Interaction of the pacemaker channel HCN1 with filamin A

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Pacemaker channels are encoded by the HCN gene family and are responsible for a variety of cellular functions including control of spontaneous activity in cardiac myocytes and control of excitability in different types of neurons. Some of these functions require specific membrane localization. Although several voltage gated channels are known to interact with intracellular proteins exerting auxiliary functions, no cytoplasmic proteins have been found so far to modulate HCN channels. By use of yeast two-hybrid technique, here we show that filamin A interacts with HCN1, an HCN isoform widely expressed in the brain, but not with HCN2 or HCN4. Filamin A is a cytoplasmic scaffold protein with actin-binding domains whose main function is to link transmembrane proteins to the actin cytoskeleton. Using several HCN1 C-terminal constructs, we identified a filamin A-interacting region of 22 aminoacids located downstream the cyclic nucleotide-binding domain; this region is not conserved in HCN2, HCN3 or HCN4. We also verified by immunoprecipitation from bovine brain that the filamin A-HCN1 interaction is functional in vivo. In filamin A-expressing cells (filamin+), HCN1 (but not HCN4) channels were expressed in hot-spots, whereas they were evenly distributed on the membrane of cells lacking filamin A (filamin−), indicating that interaction with filamin A affects membrane localization. Also, in filamin− cells the gating kinetics of HCN1 were strongly accelerated relative to filamin+ cells. The interaction with filamin A may contribute to localize HCN1 channels to specific neuronal areas and to modulate channel activity.
INTRODUCTION

The hyperpolarization activated pacemaker current $I_f$/$I_h$ has an established role in underlying generation and neurotransmitter-mediated modulation of cardiac pacemaker activity (1) and control of excitability and other functions, including integration of synaptic activity and modulation of synaptic strength, in several different types of neurons (2,3).

The molecular correlates of $f$/h- channels are the Hyperpolarization-activated Cyclic Nucleotide gated channels, of which 4 isoforms (HCN1-4) are known. When expressed alone in heterologous systems, HCN channels elicit currents whose properties are qualitatively similar to those of native $I_f$/I$\text{h}$ currents, although they differ quantitatively in kinetics and cAMP sensitivity (4,5).

Recently, several studies have addressed the problem of the distribution of HCN isoforms in different cell types. HCN1, the first HCN isoform to be cloned (6), is extensively expressed in the brain, with a selective distribution is specific brain areas. It is expressed for example in the layer 5 neocortical neurons, in the CA1 and, to a lower degree, CA3 hippocampal regions, in the molecular cell layer of the cerebellum, and in the superior colliculus (7,8,9,3). Interestingly, in some of the tissues of expression such as the neocortex, the retina, the hippocampus and the taste receptors, the HCN1 isoform was found to be present in specific cell types and/or in specific subcellular compartments (e.g. the inner segment of rods and the cortical dendrites of CA1 hippocampal neurons) both at the RNA and protein levels (10,11,12,13,14).

Correct functioning of various ion channels depends upon the interaction with auxiliary subunits (15) and with scaffolding proteins, which colocalize channels and regulatory components in subcellular compartments for improved efficiency of channel modulation (16,17,18). The aim of this work was to investigate if HCN1 channels interact with partner proteins in the brain and to check if existing associations with any such proteins have a role in specific cellular localization.
of the channels and modulation of channel function. We used the C-terminus of mHCN1, since this contains specific sequences for protein-protein interaction (including PDZ and SH3 domains), to screen a mouse brain cDNA library by yeast two-hybrid assay. We found that HCN1 associates with several proteins and specifically with filamin A, a high molecular mass cytoskeletal protein known to anchor different transmembrane proteins to the actin cytoskeleton and to act as a scaffold for various signaling proteins.

EXPERIMENTAL PROCEDURES

Yeast two–hybrid system

We used the “Matchmaker two-hybrid system 3” to screen a library of mouse brain cDNA inserted in pACT2 (Clontech, Palo Alto CA), with a portion of the C-terminus of mHCN1 in pGBKT7 as a bait (see below). The surviving clones were identified by PCR; the primers for amplification were mapped in pACT2:

(FOR 5’-CTATTCGATGATGAAGATACCCCACCAAACCC-3’);
(REV 5’-GTGAACTTGCGGGGTTTTTCAGTATCTACGA-3’).

The proteins thus amplified included: filamin A (retrieved 8 times); Ral A (a protein of the Rho family of GTPases, (19)) (retrieved twice); GDI (GDP Dissociation Inhibitor, (20)) (retrieved once). Filamin A was selected for further analysis.

Constructs

To identify the shortest filamin A-interacting site of mHCN1, we made several constructs of mHCN1 C-terminal portions in pGKT7, as outlined below.

