Hyperpolarization-Activated Cation Channels: From Genes to Function

MARTIN BIEL, CHRISTIAN WAHL-SCHOTT, STYLIANOS MICHALAKIS, AND XIANGANG ZONG

Center for Integrated Protein Science CIPS-M and Zentrum für Pharmaforschung, Department Pharmazie, Pharmakologie für Naturwissenschaften, Ludwig-Maximilians-Universität München, Munich, Germany

I. Introduction
II. A Short Overview on Basic Biophysical Properties of $I_h$
   A. Channel gating by membrane hyperpolarization
   B. Modulation by cyclic nucleotides
   C. Ion selectivity
   D. Pharmacological profile
III. The HCN Channel Family
   A. Transmembrane segments and voltage sensor
   B. Pore loop and selectivity filter
   C. Cyclic nucleotide-binding domain and C-linker
   D. Dual channel gating
   E. Functional differences between HCN channel types
IV. Regulation of HCN Channels
   A. Regulation by acidic lipids
   B. Regulation by protons
   C. Regulation by chloride
   D. Regulation by Src kinase-mediated tyrosine phosphorylation
   E. Regulation by p38-mitogen-activated protein kinase
   F. Transmembrane and cytosolic proteins interacting with HCN channels
V. Tissue Expression of HCN Channels
VI. Physiological Roles of HCN Channels in Neurons
   A. Principles
   B. Role of $I_h$ in dendritic integration
   C. Role of $I_h$ in working memory
   D. Role of $I_h$ in constraining hippocampal LTP
   E. Role of $I_h$ in motor learning
   F. Role of $I_h$ in synaptic transmission
   G. Role of $I_h$ in resonance and oscillations
   H. Role of $I_h$ in the generation of thalamic rhythms
VII. Role of $I_h$ Channels in Cardiac Rhythmicity
   A. HCN4
   B. HCN2
   C. Conclusions and open questions
VIII. Role of $I_h$ in Disease
   A. Inherited channelopathies
   B. Transcriptional channelopathies
IX. HCN Channels as Novel Drug Targets
   A. Heart rate-reducing agents
   B. Blockers of neuronal $I_h$
X. Conclusions and Future Directions

Biel M, Wahl-Schott C, Michalakis S, Zong X. Hyperpolarization-Activated Cation Channels: From Genes to Function. Physiol Rev 89: 847–885, 2009; doi:10.1152/physrev.00029.2008.—Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels comprise a small subfamily of proteins within the superfamily of pore-loop cation channels. In mammals, the HCN channel family comprises four members (HCN1-4) that are expressed in heart and nervous system. The current produced by HCN channels has been known as $I_h$ (or $I_f$ or $I_q$). $I_h$ has also been
I. INTRODUCTION

Since their first discovery in sinoatrial node cells (49, 51, 113, 116, 430) and neurons (169, 234) in the late 1970s and early 1980s, hyperpolarization-activated currents ($I_h$) have sparked continuing interest of physiologists and researchers working in biomedical disciplines. The exceptional position of $I_h$ among ionic currents mainly arises from its unique ion selectivity and gating properties. $I_h$ is a mixed cationic current carried by Na$^+$ and K$^+$. Since its reversal potential is around $-20$ mV at physiological ionic conditions (114), $I_h$ is inwardly directed at rest and, hence, depolarizes the membrane potential. However, unlike the vast majority of cellular conductances that are activated upon membrane depolarization, $I_h$ is activated by hyperpolarizing voltage steps to potentials negative to $-55$ mV, near the resting potential of cells. In addition, activation of $I_h$ is facilitated by cAMP in a direct, protein kinase A (i.e., phosphorylation)-independent fashion. Because of its quite unusual biophysical profile, the hyperpolarization-activated current was designated “funny” current ($I_f$) in heart (49), while other researchers used the term $I_h$ (for “queer”) instead of $I_f$ for the neuronal current (169). In this review, we will generally use the term $I_h$.

Many physiological roles have been attributed to $I_h$. First and foremost, the properties of $I_h$ suggested that the current plays an important role in the initiation and regulation (49, 117) of the heart beat (“pacemaker current”) (51, 113, 430). However, even now, 30 years after this hypothesis has been raised, strong controversy over the exact role of $I_h$ in cardiac pacemaking exists. Genetic studies in men and mice (see sect. vii) will be very instrumental in solving this physiological conundrum (171, 174, 226, 370). $I_h$ is also involved in the control of rhythmic activity in neuronal circuits (128, 221, 222, 258, 261, 355) (e.g., in thalamus) and contributes to several other basic neuronal processes, including determination of resting membrane potential (98, 125, 226, 230, 266, 291–293, 300), dendritic integration (239–241, 423), synaptic transmission (32, 33), and the temporal processing of visual signals in the retina (105, 106, 209, 247).

The ion channels underlying $I_h$ have been discovered about a decade ago (153, 192, 211, 227, 228, 251, 332, 333, 341, 344, 400). With reference to their complex dual gating mode (7, 93, 112), these proteins were termed hyperpolarization-activated cyclic nucleotide-gated (HCN) channels.

In mammals, the HCN channel family comprises four distinct members (HCN1-4). Currents obtained after heterologous expression of the cDNAs of HCN1-4 channels reveal the principal features of native $I_h$, confirming that HCN channels indeed represent the molecular correlate of $I_h$.

In the first three sections of this review, we give a brief overview on the biophysical properties of $I_h$/HCN currents and correlate these properties with structural domains of HCN channels. Readers who are interested in getting a more detailed and complete overview on the mentioned topics are referred to a number of excellent reviews that extensively deal with these issues and also describe the discovery of $I_h$ and the advance in the $I_h$ field from a more historical point of view (29, 40, 82, 93, 152, 226, 300, 324, 334). Section iv is a first important focus of this review, presenting recent data on the cellular regulation of HCN channels. Section v briefly summarizes the data available on the tissue expression of HCN channels. The major focus of this review is on the roles of HCN channels in neuronal function and cardiac rhythmicity (sects. vi and vii). We discuss these issues in the context of recent results from the analysis of HCN channel knockout mice. Finally, we give an overview on the role of HCN channels in the pathologies of human diseases (sect. viii) and discuss pharmacological agents interacting with these proteins (sect. ix).

II. A SHORT OVERVIEW ON BASIC BIOPHYSICAL PROPERTIES OF $I_h$

Native $I_h$ as well as the currents induced by heterologously expressed HCN channels are characterized by four major hallmark properties: 1) channel activation by membrane hyperpolarization, 2) facilitation of channel activation by direct interaction with cAMP, 3) permeation of Na$^+$ and K$^+$, and 4) a specific pharmacological profile that includes sensitivity to external Cs$^+$ concurred with relative insensitivity to Ba$^{2+}$ (Fig. 1).
A. Channel Gating by Membrane Hyperpolarization

In general, \( I_h \) currents activate with hyperpolarizing steps to potentials negative to \(-50\) to \(-60\) mV (Fig. 1A). Unlike most other voltage-gated currents, \( I_h \) does not display voltage-dependent inactivation. Typically, two kinetic components can be distinguished upon activation of \( I_h \): a minor instantaneous current (\( I_{\text{INS}} \)) (236, 237, 312), which is fully activated within a few milliseconds, and the major slowly developing component (\( I_{\text{SS}} \)) that reaches its steady-state level within a range of tens of milliseconds to several seconds under fully activating conditions. While there is no doubt that \( I_{\text{SS}} \) is generated by cations passing the well-characterized pore of HCN channels, the ionic nature of \( I_{\text{INS}} \) is a matter of current dispute. \( I_{\text{INS}} \) is not consistently observed in all measurements of \( I_h \), and if so, its amplitude is usually small.

However, in some neurons, a large \( I_{\text{INS}} \) lacking time-dependent \( I_{\text{SS}} \) has been reported (12, 100, 326). Speculations on the nature of \( I_{\text{INS}} \) reach from models where this current represents a leak conductance or an experimental artifact to models in which \( I_{\text{INS}} \) is caused by a second pore that is found within the same HCN channel that produces \( I_{\text{SS}} \) or a second channel population associated with HCN channels (236, 237, 312). Depending on the cell type, the activation of \( I_{\text{SS}} \) can be empirically described by either a single (120, 136, 200, 261, 264, 355, 393, 425) or double exponential function (17, 104, 131, 175, 248, 402). As mentioned above, kinetics of \( I_{\text{SS}} \) are quite variable (111, 118). While most \( I_h \) channels activate quite slowly with time constants (\( \tau_{\text{act}} \)) ranging between hundreds of milliseconds and seconds (131) (Fig. 1B, inset), there are also currents with profoundly faster activation (17, 169, 175, 234, 248, 264), for example, in hippocampal CA1 neurons, where \( \tau_{\text{act}} \) values in the range of \( 30\)–\(50\) ms have been found (146, 300, 324). The diversity of \( \tau_{\text{act}} \) probably results from an interplay of several factors. First of all, the differences reflect the diverse intrinsic activation properties of distinct HCN channel isoforms underlying the \( I_h \) of a given cell type. Second, there is growing evidence that the cellular microenvironment that fine-tunes HCN channel activity (e.g., auxiliary subunits, concentration of cellular factors, etc.) can profoundly vary from cell type to cell type. Third, \( I_h \) measurements are highly sensitive to experimental conditions (e.g., pH, temperature, patch configuration, expression system, ionic composition of solutions, etc.), a circumstance that may explain that even for the same channel different kinetics have been found by different laboratories. As mentioned above, \( I_h \) activates at around resting membrane potential. The voltage dependence of activation shows a typical S-shaped dependence that can be fit using Boltzmann functions (20, 25, 131, 175, 200, 216, 234, 253, 261, 354, 357, 393) (Fig. 1B). Such fits reveal half-maximal activation ("midpoint") potentials (\( V_{0.5} \)) of around \(-70\) to \(-100\) mV in most cell types. However, like for \( \tau_{\text{act}} \), \( V_{0.5} \) values can vary profoundly in vivo (28, 111, 118). For example, \( V_{0.5} \) of \( I_h \) recorded in sinoatrial node cells is in the range of \(-60\) to \(-70\) mV (3–5, 121, 124, 167) while currents found in ventricular cardiomyocytes activate at much more hyperpolarized voltages with \( V_{0.5} \) being negative to \(-90\) to \(-140\) mV (138, 319, 346). Like for \( \tau_{\text{act}} \), the strikingly large range of \( V_{0.5} \) values probably results from the combination of intrinsic parameters such as the HCN channel isoform and modulatory factors present in a given cell type, and extrinsic...
factors such as the experimental settings during $I_h$ measurements.

B. Modulation by Cyclic Nucleotides

The second key feature of $I_h$ is its regulation by cyclic nucleotides (122; Fig. 1B). Hormones and neurotransmitters that elevate cAMP levels facilitate activation of $I_h$ by shifting $V_{0.5}$ values to more positive values and by accelerating the opening kinetics (25, 41, 49, 50, 122, 136, 151, 203, 216–218, 260, 262, 303, 391). It has been shown that the acceleration of the opening kinetics with cAMP can be attributed to the shift in voltage dependence of activation (412). Thus, in the presence of high cAMP concentrations, $I_h$ channel opening is faster and more complete than at low cAMP levels (Fig. 1B, inset). Conversely, neurotransmitters that downregulate cAMP inhibit $I_h$ activation by shifting its activation curve to more hyperpolarized voltages (117, 123, 124, 147, 190, 299, 315, 318). The range of $V_{0.5}$ shift induced by saturating cAMP concentrations is quite large (0–20 mV), depending on the cell type (25, 41, 49, 50, 122, 136, 151, 203, 216–218, 260, 262, 303, 384, 391) and the expressed $I_h$ channel isofrom (192, 227, 228, 333, 341). The regulation by cellular cAMP level is of key importance for the specific role that $I_h$ channels fulfill in physiological settings, since it enables these proteins to operate as transmembrane integrators of electrical (membrane potential) and chemical (hormones and neurotransmitters) inputs. Most notably, the cAMP-mediated modulation of $I_h$ channel activity is considered to play a major role in the up- or downregulation of the heart rate during sympathetic stimulation and muscarinic regulation of heart rate at low vagal tone (49, 50, 117, 122–124).

As mentioned above, it was this specific physiological function that earned $I_h$ the name “pacemaker” current. There is also good evidence that cAMP-dependent modulation of $I_h$ is of crucial significance in some neuronal circuits, e.g., in sleep-related thalamocortical circuits (231). There are a few studies showing that $I_h$ can also be regulated by nitric oxide (NO)-mediated increase of cGMP levels in brain (302) and heart (283). However, so far the physiological role of this kind of modulation remains unclear. Like cAMP, cGMP shifts the voltage dependence of channel activation to more positive values (Fig. 1B) (153, 227). While the extent of the shift is similar for both cyclic nucleotides (i.e., both cyclic nucleotides have the same efficacy at least in mammalian $I_h$), the apparent affinities of $I_h$ are ~10- to 100-fold higher for cAMP (range of $K_a$ = 60–500 nM; Fig. 1C; Ref. 227) than for cGMP ($K_a$ = 6 μM for HCN2; Ref. 227). The principal mechanism by which cyclic nucleotides regulate $I_h$ channel gating was uncovered by DiFrancesco and Tortora (122) in the early 1990s using current recordings in excised patches of sinoatrial node cells. It came as a big surprise that in contrast to many other ion channels that are regulated by cAMP via protein kinase A (PKA)-mediated serine or threonine phosphorylation (304, 337, 433), $I_h$ channels are activated by cAMP independent of phosphorylation (122). Like in the structurally related cyclic nucleotide-gated (CNG) channels (39, 93, 201), $I_h$ channels are activated by cAMP binding to a cyclic nucleotide-binding domain (CNBD) on the COOH terminus of the channel (443). The molecular details underlying the modulation of $I_h$ channels by cAMP have been extensively studied when cloned $I_h$ (HCN) channels were available and will be discussed later. There also are some reports on PKA-mediated phosphorylation of $I_h$ channels (79, 403); however, phosphorylation could not yet be confirmed on the molecular level in HCN channels. Moreover, the physiological relevance of PKA-induced $I_h$ phosphorylation remains elusive.

