formation of stable channel complexes in the plasma membrane. We also identified HCN1/HCN2 heteromers in mouse brain indicating that heteromeric channels exist in vivo. Surprisingly, HCN2 and HCN3 did not coassemble to heteromeric channels. This finding indicates that heteromerization requires specific structural determinants that are not present in all HCN channel combinations. Using N-glycosidase F we show that native as well as recombinant HCN channels are glycosylated resulting in a 10–20-kDa shift in the molecular weight. Tunicamycin, an inhibitor of N-linked glycosylation, blocked surface membrane expression of HCN2. Similarly, a mutant HCN2 channel in which the putative N-glycosylation site in the loop between S5 and the pore helix was replaced by glutamine (HCN2N380Q) was not inserted into the plasma membrane and did not yield detectable whole-cell currents. These results indicate that N-linked glycosylation is required for cell surface trafficking of HCN channels. Cotransfection of HCN2N380Q with HCN4, but not with HCN3, rescued cell surface expression of HCN2N380Q. Immunoprecipitation revealed that this rescue was due to the formation of a HCN2N380Q/HCN4 heteromeric channel. Taken together, our results indicate that subunit heteromerization and glycosylation are important determinants of the formation of native HCN channels.

The hyperpolarization-activated cation current $I_h$ (or $I_{h}$) plays a key role in the control of important biological processes such as heart beat (1), sleep-wake cycle (2), transduction of sour taste (3), and synaptic plasticity (4). $I_h$ is encoded by the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel gene family (for review, see Refs. 5–7). In mammals, the HCN channel family comprises four homologous members (HCN1–4). Structurally, HCN channels belong to the superfamily of voltage-gated cation channels. Like other members of this family HCN channels are supposed to form tetramers with fundamental building blocks consisting of six hydrophobic segments (S1–S6), a positively charged S4 sensor, and an ion-conducting hairpin between S5 and S6. In the cytosolic carboxyl terminus each of the four HCN channel subunits carries a cyclic nucleotide-binding site mediating modulation by cAMP. Expression of HCN1–4 cDNAs in heterologous expression systems yields currents with the hallmark properties of HCN/h currents. Functional heterogeneity of HCN1–4 at least partly explains the diversity of native $I_h$ in different cell types, especially in neurons. Further complexity of $I_h$ may be generated in vivo by post-translational modification of channel subunits (e.g. glycosylation), by the presence of auxiliary subunits (8) and the formation of heteromeric HCN channels. The coassembly of subunits to functional heteromers is a mechanism that is found in a number of voltage-gated channel families, such as voltage-gated K+ channels (9) or cyclic nucleotide-gated channels (10). Coexpression studies in Xenopus oocytes and in HEK293 cells (11–13) and studies using dominant-negative channel constructs (14, 15) provided initial evidence that HCN channels may also exist as heteromers. So far, heteromerization of HCN channel subunits has been investigated only for a limited number of subunit combinations. Moreover, the present studies solely rely on the electrophysiological analysis of $I_h$ currents obtained after coexpression of HCN channel subunits. In par-

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ticular, there are no data showing HCN subunit interaction directly at the protein level. To address these important issues, we have studied the assembly of all dimeric combinations of HCN channel subunits using fluorescence confocal microscopy and communoprecipitation. We provide evidence that most subunits can coassemble to heteromers. As an exception from this behavior we find that the mHCN2 subunit does not form functional channels with HCN2. Finally, we identify N-glycosylation of HCN channel subunits as a crucial factor for controlling HCN channel trafficking.