1. aa 657-910: we cut pEGFPC1-mHCN1 with AccI (blunt) and BamHI; the resulting fragment was inserted into pGBKT7 EcoRI (blunt) and BamHI;
2. aa 657-858 and aa 859-910: the inserts coding for these two stretches were obtained by cutting pGBK7-mHCN1 C-terminus (construct 1) with EcoRI, AflIII, and with AflIII and BamHI, respectively.

3. aa 657-791: we inserted a stop codon (TGA) in construct 1 (aa 790) using three PCR cycles. The primers used were F1 and R3 (1st cycle); F3 and R2 (2nd cycle); F1 and R2 (3rd cycle) (see below sequence of primers);

4. aa 657-715: construct 1 was cut with EcoRI and PvuII and the fragment reinserted into pGBK7 (EcoRI and SmaI);

5. aa 657-693: construct 1 was cut with PstI and the vector ligated;

6. aa 694-715: two partially overlapping oligos covering this region and carrying two restriction sites were annealed in vitro, blunted with a Klenow polymerase and digested with NcoI/BamHI; the final product was cloned into the pGBK7 plasmid;

7. aa 657-910 with the mutation PSLP/ASAA (aa 814-817). We used three PCR cycles using the primers: F1 and R1 (1st cycle); R2 and R2 (2nd cycle); F1 and R2 (3rd cycle).

8. aa 657-715 with the mutation PP/AA (aa 695-696): the C-terminus of HCN1 was amplified by PCR from aa 694 to 715 using a mutagenic forward primer; the product was then cloned into the PstI restriction site of aa 657-715.

   The primers used for protocols 3 and 7 were:

   F1: 5'-ATCTCAGAGGAGGACCTGCATA-3'; R1: 5'-CTCATGGGCCAGAAGCCTG-3';
   F2: 5'-CAGGCTTCTGCGGCCCATGAG-3'; R2: 5'-GTTATGCGGCCGCTGCAGGT-3';
   F3: 5'-TGAAGTGCACAAGTGAAGCACA-3'; R3: 5'-TGTGCTTCACTTGTGCACTTCA-3'.

   All constructs were sequenced by MWG AG (Ebersberg). We used the above constructs to transform the AH109 yeast strain, while the Y187 yeast strain was transformed with pGADT7-filamin A; the two strains were then mixed and plated according to the “Yeast Mating” method,
in order to verify the interaction found by the screening procedure and identify minimum interacting sites. For positive- and negative-interaction controls, we used the large T-antigen vector with the p53 and the laminin vectors, respectively.

**In vitro transcription/translation**

We performed transcription/translation of the mHCN1 constructs generated by the above protocols and of filamin A by TnT Quick Coupled Transcription/Translation System (Promega). All mHCN1 constructs were inserted in pGBK7 and filamin A was inserted in pGADT7 (Clontech). $[^{35}\text{S}]$Methionine radio-labelled protein was obtained by an *in vitro* system based on mammalian reticulocyte lysate (TnT Quick Master Mix, Promega) to which plasmidic cDNA and $[^{35}\text{S}]$Methionine (Amersham Bioscences) were added. Each sample (final volume of 50 µl) was incubated for 60-90 min at 30 °C.

**Cell transfection**

The A7 (filamin$^+$ or ABP$^+$, Actin-Binding Protein$^+$) and M2 (filamin$^-$ or ABP$^-$) cells (21) were incubated in a culture medium (MEM, Gibco) containing 10% FBS (Gibco), 1% MEM non-essential AA solution (Sigma), 2% MEM Vitamin solution (Sigma), 1% Antibiotic Antimycotic solution (Sigma). The day before transfection cells were detached by gentle shaking in a 0.5 g/l trypsin solution and plated on 35 mm petri dishes at 70% confluence. A7 and M2 cells were transiently transfected with pEGFPC1-mHCN1 or pEGFPC1-hHCN4 (2 µg in each petri dish) using a Lipofectamine-based protocol (Invitrogen). After growing for 2 days at 37 °C and 5% CO$_2$, the cells were separated by trypsinization and re-plated at a lower density for electrophysiology and fluorescence.
**GFP fluorescence**

Cells transfected as described above were treated with cycloheximide (Sigma) 50µg/ml and kept for 4 hours at 37°C in a 5% CO₂ incubator to block protein synthesis (22). The cells were then washed with PBS at 4 °C three times and fixed with paraformaldehyde (4%) for 5 minutes at 4 °C; this was followed by a treatment with a quenching solution (0.1 M glycine/PBS) for 30 min at 4 °C. Cells were washed again with a 20 mM phosphate buffer and harvested on glass coverslips with DAPI mounting medium. After fixation, cells were observed by confocal microscopy (TCS Laica) with sections at approximately 4 µm intervals.