C. Ion Selectivity

$I_h$ is a mixed cation current that is carried by both K$^+$ and Na$^+$ under normal physiological conditions (110, 116, 145, 227, 333, 424). The ratio of the Na$^+$ to K$^+$ permeability of the channel ($P_{Na}/P_K$) ranges from 1:3 to 1:5, yielding values for the reversal potential between −25 and −40 mV (Fig. 1D) (20, 25, 31, 60, 95, 103, 113, 131, 175, 180, 200, 203, 227, 253, 261, 264, 301, 333, 341, 354, 357, 383, 391, 393, 402, 424). As a consequence, activation of $I_h$ at resting membrane potentials results in an inward current carried mainly by Na$^+$, which depolarizes the membrane toward threshold for firing of action potentials. $I_h$ is almost impermeable to Li$^+$ (180, 424) and is blocked by Cs$^+$ (109, 137, 227). K$^+$ is not only a permeating cation, it also affects the permeation of Na$^+$ (20, 95, 103, 109, 132, 145, 175, 200, 227, 234, 248, 250, 261, 354, 357, 383, 385, 424). Both the current amplitude and the $P_{Na}/P_K$ ratio of $I_h$ channels depend on the extracellular K$^+$ concentration (Fig. 1D) (227). An increase in extracellular K$^+$ concentration results in a strongly increased current amplitude and in a slightly reduced selectivity for K$^+$ over Na$^+$ (145, 424). The interdependence of Na$^+$ and K$^+$ permeation of $I_h$ channels is illustrated by the finding that the channels conduct little, if any, Na$^+$ in the absence of K$^+$. In contrast, current modifications upon reduction of extracellular Na$^+$ levels are merely the result of the altered driving force (116, 300). Although $I_h$ channels do not conduct anions, their conductance is sensitive to external Cl$^-$ levels (144, 411) (see sect. vC for further discussion). For a long time, only monovalent cations were expected to permeate through the $I_h$ channels. However, there is recent evidence for a small but significant Ca$^{2+}$ permeability of these channels (440, 441). At 2.5 mM external Ca$^{2+}$, the fractional Ca$^{2+}$ current of native $I_h$ as well as that of heterologously expressed HCN2 and HCN4 channels is

Physiol Rev • Vol. 89 • July 2009 • www.prv.org
The functional relevance of Ca\(^{2+}\) entry through \(I_h\) channels is not clear at the moment. It was assumed that in dorsal root ganglion neurons, Ca\(^{2+}\) influx through \(I_h\) channels at negative potentials contributes to activity-evoked secretion (441).

There is an ongoing controversy on the size of the single-channel conductance of \(I_h\). Originally, single-channel conductance was found to be very low, in the range of \(~1\) pS (110). This estimate is in good agreement with very recent data (210). However, single-channel conductances that are 10–30 times higher have been reported for cloned HCN channels (267) as well as for native cardiac (267) channels, that are 0.5% (440, 441). The functional relevance of Ca\(^{2+}\) influx through \(I_h\) channels is not clear at the moment. It was assumed that in dorsal root ganglion neurons, Ca\(^{2+}\) influx through \(I_h\) channels at negative potentials contributes to activity-evoked secretion (441).

In all mammals investigated so far, four homologous HCN channel subunits (HCN1-4) exist. HCN1-4 are also present in the genome of fishes (e.g., Tetraodon nigroviridis and Takifugu rubripes). Analysis of genomic sequences suggests that HCN2–4 genes underwent duplications in the fish lineage increasing the number of potential HCN species (194). HCN homologs have also been cloned from several invertebrates including arthropods [e.g., spiny lobster (157), insects (158, 211, 251)] and sea urchins (149, 153). The overall characteristics of the \(I_h\) currents from these species are strikingly similar to \(I_h\) currents from mammals. Notably, HCN channels are not present in C. elegans and yeast and also have not been found in a prokaryotic genome.

Like other pore-loop channels, HCN channels are complexes consisting of four subunits that are arranged around the centrally located pore (Fig. 2). These subunits form four different homotetramers with distinct biophysical properties (192, 227, 228, 333, 341). There is evidence that the number of potential HCN channel types is increased in vivo by the formation of heterotetramers (8, 85, 135, 278, 397, 420, 428). So far, splicing variants of vertebrate HCN channels were not identified. In contrast, alternative splicing of HCN channel transcripts was reported for some invertebrates, including spiny lobster, C. elegans, and yeast, and also have not been found in a prokaryotic genome.

\[\text{FIG. 2. Structure of HCN channels. HCN channels are tetramers.}\]

HCN channels represent the molecular correlate of \(I_h\). Together with CNG channels and the Eag-like K\(^{+}\) channels (39, 93, 201), HCN channels form the subgroup of cyclic nucleotide-regulated cation channels within the large superfamily of the pore-loop cation channels (437).

HCN channels have been cloned from vertebrates and several invertebrates (153, 192, 211, 227, 228, 251, 332, 333, 341, 344, 400) but are missing in Caenorhabditis elegans, yeast, and prokaryotes.

\[\text{D. Pharmacological Profile}\]

\(I_h\) channels are almost completely blocked by low millimolar concentrations of Cs\(^{+}\) (Fig. 1, E and F) (109, 137, 227). Inwardly rectifying K\(^{+}\) currents that activate over a similar voltage range as \(I_h\) are also blocked by this cation (99, 288, 289). However, in contrast to these currents, \(I_h\) is insensitive to millimolar concentrations of external Ba\(^{2+}\) and tetraethylammonium (TEA) (227). \(I_h\) is also insensitive to 4-aminopyridine, a blocker of voltage-gated K\(^{+}\) channels (227). A number of organic blockers have been described to block \(I_h\) channels. Among these, the most specific ones are bradycardic agents such as ivabradine that block the \(I_h\) channels in the low micromolar range (see sect. IX).

\[\text{III. THE HCN CHANNEL FAMILY}\]

HCN channels represent the molecular correlate of \(I_h\). Together with CNG channels and the Eag-like K\(^{+}\) channels (39, 93, 201), HCN channels form the subgroup of cyclic nucleotide-regulated cation channels within the large superfamily of the pore-loop cation channels (437). HCN channels have been cloned from vertebrates and several invertebrates (153, 192, 211, 227, 228, 251, 332, 333, 341, 344, 400) but are missing in Caenorhabditis elegans, yeast, and prokaryotes.
**Drosophila melanogaster**, and *Apis mellifera* (156, 159, 298). Each HCN channel subunit consists of three principal structural modules: the transmembrane core and the cytosolic NH₂-terminal and COOH-terminal domains (Fig. 2). The transmembrane core harbors the gating machinery and the ion-conducting pore while the proximal part of the cytosolic COOH-terminal domain consisting of the CNBD and the peptide that connects the CNBD with the transmembrane core (the "C-linker") confers modulation by cyclic nucleotides (93) (Fig. 2). The transmembrane core and the proximal COOH terminus allosterically interact with each other during channel gating and reveal a high degree of sequence conservation within the HCN channel family (sequence identity of ~80–90% between HCN1-4) (28, 202). In contrast, cytosolic NH₂ termini and the sequence downstream of the CNBD vary considerably in their length and share only modest to low homology between various HCN channels (28).

### A. Transmembrane Segments and Voltage Sensor

The transmembrane channel core of HCN channels consists of six α-helical segments (S1-S6) and an ion-conducting pore loop between S5 and S6 (Fig. 2). A highly conserved asparagine residue in the extracellular loop between S5 and the pore loop is glycosylated (N380 in murine HCN2; Fig. 2). This posttranslational channel modification was shown to be crucial for normal cell surface expression (278). The voltage sensor of HCN channels is formed by a charged S4-helix carrying nine arginine or lysine residues regularly spaced at every third position (81, 399). Positively charged S4 segments are found in all voltage-dependent members of the pore-loop cation channel superfamily (436). However, inward movement of S4 charges through the plane of the cell membrane leads to opening of HCN channels while it triggers the closure of depolarization-activated channels such as the Kv channels (245). The molecular determinants underlying the different polarity of the gating mechanism of HCN and depolarization-gated channels remain to be clarified. However, there is initial evidence that the loop connecting the S4 with the S5 segment plays a crucial role in conferring the differential response to voltage (102, 224, 314) (Fig. 2).

Recently, it was shown that the voltage-dependent activation of the HCN channel cloned from sea urchin sperm (spHCN) can shift between two modes depending on the previous activity (53, 133, 246). In mode I, gating charge movement and channel opening occur at very negative potentials, while in mode II, both processes are shifted to >50 mV more positive potentials (133). The transition from mode I to mode II is favored in the open state, while the transition from mode II to mode I preferentially occurs in the closed state. The shift between these two modes also affects the kinetics of the channel activation and deactivation. The fact that these channels are differentially sensitive to the direction of the voltage change and that the activation curves in mode I and mode II are separated by up to 50 mV is defined as "voltage hysteresis." This interesting gating behavior of spHCN is probably the result of a slow conformational change attributable to lateral movement of S4 that stabilizes the inward position of S4 upon hyperpolarization (52, 53, 133, 245, 246, 405). HCN1 channels behave similarly as spHCN channels with respect to hysteresis behavior and mode shift (246). In contrast, for HCN2, the voltage hysteresis is less pronounced, but the effects of the mode shift on the deactivation kinetics are present. For HCN4, only the changes in deactivation kinetics are observed under certain conditions using high K⁺ concentrations in the extracellular recording solution. It has been suggested that the mode shift is important for short-term, activity-dependent memory in HCN channels (53).

### B. Pore Loop and Selectivity Filter

As pointed out before, *Iₜ* channels slightly select K⁺ over Na⁺ and carry an inward Na⁺ current under physiological conditions (Fig. 2). Given this particular ion selectivity profile, it came as a big surprise that the pore loop sequence of HCN channels is closely related to that of highly selective K⁺ channels (436). Notably, the pore contains the glycine-tyrosine-glycine (GYG) motif that in K⁺ channels forms the selectivity filter for K⁺ (129, 448) (Fig. 2). Thus, based on sequence analysis, HCN channels are expected to be selective for K⁺ and to exclude Na⁺ from permeation. Also, conduction of divalent cations is not consistent with current models, since a ring of acidic residues (glutamate or aspartate) that is present in the pore of Ca²⁺-permeable channels is missing in HCN channels (343, 431, 442). Several attempts have been made, mainly using site-directed mutagenesis approaches, to identify residues in the pore of HCN channels that confer the unique permeation properties of these channels (19, 135, 237, 278, 428). However, these efforts were quite unsuccessful. A major problem is that the pore region turned out to be extremely sensitive to mutagenesis and that even subtle mutations can lead to nonconducting channels. Clearly, a high-solution crystal structure will be required to establish a conclusive model of ion permeation in HCN channels.

### C. Cyclic Nucleotide-Binding Domain and C-Linker

Regulation of HCN channels by cAMP is mediated by the proximal portion of the cytosolic COOH terminus (443) (Fig. 2). This part of the channel contains a cyclic
nucleotide-binding domain composed of ~120 amino acids (CNBD) and an 80-amino acid-long C-linker region that connects the CNBD with the S6 segment of the channel core (Figs. 2 and 3). Evolutionarily, the CNBD is an ancient protein domain that has been conserved across a wide range of proteins, including bacterial transcription factors (e.g., CAP; Ref. 419), cAMP- and cGMP-dependent protein kinases (307, 387), cAMP-dependent guanine nucleotide exchange factors (Epac; Ref. 321), and CNG channels (39, 201). The determination of the crystal structure of the C-linker-CNBD of HCN2 in its cAMP- and cGMP-bound form (443) and of spHCN in its cAMP-bound form (142) has provided important insights into the molecular determinants of cyclic nucleotide-dependent channel modulation. So far, high-resolution structures of unliganded CNBDs from HCN channels are not available. The structures of the two liganded C-linker-CNBD domains of HCN2 were virtually identical, with the only difference that cAMP binds in its anti conformation while cGMP binds in the syn conformation (443). The CNBD shows a highly conserved fold consisting of an initial α-helix (A-helix), followed by an eight-stranded antiparallel β-roll (β1-β8), a short B-helix, and a long C-helix (Figs. 2 and 3). The C-linker consists of six α-helices (A’–F’) (Figs. 2 and 3). Four C-linker CNBDs are assembled to a complex with a fourfold axis of symmetry that is consistent with the tetrameric architecture of pore-loop channels. Most of the subunit-subunit interactions in the tetramer are mediated by amino acid residues in the C-linker. The binding pocket for cAMP is formed by a number of residues at the interface between the β-roll and the C-helix (142, 443, 446). Within the binding pocket of HCN2, a group of seven amino acids interacts with ligand (446) (Fig. 3).

Three of these residues are located in the β-roll (R591, T592, and E582 in HCN2) and four in the C-helix (R632, R635, 1636, and K638 in HCN2). Among these, only one key residue of the C-helix (R632) was identified to control the efficacy by which cyclic nucleotides enhance channel opening (446). Four residues in the C-helix (e.g., R632, R635, 1636, and K638) contribute to the higher selectivity of HCN2 channels for cAMP than for cGMP (446). However, it is not clear how these residues generate cAMP selectivity. It was suggested that cAMP selectivity at least partially is not due to its preferential contacts with the protein, but rather reflects the greater hydration energy of cGMP relative to cAMP. This could result in a greater energetic cost for cGMP binding to the channel (447).

The CNBD is not only an important modulatory domain in HCN channels, like in the structurally related Eag-like K+ channels (18), it may also be important for normal cell surface expression of these proteins (6, 313). Recently, a four-amino acid motif (EEYP) in the B-helix of HCN2 was identified that strongly promotes channel export from the endoplasmatic reticulum and targeting to the cell membrane (287).

D. Dual Channel Gating

Unlike CNG channels, which obligatorily require binding of cAMP to open (39, 201), HCN channels are principally operated by voltage, i.e., membrane hyperpolarization is necessary and sufficient to activate these channels. cAMP and cGMP can be considered as stimulatory modulators (or coagonists) of HCN channels that do not open the channels in the absence of membrane hyperpolarization but rather facilitate voltage-dependent activation by shifting the voltage dependence of activation to more positive voltages (122). Electrophysiological studies in excised patches from sinoatrial node cells in-

**FIG. 3.** Sequence alignment of HCN2 and spHCN COOH terminus. Residues that have been reported by Zhou and Siegelbaum (446) to interact with cAMP are indicated in red. Residues that have been identified by Flynn et al. (142) to control cGMP efficacy in spHCN are indicated by arrows. Tyrosine residues that are phosphorylated by cSrc kinase are shown in blue (Y453 in HCN2 corresponds to Y531 in HCN4). α-Helices are indicated as green boxes, and the eight β-sheets are indicated as green arrows and numbered from 1–8.
dicated that the modulatory effect of cAMP is due to its direct binding to the channel rather than being conferred by protein phosphorylation (122). The identification of CNBDs in the primary sequence of HCN channels strongly supported this model and suggested that mechanistically there were probably similarities between the action of cyclic nucleotides in HCN and CNG channels. As discussed in the previous section, crystal structure data along with site-directed mutagenesis and molecular dynamics simulations allowed to identify structural determinants controlling cAMP and cGMP binding (142, 412, 414, 443, 446, 447).