EXPERIMENTAL PROCEDURES

Construction of HCN Channel Fusion Proteins and Mutants—For immunofluorescence studies full-length HCN channels were fused to the COOH termini to EGFP or DsRed (RFP) domains to yield murine HCN1-EGFP (mHCN1-EGFP), mHCN2-EGFP, mHCN2-RFP, mHCN2-DsRed-EGFP, mHCN3-EGFP, mHCN3-RFP, human HCN4-EGFP (hHCN4-EGFP), and hHCN4-RFP. In addition, the following HCN channels containing a carboxy-terminal myc-tag were constructed: mHCN1-myc, mHCN2-myc, mHCN3-myc, and hHCN4-myc. All chimeric channels were generated by deleting the respective STOP codons above). Beads were pelleted by centrifugation (15 min, 4 °C), washed three times with cold HNTG buffer, and proteins were visualized after boiling for 5 min in Laemmli sample buffer supplemented with 100 mM dithiothreitol by SDS-PAGE and subsequent fluorophorescence. The plasmid pECFP-Mem (Clontech) was transfected into HEK293 cells by calcium phosphate. Seventy-two hours after transfection cells were washed with PBS, harvested with a cell scraper, and centrifuged (10 min, 1,000 × g). Pellets were resuspended in ice cold MOPS lysis buffer (0.3 mM sucrose, 20 mM MOPS, 1 mM EDTA supplemented with protease inhibitors: 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml antipain, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM orthophenanthroline, 1 mM benzamidine, 1 mM iodoacetamide, and homogenated at 30 °C using a glass rod and froder plunger. Homogenates were centrifuged for 10 min at 5,000 × g. The pellet was rehomogenized and spun once more for 10 min. The combined supernatants were then centrifuged (45 min, 4 °C, 100,000 × g). The pellet comprising the total membrane fraction was resuspended in MOPS lysis buffer and stored at −80 °C. Membrane preparation of total mouse brain was performed equally.

Membrane Preparation and Deglycosylation Assay—HEK293 cells were transiently transfected using calcium phosphate. Seventy-two hours after transfection cells were washed with PBS, harvested with a cell scraper, and centrifuged (10 min, 1,000 × g). Pellets were resuspended in ice cold MOPS lysis buffer (0.3 mM sucrose, 20 mM MOPS, 1 mM EDTA supplemented with protease inhibitors: 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml antipain, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM orthophenanthroline, 1 mM benzamidine, 1 mM iodoacetamide, and homogenated at 30 °C using a glass rod and froder plunger. Homogenates were centrifuged for 10 min at 5,000 × g. The pellet was rehomogenized and spun once more for 10 min. The combined supernatants were then centrifuged (45 min, 4 °C, 100,000 × g). The pellet comprising the total membrane fraction was resuspended in MOPS lysis buffer and stored at −80 °C. Membrane preparation of total mouse brain was performed equally.

Deglycosylation was carried out by first boiling the membrane protein fraction in 100 mM β-mercaptoethanol, 0.5% SDS for 5 min. The mixture was then transferred to a solution composed of 15 mM Tris-Cl, pH 8.0, 20 mM orthophenanthroline, and 1% Triton X-100. Finally 5 units of N-glycosidase F (Roche Diagnostics) were added, and the reaction was incubated overnight at 37 °C. For coimmunoprecipitation HEK293 cells were seeded on 6-well plates (0.6 g/ml leupeptin, 1 mM orthophenanthroline, 1 mM benzamidine, 1 mM iodoacetamide, and homogenated at 30 °C using a glass rod and froder plunger. Homogenates were centrifuged for 10 min at 5,000 × g. The pellet comprising the total membrane fraction was resuspended in MOPS lysis buffer and stored at −80 °C. Membrane preparation of total mouse brain was performed equally.

Electrophysiology—Currents were measured at room temperature 2–3 days after transfection using whole cell patch clamp technique. The extracellular solution was composed of (in mM): 110, NaCl; 0.5, MgCl2; 1.8, CaCl2; 5, HEPES; 30, KCl; pH 7.4 adjusted with NaOH. The intracellular solution contained (in mM): 130, KCl, 10, NaCl, 0.5, MgCl2, 1, EGTA; 5, HEPES; pH 7.4 adjusted with KOH. Data were acquired at 10 kHz using an Axopatch 200B amplifier and pClamp 8 (Axon Instruments). Voltage clamp data were stored on the computer hard disk and analyzed off-line by using Clampfit 8 (Axon Instruments). For determination of current densities currents were evoked by a hyperpolarizing voltage pulse to −140 mV for 2.25 s from a holding potential of −40 mV. Current amplitudes were measured at the peak of the tail currents immediately after the final step to −140 mV and were normalized with respect to cell membrane capacitance (Cm) to obtain current densities. For determination of half-maximum activation voltage (V1/2) steady-state activation curves were determined by hyperpolarizing voltages (−140 to −30 mV) from a holding potential of −40 mV for 2.25 s followed by a step to −140 mV. Tail currents immediately after the final step to −140 mV were normalized to maximum current at −140 mV and plotted as a function of the preceding membrane potential. The data points were fitted with the Boltzmann function: (I − Ioff)/Ioff = [1 − exp(−(Vm − V1/2)/k)], where Ioff is an offset caused by a nonzero holding current, Vm is the test potential, V1/2 is the membrane potential for half-maximal activation, and k is the slope factor. Time constants of channel activation (τact) of wild type and mutant HCN2 channels were determined by monoeponential function fitting the current evoked during hyperpolarizing voltage pulses to −140 mV. As has been described earlier (18) the initial lag in the activation of HCN channel currents was excluded from the fitting procedure.