**Immunoprecipitation and membrane preparation**

We homogenized 10g of adult bovine brain in 30 ml of a solution containing 10 mM Tris-HCl, sucrose 320 mM, 0.5% v/v protease inhibitor (Sigma), pH 7.4, with a tissue grinder and a loose fitting Dounce homogenizer (10 strokes). After a first centrifugation (2,000 rpm for 10 min at 4 °C), the pellet (P1) was discarded and the supernatant was centrifuged at 20,000 rpm for 20 min at 4 °C. The newly formed pellet (P2) was dissolved into 15 ml of a solution containing 10 mM Tris-HCl, 0.5 % v/v protease inhibitor, 1% v/v Triton X-100 (Sigma), pH 7.4 and stirred for 20 min on ice. After a further centrifugation (20,000 rpm for 20 min at 4°C), the insoluble pellet (P3) was eliminated and the supernatant used for the immunoprecipitation protocol.

For immunoprecipitation, 400 µg of soluble membrane proteins were dissolved in 0.5 ml of water and incubated with Ab anti-filamin (MAB 1680, Chemicon) overnight at 4 °C on a rotary shaker. The following day we added 25 µg protein G-sepharose beads (P-3296, Sigma), incubated for 2 hours and centrifuged at 13,000 rpm for 10 min at 4 °C; the pellet was then
washed 3 times with a buffer solution containing 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, Triton X100 1%, 0.5 % sodium deoxicholate, 0.1% SDS, pH 7.4. The proteins were separated by SDS-PAGE, transferred to nitrocellulose and incubated with anti-HCN1 antibody (Alomone Labs) for blotting.

A7 and M2 cells used for immunoprecipitation were grown in 10 cm petri dishes, transfected as described above, washed in cold PBS and scraped from the bottom; extracts from 3 dishes were resuspended in 500 µl of PBS containing 0.4% v/v protease inhibitor (Sigma) and homogenized; immunoprecipitation was carried out as described above for brain extracts.

For the immunoprecipitation of proteins obtained by the in vitro transcription/ translation procedure described above, individual mHCN1 constructs were either used alone or mixed (as baits) with filamin A (as prey) for 1 hour at room temperature. Protocols for immunoprecipitation with anti- HA (Santa Cruz) and anti- C-Myc antibodies (Sigma) were as described above. The gel was treated with a gel fixation solution (50% methanol, 10% glacial acetic acid, 40% water) and exposed to autoradiography to reveal the presence of [$^{35}$S]Methionine labelled proteins.

Electrophysiology

M2 and A7 cells were washed once in Tyrode solution, placed under a fluorescent microscope and superfused at room temperature with Tyrode. Only fluorescent cells were chosen for recording. The Tyrode solution was composed as follows (in mM): NaCl, 140; KCl, 5.4; CaCl$_2$, 1.8; MgCl$_2$, 1; D-glucose, 5.5; Hepes-NaOH, 5; pH 7.4. During whole-cell recording, the perfusing solution was switched to a high K$^+$ solution containing (mM): NaCl, 110; KCl, 30; CaCl$_2$, 1.8; MgCl$_2$, 0.5; Hepes-NaOH, 5; 1 BaCl$_2$, 1; MnCl$_2$, 2; NiCl$_2$, 0.1; nifedipine, 0.02; pH 7.4. The intracellular-like solution contained (mM): KCl, 130; NaCl, 10; EGTA-KOH, 1; MgCl$_2$,
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0.5; ATP (Na-salt), 2; creatine phosphate, 5; GTP (Na-salt), 0.1; Heps-KOH, 5; pH 7.2. From a holding potential of -20 mV, hyperpolarizing steps to the range -25 to -125 mV followed by a step at -135 mV were applied to measure activation curves in standard two-step protocols. Tail currents at -135 mV were normalized to plot activation curves. Single activation curves were fitted to the Boltzmann equation \( y=\frac{1}{1+\exp((V-V_{1/2})/s)} \), where \( V \) is voltage, \( V_{1/2} \) is half-maximum activation potential and \( s \) is the inverse slope factor, to yield \( V_{1/2} \) and \( s \) values, which were then averaged. Deactivation traces were recorded by fully activating the current at -135 mV and then stepping to the range -55 to 45 mV for time long enough to reach steady-state. Time constants were calculated by fitting activation/ deactivation traces to a monoexponential function after an initial delay (5) and values averaged and plotted against voltage. Conductances were normalized to cell capacitance, measured by a specific 10 mV step protocol. Values are given as mean ± sem throughout. Statistical analysis was performed by t-test comparison with a significance level set to P=0.05.

RESULTS

cDNA screening by yeast two-hybrid system

We screened over 4 million proteins using the final portion of mouse HCN1 (mHCN1) C-terminus (aa 657-910) as a bait and found about 40 hypothetical interacting partners. Of these 40 proteins, some were retrieved more often than others. To check for true vs false positives, we performed a yeast-mating assay on a high-stringency medium, which led to the identification of three proteins which were confirmed to bind to the HCN1 bait: Ral A (Ras-like GTPase, a protein of the Rho family of GTPases) (19), GDI (GDP Dissociation Inhibitor) (20) and filamin A, retrieved 2, 1 and 8 times respectively. We focussed our attention on filamin A. The interaction
of filamin A with HCN1 in yeast is shown in Fig 1A.