There is now good evidence that “disinhibition” is the fundamental principle underlying the cAMP-dependent modulation of HCN channels. The C-linker-CNBD is an autoinhibitory domain that in the absence of cAMP lowers open probability. Binding of cAMP increases channel activity by removing tonic channel inhibition that is conferred by this domain (412, 414). The exact sequence of molecular events leading from initial cyclic nucleotide binding to facilitation of voltage-gated channel opening is still an unsolved issue. Addressing this problem is complicated by the fact that in the available crystal structures the C-linker seems to be caught in the resting conformation (i.e., the conformation that is occupied in the absence of cAMP) while the CNBD is in its active (i.e., cAMP-bound) conformation (93, 94, 198). According to a recent model (93), in the absence of cAMP, the C-linker is thought to be in a “compact” conformation that produces an inhibitory effect on channel opening. Binding of cAMP in turn induces a conformational change in the CNBD involving the C-helix. The conformational change in the C-helix is then coupled to the C-linker that occupies a more “loose” conformation leading to an alteration of the intersubunit interface between helices of neighboring subunits. The resulting change in the quaternary conformation removes the inhibition of the COOH terminus and destabilizes the closed state, thus promoting the opening of the channel. One would expect that the described activation process comes along with large translocations of subdomains in the C-linker-CNBD. However, recent data suggest that this may not be necessarily the case. Fluorescent resonance energy transfer (FRET) measurements in a CNG channel suggested that movements within the C-linker are probably subtle, involving only limited rearrangements (386).

The finding that cAMP facilitates activation of HCN channels by affecting their voltage dependence suggests that the gating mechanism operated by hyperpolarization and the one operated by cAMP are closely related to each other, if not identical. In other words, HCN channels behave in the presence of a given cAMP concentration simply as if they were experiencing a stronger voltage drop. Several kinetic models have been proposed to describe the tight interconnection between voltage- and cyclic nucleotide-dependent gating (for a recent overview, see Refs. 28, 93). Among these, cyclic allosteric models derived from the classic Monod-Wyman-Changeux (MWC) model developed for hemoglobin (274), currently give the best approximation to HCN channel behavior (7, 112). In its basic form, the MWC model assumes that each of the four subunits of the tetrameric channel is independently gated by voltage. Every time a voltage sensor switches to the activated state, the probability for channel opening increases. The opening/closing reactions occur allosterically and involve concerted transitions of all four subunits. This transition occurs if the channel is unliganded, partially liganded, or fully liganded and is energetically stabilized by a constant amount for each cAMP bound. The model further assumes that cAMP has a higher binding affinity to open than to closed channels (7, 112, 413). The model reproduces the characteristic features of HCN channel gating like the voltage dependence and the activation by cyclic nucleotides. In addition, several kinetic features are well described by the model, including the delay in current activation and deactivation. However, in its original form, the model also has several limitations. For example, there is evidence (86) that the final allosteric open/close transition in HCN2 channels is not voltage dependent as suggested previously (7). In addition, the mode shift and the voltage hysteresis of HCN channels cannot be explained by simple MWC models. To solve these issues, several extensions of the cyclic allosteric model have been proposed among which the dimer-of-dimers model (396), the circular four-state model by Männikkö et al. (246), and an extension of the MWC model made by Zhou and Siegelbaum (446) are the most recent ones. Despite the progress that clearly has been made in mathematically modeling HCN channel gating, it is obvious that these approaches have to be accompanied by new high-resolution structures of HCN channels in closed and open state(s) to achieve a full understanding of channel behavior.

E. Functional Differences Between HCN Channel Types

All four mammalian HCN channel subtypes have been expressed in heterologous expression systems and shown to induce currents that display the principal biophysical properties of native I_{h} (153, 192, 211, 227, 228, 333, 341, 400). However, the individual HCN currents quantitatively differ from each other with respect to their respective activation time constants (τ), their steady-state voltage dependence, and the extent of cAMP-dependent modulation. Before we will discuss these issues in more detail, it is important to recall that at least some of the reported differences may not reflect the intrinsic biophysical diversity of individual HCN channel types. Instead,
these differences rather can be attributed to the fact that measurements of $I_h$ are very sensitive to experimental parameters and that the same HCN channel may be embedded in quite diverse modulatory networks, depending on the expression system or cell type (111, 118). The intrinsic sensitivity to external and internal factors also makes it difficult to unambiguously match the $I_h$ characterized in a native type with $I_h$ currents induced by a heterologously expressed HCN channel isoform. Assembly of different HCN channel subtypes to heterotetrameric channel complexes may further increase heterogeneity of $I_h$ channels.

Within the HCN channel family, HCN1 is the subtype with the fastest kinetics (191, 331, 372). Like in native $I_h$ activation kinetics of HCN1 is strongly voltage dependent, with $\tau_{act}$ values ranging from 30 to 300 ms at $-140$ to $-95$ mV (191, 331, 372). HCN1 is also the HCN channel type with the most positive $V_{0.5}$ value (range $-70$ to $-90$ mV) (8, 28, 372). On the other hand, compared with HCN2 and HCN4, HCN1 reveals only a weak shift of the activation curve in the presence of saturating cAMP concentrations (+2 to $+7$ mV) (8, 372, 409, 412, 414). Taken together, these findings suggest that HCN1 activation is energetically favored compared with the other channels. One reason for this finding is that the tonic channel inhibition produced by the C-linker-CNBD is weaker in HCN1 than in HCN2 or HCN4. Indeed, deletion of the CNBD in HCN1 or HCN2 which is equivalent with a total removal of tonic inhibition resulted in channels with almost identical $V_{0.5}$ values (412). However, HCN1 lacking a CNBD still activates five- to sixfold faster than HCN2 lacking this domain. This shows that gating differences between both channels are not solely determined by the different degree of tonic inhibition conferred by the CNBD but that the opening of the core HCN2 channel is also inherently slower than that of HCN1 (412).

While HCN1 is the fastest member within the HCN channel family, HCN4 is by far the channel with the slowest opening kinetics (192, 228, 341). $\tau_{act}$ values for this channel range between a few hundred milliseconds at strongly hyperpolarized voltages ($-140$ mV) up to many seconds at normal resting potential ($-70$ mV) (192, 228, 341). HCN2 occupies an intermediate position with $\tau_{act}$ ranging from 150 ms to 1 s (228, 372). $V_{0.5}$ values between $-70$ and $-100$ mV have been reported for HCN2 and HCN4 (8, 28, 372). Both channels also have in common that their steady-state activation curves are very sensitive to cAMP with shifts of $V_{0.5}$ of 10–25 mV (8, 192, 228, 277, 341, 409, 412, 414, 443).

So far, electrophysiological measurements of HCN3-mediated currents have been only reported by a few groups (80, 271, 372). A major obstacle to a full characterization of this particular channel type is that the HCN3 protein tends to accumulate in intracellular compartments and reveals only weak cell surface expression (170; unpublished observations). $V_{0.5}$ of HCN3 ranges between $-80$ and $-95$ mV. Activation constants of 250–400 ms at $-140$ mV place the kinetics of HCN3 in between those of HCN2 and HCN4 (271, 372). Remarkably and unlike in all other HCN channels, cyclic nucleotides do not induce a positive shift of $V_{0.5}$ in HCN3. Human HCN3 was found to be insensitive to cAMP or cGMP (372), while the murine channel seems to be even slightly inhibited by cyclic nucleotides (shift of $V_{0.5}$ by $-5$ mV) (271). The structural determinants underlying this unexpected behavior are unclear. Interestingly, when the CNBD of HCN4 is replaced by the corresponding domain of HCN3, cAMP sensitivity is fully maintained, suggesting that the CNBD of HCN3 is principally able to bind cAMP and to mediate cAMP-dependent gating (372). Thus, within the HCN3 channel, the CNBD may be functionally silenced by a structural change in channel domains that communicate cAMP binding with channel gating. A similar mechanism may explain the lack of cAMP sensitivity in members of the Erg K$^+$ channel family that also contain a CNBD (54, 184, 443).

The properties of an HCN channel cloned from sea urchin sperm (spHCN) differ from mammalian HCN channels (153). In the absence of cAMP, the spHCN current develops with a sigmoidal time course, and then decays to a much lower degree. In contrast, in the presence of saturating cAMP, hyperpolarizing voltage steps produce large currents of sigmoidal waveform lacking inactivation, which are virtually identical to those of mammalian HCN currents. The large augmentation of spHCN channels by cAMP is produced by removal of inactivation with little or no shift in steady-state voltage dependence (142, 153, 347). Mutation of a conserved phenylalanine residue in the S6-helix of spHCN to a leucine (F459L; F431 in HCN2) is sufficient to convert spHCN to a channel that behaves very much like HCN2, i.e., it shows no inactivation and its voltage dependence is positively shifted by cAMP (347). The ability to accomplish this conversion with a single point mutation in S6 argues strongly that the two channel types use the same fundamental mechanisms for gating and that the different phenotypes represent differences in the precise relationship between the energetics and kinetics of the different gating processes.
CNBD. Replacement of a valine in the β-sheet to threonine (V62IT) in conjunction with another replacement in the C-helix (I665D) (Fig. 3) is sufficient to convert cGMP into a full agonist of spHCN. The V62IT mutation probably leads to binding of cGMP in the syn conformation (like in HCN2), while the aspartate in the C-helix is necessary to stabilize the movement of the C-helix after primary cGMP binding, a process which is coupled to the opening conformational change in the channel pore. All four mammalian HCN channels carry a threonine residue at the position equivalent to V621 in spHCN, which may explain why cGMP is a full agonist of these channels. Interestingly, however, mammalian channels also harbor an isoleucine at the position equivalent to I665 in spHCN. Flynn et al. (142) speculated that in mammalian HCN channels the opening allosteric conformational change may be less energetically costly than in spHCN. Therefore, unlike in spHCN, in mammalian channels an interaction with an aspartic acid residue in the C-helix may not be required to promote full activation.

**IV. REGULATION OF HCN CHANNELS**

HCN channels are tightly regulated by interacting proteins as well as by low molecular factors (e.g., protons, chloride ions) in the cytosol and the extracellular space (Fig. 4). These molecules control the functional properties of the channels in the plasma membrane, regulate their cell surface expression (i.e., the number of functional channels in the membrane), and control their targeting to defined cellular compartments. In the following section, we give an overview on HCN channel regulators that have been identified in the last couple of years.

**A. Regulation by Acidic Lipids**

It has been long known that native Ih as well as heterologously expressed HCN currents display a rapid rundown when measured in excised patches or during prolonged whole cell recordings (43, 85, 118, 119, 122, 227). This effect is caused by an ~30 to 50 mV hyperpolarizing shift of the activation curve. It was speculated that washout or depletion of cAMP may account for up to 20 mV of this shift; however, the mechanistic basis of the rest of the voltage shift was unclear (85, 119). Recently, two groups provided convincing evidence that phosphatidylinositol 4,5-bisphosphate (PIP2) is probably the missing factor that underlies the so far unexplained gap in the voltage shift (308, 449).

PIP2 is not a novel entry in the list of ion channel regulators but has been found to control numerous ion transporters and ion channels including many K+ channels, TRP channels, and Ca2+ channels (for recent reviews, see Refs. 150, 378). In HCN channels, PIP2 acts as an allosteric activator from the intracellular site that facilitates channel activation by shifting V0.5 ~20 mV toward more positive potentials. Importantly, this action is independent of the presence of cyclic nucleotides. As a result, PIP2 adjusts HCN channel opening to a voltage range relevant for the physiological role of Ih channels. PIP2-mediated regulation of HCN channels may be of physiological significance for the function in neuronal circuits, as enzymatic degradation of phospholipids reduces channel activation and slows down firing frequency of neurons. Different levels of PIP2 may also contribute to the profound variations in the half-maximal activation voltages of Ih in cardiac cells of different developmental stage or distinct regional distribution (77, 316, 325). The molecular determinants conferring the effect of PIP2 on HCN channel gating are not yet known. Most likely, HCN channels are activated by an electrostatic interaction between the negatively charged head groups of phosphoinositides and the channel protein. A similar mechanism for PIP2 modulation of gating was identified in voltage-gated and inwardly rectifying K+ channels (30, 186, 297, 349). It was proposed that the interaction between PIP2 and Ih channels relieves an inhibition of channel opening conferred by an inhibitory channel domain. This “PIP2 responsive” domain is clearly different from the CNBD because the PIP2 effect is still present in channels lacking the CNBD.

There is recent evidence that in addition to PIP2, other acidic lipids may also serve as allosteric modulators of HCN channels. Fogle et al. (143) showed that phosphatidic acid and arachidonic acid, which are products of diacylglycerol kinase/phospholipase A2 signaling pathways, directly facilitate HCN channel gating by shifting the V0.5 to more positive values (shift of 5–10 mV in HCN2). The molecular mechanism of this facilitation re-
remains to be elucidated, but there is initial evidence that it involves direct binding of both metabolites to the channel and is independent of cAMP pathways. It was speculated that the regulation of the voltage dependence of $I_h$ by acidic lipids may be involved in the fine tuning of subthreshold properties of excitable cells (143).

### B. Regulation by Protons

The activity of HCN channels depends on both intracellular (281, 451) and extracellular (369) concentrations of protons. Intracellular protons shift the voltage dependence of channel activation to more hyperpolarized potentials and slow down the speed of channel opening. In the murine HCN2, a protonable histidine residue (His321) localized at the boundary between the voltage-sensing S4-helix and the cytoplasmatic S4-S5-linker has been identified to confer intracellular pH ($pH_i$) sensitivity (451) (Fig. 4). At acidic ($pH_i$ 6.0) and alkaline $pH_i$ ($pH_i$ 9.0), the midpoint potential of HCN2 activation is shifted by $\sim 10$ mV to more hyperpolarized and depolarized potentials, respectively, compared with physiological $pH_i$ ($pH_i$ 7.4) (451). The modulation of HCN channels by intracellular protons may have an important physiological impact on the modulation of HCN channel activity in the brain (281), for example, for the regulation of thalamic oscillations and the respiratory frequency. The protective action of carbonic anhydrase inhibitors in generalized seizures has been attributed to the high sensitivity of HCN channels to $pH_i$ (282). Inhibition of the carbonic anhydrase causes an increase in $pH_i$ and augments $I_h$ in thalamocortical neurons. As a result, these neurons are depolarized, and their engagement in synchronized paroxysmal discharges is reduced. Inhibition of HCN channels by intracellular acidosis could also be pathophysiologically relevant during cardiac ischemia and heart failure (70, 451).

Acidic extracellular pH ($pH_e < 5.0$) was found to activate $I_h$ in a subset of rat taste cells. It was assumed that this mechanism contributes to sour taste transduction in these cells (369). Heterologous expression of the two HCN channel isoforms present in taste cells, HCN1 and HCN4, verified the findings from native cells. Acidification to $pH 3.9$ profoundly sped up channel kinetics, shifted the threshold of activation by up to $+50$ mV, and induced a depolarizing shift of $V_{0.5}$ by up to $+35$ mV with respect to neutral pH. The structural determinants in the HCN1 or HCN4 protein underlying regulation by external pH have not been reported so far.

### C. Regulation by Chloride

Although $I_h$ is a pure cationic current, its conductance is affected by the concentration of small anions, notably by $Cl^{-}$ (144, 411). Frace et al. (144) showed that the amplitude of rabbit sinoatrial $I_h$ decreased when extracellular $Cl^{-}$ was replaced by larger anions such as aspartate. The molecular basis of the regulation by external $Cl^{-}$ was studied in heterologously expressed HCN channels (411). The effect of $Cl^{-}$ was found to be pronounced for HCN2 and HCN4, while it is rather weak for HCN1 (411). A single amino acid residue in the pore region was identified as molecular determinant of extracellular $Cl^{-}$ sensitivity (411) (Fig. 4). Channels with high $Cl^{-}$ sensitivity (HCN2 and HCN4) carry an arginine residue at this position (R405 in HCN2; R483 in HCN4), while HCN1 carries an alanine (A352). The regulation of HCN channels by $Cl^{-}$ is probably relevant for heart (patho) physiology. A reduction of the amplitude of sinoatrial $I_h$ could be involved in the generation of arrhythmias observed in hypochloremia.

