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The fast and slow ups and downs of HCN channel regulation

Alan S. Lewis[1], **Chad M. Estep**[1], and **Dane M. Chetkovich**[1,2,*]

[1]Davee Department of Neurology and Clinical Neurosciences, Northwestern University; Chicago, IL USA

[2]Department of Physiology; Feinberg School of Medicine; Northwestern University; Chicago, IL USA

Abstract

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (h channels) form the molecular basis for the hyperpolarization-activated current, I_h and modulation of h channels contributes to changes in cellular properties critical for normal functions in the mammalian brain and heart. Numerous mechanisms underlie h channel modulation during both physiological and pathological conditions, leading to distinct changes in gating, kinetics, surface expression, channel conductance or subunit composition of h channels. Here we provide a focused review examining contemporary mechanisms of h channel regulation, with an emphasis on recent findings regarding interacting proteins such as TRIP8b. This review is intended to serve as a comprehensive resource for physiologists to provide potential molecular mechanisms underlying functionally important changes in I_h in different biological models, as well as for molecular biologists to delineate the predicted h channel changes associated with complex regulatory mechanisms in both normal function and in disease states.

Keywords

HCN channels; channel trafficking; TRIP8b; I_h; I_f; epilepsy; long QT syndrome; voltage-gated ion channel

Introduction

Voltage-gated ion channels (VGICs) are expressed by many cell types, but are of critical importance in excitable cells such as neurons or cells of the sinoatrial node (SAN) in the heart. Most VGICs are activated upon membrane depolarization, yet the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel (h channel) is activated by hyperpolarization[1–5] and underlies the hyperpolarization-activated current, I_h (I_f or I_q in the heart, for *funny* or *queer* current, respectively; when discussing the neuronal or cardiac current I_h or I_f will be used, respectively), observed in many types of neuronal[4,6] and cardiac[3, 7, 8] cells. Four HCN pore-forming α-subunits have been identified, numbered HCN1–4,[9–11] and their expression profiles are cell type-specific. h channels are permeable

*Correspondence to: Dane M. Chetkovich; d-chetkovich@northwestern.edu.

primarily to Na^+ and K^+ ions, and have roughly four times greater permeability for K^+ compared to Na^+, yet upon activation h channels conduct a net inward current.[1] Because h channels are activated near typical resting membrane potential (RMP), I_h significantly contributes to the determination and stabilization of RMP as well as input resistance. In neurons, the presence of h channels in dendrites endows specific functional significance and helps regulate cellular input-output properties. Importantly, dendritic h channels regulate dendritic integration of excitatory post-synaptic potentials (EPSPs) and reduce temporal summation of distal inputs.[12–15] Furthermore, dendritic I_h influences additional ionic conductances, such as T-type and N-type voltage-gated Ca^{2+} channels[16] and the delayed rectifier M-type K^+ channel.[17] Such interactions have important functional consequences; for instance, HCN1 knockout mice demonstrate enhanced spatial learning and long-term potentiation (LTP), implicating HCN1 and I_h as an inhibitory constraint on these properties.[18] These findings are partially explained by increased resting inactivation of Ca^{2+} channels of hippocampal area CA1 by HCN1-mediated I_h.[16]

h channels play important functional roles in both the central and peripheral nervous systems as well as in the heart in healthy and disease states. Because they are activated at hyperpolarized potentials, h channels can play a role in rhythmogenesis, which has been studied extensively in thalamocortical (TC) neurons and cells of the SAN.[6] The contribution of I_h to rhythmicity may be exemplified in TC neurons, whereby membrane depolarization from activation of I_h activates a low-threshold t-type Ca^{2+} current I_t, which ultimately triggers a fast burst of Na^+/K^+ spikes. Deactivation of I_h by this depolarization as well as inactivation of I_t leads to a hyperpolarizing overshoot, and ultimately reactivation of I_h to renew the cycle.[6] This intrinsic oscillatory mechanism of TC neurons is modified by reciprocally connected inhibitory GABAergic input from neighboring neurons of the reticular thalamic nucleus (RTN), which ultimately can be observed as delta and spindle waves in the electroencephalogram (EEG) of mammals in non-rapid eye movement sleep.[19] I_f is also believed to contribute to rhythmicity and regularity in cardiac pacing, yet its role has not proven as straightforward as in neurons.[5,8,20]

Just as it is clear that h channels and I_h play rhythmogenic roles in homeostatic functions such as sleep and cardiac rhythm, research over the past decade has implicated h channel dysfunction in the pathophysiology of neurological and cardiac disorders, not surprisingly many involving dysrhythmia and altered cellular excitability. Changes in I_h have been demonstrated in multiple rodent models of epilepsy, including absence epilepsy and temporal lobe epilepsy (TLE).[21] Mice lacking the HCN2 subunit demonstrate severe absence epilepsy, with significant changes in firing behavior of TC neurons and a shift from tonic to burst firing modes,[22,23] and rat models of absence epilepsy have also demonstrated altered regulation of h channels in layer V cortical neurons.[24] Numerous studies have found h channel misregulation in TLE, although the form of h channel dysfunction differs in different studies and includes changes inkinetics, protein expression, channel heteromerization and channel localization.[25–30] Additionally, h channels have been implicated in neuropathic pain, whereby blockade of I_h with the selective antagonist ZD7288 significantly reduced allodynia following spinal nerve ligation.[31]