Fig. 1 near here

Filamin A, or Actin-Binding Protein (ABP), is a large 280 kDa cytoskeletal protein carrying an actin-binding site at the N-terminus, 24 Immunoglobulin (Ig)-like repeats and two hinge regions linking repeats 15-16 and repeats 23-24 (23). Filamin A acts as a dimer and, due to its specific structure, behaves as a scaffold protein. For example it can assemble broad macrocomplexes by binding a variety of proteins such as membrane receptors (e.g. D2 and D3 dopamine receptor), phosphatases (e.g. SHIP-2), ion channels (e.g. Kv4.2 potassium channel), monomeric G-proteins (e.g. RalA) and structural proteins (e.g. β1-integrin and caveolin-1) (17,19,23,24;25,26,27,28). Figure 1B shows the schematic structure of filamin A and the shortest C-terminal segment rescued by the HCN1 bait with the screening protocol (bottom segment, repeats 23-24).

**Immunoprecipitation from bovine brain**

We proceeded to test if filamin A and HCN1 also interact in vivo in a mammalian system, and specifically in the CNS where both proteins are highly expressed. Using specific anti-filamin A antibodies (see Methods), we immunoprecipitated filamin A from 400 µg of membrane proteins extracted from bovine brain. The immunoprecipitated and total membrane proteins were loaded onto SDS-PAGE and transferred to nitrocellulose for Western blot analysis. In Fig 2, the filamin A-immunoprecipitated proteins (lane 1) yielded a strong band near 116 kDa, corresponding to the expected molecular weight of the HCN1 isoform (12), as detected by isoform specific antibodies. As a control, the same signal was confirmed in the total membrane proteins before immunoprecipitation (lane 2). For further control, we verified the presence (lane 3) and specificity (lane 4, primary antibody omitted) of filamin A signal in the filamin A-
immunoprecipitated proteins; also, HCN1 was not detected by incubating the same extract with a preimmune serum (lane 5) or by omitting the primary HCN1 antibody (lane 6, compare with lane 7 in the presence of primary antibody).

Fig. 2 near here

**Protein–protein interaction assay: mating and in vitro transcription-translation**

In order to identify the minimum filamin A-interacting portion of mHCN1 and to investigate if HCN isoforms other than HCN1 could bind filamin A, we performed a yeast-mating assay (Fig 3). This was achieved by transforming the (MATα) Y187 yeast strain with filamin A and the (MATa) AH109 strain with different C-terminal portions of either mHCN1 or with the C-termini of mHCN2 or hHCN4 (see Table 1 and Fig. 3 legend). Yeasts were then plated in high stringency conditions, allowing yeast growth only under condition of strong interaction between constructs.

Fig. 3 near here

In Fig. 3 A to K, a collection of yeast-mating combinations of filamin A and HCN C-terminal sequences is shown. Panel A represents the C-terminal portion of mHCN1 used as a control for screening (aa 657-910, where 910 is the last aminoacid of the mHCN1 protein), which clearly interacts with filamin A. This portion contains a PXXP motif conserved among the HCN isoforms 1, 2, 3 and 4 (812 PSLP 815 in mHCN1), a sequence known to bind SH3 domains and to be involved in some cases in filamin A binding (6,17), although filamin A does not contain SH3 domains. We therefore first checked if this putative consensus site could be responsible for the interaction between mHCN1 and filamin A, and replaced by mutagenesis aminoacids PSLP with ASAA, a substitution known to affect filamin A - protein interactions (17) (Fig 3B). The yeasts were able to grow normally indicating that this sequence is not necessary for interaction.
We also noted that the C-terminal part of mHCN1 includes a consensus sequence, conserved among all HCN isoforms (not shown), for PDZ domains (aa 907-910, XSXL), which are known to be involved in the organization of supramolecular complexes and protein scaffolding (29). To check for a potential interacting site with filamin A in this region, we generated a small fragment from the C-terminal part of the protein (aa 859-910) containing the PDZ-consensus site. As shown in Fig. 3 C, D, this fragment did not interact with filamin A, whereas its complementary sequence (aa 657-858) did, indicating that the PDZ consensus site is not responsible for this interaction.

We then proceeded to shorten the test sequence of mHCN1 from the control aa 657-910 portion and prepared four truncated constructs in order to identify a shorter interacting region, as shown in panels E, F, G and H of Fig. 3 (see also Table 1). This approach revealed that the 22 aa-long sequence comprised between aa 694 and 715 (SPPIQSPLATRTFHYASPTASQ) is necessary for the interaction. Since proline-rich sequences may contribute to protein-protein interactions (for example with WW domains), we mutated the di-proline tract 695-696 to AA in the sequence aa 657-715; the mutation did not disrupt the interaction (Fig. 3I), ruling against an involvement of this tract in the binding with filamin A.