$Cl^{-}$ regulates HCN channel function also from the intracellular site. In a recent study, it was shown that intracellular $Cl^{-}$ acts as a physiological suppressor of the instantaneous component of $I_h$ ($I_{INS}$) (272). An increase of intracellular $Cl^{-}$ from physiological concentrations (10 mM) to high concentrations (140 mM) almost completely abolished $I_{INS}$ while it had no significant effect on the steady-state component of $I_h$. The physiological relevance of this regulation remains to be determined.

### D. Regulation by Src Kinase-Mediated Tyrosine Phosphorylation

On the basis of experiments with pharmacological blockers, it was assumed that cardiac $I_h$ is regulated by tyrosine kinases of the Src family (426). This hypothesis was corroborated by yeast two-hybrid screens that provided evidence for direct interaction between Src and HCN1 (332), as well as between Src and HCN2 (450). Direct binding of Src to HCN2 (450) and to HCN4 (13) was also shown by communoprecipitation in heterologous expression systems and native tissue.

Mapping experiments in HCN2 revealed that Src binds via its SH3 domain to the C-linker-CNBD and phosphorylates the channel at this domain (450). As a consequence, the activation of the channel is accelerated. Conversely, inhibition of Src by pharmacological agents or cotransfection of a dominant-negative Src mutant slows down channel kinetics probably via dephosphorylation by not yet defined cellular phosphatases. The residue conferring modulation by Src (Y476) has been identified in HCN2 by mass spectrometry. The residue is localized in the B'-helix of the C-linker (Figs. 3 and 4) and is conserved in all HCN channel isoforms, suggesting that regulation by Src may be a commonality of the HCN channel class. In agreement with this notion, replacement of Y476 by phenylalanine in either HCN2 or HCN4 yields to channels that are no longer regulated by Src. A recent study
identified an additional tyrosine residue in HCN4 (Y531 in A′-helix of the C-linker) that may also be involved in Src-mediated channel regulation (223) (Fig. 3). In agreement with findings of Arinburg et al. (13), this study also found that Src not only speeds up channel kinetics of HCN4 but also induces a +10 to 15 mV shift of the V_0.5. At this point, one can only speculate on the mechanism by which Src-mediated phosphorylation facilitates channel opening. It is tempting to assume that the presence of the bulky negatively charged phosphate group in the B′-helix of the C-linker destabilizes the interaction between neighboring C-linkers within the HCN channel tetramer. As a consequence, the C-linker tetramer would occupy a less compact conformation and would impose a weaker inhibitory impact on the channel gate as if present in the more compact dephosphorylated conformation.

Regulation of I_h by tyrosine phosphorylation through Src kinase has been demonstrated under physiological conditions in sinoatrial pacemaker cells in the murine (450) and rat heart (13, 439) as well as in neurons (450). These results support the notion that the control of the phosphorylation status is indeed an important regulatory mechanism to adjust the properties of I_h to the specific requirements of different types of neurons and heart cells.

E. Regulation by p38-Mitogen-Activated Protein Kinase

In addition to tyrosine kinases, HCN channels are also regulated by the serine/threonine kinase, p38-mitogen-activated protein (MAP) kinase (310). In hippocampal pyramidal neurons, activation of p38-MAP kinase significantly shifts the voltage-dependent activation towards more positive potentials. This regulation may functionally affect temporal summation and neuronal excitability. It is not clear whether p38 induces the observed effects by direct phosphorylation of the HCN channel protein or by phosphorylation of another protein interacting with these channels.

F. Transmembrane and Cytosolic Proteins Interacting With HCN Channels

There is growing evidence that ion channels usually are macromolecular protein complexes that in addition to the principal pore-forming subunit contain auxiliary proteins that are required for the fine-tuning of electrophysiological properties, the functional coupling to signaling pathways, and trafficking to specific cellular compartments. HCN channels are no exception from this rule. In the last couple of years, several proteins interacting with HCN channels have been identified (Fig. 4).

1. Regulation by MiRP1

The MinK-related protein MiRP1 (encoded by the gene KCNE2) was reported to interact with several HCN channel types (101, 317, 438). MiRP1 is a member of a family of single transmembrane-spanning proteins and is an established auxiliary subunit of the HERG delayed rectifier K⁺ channel (2, 390, 445). MiRP1 was found to interact with HCN2 in rat neonatal cardiomyocytes and canine sinoatrial node tissue (317). Overexpression studies in Xenopus oocytes (438) and neonatal rat cardiomyocytes (317) showed that MiRP1 increases current densities and accelerates activation kinetics of HCN2. In contrast, MiRP1 did not affect voltage dependence of activation. In overexpression systems, MiRP1 was also found to interact with HCN1 (438) and HCN4 (101). MiRP1 increased current densities of HCN4, but unlike in coexpression experiments with HCN1 or HCN2, it slowed down kinetics and induced a negative shift of the activation curve of this channel (101). Collectively, these results may suggest that MiRP1 is an auxiliary subunit of HCN channels. However, extreme care should be taken before a final decision on this issue is made. It will be necessary to verify the interaction between MiRP1 and HCN channels including its functional implications in native tissues. Furthermore, the specificity of antibodies used for immunoprecipitations must be confirmed in adequate knockout models.

2. Regulation by KCR1

Very recently, another transmembrane protein (KCR1) was reported to interact with HCN2 and native cardiac I_h channels (268). KCR1 is a plasma membrane-associated protein with 12 putative transmembrane regions that like MiRP1 can associate with the HERG K⁺ channel (183, 213). Upon overexpression in CHO cells, KCR1 reduces HCN2 current densities and affects single-channel current parameters of this channel. Overexpression in rat cardiomyocytes also reduced current densities of native I_h and suppressed spontaneous action potential activity of these cells. From these experiments, it was concluded that KCR1 is an inhibitory auxiliary subunit of HCN channels and serves as a regulator of cardiac automaticity. As discussed for MiRP1, further experiments will be required to verify this hypothesis.

3. Regulation by neuronal scaffold proteins

Several scaffold proteins interact with the COOH terminus of HCN channels. These proteins were mainly identified in neurons and may regulate channel targeting to distinct subcellular compartments (e.g., dendrites or synapses). A brain-specific protein termed TRIP8b (TPR-containing Rab8b interacting protein) interacts through a conserved tripeptide sequence in the COOH terminus of HCN channels.
HCN channels (335). TRIP8b is thought to play an important role in the trafficking of vesicles to their final targets (444). TRIP8b colocalizes with HCN1 in dendrites of cortical and hippocampal pyramidal cells. Functional coexpression of TRIP8b with HCN protein, either in native cells or heterologous systems, results in a strong downregulation of HCN channels in the plasma membrane. On the basis of these experiments and the analysis of HCN1 knockout mice, it was speculated that TRIP8 is involved in the generation of somatodendritic HCN channel gradients in cortical layer V pyramidal neurons (335). HCN2, but not other HCN channel types, interacts with the neuronal scaffold protein tamalin, mostly through a PDZ-like binding domain (205). In addition, HCN2 interacts with the scaffold proteins Mint2 and S-SCAM via distinct protein-binding domains at the COOH-terminal tail. In COS-7 cells, HCN2 levels were increased upon coexpression with Mint2, suggesting that this protein is a positive regulator of cell surface expression of HCN channels (205).

4. Regulation by filamin A

HCN1, but not HCN2 or HCN4, was found to bind filamin A via a 22-amino acid sequence downstream of the CNBD (161). Filamin A is a putative cytoplasmic scaffold protein that binds actin and thereby links transmembrane proteins, among those are the K+ channels Kv4.2 and Kir 2.1, to the actin cytoskeleton (305, 329). On the basis of heterologous overexpression in filamin A-expressing and filamin A-deficient cell lines, it was proposed that filamin A causes clustering and slows down activation and deactivation kinetics of HCN1 (161).

5. Regulation by caveolin-3

Ih channels localize to membrane lipid rafts in sinoatrial myocytes and in HEK293 cells expressing HCN4, the major HCN channel isoform contributing to native sinoatrial Ih (26). Immunoprecipitation experiments indicate an interaction between HCN4 and caveolin-3, which is a marker protein for so-called caveolae (27). Caveolae represent a morphologically distinct type of lipid rafts. In cardiomyocytes and pacemaker cells, several elements of the β-adrenergic signaling pathway including β1-adrenoceptors that regulate HCN channel activity are localized in caveolae. It was proposed that clustering of HCN4 in caveolae is essential for normal function and regulation of this channel. Indeed, disruption of lipid rafts by cholesterol depletion caused a redistribution of HCN channels within the membrane and modified their kinetic properties (26).

V. TISSUE EXPRESSION OF HCN CHANNELS

Throughout the nervous system and the heart, the hyperpolarization-activated current Ih as well as HCN channel expression has been identified. In addition, Ih was also found in some other tissues such as B cells of pancreatic islets (134), enteric (37, 179, 429), lymphatic smooth muscle (254), uterine smooth muscle (296), smooth muscle cells of the bladder (163) or portal vein (164), testis (341), and the enteric nervous system (427). However, HCN channel transcripts have only been reported in some of these tissues or cell types (134, 164, 341, 427). Moreover, in most of these tissues, HCN channels are expressed at low levels, making it very difficult to define the physiological role of Ih if there is any. Another problem is that in several cell types, HCN channels have been detected only on the mRNA level, e.g., using RT-PCR or in situ hybridization, while protein expression has not been shown so far. Given these drawbacks, in this review we will concentrate on HCN channels in heart and nervous system. We will also give only a rough outline of tissue distribution of HCN1-4. Readers who are interested in a more complete description of the expression pattern are referred to a number of excellent studies that have reported on this issue in great detail (126, 226, 275, 276, 294, 331, 332, 344, 345).

Briefly, in the brain, HCN1 is expressed in the neocortex, hippocampus, cerebellar cortex, and brain stem (270, 275, 294, 331, 332). In addition, HCN1 expression was reported in the spinal cord (270). HCN2 is distributed nearly ubiquitously throughout most brain regions, with the highest expression in the thalamus and brain stem nuclei (275, 294, 331). In contrast, HCN3 is expressed at very low levels in the central nervous system. Moderate to high expression has only been detected in the olfactory bulb and in some hypothalamic nuclei (275, 294). HCN4 is strongly expressed in some parts of the brain, e.g., in various thalamic nuclei and in the mitral cell layer of the olfactory bulb (275, 294, 331). In other brain regions, the expression of HCN4 is much lower. All four HCN channel isoforms are expressed differentially in the retina (60, 193, 209, 276, 280). In the peripheral nervous system, all four HCN subtypes have been reported in the dorsal root ganglion, with HCN1 being the most abundant one (80).

All four HCN channel isoforms have been detected in the heart. The expression levels of these isoforms strongly depend on the cardiac region and, in addition, seem to vary between species. In the sinoatrial node, in all species analyzed so far (e.g., human, rabbit, guinea pig, mouse, and dog), HCN4 is the major isoform accounting for ~80% of Ih (276, 344, 388). The remaining fraction of this current is species dependent. In rabbits, this fraction of Ih is dominated by HCN1 (344), whereas in mice and humans, HCN2 accounts for this fraction (226, 388). In addition, in the mouse sinoatrial node, a significant amount of HCN1 mRNA was identified (249).

In other parts of the cardiac conduction system, HCN4 is also the major isoform, with expression in the atrioventricular node (126) and the Purkinje fibers (345).
HCN1 expression has been reported in the atrioventricular node (249). For HCN3, either no expression (276) or only very low expression levels have been reported in the conduction system (249). HCN channels are also present in atrial and ventricular myocytes. In these cells, HCN2 is the dominant isoform displaying a rather ubiquitous distribution. Transcripts of HCN1, HCN3, and HCN4 have also been detected in heart muscle (344, 375). In general, expression levels of HCN channels are low in normal heart muscle compared with cells of the conduction system. However, upregulation of HCN channels may occur during heart diseases (72, 73, 139, 374) (see also sect. viii).

VI. PHYSIOLOGICAL ROLES OF HCN CHANNELS IN NEURONS

A. Principles

$I_h$ displays a set of unique biophysical features that is essential to control excitability and electrical responsiveness of cells. With respect to these physiological roles, two properties of $I_h$ are of particular relevance. First, $I_h$ channels are partially open at rest. As a consequence, the channels contribute to the setting of the membrane potential in many cells (98, 125, 226, 230, 266, 291–293, 300). Second, $I_h$ channels possess an inherent negative-feedback property because they can counteract both membrane hyperpolarization and depolarization by producing a depolarizing inward current (due to $I_h$ channel activation) or by facilitating hyperpolarization (resulting from $I_h$ channel deactivation), respectively. Thus $I_h$ can actively dampen both inhibitory and excitatory stimuli arriving at the cell membrane and thus helps to stabilize the membrane potential (31, 253, 291, 354). We will now discuss in some more detail these two key properties of $I_h$ which together provide the Leitmotiv for the specific functions of this current in various physiological settings.

1. Activation of $I_h$ at the resting membrane potential

In sinoatrial pacemaker cells of the heart as well as in many neurons of the central nervous system, $I_h$ channels are constitutively open at voltages near the resting membrane potential (12, 226, 291–293, 326), pass a depolarizing noninactivating inward current, and hence set the membrane potential to more depolarized voltages. In addition, constitutively open $I_h$ channels per se stabilize the resting membrane potential by lowering the membrane resistance ($R_m$), which is defined as the ratio of a voltage change and the required current (226, 291–293). Therefore, in the presence of $I_h$, any given input current evokes a smaller change in membrane potential than in the absence of $I_h$. Constitutively open $I_h$ channels seem to function as a slow “voltage clamp” (291), tending to stabilize the membrane potential by opposing depolarizing or hyperpolarizing inputs (185). This effect suppresses low-frequency fluctuations in membrane potential (291). In dendrites, constitutively open $I_h$ channels influence the passive propagation of excitatory postsynaptic potentials (EPSPs) (239–241). In the absence of active voltage-dependent ion channels, the passive properties of a dendrite (Fig. 5A) attenuate the amplitude and slow down the kinetics of an EPSP (Fig. 5B) as it spreads from its site of origin in the dendrites to the soma (Fig. 5, A–C). This process is comparable to a wave traveling across water. Just as a wave moving across water widens and diminishes in height over distance, EPSP signals can degrade with distance along the neuronal membrane (Fig. 5B). The presence of $I_h$ in dendrites lowers $R_m$ and thereby further increases the amplitude attenuation of EPSPs. In addition, $I_h$ counteracts kinetic filtering of propagating EPSPs also by lowering $R_m$. Therefore, the EPSP upstroke and decline are faster (Fig. 5C).