All values are given as mean ± S.E. (n = number of experiments).

RESULTS

Colocalization of Heterologously Expressed HCN Channel Subunits—To investigate the colocalization of HCN channel subunits, the four HCN channel subunits were COOH-termi-
almost no fluorescence is seen. The merged image (Fig. 1D) shows a complete overlap of HCN1 and HCN2 expression (yellow). Similarly, overlapping expression in the plasma membrane was observed for the following subunit combinations: HCN1/HCN3 (Fig. 1E), HCN1/HCN4 (Fig. 1F), HCN2/HCN4 (Fig. 1G), and HCN3/HCN4 (Fig. 1H). In contrast, plasma membranes of cells coexpressing HCN2 and HCN3 consistently revealed a patchy appearance with areas in which either green or red fluorescence dominated. Fig. 1I shows a merged image of two attached HEK293 cells coexpressing HCN2-EGFP and HCN3-RFP. Green and red fluorescence is present in the plasma membrane. Unlike in other HCN channel coexpressions there is no overlap in the two signals indicating that HCN2 and HCN3 are not colocalized. The calibration bar indicates 10 μm.

Commmunoprecipitation of HCN Channel Subunits—The overlap of fluorescence could be due to the colocalization of homomeric HCN channels or could reflect the presence of heteromeric HCN channels. To discriminate between these two possibilities, we performed communoprecipitation experiments. For the experiments shown in Fig. 2A wild type HCN1 was transiently coexpressed with either myc-tagged HCN2, HCN3, or HCN4. Cell lysates were immunoprecipitated with a monoclonal anti-myc antibody and then subjected to Western blot analysis with an anti-HCN1 antibody. In all three experiments a major band of about 110 kDa was recognized by the anti-HCN1 antibody. This is in agreement with the size of the HCN1 protein as predicted from the primary sequence (102 kDa). In immunoprecipitations with a control antibody no specific band was detected. In some experiments a band of about 125 kDa was detected in addition to the 110-kDa band (see HCN2/1 lane). This band very likely represents the glycosylated HCN1 protein because it disappeared after treatment with N-glycosidase F (see Fig. 4A). In total brain lysates, which were immunoprecipitated with a polyclonal anti-HCN2 antibody (16) and probed with anti-HCN1 antibody, only the 125-kDa band was detected indicating that the glycosylated form is the major HCN isoform in vivo (Fig. 2B). Formation of heteromeric channels was also demonstrated for HCN3/HCN4 and HCN2/HCN4 channels (Fig. 2C) and for the coexpression of HCN4/HCN2 (Fig. 2D). In contrast, formation of a stable HCN3/HCN2 channel complex was not detected (Fig. 2D, third lane). The lack of biochemically detectable interaction between HCN2 and HCN3 is in complete agreement with the fluorescence data (Fig. 1D).

We further analyzed the properties of I\textsubscript{h} currents obtained after coexpression of HCN2 and HCN3. To ensure that cells subjected to measurement contained both subunits we used HCN2-EGFP and HCN3-RFP for this set of experiments. Only cells that revealed both green fluorescence (expression of HCN2) and red fluorescence (expression of HCN3) were analyzed. Fig. 3 shows representative current traces of a HCN2-expressing cell and a cell that coexpresses HCN2/HCN3. In cells cotransfected with HCN2/HCN3 we consistently observed an about 40% reduction in current density (184 ± 23 pA/pF for HCN2-EGFP (n = 34) versus 105 ± 29 pA/pF for HCN2-EGFP/HCN3-RFP (n = 18)). Values for V\textsubscript{0.5} (HCN2-EGFP: -103 ± 0.9 mV (n = 16); HCN2-EGFP/HCN3-RFP: -99.2 ± 2.3 mV (n = 5)) and \textit{t}\textsubscript{act} (HCN2-EGFP: 406 ± 21.3 ms (n = 20); HCN2-EGFP/HCN3-RFP: 498 ± 68.2 ms (n = 8)) were not statistically different between both HCN channel combinations.