The sequence aa 694-715 is not conserved in any of the other HCN isoforms (HCN2, HCN3, HCN4). This was verified by performing a multiple sequence alignments with Clustal-W as well as a search of homologous sequences with Blast; neither methods provided significant alignment scores (not shown). In agreement with this observation, the HCN2 and HCN4 regions homologous to the HCN1 aa 657-910 control sequence (spanning from the C-terminal ends of the cyclic-nucleotide binding domain, CNBD, downstream to the C-termini of HCN2 or HCN4, see Methods) did not interact with filamin A (Fig. 3 J,K). The filamin A-interacting sequence was downstream the CNBD in mHCN1 (Fig. 3L).
As a way to confirm the results above, we also checked for protein-protein interactions using \textit{in vitro} transcription and translation of the various constructs to which either a HA or C-myc tag was fused in frame (see Methods for details).

Fig. 4 near here

In Fig 4A, filamin A translated \textit{in vitro} with $^{35}$S-Methionine was immunoprecipitated using an anti-HA antibody and its presence revealed by autoradiography (left lane, as labelled); similarly, the mHCN1 control sequence (aa 657-910) fused to C-myc was translated \textit{in vitro} and immunoprecipitated using an anti-C-myc antibody, and its presence revealed (center lane, as labelled). The two proteins were then mixed together and the immunoprecipitation was performed with the anti-C-myc antibody (right lane). Autoradiography confirmed the presence of filamin A in the pellet. In panels B-G, similar experiments were carried out using the same constructs as in Fig. 3 C-H (as indicated). Once again, the region between amino acids 694 and 715 was the shortest one yielding positive interaction. The data from the yeast mating assay and the \textit{in vitro} transcription-translation assay are summarized in Table 1.

Table 1 near here

To investigate the role of the interaction between filamin A and mHCN1, we performed fluorescence and electrophysiological experiments by transiently transfecting the mHCN1 isoform in frame with GFP in a melanoma cell line lacking filamin A (filamin$^-$ or M2 cells) and compared the results with those obtained with the same cell line stably expressing the filamin A clone (filamin$^+$ or A7 cells) (21,17).

We first verified by immunoprecipitation that filamin A is expressed in A7, but not in M2 cells (Fig. 5A, top), and that mHCN1 and filamin A, as expected, co-immunoprecipitate in A7, but not in M2 cells (5A, left); the interaction was specific, since no signal was detected when
untransfected cells were incubated with the anti-HCN1 antibody (5A, bottom right).

In Fig. 5 B,C, microphotographs of typical A7 (B) and M2 cells (C) expressing the mHCN1-GFP protein are shown. There is a significant level of protein expression in both cell types. In panels D, E, cells were treated with cyclohexamide for 4 hours to block protein synthesis and consequently reduce the fluorescence produced within ER and Golgi regions (see Methods for details). Under these conditions, the mHCN1 fluorescence signal was concentrated in discrete spots on the plasma membrane of A7 cells (D), whereas in M2 cells it was evenly distributed on the membrane (E). The same protocol was applied after transfection of hHCN4-GFP in A7 and M2 cells; in this case, as apparent in panels F and G of Fig. 5, channels were expressed evenly on the membrane of both cell types.

These results indicate that filamin A contributes to a channel clustering process which takes place while HCN1 channels are inserted into the plasma membrane. At the same time, since lack of filamin A does not appear to inhibit membrane insertion of channels, the data suggest that this protein is not involved in HCN1 channel trafficking from the ER to the plasma membrane.

In order to identify potential effects of the mHCN1- filamin A association on the biophysical properties of mHCN1 channels, we measured whole-cell mHCN1 currents by patch-clamp in both A7 and M2 cells. In Fig 6A, typical currents elicited by hyperpolarization to the voltage range -25 to -125 mV (20 mV steps) are shown for A7 (left) and M2 cells (right).

On average, the normalized conductance was approximately twice smaller in A7 than in M2 cells (0.28 ± 0.03 pS/pF, n=11 and 0.58 ± 0.12 pS/pF, n=12, respectively, P < 0.05). The cell capacitance did not differ between the two groups (A7, 47 ± 4.4 pF, n=11; M2, 46.8 ± 4.7 pF, n=12). In Fig 6B, the mean activation curves measured for the two types of cells are shown. In
cells lacking filamin A (M2 cells, open circles), the activation curve was shifted to the positive
direction relative to filamin A-expressing cells (A7, filled squares) by 7.6 mV. Fitting individual
curves to the Boltzmann equation yielded half-activation voltages $V_{1/2} = -71.2 \pm 2.1$ mV and
$-63.6 \pm 1.3$ mV (significantly different) and inverse slope factors $s = 9.5 \pm 1$ and $10.24 \pm 0.8$ mV
(non-significantly different) for A7 ($n=7$) and M2 ($n=8$) cells, respectively. Time constants of
activation and deactivation were measured and averaged (Fig. 6C). Both activation and
deactivation time constants were much slower (1.4 to 3-fold) in A7 cells than in M2 cells. Both
the activation curve and the activation/ deactivation time constant curves in A7 cells were similar
to those measured previously for mHCN1 expressed in HEK293 cells (5).