2. Negative-feedback properties of $I_h$

As mentioned above, voltage-dependent activation and deactivation of $I_h$ actively oppose deviations of the membrane potential away from the resting membrane potential (31, 253, 291, 354). This property is a consequence of the unusual relation between the activation curve and the reversal potential of $I_h$. In contrast to voltage-gated Ca$^{2+}$ and Na$^+$ channels (Fig. 5E), the reversal potential of $I_h$ falls close to the base of its activation curve (189) (Fig. 5E). If membrane hyperpolarization increases the fraction of open $I_h$ channels, the depolarizing inward current drives the membrane potential back towards the reversal potential of $I_h$ and thereby to the initial value close to the resting membrane potential (Fig. 5D). This characteristic effect is called “depolarizing voltage sag” (300, 324). Conversely, a depolarizing input causes deactivation of the $I_h$ that was active at rest. The loss of a tonic depolarizing current causes a hyperpolarization (“hyperpolarizing voltage sag”; Refs. 300, 324) again returning membrane potential toward rest (Fig. 5D, right). $I_h$ is involved in the control of a variety of basic and complex neuronal functions, including dendritic integration, long-term potentiation, learning, and neuronal pacemaking, just to mention a few of them. In the following sections we will review the role of $I_h$ in these diverse functions in more detail. Wherever possible, we will discuss the role of $I_h$ in the light of recent findings obtained from the analysis of HCN channel-deficient mouse lines. These studies have allowed defining the specific...
contribution of distinct HCN channel subtypes to different physiological functions.

B. Role of $I_h$ in Dendritic Integration

Dendritic integration is a process that is crucial for signal processing in most neurons. Typical isolated EPSPs are too small to bridge the gap between the resting membrane potential and the action potential threshold. Therefore, generation of action potentials at the soma usually requires the integration of multiple synaptic inputs. To ensure high fidelity of information processing, integration of EPSPs must be tightly controlled both in space and time. Dendritic integration has been extensively studied in CA1 hippocampal and neocortical pyramidal neurons (241). There is good evidence that in these neurons $I_h$ and most notably the $I_h$ component encoded by HCN1 has an important role in regulating dendritic integration (239–241, 394, 423). The way incoming EPSPs are summed up in time largely depends on kinetic filtering by passive cable properties of the dendrites. As mentioned above, dendritic filtering slows down the time course of EPSPs resulting in somatic EPSPs that rise and decay more slowly if they are generated in distal dendrites compared with proximal dendrites (Fig. 5, B and C). As a consequence of filtering, one would expect that repetitive EPSPs arising from more distal synapses should summate at the soma to a greater extent and over a longer time course than EPSPs generated in more proximal dendrites. However, in many neurons, including CA1 (239, 240) and neocortical layer 5 pyramidal cells (38, 377, 423), this localization dependence of temporal summation is not observed. This discrepancy is probably explained by a gradient of $I_h$ whose density rises progressively by more than sixfold with distance from the soma (225, 239–241, 423) (Fig. 6). This gradient efficiently counteracts dendritic filtering. During the rising phase of an EPSP, $I_h$ actively opposes changes in membrane potential. Cartoon of a current-clamp experiment in a neuron. In the presence of $I_h$, a hyperpolarizing current step (−200 pA) induces a depolarizing voltage sag (arrow, left panel) and a depolarizing current step (200 pA) induces a hyperpolarizing voltage sag (arrow, right panel). Blockade of $I_h$ by Cs+ eliminates the sag (red traces). E: the reversal potential ($V_{\text{rev}}$) of $I_h$ falls near the base of its activation curve. Therefore, $I_h$ actively opposes changes in membrane voltage. Partial activation of the current drives the membrane potential toward the reversal potential on the base of the activation curve (left, arrow in the activation curve) and thus is self-limiting and stabilizing for the membrane potential. In contrast, voltage-gated currents, whose reversal potential falls near the top of their activation curve (e.g., $I_{\text{Na}}$; right), are destabilizing and self-regenerative. Partial activation drives the membrane potential towards the reversal potential at the top of the activation curve (right, arrow in the activation curve).

**FIG. 5.** Basic properties of $I_h$. A: equivalent electrical circuit of an idealized cylindrical segment of a dendrite. Three electrical components are responsible for the passive features of a dendrite: the specific membrane resistance ($R_m$), the specific membrane capacitance ($C_m$), and the intracellular resistance ($R_i$). For simplicity, the resistance of the extracellular space has been neglected. B: the passive properties of a dendrite attenuate the amplitude (arrow) of an excitatory postsynaptic potential (EPSP) as it spreads from dendrites to the soma (EPSP at the distal synapse, black line; the same EPSP after propagation to the soma, red line). C: EPSP recorded at the soma after propagation from a distal dendrite before and after blockade of $I_h$ with ZD 7288. In the presence of $I_h$ (black line), the EPSP rise and decay faster than after blockade of $I_h$ with ZD 7288 (red line). D: $I_h$ actively opposes changes in membrane voltage. Cartoon of a current-clamp experiment in a neuron. In the presence of $I_h$, a hyperpolarizing current step (−200 pA) induces a depolarizing voltage sag (arrow, left panel) and a depolarizing current step (200 pA) induces a hyperpolarizing voltage sag (arrow, right panel). Blockade of $I_h$ by Cs+ eliminates the sag (red traces). E: the reversal potential ($V_{\text{rev}}$) of $I_h$ falls near the base of its activation curve. Therefore, $I_h$ actively opposes changes in membrane voltage. Partial activation of the current drives the membrane potential toward the reversal potential on the base of the activation curve (left, arrow in the activation curve) and thus is self-limiting and stabilizing for the membrane potential. In contrast, voltage-gated currents, whose reversal potential falls near the top of their activation curve (e.g., $I_{\text{Na}}$; right), are destabilizing and self-regenerative. Partial activation drives the membrane potential towards the reversal potential at the top of the activation curve (right, arrow in the activation curve).
decay faster and are therefore shorter (Fig. 5C). As a consequence, after propagation to the soma, the temporal summation is more dampened for distal than for proximal inputs. Therefore, the temporal summation of all inputs reaching the soma is about equal (Fig. 6A). However, the normalization of somatic EPSP time course by $I_h$ comes at a cost. As mentioned in section VI, the activation of $I_h$ increases the dendrosomatic attenuation of EPSP amplitude and thus the dependence of somatic EPSP amplitude on synapse location (Fig. 5B).

Collectively, these results suggest that the proposed role of $I_h$ in dendritic integration critically depends on an increasing somatodendritic $I_h$ gradient. However, this may not apply to all types of neurons. For example, there is a shallower $I_h$ gradient or even a reversed $I_h$ distribution in a subset of CA1 pyramidal cells that show a similar dendritic integration and temporal summation as pyramidal cells with a distal enrichment of $I_h$ (61, 160). Moreover, location independence of EPSP summation is observed in cerebellar Purkinje cells, although $I_h$ exhibits a uniform dendritic density in these neurons (11). Thus normalization of temporal summation may occur in a variety of $I_h$ distribution patterns. Using a modeling approach, Angelo et al. (11) concluded that it is the total number of $I_h$ channels, not their distribution, that governs the degree of temporal summation of EPSPs. Finally, other voltage-gated ion channels also have important roles in dendritic integration. For example, a gradient of A-type potassium channels rising from proximal to distal dendrites (34, 181, 199, 377) may be involved in dendritic
integration. In addition, it has been shown that the density of AMPA glutamate receptors is increased in distal dendrites of several neurons, increasing the strength of distal synapses (10, 242, 352). Thus the relative importance of individual ion channels and receptors to the complex process of dendritic integration may vary among different neurons.

C. Role of $I_h$ in Working Memory

A recently discovered role of $I_h$ is the control of the spatial working memory (415) (Fig. 6B). This form of memory depends on prefrontal cortical networks that have the unique ability to represent information that is no longer present in the environment and to use this “representational knowledge” to guide behavior. In monkeys performing a spatial working memory task, prefrontal cortical neurons increase firing in response to visual stimuli presented in the preferred direction, while they decrease firing when stimuli are presented in the nonpreferred direction. This property is called spatial tuning. It was proposed that spatial tuning is regulated by the opposing effects of adrenoceptors and the D1-receptor on intracellular cAMP signaling (15, 407). It has been suggested that HCN channels are important downstream targets for cAMP in this signaling pathway (415). HCN channels colocalize and functionally interact with $\alpha_2$-adrenoceptors on dendritic spines of prefrontal neurons. The functional interaction was explained according to the following model (Fig. 6B) (415): $\alpha_2$-adrenoceptors are activated by relatively low levels of norepinephrine released during alert and wakefulness (16). The activation of $\alpha_2$-adrenoceptors inhibits the production of cAMP via $G_i$ signaling and thus reduces the open probability of HCN channels. As a result, $R_m$, the efficiency of synaptic input, and the network activity of these neurons increase. On the basis of this model, it was speculated that the reduced HCN channel activity on spines receiving inputs from neurons with similar spatial properties increases the firing to preferred spatial directions and thereby augments relevant information (15). In contrast, activation of D1-receptors increases the production of cAMP via $G_s$ signaling. As a result, HCN channels open, reduce the membrane resistance, and, thereby, decrease inputs to the spines (Fig. 6B). This could selectively disconnect spinoous inputs to the dendrites. Thus activation of D1-receptors on spines receiving inputs from neurons with dissimilar spatial properties could suppress irrelevant information (“noise”) from the nonpreferred direction (15, 407, 422). So far, the regulation of HCN channels by D1-receptors has not directly been demonstrated and therefore remains hypothetical (15). During stress, higher concentrations of norepinephrine may be present and activate low-affinity $\alpha_1$- and very-low-affinity $\beta_1$-adrenoceptors giving rise to strongly increased cAMP levels (16). In this situation, cAMP levels could significantly increase the open probability of HCN channels and functionally disconnect spinoous inputs to the dendrites (15). Thus connections of the prefrontal cortex would be functionally cut off.

An alternative modulation of HCN channels by $\alpha_2$-adrenoceptors in prefrontal neurons has been proposed by a different group (71). Carr et al. (71) suggested that $\alpha_2$-adrenoceptor activation suppresses HCN channels in prefrontal neurons through a cAMP-independent signaling pathway that appears to be mediated by PLC-PKC signaling.

D. Role of $I_h$ in Constraining Hippocampal LTP

Mice in which the HCN1 gene has been selectively deleted from forebrain show an improvement of hippocampal-dependent learning and memory formation, compared with wild-type mice (292, 394) (Fig. 6D). Electrophysiological recordings indicated that this surprising effect is concurred and probably also caused by a specific enhancement of long-term synaptic plasticity (LTP) and dendritic excitability in hippocampal CA1 neurons. CA1 neurons provide the major output of the hippocampus and receive two major sources of excitatory synaptic inputs (Fig. 7A). One set of inputs, the Schaffer collaterals, comes from hippocampal CA3 pyramidal neurons and terminates on regions of CA1 neuron dendrites that are relatively close to the cell body, an area with only moderate HCN1 expression. The second set of inputs, the perforant path, represents a direct connection from layer III neurons of the entorhinal cortex and terminates on the distal dendritic regions of the CA1 neurons, an area where HCN1 expression is very high. At the more proximal Schaffer collateral pathway, both synaptic transmission and long-term plasticity are relatively unaffected by the deletion of HCN1, consistent with the relatively low expression of HCN1 in this dendritic region (292)(Fig. 6D). In contrast, at distal perforant path synapses, a significant enhancement in the integration of synaptic potentials and an increase in LTP was observed, consistent with the relatively high expression of HCN1 in this region (292) (Fig. 6D). From these results, it was concluded that HCN1 channels impair spatial learning because they exert an inhibitory constraint on dendritic integration and synaptic long-term plasticity at the perforant path inputs to CA1 pyramidal neurons. It was proposed that high levels of HCN1 may interfere with generation of Ca$^{2+}$ spikes, a process that is critical for the induction of LTP at dendritic synapses (394). These Ca$^{2+}$ spikes are triggered by synaptic activation of AMPA- and NMDA-receptors (394). The activation of these glutamate receptors generates an initial depolarization that activates most likely T-type
The role of \( I_h \) in motor learning has been recently uncovered in mice with a global deletion of HCN1 (293) (Fig. 6C). Deletion of HCN1 causes profound learning and memory deficits in behavioral tests that require complex and repeated coordination of motor output by the cerebellum (e.g., in visible platform, water maze, and rotarod tasks). In contrast, the deletion of HCN1 does not modify motor behavior that also involves cerebellar synaptic plasticity. The actions of HCN1 were tested in cerebellar Purkinje cells, the key component of the cerebellar circuit required for learning of correctly timed movements (293). In these spontaneously spiking neurons, HCN1 mediates a depolarizing inward current that counteracts inputs that hyperpolarize the membrane below the threshold for spontaneous spiking. By stabilizing the integrative properties of Purkinje cells, HCN1 enables the Purkinje cells to maintain an input-output relation that is independent of the neuron’s previous history of activity (Fig. 6C). This function of HCN1 is required for reliable encoding of information and ensures accurate decoding of input patterns.

G. Role of \( I_h \) in Resonance and Oscillations

1. Resonance

The term resonance describes the property of a neuron to selectively respond to inputs at a preferred frequency (189). In electrophysiological experiments, resonance can be induced by applying a current stimulus with linearly increasing frequency (so-called ZAP current). In support of this hypothesis, long-term facilitation of synaptic transmission in crustacean motor terminals was shown to be conferred by cAMP-dependent upregulation of \( I_h \) (32, 33). The downstream pathway coupling \( I_h \) activation to synaptic release is not yet known. However, there is initial evidence that \( I_h \) channels may directly interact with the release machinery, perhaps mediated by the cytoskeleton (32, 33). In vertebrates, the functional relevance of \( I_h \) in regulating synaptic transmission is currently disputed. Mellor et al. (263) reported that presynaptic \( I_h \) channels are involved in the generation of LTP in hippocampal mossy fiber synapses that terminate on CA3 pyramidal cells. However, this finding was challenged by another study that showed in the same system that LTP is independent of \( I_h \) (89).
bined with properties of a high-pass filter (65, 69, 187, 189) (Fig. 7C). The low-pass filtering is caused by the membrane time constant $\tau_m$. The high-pass filtering is caused by the action of several voltage-gated currents including $I_h$. To act as a high-pass filter, these currents need to be able to actively oppose changes in membrane voltage (Fig. 5, D and E). In addition, these currents have to activate slowly relative to the membrane time constant. Both requirements are met for $I_h$. At low frequencies, the $I_h$ channels have time to activate and actively oppose changes in membrane potential. At high frequencies, there is not enough time for the $I_h$ channels to open. As a result, high-frequency changes are not suppressed, rendering the neuron responsive for fast trains of spikes. Resonance arises at intermediate frequencies where input-induced voltage changes are too high to be opposed by $I_h$ and too low to be filtered by $\tau_m$. The importance of $I_h$ for the generation of membrane resonance has been demonstrated for several neurons, for example, in neocortical pyramidal cells of the rat (398), in subicular pyramidal neurons of the rat (416), and in neurons from the sensorimotor cortex of juvenile rats (188).