N-Glycosylation Is Required for Surface Membrane Expression of HCN Channels—The experiments shown in Fig. 2 suggested that HCN channels expressed in HEK293 cells exist in two isoforms, a predominant isoform with a molecular mass being in the range of the theoretical molecular mass and a larger isoform with a significantly higher molecular mass. In contrast, the larger isoform was almost exclusively detected in brain lysates. To investigate whether the size difference is due to N-linked glycosylation we treated membrane fractions containing HCN channels with N-glycosidase F (Fig. 4). After incubation with this enzyme antibodies directed against HCN1 (Fig. 4A) or HCN2 (Fig. 4B) only detected the smaller band, whereas the larger band almost completely disappeared. The primary sequence of all four HCN channels contains a conserved consensus site for N-glycosylation in the extracellular loop between S5 and the pore helix. Replacement of the corresponding residue in HCN2 by glutamine (HCN2\textsubscript{N\textsubscript{380Q}}) resulted in a channel that migrates at about 100 kDa independent of a treatment with N-glycosidase F verifying that the channel is specifically glycosylated at position 380 (Fig. 4B, last lanes).

Next we set out to investigate the functional role of glycosylation in HCN channels. HEK293 cells transfected with HCN2\textsubscript{N\textsubscript{380Q}} exhibited no current over the whole voltage range in which wild type HCN2 is activated (Fig. 5, A–C). The lack of current in cells transfected with HCN2\textsubscript{N\textsubscript{380Q}} could be due to a severe impairment of surface expression or, alternatively, could be caused by altered properties of deglycosylated channels integrated in the cell membrane. To differentiate between these two possibilities we analyzed the subcellular localization of HCN2\textsubscript{N\textsubscript{380Q}}-EGFP by confocal microscopy. Unlike HEK293 cells expressing the HCN2-EGFP channel HEK293 cells transfected with HCN2\textsubscript{N\textsubscript{380Q}}-EGFP no longer showed labeling of the cell membrane (Fig. 6, A and B). Rather, the mutant protein was diffusely distributed in the intracellular compartment. Similarly, when HEK293 cells expressing HCN2-EGFP were
treated with tunicamycin, an inhibitor of N-linked glycosylation, cell surface expression of HCN2 was completely prevented (Fig. 6C). This finding indicates that the impairment of cell surface expression of HCN2N380Q-EGFP is not caused by incorrect folding due to the replacement of N380Q but is due to the lack of carbohydrate chains in the protein. When HCN2N380Q-EGFP was coexpressed together with HCN4-RFP the mutant protein was highly enriched in the plasma membrane (Fig. 6D) indicating that HCN4 rescued the wild type phenotype by forming a heteromeric complex. Indeed, the coassembly of HCN4 and HCN2N380Q could be confirmed by coimmunoprecipitation (Fig. 6F). Finally, when HCN2N380Q-EGFP was coexpressed with HCN3-RFP no increase in surface expression was observed (Fig. 6E). The mutant protein again showed intracellular accumulation. Thus, HCN3 is not able to normalize channel trafficking of HCN2N380Q, most likely because it cannot form a stable heteromeric complex with this subunit in the cell membrane.