In order to verify whether the changes in current density and in kinetics in Fig. 6 were
actually attributable to the specific interaction between mHCN1 channels and filamin A, we
analyzed the properties of hHCN4 channels expressed in A7 and M2 cells. We did not find
significant differences in any of the properties of hHCN4 currents: normalized conductances
were $0.12 \pm 0.04$ (n=5) in A7 and $0.13 \pm 0.07$ pS/pF (n=6) in M2 cells; half-activation voltages
and inverse slope factors were (mV) -86.0 and 11.8 in A7 (n=5), and -84.4 and 10.4 in M2 cells
(n=9), respectively; also, time constants of activation/ deactivation were not significantly
different in A7 and M2 cells in the range -135 to +45 mV (not shown).

These data indicate that protein-protein interaction of mHCN1 channels with filamin A strongly
affects the channel kinetic properties.

**DISCUSSION**

HCN channels are members of a superfamily of ion channels which include voltage gated
potassium and cyclic-nucleotide gated (CNG) channels. Many channels belonging to this
superfamily interact with and/or are functionally modified by accessory proteins, including β-
subunits able to modulate ion channel activity and structural proteins involved in the organization
of channels and interacting elements into macromolecular complexes. For example, it is known
that several KCNQ channel isoforms interact with the β-subunits KCNE1 or KCNE2 to generate
currents similar to those recorded in native tissue (30); also, Kv1 channels interact with PSD95
while Kv4.2 and Kir2.1 channels have been reported to link to filamin A (17,18,31).

Heterologous expression of individual HCN isoforms does not normally results in current
whose properties reproduce entirely those of the native pacemaker (If/Ih) currents (4,32). Part of
this difference may be attributable to the existence of a “context” dependence of channel
properties, i.e. a dependence on conditioning mechanisms such as phosphorylation of the channel
protein, or the interaction with auxiliary/ cytoskeletal proteins. Phosphorylation-dependent
processes, for example, can modify channel kinetics (33) and the density of expressed channels
(34); also, it has been shown recently that HCN channels are modulated by interaction with the
KCNE2 subunit (35,36), even though the relevance of this interaction is still uncertain (Altomare
et al., 2003). The existence of a “context” dependence of HCN properties is further supported by
evidence that some of the kinetic features of a given isoform vary in different expression systems
(37).

One way by which intracellular processes can affect channel function is by protein-
protein interactions. In order to find possible partners for HCN channels we used the yeast two-
hybrid assay and screened a cDNA library of mouse brain. To select a bait we used the HCN1
subunit because of its extensive expression in various regions of the central nervous system (e.g
neocortex, hyppocampus, cerebellum (11,14).

Library screening and yeast-mating procedures, the latter used to confirm screening data
and remove false positives, indicated that the C-terminus mHCN1 bait interacts with three proteins: filamin A, Ral A and GDI (an inhibitor of Ral A activation). Interestingly, all of these proteins are involved in the organization of the actin cytoskeleton and related plasticity processes. For example, it has been reported that Ral A interacts directly with repeat 24 of filamin A (19,27), the same region which we have found to interact with HCN1.

The proximity of the interacting sites on the filamin A molecule may explain our finding that RalA and HCN1 interact. This result suggests that Ral A, filamin A and HCN1 are strictly packed in a macromolecular complex. It is also interesting to note that monomeric G-proteins of the Rho family, of which Ral A is a member, are involved in the modulation of ionic channels such as NMDA and GABA-A receptors (38,39). We found that the interaction between HCN1 and filamin A is conserved in \textit{in vivo} conditions, as shown by co-immunoprecipitation of the two proteins from bovine brain (Fig. 2).

It is known that filamin A interacts with several membrane proteins, among which the K$^+$ channels Kv4.2 and Kir 2.1, which bind to filamin A at their C-terminal ends; Kv4.2 is reported to bind via the sequence PTPP (17,31). Although the sequence PXXP is conserved in mHCN1, however, we found that it is not responsible for the interaction between filamin A and mHCN1. Using the yeast-mating procedure, we restricted the mHCN1 C-terminus region interacting with filamin A to a stretch of 22 amino acids downstream the CNBD (aa 694-715). This region comprises a di-proline sequence (aa 695-696), which could potentially contribute to protein-protein interaction with filamin A; this was however ruled out by the evidence that its mutation (PP-AA) did not disrupt the binding to filamin A (Fig. 3I).

A region homologous to this was not found in the C-termini of either the HCN2 or HCN4 isoforms, as verified by the lack of interaction with filamin A in the yeast mating assay of Fig. 3.
Although we did not investigate the interaction between HCN3 and filamin A, no region homologous to the HCN1 binding site was found by Clustal-W or Blast screening (not shown).