2. Oscillations

Sustained rhythmic oscillations are a hallmark feature of neuronal circuits in various brain regions (62, 65). Oscillations arise from synchronized activity of neurons and are thought to play an essential role in information processing in neuronal networks. There are several types of oscillations in different frequency bands, called delta, theta, gamma, and fast “ripple” oscillations (62, 65). One oscillation, the theta frequency oscillation (4–12 Hz), is prominent in all areas of the hippocampal formation. These oscillations may be important for various cognitive processes including processing, encoding, and storing of spatial information (62) as well as for memory formation and retrieval (63, 219). Hippocampal theta oscillations have been proposed to originate from reciprocal interactions between rhythmic inputs from the medial septum-diagonal band of Broca, the entorhinal cortex, and other subcortical structures (Fig. 7A; Refs. 66, 306, 406). The inputs from septal neurons to the hippocampus seem to be mainly responsible for the generation of the theta rhythm (62, 306). In this process, the perforant path and various other brain regions are also involved (155, 327).

In mice with a forebrain-restricted deletion of HCN1, changes in hippocampal-dependent network oscillations have been described (292). While low- and high-frequency network oscillations appeared to be unchanged in knockout mice, there was a significant enhancement in the theta frequency band (4–9 Hz), during rapid-eye-movement (REM) sleep and during wheel running (292). Consistent with these results, in CA1 neurons responses to low-frequency inputs at the theta range are preferentially increased in HCN1-deficient mice. HCN1 channels can therefore be thought of as a partial brake that contributes to resonance by preferentially dampening low-frequency components of input waveforms at frequencies below the theta range. Releasing the brake in knockout animals causes a general enhancement in the voltage response to low-frequency oscillatory currents.

In addition to the important role of $I_h$ in the generation of theta oscillation, $I_h$ is also involved in the generation of other types of oscillations. There are several studies that demonstrated the importance of $I_h$ for $\gamma$-oscillations in the hippocampus (97, 140), synchronized oscillations in the inferior olive (21), and subthreshold oscillations in the entorhinal cortex (108, 166). Moreover, $I_h$ has been suggested to influence network oscillations that play an important role in learning and memory (140, 185, 230, 235).

H. Role of $I_h$ in the Generation of Thalamic Rhythms

Synchronized neuronal oscillations are produced in thalamocortical networks during sleep (64, 255, 363, 364), sensory processing (162), and seizures (256). The generation and synchronization of complex oscillations (e.g., spindle waves and slow waves) requires extensive synaptic interactions within the thalamocortical network (361) (Fig. 8A). In contrast, there are other neuronal rhythms, for example, the delta frequency rhythm that can be generated in single cells (255, 361, 367). We will first discuss the role of $I_h$ in the generation of characteristic firing patterns that arise in single thalamocortical cells and then discuss the role of $I_h$ in oscillations that require more extensive synaptic interactions. In our discussion, we focus on data from in vitro slice preparations due to the current paucity of in vivo data.

1. Single cell oscillations in thalamic relay neurons

In vitro (22–24, 96, 231, 232, 259) and in vivo (9, 23, 107, 238, 328, 363) studies have shown that thalamocortical neurons fire in two distinct firing modes called transmission mode and burst mode (22–24, 96, 231, 232, 259) (Fig. 8B). During wakefulness and REM sleep, thalamocortical neurons are depolarized by afferent inputs and switch to the transmission or “single spike” mode (195, 196, 257) (Fig. 8, B and D). During this mode information is gated through the thalamus and forwarded to the cortex (255, 367). The transmission mode is characterized by the generation of repetitive single Na$^+$ spikes at depolarized potentials where T-type Ca$^{2+}$ channels are inactivated (Fig. 8D). During this mode, sufficient excitation repetitively discharges thalamocortical neurons. The output frequency of these neurons increases with increasing depolarization induced by afferent excitatory inputs (165).
During the burst mode, thalamic networks display a monotonous repetitive firing pattern at hyperpolarized membrane potentials (178, 195, 196, 368, 389). This firing mode is characteristically seen during non-REM sleep or epileptic discharges and has been considered to decrease transfer of sensory information to the cortex (368).

In vitro studies have demonstrated that thalamic neurons fire in the “burst mode” through the interaction of a low-threshold Ca\(^{2+}\) current (\(I_T\)) and \(I_h\) (Fig. 8, B and C) (255, 300). The following model has been proposed: the activation of \(I_h\) by hyperpolarization beyond \(-65\) mV slowly depolarizes the membrane potential until a re-bound low-threshold Ca\(^{2+}\) spike is generated by the activation of \(I_T\) at more depolarized potentials. Because these “slow spikes” are depolarizing and last for tens of milliseconds, typically a series of Na\(^{+}\) spikes rides on them. The inactivation of \(I_T\) terminates the low-threshold Ca\(^{2+}\) spike. During the spike, \(I_h\) is deactivated. As a result, the termination of the spike is followed by a hyperpolarizing “overshoot.” In turn, \(I_h\) is activated, the cycle repeats, and continuous rhythmic burst firing is sustained. The intrinsic interplay of these currents promotes delta rhythmicity in single thalamocortical neurons (128, 221, 222, 258, 261, 355). These single cell oscillations are synchronized in large neuronal circuits and can be recorded in the EEG as delta waves during non-REM sleep (128, 295, 366, 367).

Electrophysiological recordings in thalamic slice preparations of HCN2-deficient mice revealed that the current produced by HCN2 constitutes the major component underlying thalamocortical \(I_h\) (226). Deletion of HCN2 reduced the current amplitude of \(I_h\) by \(-80\%\) and resulted in a 12 mV hyperpolarizing shift of the resting membrane potential. Thalamocortical neurons of HCN2-deficient mice displayed a higher susceptibility to fire in the burst mode when experiencing excitatory inputs than wild-type neurons (Fig. 9). Macroscopically, the altered thalamic firing behavior of HCN2-deficient mice concurred with the presence of spike-and-wave discharges, the clinical hallmark of absence epilepsy (Fig. 9A; see also sect. viii). The higher incidence of burst firing in HCN2-deficient mice (Fig. 9B) may simply result from the fact that the ratio of T-type channels present in the closed (but activatable) versus inactivated state depends on the resting membrane potential. In HCN2-deficient neurons that display a more hyperpolarized resting membrane potential, the fraction of T-type channels present in the closed state will be higher than in wild-type cells where more T-type channels will be in the inactivated state. Thus the susceptibility of HCN2-deficient neurons to produce a Ca\(^{2+}\) spike is higher than that of wild-type neurons because the T-type channels present in these cells are easier to activate by excitatory inputs.

**Fig. 8.** The role of \(I_h\) in the generation of thalamic oscillations. A, top: coronal section through the mouse brain. The cortex, the reticular thalamic nuclei, and the thalamus are indicated. Bottom: schematic diagram of the thalamocortical loop: +, excitatory glutamatergic synaptic contacts; –, inhibitory GABAergic contacts. For detailed information, see text. B: firing modes of thalamocortical neurons. C and D: higher temporal resolution of sections shown in B. [Modified from McCormick and Bal (255).]
Network oscillations

$I_h$ is involved in the generation of a number of rhythmic oscillations in thalamocortical networks. During the early state of non-REM sleep, synchronized oscillations are generated in the thalamocortical network that give rise to spindle waves in the surface EEG (22, 236, 367). Spindle waves are characterized by a crescendo-decrescendo type of oscillation at 6–14 Hz that lasts for 1–4 s followed by a refractory period of 5–20 s that terminates the oscillations (91, 204, 363, 367). These waves are assumed to have an essential functional role in synaptic plasticity of cortical and thalamic neurons (368). Spindle waves have been investigated in vivo (9, 92, 279, 362, 364, 365) and also in slice preparations (410). These studies indicate that spindle waves are generated by a cyclic interaction between excitatory thalamocortical cells and inhibitory thalamic reticular neurons (23, 24, 363, 367, 410) (Fig. 8A). Thalamic reticular cells are the pacemakers for the generation of spindle wave oscillations (148, 359). In these neurons, rhythmic bursts are generated by low-threshold $\mathrm{Ca}^{2+}$ spikes. Burst firing in thalamic reticular neurons induces rhythmic inhibitory postsynaptic potentials (IPSPs) in thalamocortical cells. These IPSPs hyperpolarize thalamocortical cells, remove inactivation of the low-threshold $\mathrm{Ca}^{2+}$ current, and at the same time activate $I_h$. The resulting depolarization triggers a rebound $\mathrm{Ca}^{2+}$ spike and a burst of action potentials (Fig. 8C). These bursts reexcite the thalamic reticular neurons and also stimulate cortical pyramidal cells. The nearly simultaneous occurrence of spindle waves over widespread cortical territories is produced by network synchronization involving the cortex and the thalamus (358). This synchronized activity underlies the presence of spindle waves in the EEG.

The silent period between spindle waves is largely determined by persistent $I_h$ activation in thalamocortical cells (22, 236, 367). This persistent activation of $I_h$ results from an increase in intracellular $\mathrm{Ca}^{2+}$ primarily triggered by the rebound low-threshold $\mathrm{Ca}^{2+}$ bursts that occurred during the spindle wave generation. The $\mathrm{Ca}^{2+}$ most likely activate a $\mathrm{Ca}^{2+}$-sensitive isoform of adenyl cyclase, producing an increase in cAMP synthesis that enhances $I_h$ (231). The persistent activation of $I_h$ in turn suppresses the next spindle wave until $I_h$ is slowly decayed. Spindle waves disappear during wakening or REM sleep. The transition to these states is regulated by several neurotransmitters such as norepinephrine and serotonin that both increase intracellular cAMP and lead to a consecutive upregulation of $I_h$ (255).

VII. ROLE OF $I_h$ CHANNELS
IN CARDIAC RHYTHMICITY

The heart beat originates from specialized pacemaker cells in the sinoatrial (SA) node region of the right atrium. These cells generate a special kind of action potential ("pacemaker potential") that is characterized by the presence of a progressive diastolic depolarization (DD) in the voltage range between $-65$ to $-45$ mV (Fig. 10; see also Ref. 244 for an excellent recent overview on heart automaticity). After the repolarization phase, it is the DD that drives the membrane potential of a SA node cell back toward threshold of $\mathrm{Ca}^{2+}$ channel activation, thereby maintaining firing. The DD is generated by the concerted action of several currents, among which $I_h$ is considered to play a prominent role because it is activated at negative potentials and, therefore, potentially could serve as primary initiator of the DD. In addition to $I_h$, other inward currents including $\mathrm{Ca}^{2+}$ currents ($I_{\text{CaT}}$ and $I_{\text{CaL}}$) (168, 243, 244, 371), as well as the sustained inward current ($I_n$) (273) whose molecular correlate is not yet known may contribute to the DD. Furthermore, it was proposed that
the DD may be initiated by the decay of outward rectifier K\(^+\) currents (I\(_{Kr}\) and I\(_{Ks}\)) or by mechanisms involving intracellular Ca\(^{2+}\) release through ryanodine (214, 215, 408) or inositol 1,4,5-trisphosphate (IP\(_3\)) receptors (265). I\(_h\) is not only involved in principal rhythm generation, it also plays a key role in heart rate regulation by the autonomic nervous system. Sympathetic stimulation activates I\(_h\) and, hence, accelerates heart rate via \(\beta\)-adrenoceptor-triggered cAMP production (49, 50, 122) (Fig. 10, C and D) while low vagal stimulation lowers heart rate via inhibition of cAMP synthesis and an ensuing inhibition of I\(_h\) activity (117, 123, 124). High vagal tone most likely lowers the heart rate mainly via the activation of I\(_{KACH}\) (117, 421).

### A. HCN4

Recent genetic mouse models have made it possible to evaluate the proposed roles of I\(_h\) in cardiac rhythmicity under in vivo conditions. As mentioned above, HCN4 makes up \(\sim 80\%\) of SA node I\(_h\) and has been considered to be crucial for the generation of the heart beat (171, 172, 370). In support of this notion, mice with global- or heart-specific disruption of the HCN4 gene die in utero between embryonic days 9.5 and 11.5 (370). Embryonic hearts of HCN4-deficient mice analyzed before embryonic day 10 show a reduction of the beating frequency of \(\sim 40\%\). Importantly, these hearts do not respond to \(\beta\)-adrenergic stimulation. Thus one may conclude that in the embryonic heart HCN4 is not required for principal heart beat generation, at least not before embryonic day 11.5, but that this channel is absolutely crucial for autonomous heart rate regulation (370). In support of this conclusion, hearts of mice carrying a mutation in the CNBD that abolishes high-affinity cAMP binding (HCN4R669Q) also fail to respond to \(\beta\)-adrenergic stimulation (171). Further analysis revealed that HCN4-deficient mice probably die from a developmental defect of the SA node (370). These mice develop normal embryonic pacemaker cells that produce primitive pacemaker potentials but fail to generate adult-type pacemaker cells. Obviously, the latter cells are crucially required to drive the heart beat after embryonic day 11. Interestingly, HCN4R669Q mice are able to generate this kind of pacemaker cells, but like HCN4-deficient mice also die around embryonic day 11.5 (171). Together, the mouse studies indicate that the HCN4 protein is required 1) for the formation of adult pacemaker cells during embryonic heart development and 2) for conferring cAMP-dependent upregulation of embryonic heart rate. Both HCN4-mediated functions are dispensable in early heart function and early embryonic heart development but are needed for the transition to late embryonic stages.

Given its vital role in embryonic heart, it came as a big surprise that mice in which HCN4 was deleted in adult SA node using a temporally controlled knock-out ap-
approach were viable and displayed a rather mild cardiac phenotype (172) (Fig. 11A). Like in embryonic pacemaker cells of global HCN4-deficient mice, $I_h$ in SA node cells of the adult HCN4-deficient mice was reduced by $\sim$80% (172). However, the lack of this current component did neither lead to a major impairment of pacemaker potential generation nor did it interfere with $\beta$-adrenergic regulation of heart rate (Fig. 11A). Unlike global knockout mice, adult HCN4 knockout mice revealed a normal basal heart rate and normal sympathetic and vagal heart rate modulation. However, adult HCN4-deficient mice displayed a cardiac arrhythmia characterized by recurrent sinus pauses (Fig. 11B). Pacemaker cells of adult HCN4 knockout mice were hyperpolarized by $\sim$8 mV and in most cases did not fire spontaneously under basal conditions. However, this functional impairment could be compensated by $\beta$-adrenergic stimulation. In conclusion, these findings suggest that in the adult SA node, HCN4 may serve as a kind of a stabilizer of the pacemaker potentials. In most situations, and particularly during sympathetic stimulation, HCN4 does not seem to be required to promote stable pacemaking. However, after an increase in repolarizing currents (e.g., vagal stimulation or transition from activated to basal cardiac state), HCN4 is activated and provides a depolarizing current, keeping the system well-balanced. The loss of this “depolarization reserve” may explain the induction of recurrent sinus pauses in HCN4 knockout mice.