**DISCUSSION**

Sequence analysis indicates that HCN channels are members of the family of voltage-gated cation channels. Hence, these channels almost surely possess a tetrameric structure. Recent immunohistochemical and in situ hybridization studies (19–21) indicated that in some cell types homotetrameric HCN channels exist. On the other hand, the overlapping expression pattern of the four HCN channels in brain and heart (20, 22) as well as comparative electrophysiological studies (12, 13, 20) support the notion that heterotetrameric HCN channels may be formed in vivo as well. So far, however, biochemical data on the assembly of HCN channel subunits were missing. Moreover, it was unclear whether all the members of the HCN channel family can freely assemble with each other or whether some combinations are excluded. In this study we have addressed this issue by a combination of fluorescence confocal microscopy, coimmunoprecipitation, and electrophysiology. Our data indicate that HCN channel subunits assemble to a variety of different heteromers. Only one pair of channel subunits, HCN2 and HCN3, makes an exception and does not form a functional heteromer. Although HCN2 and HCN3 subunits could be easily detected in the plasma membrane, there was no obvious colocalization of the two proteins. In accordance with this finding HCN2 and HCN3 are not coimmunoprecipitated by specific antibodies. Interestingly, cells coexpressing HCN2 and HCN3 exhibited a significantly reduced current density compared with cells expressing HCN2 alone. One explanation for this result is that in the presence of HCN3 less functional HCN2 channel protein is inserted into the plasma membrane. Homomeric HCN3 channels can probably not compensate for the resulting reduction in current amplitude because for unknown reasons HCN3 produces notoriously small currents. We cannot exclude that HCN3 exerts some direct inhibitory effect on HCN2 surface trafficking, e.g., by forming a transient intracellular complex that is not stable enough to be detected in immunoprecipitation experiments. It is not known whether all HCN channel heteromers that assemble in HEK293 cells also exist in vivo. The partially overlapping expression pattern of these channels in brain and heart indicates that at least in some cell types heteromerization might occur. The coimmuno-

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**Fig. 2. Detection of heteromeric HCN channels by coimmunoprecipitation.** A, lysates from HEK293 cells transiently cotransfected with HCN1 and myc-tagged HCN2, myc-tagged HCN3, or myc-tagged HCN4 were immunoprecipitated (IP) with either anti-myc antibody (m) or control IgG (c). Western blots (WB) were probed with anti-HCN1 antibody. As a size control the lane at the right (Ø) was loaded with a membrane fraction (10 μg of protein) of HCN1-expressing HEK293 cells. The sizes of the HCN1-specific immunosignals are indicated. B, detection of heteromeric HCN1/HCN2 channels in mouse brain. Mouse brain lysates were immunoprecipitated with either anti-HCN2 antibody (AS2) or control IgG (c), blotted, and probed with anti-HCN1 antibody. A specific band of about 125 kDa was recognized only after IP with AS2. The size of the immunosignal is identical to that detected by anti-HCN1 in total brain lysate not subjected to IP (Ø, 75 μg of protein). C, detection of heteromeric HCN3/HCN4 and HCN2/HCN4 channels. IPs with anti-myc or control antibody were performed from lysates of HEK293 cells cotransfected with HCN4 and myc-tagged HCN2 or myc-tagged HCN3, followed by immunoblotting with anti-HCN4. Ø, membrane fractions (30 μg of protein) of HCN4 transfected HEK293 cells not subjected to IP. D, HCN2 assembles with HCN4 but not with HCN3. After IP with anti-myc antibody a specific immunosignal was observed with the anti-HCN2 antibody in HCN4/HCN2 but not in HCN3/HCN2 coexpressing cells. Ø, membrane fractions (10 μg of protein) of HCN2 transfected HEK293 cells not subjected to IP.

**Fig. 3. Inhibitory effect of HCN3 on HCN2 mediated current.** Representative whole-cell current traces of HEK293 cells transfected with either HCN2-EGFP alone or with a combination of HCN2-EGFP and HCN3-RFP. Cell capacities were 10 and 9 pF for the HCN2-EGFP and HCN2-EGFP/HCN3-RFP expressing cell, respectively. Currents were evoked by stepping from a holding potential of −40 mV to various test potentials ranging from −140 to −30 mV in 10-mV steps.
precipitation of HCN1/HCN2 heteromers from total brain lysates as demonstrated in our study clearly supports this notion. The functional relevance of heteromeric HCN channels is not understood so far. It is very likely that cells utilize heteromerization as a powerful mechanism to generate a large number of different channels with distinct electrophysiological and biochemical characteristics based on a relatively small number of genes. While our results point out that heteromers exist in cells further experiments will be necessary to pinpoint the stoichiometry of these channels. Recently, in the related cyclic nucleotide-gated channel from rod photoreceptors an asymmetrical 3:1 stoichiometry of the two subunits present in the tetramer was determined (23–25). It will be interesting to see whether or not this stoichiometry is also conserved in HCN channels.

Finally, complexity of HCN channels could be further increased by the expression of channels containing three different subunits. Such a stoichiometry has been identified in the olfactory cyclic nucleotide-gated channel (26, 27).