What is the function of the interaction between filamin A and HCN1? Binding of filamin A could be a factor affecting clustering of HCN1 channels in restricted regions of the cell membrane. As shown by the confocal images in Fig. 5 D,E, while HCN1 channels are normally distributed in a “hot spot”-like arrangement, in cells lacking filamin A dense, concentrated expression areas are lost and channels are evenly distributed on the membrane. Clusterization was due to the specific HCN1-filamin A interaction since it was not observed in either filamin− or filamin+ cells transfected with hHCN4 (Fig. 5 F, G). Clusterization was not due to the lower level of mHCN1 protein expression in A7 relative to M2 cells, since no clusterization was observed with hHCN4, which expressed equally well in A7 and M2 cells, but at a much lower level (less than 50%) than mHCN1 in A7 cells as quantified by measurement of mean normalized conductances.

Since filamin A binds D2 and D3 receptors and has been proposed to be functionally linked to downstream signaling components (26), our results suggest the intriguing possibility that filamin A contributes to the organization of subcellular macrocomplexes in which receptors, second messengers and effectors are in close proximity. Additional support to a function in channel localization and clustering comes from evidence that filamin A binds caveolin 1, a structural protein responsible for the organization of caveolae (25). Caveolae represent a type of lipid rafts, spatially delimited membrane microdomains known to compartmentalize membrane proteins in macromolecular complexes. For example, proteins of the β-adrenergic transduction pathway localize to lipid rafts in cardiac membranes (40).
Of the 4 HCN isoforms known, HCN1 is the least sensitive to cAMP, and shifts of the channel activation curve caused by saturating cAMP concentrations are of the order of only a few mV (11,4,41,32). Since we find that only HCN1 binds filamin A, this appears to contrast with the hypothesis that interaction with filamin A serves the purpose of concentrating elements of the cAMP second messenger cascade in restricted locations for improved channel cAMP-dependent modulation. However, most tissues expressing HCN channels possess more than one isoform (8), and heteromers can be formed by co-assembly of HCN1 and HCN2 (41,42), or HCN1 and HCN4 (32). An intriguing possibility is therefore that HCN1 subunits endow heteromeric channels with an “anchoring” function whose aim is to localize channels in specific membrane subdomains, while the “modulating” function would rely on the cAMP sensitivity of HCN2 or HCN4 subunits.

From our data in Fig. 5 C,E,F,G it also appears that the interaction with filamin A is not necessary for channel translocation from the endoplasmic reticulum to the plasma membrane. Indeed, block of protein synthesis by cycloheximide did not cause accumulation of fluorescence in the endoplasmic reticulum of M2 cells, indicating that channels can reach the membrane or its proximity even in the absence of filamin A.

The lack of interaction between filamin and mHCN1 caused not only a redistribution of the channel in the membrane but also a change in the properties of the expressed current. In particular, in filamin− cells the HCN conductance was twice that in filamin+ cells, and both channel activation and deactivation were approximately twice faster (Fig. 6). In agreement with the view that HCN1 properties are modified by specific interaction with filamin A, we found no differences in the properties of HCN4 channels expressed in A7 or M2 cells. An increased conductance is consistent with the idea that filamin A concentrates channel expression in specific, locally restricted areas, thus limiting random access of channels to the
membrane and thus decreasing the mean channel density.

On the other hand, we do not have a ready explanation for the slowing action of filamin A binding to channels. However, there is established evidence that the C-terminus is involved in determining the kinetics of HCN channels (4,43,44), and the slowing due to binding of the C-terminus to filamin A might reflect an interference with the mechanism by which C-termini affect gating.

In conclusion, our results show that HCN1, but not HCN2 or HCN4, binds to an ubiquitous isoform of filamin, filamin A. The interaction involves the C-terminal end of HCN1 and the last two Ig-like repeats at the C-terminal end of filamin A, the site of the protein dimerization domain, whereas the actin-binding domains of the protein remain free. Binding to filamin A slows HCN1 channel kinetics and causes channels to cluster within restricted regions of the cell membrane, thus reducing the density of channel expression and whole-cell conductance. A possible function of HCN1 channel compartmentation is to increase the efficiency of channel control by modulating agents.

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FIGURE LEGENDS

Fig 1. Yeast two-hybrid interaction of filamin A with mHCN1. A, yeast mating procedure used to verify the interaction between the mHCN1 C-terminus (bait, aa 657-910) and filamin A (prey) yielded positive coupling (panel 1); positive (T large antigen + p53) and negative (T large antigen + laminin) controls are also shown in panels 2 and 3, respectively. B, the drawing represents the schematic structure of filamin A which comprises 24 Ig-like repeats, the actin-binding domain (ABD) and two hinge regions; the segments found to interact with mHCN1 by the yeast two-hybrid screening protocol and the numbers of times they were retrieved are also shown.