**B. HCN2**

With respect to the stabilizing function, HCN2 that makes up $\sim$20% of SA node $I_h$ in mice may serve as a complementary channel to HCN4 (226) (Fig. 11C). HCN2-deficient mice also reveal a sinus dysrhythmia that is characterized by varying peak-peak intervals in the electrocardiogram (226). Other parameters of the sinus rhythm including autonomous heart rate regulation are normal in these mice. Like for HCN4, the maximal diastolic potential (MDP) of HCN2-deficient SA node cells is slightly hyperpolarized. It is important to note that HCN2 obviously is not crucial for the development of cardiac conduction system since HCN2-deficient mice do not display increased embryonic lethality.

**C. Conclusions and Open Questions**

In conclusion, genetic mouse studies indicate that the two components of SA node $I_h$ carried by HCN4 and HCN2 are required for maintaining a stable cardiac rhythm. However, neither HCN2 nor HCN4 is needed for principal pacemaking and for autonomous rate regulation in the adult heart. Further experiments (e.g., analysis of HCN2/4 double knockout mice) will be required to solidify these conclusions. Importantly, it remains to be elucidated why heart rate regulation in early embryos requires HCN4 but is independent of this channel in adult animals.

---

**FIG. 11. Effect of deletion of HCN4 and HCN2 on cardiac pacemaking.** A: HCN4 is not required for upregulation of the heart rate. Isoproterenol (0.5 mg/kg) injected at time 0 increased the heart rate of control ($n = 8$; black curve) and knockout ($n = 8$; red curve) animals to similar maximum levels. B: example ECG traces from control (top) and knockout mice (bottom) at 0.5 h (I) and 1.5 h (II) after isoproterenol injection. All traces are displayed at the same scale. C: sinus dysrhythmia in HCN2-deficient mice. Telemetric ECG recordings (lead II) were obtained simultaneously from wild-type (con) and HCN2-deficient mice (HCN2-KO) at rest.
In this context, it remains to be determined which current(s), if not $I_h$, are the downstream target(s) of cAMP signaling in adult SA node cells. Finally, it should be considered that the importance of HCN channels for cardiac rhythm initiation and frequency modulation may be different between species.

The analysis of human HCN4 channelopathies supports this notion (269, 290, 338, 395). So far, four different heterozygous HCN4 mutations have been identified in humans (269, 290, 338, 395) (Fig. 2). The mutations lead to loss of cAMP-dependent modulation (HCN4-573X) (338), hyperpolarizing shift of the activation curve (S672R and G480R) (269, 290), or a severe reduction of cell surface expression (D553R) (395). Interestingly, all patients suffering from these mutations have in common that they display a more or less severe bradycardia, a clinical phenotype that is not observed in HCN channel-deficient mouse models. Another issue that is difficult to explain at this moment is the effect of bradycardic agents, such as cilobradine, ivabradine, and zatebradine (373), as well as of clonidine (208). It was proposed that these agents lower heart rate by blocking $I_h$ channels and reducing the firing frequency of SA node cells. In agreement with this hypothesis, cilobradine lowers heart rate in wild-type mice but does not do so in adult HCN4 knockouts (174).

On the other hand, given that HCN4 confers the heart-rate lowering effect of cilobradine and related substances, why then do HCN4-deficient mice (which corresponds to a 100% pharmacological block) display no basal bradycardia? At present, there is no satisfying answer to this conundrum; however, solving this issue will undoubtedly profoundly advance our understanding of the molecular basis of rhythmicity in normal and diseased heart.

VIII. ROLE OF $I_h$ IN DISEASE

Given the widespread expression and physiological importance of HCN channels in nervous system and heart, it is obvious to assume that impaired expression or malfunction of these channels is associated with the genesis of human diseases. Indeed, evidence has accumulated over the last couple of years that $I_h$ is implicated in the pathologies of at least three classes of diseases: epilepsies, neuropathic pain disorders, and cardiac arrhythmias.

A. Inherited Channelopathies

In a classical sense, the term channelopathy refers to diseases that are caused by inherited genomic mutations in an ion channel gene that lead to loss, impaired expression levels, or functional defects of the channel protein. Interestingly, despite numerous efforts 10 years after the discovery of the HCN channel genes, only four inherited HCN channel mutations have been described (Fig. 2). All four are localized in the HCN4 gene and are associated with the induction of sinus bradycardia (see sect. viiC and Ref. 174). All patients identified so far are heterozygous for the respective HCN4 mutation. A likely explanation for this finding may be that like in mice disruption (370) or functional impairment (171) of both HCN4 alleles is associated with embryonic lethality due to the failure to develop a mature cardiac conduction system. Neuronal phenotypes have not been reported in the four groups of patients. This is surprising given the expression of HCN4 in CNS. So far, disease-causing mutations in human HCN1-3 genes have not been reported. On the basis of the analysis of murine HCN knockout models, it is expected that mutations in these genes would be implicated in complex cardiac (HCN2) and/or neuronal (HCN1, HCN2) phenotypes. However, one would also predict that even the total loss of these individual channels does not lead to lethality in mice. Thus one could assume that mutations in HCN1-3 proteins have much more impact in humans than in mice and are not detected because they are lethal. Alternatively, the mutations exist but have escaped detection so far because they occur very rarely. More systematical screening and sequencing approaches in patients will be required to clarify this issue.

B. Transcriptional Channelopathies

Transcriptional (or acquired) channelopathies result from pathological alterations of the expression or localization of a normal (nonmutated) ion channel (417). Such disorders occur more frequently than inherited monogenetic channelopathies, which are usually very rare. However, the analysis of transcriptional channelopathies is also much more difficult and prone to misinterpretations than the analysis of monogenetic diseases. The major obstacle in the analysis is that expression and cellular handling of ion channels usually is an extremely dynamical process that is regulated by numerous intracellular and external factors. In general, this complexity makes it difficult to decide whether an altered expression profile of an ion channel $I$ is causative to a disease, 2) is a compensatory process in response to a disease, or 3) represents a normal physiological variation that is neither causative nor compensatory to a disease. Having these limitations in mind, we will now summarize recent evidence supporting a role of HCN channels in transcriptional diseases.

1. Epilepsy

First evidence connecting $I_h$ and epileptic seizures came from studies in a rat model of childhood febrile seizures (hyperthermia model of febrile seizure) (83, 84). At the age of 10 days, these rats are exposed to a single period of hyperthermia lasting for ~30 min. Such animals
reliably develop epileptic convulsions originating in the hippocampus and reveal an increased tendency to develop seizures at adult stage. Electrophysiological recordings revealed an enhanced $I_h$ (increase of $I_{\text{max}}$ by 40% at $-70\,\text{mV}$) in hippocampal CA1 neurons of epileptic rats. In addition, the $I_h$ current showed a slight positive shift of the $V_{0.5}$ (+3 to +5 mV) and slowed activation kinetics, compared with wild-type rats. Functionally, the enhanced $I_h$ coupled to an increased probability of rebound depolarizations and action potential firing (83). Closer inspection of the molecular basis of $I_h$ observed after febrile seizures revealed a downregulation of HCN1 in hippocampal pyramidal neurons while HCN2 was upregulated, resulting in a decrease of the ratio of HCN1 vs. HCN2 protein from 8:1 to $\sim 4:1$ (47, 48). The mechanism underlying changes in HCN channel expression levels in response to seizures is unclear. Reduction of HCN1 channel expression is probably a transcriptionally regulated process that involves activation of calmodulin (CaM) kinase II and $\text{Ca}^{2+}$ entry via AMPA receptors (322). In contrast, upregulation of HCN2 was found to be independent of CaM kinase II (322). The consequences of up- and downregulation of HCN channel subunits may be more complex than originally expected. A recent coimmunoprecipitation study indicated that altered hippocampal HCN1 and HCN2 expression levels are accompanied by a long-lasting increase in the levels of heteromeric HCN1/2 channels (48). Thus the $I_h$ of hippocampal neurons is probably conferred by a complex mixture of HCN1 and HCN2 homomers as well as by HCN1/2 heteromers. Pathological deviations from the normal ratio of the individual HCN channel types may play a critical role in the generation and the long-term maintenance of hippocampal hyperexcitability.

Altered HCN channel expression has also been found in another class of seizures, the absence epilepsies. The typical absence is clinically defined by a sudden, brief impairment of consciousness and behavioral arrest. Immediately following the absence, there appears to be little if any disruption of cognitive abilities. Absences mainly originate in the cortex; however, the thalamus is also involved in the pathology of the disease (256, 360). In particular, the generation of spike-and-wave discharges (SWDs), a diagnostic hallmark of absences, is correlated with an increased prevalence of burst firing and synchronized oscillatory activity in thalamocortical circuits (256). Given the key role of HCN channels in controlling the firing behavior of thalamocortical neurons, abnormal HCN channel expression may contribute to the generation of absences. In agreement with this hypothesis, in two rat models for absence epilepsy [WAG/Rij (58, 376) and GAERS (212)], increased levels of HCN1 were found in thalamocortical neurons. In contrast, expression of HCN2-4 was not altered. In both rat models, the $I_h$ of thalamocortical neurons displayed a reduced sensitivity to cAMP that is consistent with an increase of HCN1 levels relative to HCN2. In the WAG/Rij model, but not in GAERS, $V_{0.5}$ of $I_h$ was also shifted to more hyperpolarized voltages. It was speculated that the hyperpolarizing shift in combination with the reduced cAMP sensitivity locks thalamocortical neurons in the burst firing mode (58).

Perhaps the strongest evidence linking $I_h$ channels with absences relates to HCN2-deficient mice (226) (Fig. 9). Like other absence models, HCN2-deficient mice display frequent bilateral synchrony SWDs in the EEG that are accompanied with brief episodes of immobility (so-called behavioral arrest). In thalamocortical and thalamic reticular neurons of these mice, $I_h$ was found to be almost completely abolished (1, 226, 320, 435). The residual $I_h$ (max. 20% of wild-type $I_h$) had a very slow kinetics and a strongly hyperpolarized $V_{0.5}$. As a consequence, the resting membrane potential of thalamocortical neurons from HCN2-deficient mice was shifted to hyperpolarized potentials, which could well explain the increased burst activity and network oscillations seen in these animals (see also sect. v).

Downregulation of $I_h$ was also found in a pharmacological (kainic acid) rat model of temporal lobe epilepsy (TLE) (342). Layer III pyramidal neurons of the entorhinal cortex (EC) of these epileptic rats are hyperexcitable, giving rise to profound synchronous network activity. Electrophysiological recordings in EC neurons revealed a significant reduction of dendritic $I_h$ which was caused by reduced HCN1 and HCN2 protein levels. Mechanistically, hyperexcitability induced by downregulation of $I_h$ could be put down to the key role $I_h$ plays in dendritic integration (see also sect. v). As predicted, reduction of $I_h$ densities in ddrinides leads to an increase of the dendritic input resistance. This, in turn, enhances dendritic EPSP summation and EPSP-spike coupling.

Rat and mouse models clearly indicated that impaired HCN channel function or expression is associated with epileptiform activity. Such a clear correlation, although likely, is so far missing in humans. As mentioned, HCN channelopathies involving epilepsy have not been reported so far. However, changes in HCN channel expression have been found in the dentate gyrus from patients with temporal lobe epilepsy and severe hippocampal sclerosis (35). Since upregulation of HCN1 mRNA was found only in cases of end-stage disease, long after the onset of epilepsy, it was suggested that it is not causative for the epilepsy but rather represents a compensatory change of the brain.

In conclusion, the role of $I_h$ in epilepsies seems to be extremely complex and diverse. There is evidence that 1) both up- or downregulation can be associated with the disease, 2) different HCN types contribute to different extents to the disease, 3) the role of an HCN channel strongly depends on its cellular localization, and 4) findings from rodents may not necessarily be applicable to
human epilepsies. In most cases it remains unclear whether an observed change in $I_h$ produces hyperexcitability or represents a compensatory reaction to the state of hyperexcitability. Thus the relevance of $I_h$ in epileptogenesis cannot be addressed by a simplified general model but has to be defined for various pathophysiological settings in a very specific and distinct manner.

2. Peripheral neuropathic pain

Peripheral neuropathic pain is a complex pain condition that is characterized by spontaneous pain, hyperalgesia, and allodynia (68). The disease originates from the injury of peripheral nerves. Nerve injury can have many etiologies, among which are trauma, viral infections (e.g., Herpes zoster), metabolic diseases (e.g., diabetes mellitus), vascular diseases (e.g., stroke), autoimmune diseases (e.g., multiple sclerosis), cancer, and exposure to radiation or chemotherapy. The pathomechanism of neuropathic pain is complex and involves both peripheral and central sensitization. A hallmark of the disease is the generation of abnormal spontaneous (ectopic) discharges. These discharges have strong rhythmic components and are generated from injured dorsal root ganglion (DRG) somata or axons, as well as from adjacent uninjured nerves. Previous work has shown that altered expression levels of several types of voltage-gated Na$^+$ channels contribute to the generation and persistence of spontaneous discharges (252, 417, 418).

Several findings suggest that HCN channels also play a prominent role in neuropathic pain (for recent review, see Ref. 197). First, $I_h$ has been identified in DRG neurons, particularly in large- and medium-sized neurons where most ectopic discharges are produced (253, 339). Second, in situ hybridization and immunohistochemistry revealed the presence of HCN1-3 channels in different types of mouse and rat DRGs (80, 276). Third, increased densities of $I_h$ were found in large- and medium-sized DRG neurons after experimentally induced injuries (80, 206, 432). Fourth, low concentrations of the $I_h$ blocker ZD7288 reverse both pain behavior and the spontaneous discharges in injured nerve fibers (80, 220, 380). Taken together, these findings strongly suggest that upregulation of $I_h$ that leads to an increased excitability of the cell is causative to the generation of ectopic firing. The mechanism underlying upregulation of $I_h$ remains to be investigated in more detail. Interestingly, Chaplan et al. (80) found a downregulation of HCN1 and HCN2 in DRGs after injury, although $I_h$ densities were increased. It is unclear how the discrepancy between current densities and protein expression can be explained. Potential mechanisms include a positive shift of $V_{th}$ of the $I_h$ (leading to an increased open probability), altered HCN channel subunit assembly, and increased cell surface expression of HCN channel proteins after injury.

3. Cardiac remodeling and arrhythmia

Structural and functional remodeling of the heart muscle is a clinical hallmark of a variety of cardiovascular diseases including cardiomyopathies, infarction, chronic hypertension, and inflammation (e.g., myocarditis) (14). Initially, these adaptations are beneficial to maintain cardiac function (e.g., at pressure overload) but in later stages of the diseases they contribute to contractile abnormalities and sudden death. On the cellular level, irregular expression of ion channels involved in the control of cardiac contractility and automaticity plays an important role in remodeling (176, 285, 286, 323, 392).