A number of factors could control the formation of tetrameric HCN channels. By employing two-hybrid assays the cytosolic NH₂ termini of HCN1 and HCN2 were found to interact with each other (28). Within the NH₂ terminus a sequence of 52 amino acids localized adjacent to the S1 segment confers specific interaction (29). This sequence is highly conserved but not identical within the four HCN channels. Principally, subtle sequence differences could explain why HCN3 interacts with HCN4 and HCN1 but not with HCN2; however, other channel domains may be also influential in the control of unit assembly.

In agreement with a previous study by Santoro et al. (30) we show that HCN channels are N-linked glycosylated. Moreover, our study provides evidence that N-linked glycosylation is required for normal HCN channel surface expression. Treatment with tunicamycin, an inhibitor of N-linked glycosylation, completely abolished channel trafficking to the plasma membrane. By site-directed mutagenesis of HCN2 we could show that the observed N-linked glycosylation is conferred by a single asparagine residue. HCN2 channels in which this residue is replaced by glutamine (HCN2N380Q) are not inserted into the plasma membrane.
membrane and fail to yield functional $I_h$ currents. The presence of asparagine residues at positions equivalent to Asn$^{280}$ in HCN2 in all HCN channels strongly suggests that $N$-linked glycosylation is also needed for surface trafficking of other HCN family members. Coexpression of the glycosylation-deficient HCN2$^{N380Q}$ mutant with wild type HCN4 rescues surface expression of HCN2$^{N380Q}$ indicating that not all four subunits of the native HCN channel complex must be glycosylated to ensure surface trafficking. Moreover, the successful rescue of HCN2$^{N380Q}$ suggests that the lack of carbohydrate chains does not substantially affect proper channel folding. This notion is further supported by the coimmunoprecipitation experiments demonstrating the specific interaction between HCN4 and HCN2$^{N380Q}$. Again, the failure of HCN3 to rescue HCN2$^{N380Q}$ provides further evidence for the lack of interaction between these two subunits.

Taken together, it appears that glycosylation is not required for HCN channel function in the plasma membrane but is essential for normal cell surface targeting. With respect to the HCN1- and HCN2-linked glycosylation HCN channels are related to the HERG K$^+$-channel. Cell surface expression of this channel also depends on the glycosylation of asparagine residues in the S5-S6 loop (31). Mutations in the consensus $N$-linked glycosylation sites were found in patients suffering from long QT syndrome type 2 (LQT2) (32). Thus, it is tempting to speculate that homologous mutations in HCN channels may also give rise to channelopathies. A recent genetic study (33) and the analysis of the phenotype of HCN2 knockout mice (16) indicate that impairment or loss of HCN channel function affects a number of physiological functions ranging from cardiac rhythmicity to the control of thalamic network activity.

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FIG. 6. $N$-Linked glycosylation is required for HCN channel surface expression. A and B, confocal fluorescence images showing subcellular localization of HCN2-EGFP (A) and HCN2$^{N380Q}$-EGFP (B) in HEK293 cells. Unlike the wild type channel the glycosylation-deficient HCN mutant is distributed in the cytoplasm and is not enriched in the plasma membrane. The inset in B shows an overlay of the green fluorescence image with the blue fluorescence of the plasma membrane marker ECFP-Mem confirming the absence of HCN2$^{N380Q}$ in the cell membrane. C, HCN2-EGFP-expressing cells treated with tunicamycin reveal the same cytoplasmic localization of the HCN2 protein as demonstrated for HCN2$^{N380Q}$-EGFP. D, cotransfection of HCN2$^{N380Q}$-EGFP with HCN4-RFP. Unlike in B green fluorescence is now almost exclusively distributed in the plasma membrane indicating that HCN4 has rescued HCN2$^{N380Q}$ surface targeting. E, confocal image of a cell cotransfected with HCN2$^{N380Q}$-EGFP and HCN3-RFP. Coexpression of HCN3 does not rescue HCN2$^{N380Q}$ surface expression. Like in B green fluorescence is mainly seen in the intracellular space. The calibration bar in A–E indicates 10 μm. F, coimmunoprecipitation demonstrating that myc-tagged HCN4 forms stable complexes with both wild type HCN2 and HCN2$^{N380Q}$. Abbreviations are the same as described in the legend to Fig. 2.