Fig 2. In vivo interaction of filamin A with HCN1. Co-immunoprecipitation from bovine brain was performed to verify in vivo interaction between the two proteins. Top: proteins precipitated with the anti-filamin A antibody were loaded in lane 1 and tested for the presence of HCN1. Anti-HCN1 antibody revealed a band near 116 KDa. As a positive control, total membrane proteins of bovine brain were tested in lane 2 with the same antibody and a similar band was detected. Bottom: all lanes 3 to 7 were loaded with proteins precipitated with the anti-filamin A antibody and incubated as follows: 3, anti filamin A antibody; 4, secondary anti- mouse antibody only (without primary antibody); 5, rabbit preimmune serum; 6, secondary anti- rabbit antibody only (without primary antibody); 7, anti-HCN1 antibody (as in lane 1).

Fig 3. Yeast protein-protein interaction assay. A-K, the interaction of filamin A with various portions of mHCN1 C-terminus (A-I) or with the C-termini of mHCN2 or hHCN4 (J-K) was tested by yeast mating. Only yeasts where an interaction occurred could grow on plates with high
HCN1 interaction with filamin A

stringency medium. From A to I, the mHCN1 C-terminal sequences tested are indicated with bars atop each panel; the shortest interacting domain found for mHCN1 spanned the region aa 694-715 (H). In B, the control aa 657-910 mHCN1 sequence included the mutation 812 PSLP→ASAA 815; in I, the aa 657-715 mHCN1 sequence included the mutation 695 PP→AA 696; in neither case did the mutation inhibit the interaction. In J and K, C-terminal sequences of mHCN2 (aa 429-709) and hHCN4 (aa 756-1204), respectively, were tested and did not show interaction.

L, aminoacid sequence of the C-terminus of mHCN1 (from residue 391 to 910), showing the location of the filamin A-interacting domain (white characters, aa 694-715) and that of the CNBD (bold characters, aa 471-590).

**Fig 4.** Protein-protein interaction assay by *in vitro* transcription and translation. From A to G, the same HCN constructs as in Fig. 3 (A, C to H) were tested for *in vitro* interaction with filamin A. The constructs were fused in-frame to a tag (HA or C-myc) for immunoprecipitation and were radio-labeled during *in vitro* translation with L-[35S]-Methionine.

**Fig 5.** Specificity of mHCN1-filamin A interaction. A, coimmunoprecipitation of mHCN1 and filamin A in extracts from filamin+ (A7) and filamin- (M2) cells. Proteins were immunoprecipitated with anti-filamin A antibody from cells expressing mHCN1 (left) or from control cells (right). The presence of filamin A and HCN1 was checked by western blot. B to G, immunofluorescence from A7 and M2 cells transfected with GFP-tagged mHCN1 and hHCN4 channels. B, C, significant levels of mHCN1 fluorescence signal were detected from both A7 (B) and M2 (C) types of cells. D, E, confocal images (single sections) of mHCN1-transfected A7 and M2 cells treated with cycloheximide to block protein synthesis. mHCN1 channels were clustered.
in filamin+ cells, but were more evenly distributed on the cell membrane in filamin- cells. Nuclei were labeled with DAPI. F, G, confocal images (single sections) of hHCN4-transfected A7 and M2 cells treated with cycloheximide; even channel distribution is apparent on the membrane of both types of cells. Bar is 20 µm throughout.

**Fig 6. Characterization of mHCN1 current expressed in A7 and M2 cells.** A, representative sets of current traces recorded from filamin+ (A7, left) and filamin- cells (M2, right). On average, cells lacking filamin A showed a significant increase in conductance relative to filamin-expressing cells (see mean values in text). B, activation curves were measured in A7 (filled squares) and M2 cells (empty circles) and averaged. Lines are Boltzmann fits plotted with the mean $V_{1/2}$ and $s$ parameters. C, mean activation (filled symbols) and deactivation (open symbols) time constant curves measured in M2 (circles) and A7 cells (squares). Lines drawn through points. In cells lacking filamin A both activation and deactivation kinetics were significantly faster at all voltages. Protocols for measurements in B and C are detailed in Materials and Methods.
Table 1. **Summary of the HCN C-terminal constructs used to test for interaction with filamin A.** Data were obtained with either yeast two-hybrid or *in vitro* transcription/ transduction (T/T) assay. The first 9 rows refer to HCN1 constructs. The HCN1 constructs “aa 657-910 mut” and “aa 657-715 mut” carry the mutations 812 PSLP-ASAA 815 and 695 PP-AA 696, respectively. Positive interaction in yeast-two hybrid protocols is indicated with ++ or +++ according to rate of growth; - indicates no growth. HCN2 and HCN4 constructs were not employed in *in vitro* T/T experiments.
Fig. 1
HCN1 interaction with filamin A

Fig. 2

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Fig. 3
Fig. 4
HCN1 interaction with filamin A

Fig. 5
HCN1 interaction with filamin A

Fig. 6
Interaction of the pacemaker channel HCN1 with filamin A
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