Upregulation of $I_h$ has been described in a variety of animal models of cardiac hypertrophy and heart failure as well as in human patients suffering from these diseases (for reviews, see Refs. 76, 173, 244). A common feature of these diseases is the profound upregulation of $I_h$ channels in ventricular cardiomyocytes which normally express very low levels of these channels. In contrast, the activation threshold of the $I_h$ of diseased cardiomyocytes is usually not shifted to more positive values compared with normal ventricular $I_h$ (76, 139, 177, 182). Increased $I_h$ densities strongly raise the tendency of the ventricular muscle to develop ectopic, “pacemaker-like” action potentials and, thereby, contribute to arrhythmia and to sudden death (72, 73, 139, 182, 374, 452).

In rodents and humans, upregulation of $I_h$ is mirrored by an increased expression of HCN channels. Examination of mRNA levels in hypertrophied atrial and ventricular myocytes revealed an upregulation of HCN2 and HCN4 in different animal models (72, 73, 139, 177, 182, 374, 375, 452). In addition, upregulation of HCN4 was found in human patients with end-stage heart failure (45). The degree of hypertrophy positively correlates with increased $I_h$ density (73) and the expression levels of HCN channels (130, 336). While these findings strongly support a direct link between upregulation of HCN channels and ventricular dysfunction, the signaling pathways underlying upregulation of $I_h$ are only poorly understood so far. Both neuronal and humoral factors are probably involved in the process. Notably, high angiotensin II levels, which are well-known to promote ventricular remodeling (382), as well as aldosterone (284) seem to play a pivotal role in HCN channel upregulation. This is supported by the finding that antagonists of the type I angiotensin II receptor (AT1-receptor) not only reduce cardiac hypertrophy but also hamper $I_h$ and HCN2/HCN4 mRNA overexpression (74, 75, 177). Interestingly, $I_h$ is abundantly expressed in spontaneously active fetal and neonatal ventricular myocytes (77, 325, 434). During maturation, these cells lose their capacity to generate spontaneous activity. Both in rat and mouse, this is accompanied by a progressive decrease of $I_h$ expression (77, 434). Thus it is tempting to assume that cardiac hypertrophy provokes a reentry of
cells into a fetal program and the reinitiation of the corresponding expression patterns which includes up-regulation of HCN2 and HCN4. Recently, it was found in rat models of ventricular hypertrophy that up-regulation of HCN2 and HCN4 is accompanied by pronounced reduction of two microRNAs, miR-1 and miR-133. Conversely, forced expression of these miRNAs prevented overexpression of HCN2/4 in hypertrophic cardiomyocytes (229). These findings suggest that downregulation of these two miRNAs may play an important role in the reexpression of \( I_h \) during normal heart development but also during remodeling of the diseased heart.

IX. HCN Channels as Novel Drug Targets

A. Heart Rate-Reducing Agents

High heart rate positively correlates with an increased mortality in a variety of cardiac diseases including heart failure, arterial hypertension, and ischemic heart disease (36, 130, 340). Lowering heart rate is therefore one of the most important therapeutic approaches in the treatment of these diseases. Currently used bradycardic drugs including \( \beta \)-adrenoceptor antagonists, and some \( Ca^{2+} \)-channel antagonists efficiently reduce heart rate, but their use is also limited by adverse reactions or contraindications (44, 127). Given the key role of \( I_h/HCN \) channels in cardiac pacemaking, these channels are very promising pharmacological targets for the development of novel and more specific heart-rate reducing agents (44). Importantly, HCN channels are not expressed in vascular and airway smooth muscle. Therefore, in contrast to \( \beta \)-adrenoceptor or \( Ca^{2+} \)-channel antagonists, drugs acting on HCN channels are not expected to have major side effects on the vascular system or on pulmonary function. In the past, several agents inhibiting cardiac \( I_h \) have been developed. Early drugs identified as pure bradycardic agents include alinidine (ST567), ZD7288, zatebradine (UL-FS49), and cilobradine (DK-AH269). A more recent drug is ivabradine (S16257). The principal action of all these substances is to reduce the frequency of pacemaker potentials in the sinus node by inducing a reduction of the diastolic depolarization slope. In this section, we only briefly discuss these drugs. We refer readers who are interested in more detailed information to a number of recent reviews on this issue (28, 42, 55).

Recently, ivabradine (S16257, Procoralan) was introduced into clinical use as the first therapeutic \( I_h \) blocker. Ivabradine blocks cardiac \( I_h \) at low micromolar concentrations and has been approved as a treatment of chronic stable angina pectoris (379). Electrophysiological studies revealed that ivabradine acts by accessing \( I_h \) channels from their intracellular side and by exerting a use- and current-dependent block (56). The mechanism of channel block by ivabradine was examined in heterologously expressed HCN channels (57, 388). Ivabradine blocks HCN4 and HCN1 channels with half-block concentrations (IC\(_{50}\)) of \( \sim 1-2 \mu M \). Interestingly, ivabradine acts as open channel blocker in HCN4 (like in sinoatrial \( I_h \)), while block of HCN1 requires channels either to be closed or in a transitional state between open and closed configuration (57). The structural determinants underlying this difference are still unknown.

Zatebradine and cilobradine are derived from the L-type \( Ca^{2+} \)-channel blocker verapamil. Low micromolar concentrations of both blockers (IC\(_{50}\) between 0.5 and 2 \( \mu M \)) inhibit sinoatrial \( I_h \) as well as heterologously expressed HCN channels in a use-dependent fashion (373, 401). Like with ivabradine, zatebradine block results from drug molecules entering the channel pore from the intracellular site (115).

ZD7288 is probably the most widely used experimental blocker of \( I_h \). Like the agents discussed so far, ZD7288 blocks channels from the intracellular site (348). However, the block is use independent and is associated with an \( \sim 15 \) mV shift of \( V_{th} \) to more negative potentials and with a decrease of the maximal channel conductance (46). Recently, two amino acid residues were identified in the S6-helix of HCN2 (A425 and I432) and that confer high-affinity binding of ZD7288 (88).

The well-known \( \alpha_2 \)-adrenoceptor agonist clonidine, which is chemically related to the bradycardic agent alinidine (353), was recently shown to block sinoatrial \( I_h \) (208). In mice lacking all three types of \( \alpha_2 \)-receptors, clonidine still exerts a profound bradycardic activity, indicating that inhibition of cardiac \( I_h \) contributes significantly to the netto-bradycardic effect of clonidine. Electrophysiological recordings revealed that clonidine blocks HCN4 and HCN2 (IC\(_{50}\) of \( \sim 10 \) \( \mu M \)) and also, though with less sensitivity, HCN1 (IC\(_{50}\) of \( \sim 40 \) \( \mu M \)). Like ZD7288, clonidine also shifts the voltage dependence of the channel by \( 10-20 \) mV to more hyperpolarizing potentials (208).

With the exception of ivabradine, HCN channel blockers were not introduced to therapy so far. A major obstacle of most existing agents is that they are not specific enough for sinoatrial (mainly HCN4-mediated) \( I_h \), but also block neuronal \( I_h \) in several regions of the nervous system. For example, visual disturbances that result from block of retinal \( I_h \) have been reported for several of these drugs (78). Moreover, some of the blockers may also interact with other ion channels. For example, recently it was reported that the widely used “specific” \( I_h \) blocker ZD7288 inhibits T-type \( Ca^{2+} \) currents in rat hippocampal pyramidal cells (330).
B. Blockers of Neuronal \( I_h \)

Modulation of \( I_h \) may also be a promising approach for treatment of disease processes in the central and peripheral nervous systems. In the previous section we discussed that upregulation of \( I_h \) in DRGs and peripheral nerves is an important process in the pathogenesis of neuropathic pain. Therefore, blockers of \( I_h \) may be beneficial to analgesic therapy. In principle, the existing \( I_h \) blockers could be used to treat neuropathic pain. However, if applied systemically, all known blockers would also exert bradycardic effects. To circumvent this problem, agents with high sensitivity to the HCN channel types relevant for neuropathic pain (mainly HCN1 and HCN2) would be wishful. Conversely, these blockers should not interfere with the function of sinoatrial HCN4. Drugs displaying this pharmacological profile do not exist so far, but given the wealth of chemical structures relevant to \( I_h \) channel block it should be a feasible task to develop subtype-specific blockers. The number of potential chemical entities related to \( I_h \) block is increased by agents that have been shown to block \( I_h \) in addition to their well-known primary receptor. For example, it was recently found that loperamide, a potent \( \mu \)-opioid-receptor agonist, blocks \( I_h \) of DRGs with quite high affinity (IC\(_{50}\) between 5 and 10 \( \mu M \) depending on the DRG type) (404). In addition, capsazepine, a well-known inhibitor of the vanilloid-receptor (TRPV1), blocks HCN1 in a concentration-dependent manner (IC\(_{50}\) = 8 \( \mu M \)) (154). The development of structural analogs of the mentioned agents may yield compounds that selectively inhibit specific HCN channel types.

The use of \( I_h \) blockers has also been implicated in therapy of epilepsies. However, given the complexity and diversity of the cellular mechanisms leading to these diseases, a clear concept for a rational design of antiepileptic \( I_h \) channel modulators has not yet emerged. A major conceptual problem is that both inhibition and activation of \( I_h \) may be beneficial depending on the type of epilepsy. For example, stereotactic injection of the \( I_h \) blocker ZD7288 reduced the generation of hippocampal epileptiform discharges in rabbit (207). On the other side, there is evidence that part of the antiepileptic activity of lamotrigine (an established blocker of voltage-gated Na\(^+\) channels) is caused by an upregulation of \( I_h \) in dendrites of pyramidal neurons (309, 311). Similarly, the antiepileptic drug gabapentin, which was shown to inhibit Ca\(^{2+}\) currents by binding to the \( \alpha_2\delta \)-subunit, may also act in part via upregulation of \( I_h \) (381).

Finally, HCN channels may contribute to the clinical actions of general anesthetics. Native neuronal \( I_h \), as well as heterologously expressed HCN channels are inhibited by clinically relevant concentrations (\( \leq 0.5 \text{ mM} \)) of the inhalational anesthetics halothane and isoflurane (59, 87). Similarly, the intravenous anesthetic propofol inhibits and slows the activation of native and expressed HCN channels (67, 233).

X. CONCLUSIONS AND FUTURE DIRECTIONS

Thirty years after the first discovery of \( I_h \) and 10 years after the cloning of HCN channels, the field of hyperpolarization-activated cation channels has emerged into one of the most exciting areas of ion channel research. Starting as an electrophysiological curio (the “funny” current), \( I_h \) channels are now considered as important regulators of many fundamental processes in nervous system, as well as in the heart. Extensive studies with native and heterologously expressed HCN channels, combined with modeling approaches, biochemistry, and X-ray crystallography, have dramatically increased our knowledge on the structural determinants of HCN channel function. Moreover, genetic mouse models are now available to study the physiological role of individual HCN channel types in vivo. Despite the progress made, numerous key questions are still unsolved and will have to be addressed by future approaches. In the following, a small selection of important problems is listed.

1) What is the structural basis for the “inverse” gating and the mixed K\(^-\)/Na\(^+\) permeability of HCN channels? How is binding of cAMP to the CNBD coupled to the activation gate of the channel?

2) Which cellular mechanisms control the subunit stoichiometry of HCN channels? Can HCN channel subunits freely assemble to (hetero)tetramers or are there restraints limiting free assembly?

3) Like other transmembrane proteins, HCN channels are probably associated with cellular proteins in large macromolecular complexes. It is important to identify the constituents of these complexes in different types of neurons and heart cells to understand how HCN channels are regulated in vivo under physiological conditions and in diseased states.

4) Which factors control the expression, the cellular processing, and the targeting of HCN channels to specific cellular compartments?

5) What is the detailed role of HCN channels in various diseases? The analysis of advanced genetic mouse models in conjunction with studies in human patients will be required to reach this goal.

6) Finally, given that HCN channels are relevant as disease factors, the full potential of these proteins as drug targets has to be further explored. To this end, high-affinity, subtype-specific HCN channel blockers must be developed.

Last but not least, HCN channels themselves may be useful as therapeutic agents. Currently, many laboratories develop strategies to generate so-called “biological” pacemakers (for recent reviews, see Refs. 90, 244, 351).}
logical pacemakers are genetically engineered cells that produce spontaneous pacemaker potentials. Such cells can be generated by direct transfer of HCN channel genes into quiescent heart tissue or by transplantation of engineered cells expressing HCN channels. In any case, biological pacemakers are an attractive alternative to electrical devices to stimulate automaticity in defined regions of the heart. At this point, it is too early to pass a final judgment on the relevance, the long-term reliability, and the safety of HCN channel-based therapies. Whatever the answer to this question finally will be, the study of HCN channels has profoundly increased our knowledge on important physiological and pathophysiological processes and, undoubtedly, will do so in the future.

ACKNOWLEDGMENTS

We apologize to all our colleagues whose studies have not been cited because of space constraints.

Addresses for reprint requests and other correspondence: M. Biel, Center for Integrated Protein Science CIPS-M and Zentrum für Pharmakologie, Department Pharmazie, Ludwig-Maximilians-Universität München, Butenandtstr. 5-13, D-81377 Munich, Germany (e-mail: mbiel@cup.uni-muenchen.de); C. Wahl-Schott, Molekularische Pharmakologie, Zentrum für Pharmakologie, Department Pharmazie, Ludwig-Maximilians-Universität München, Butenandtstr. 5-13, D-81377 Munich, Germany (e-mail: Christian.Wahl@cup.uni-muenchen.de).

GRANTS

This work was supported by the Deutsche Forschungsgemeinschaft, the Center for Integrated Protein Science CIPS-M, and the EU research project NORMACOR (LSHM-CT-2006-018676) within the Sixth Framework Programme of the European Union.

REFERENCES


FUNCTION OF HCN CHANNELS


128. DiFrancesco D, Tromba C.
129. 878 BIEL ET AL.
130. Doan TN, Kunze DL.
131. Edman A, Grampp W.
186. Hagiwara N, Irisawa H. Modulation by intracellular Ca²⁺ of the hyperpolarization-activated inward current in rabbit sino-sinu-
187. Hagiwara N, Irisawa H, Kameyama M. Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-
193. Hofmann S, Stieber J, Hofmann F, Ludwig A. Temporarily controlled deletion of the pacemaker channel HCN4 in the sino-
201. Hoshi N, Takahashi H, Shahidullah M, Yokoyama S, Higashida H. KCRI, a membrane protein that facilitates functional expression of non-inactivating K⁺ currents associates with rat EAG voltage-


323. Santoro B, Grant SG, Bartsch D, Kandel ER. Interactive cloning with the SE3 domain of N-src identifies a new brain specific ion channel protein, with homology to eag and cyclic nucleotide-gated channels. Proc Natl Acad Sci USA 94: 14815–14820, 1997.


343. Santoro B, Grant SG, Bartsch D, Kandel ER. Interactive cloning with the SE3 domain of N-src identifies a new brain specific ion channel protein, with homology to eag and cyclic nucleotide-gated channels. Proc Natl Acad Sci USA 94: 14815–14820, 1997.