As summarized above, despite their relatively recent molecular discovery at the close of the 20th century, h channels are now known to play a diverse yet critical role in both normal function and may contribute to pathological conditions. Electrophysiological studies of h channels in neuronal networks have demonstrated that small changes in I_h current density, voltage gating and kinetics can have dramatic macroscopic consequences due to interaction with additional currents in complex systems.[16,17] Thus, a comprehensive understanding of how h channels are situated within a dynamic environment of molecular modifiers will permit a more mechanistic-based understanding of observed changes in I_h. Here we provide a tool for the research community studying h channels or neuronal networks involving I_h and offer a straightforward, reductionist view of distinct h channel properties and the mechanisms that affect them. This review is thus organized by observed phenomena, i.e., altered V_{50}, kinetics, etc., rather than by individual regulator of I_h, i.e., cAMP, intracellular pH, etc. Our goal is to aid researchers in translating an observed change in I_h or h channels in a given biological system to a mechanistic discovery, with the ultimate goal of identifying potential therapies for clinically significant disorders such as epilepsy, neuropathic pain and heart failure.

Mechanisms Regulating Distinct Properties of I_h and HCN Channels

Channel gating

(see Tables 1 and 2). As previously mentioned, the voltage gating of I_h is markedly different from most VGICs in that it is activated by hyperpolarization to potentials negative of −50 to −60 mV, rather than by depolarization. The mechanism underlying the activation of h channels by hyper-polarization involves the positively charged S4 voltage sensor similar to other VGICs, although how this region is coupled to channel opening by hyperpolarization rather than the more common depolarization is not yet completely understood.[1,4] HCN channels are not the only VGICs activated by hyperpolarization. The *Arabidopsis thaliana* inwardly rectifying K^+ channel KAT1 also demonstrates activation upon hyperpolarization, again with an activation mechanism reliant upon the S4 voltage sensor.[32] In contrast, the inwardly rectifying K^+ (K_{ir}) channels, a family of tetrameric two-transmembrane ion channels that differ from the six-transmembrane voltage-gated K^+ (K_v) channels, do not contain a voltage sensing S4 helix but rather activate upon hyperpolarization by removal of Mg^{2+} ions and polyamines that physically block the channel pore.[33] The rate of unblocking by Mg^{2+} and polyamines differ, thus hyperpolarization of $K_{(ir)}$ channels leads to an initial time-independent current followed by a time-dependent current. Thus, multiple families of ion channels are activated by hyperpolarization, yet the mechanism by which they are activated by voltage may differ.

When expressed individually in heterologous systems, HCN1–4 exhibit markedly different characteristics of voltage gating, with half maximal voltage of activation (V_{50}) ranging from −70 mV for HCN1 to −100 mV for HCN4.[1] Another important difference between h channel subunits is in sensitivity to the ubiquitous second messenger cAMP. HCN1 is weakly sensitive to cAMP, while HCN4 is extremely cAMP sensitive and HCN2 and HCN3 display intermediate sensitivities. cAMP shifts the voltage gating of h channels in a depolarizing direction, allowing more channels to open at less hyperpolarized membrane

potentials.[4] The structural basis of this modulation has been studied in HCN2 channels by Zagotta and colleagues, who examined the interaction of cAMP and cGMP with two different C-terminal fragments containing the cyclic nucleotide binding domain (CNBD) and the region connecting the CNBD to the channel pore (C-linker).[34] X-ray crystallographic structures demonstrated that the C-linker consists of 6 α-helices and is almost solely responsible for tetramerization of channel subunits, while the CNBD binds cyclic nucleotides and consists of four α-helices with a β-roll between two helices that encompasses eight β-strands and is similar to the CNBDs of cAMP-binding proteins in *Escherichia coli* and cAMP-dependent protein kinase (PKA).[34] Experiments performed by Wainger et al. involving deletion of specific regions of HCN1 and HCN2 followed by assessment of cAMP-induced changes in voltage gating have synergized with these structural data to provide a model for the mechanism by which cAMP alters h channel voltage gating.[35] Deletion of the HCN2 CNBD C helix eliminated sensitivity to cAMP, yet did not change the V_{50} in the absence of cAMP.[35] However, deletion of the entire HCN2 CNBD significantly depolarized the V_{50} in the absence of cAMP to a level near that of wild type channels in the presence of cAMP.[35] Taken together with the structural data, the CNBD appears to exert an inhibitory effect on channel gating that is removed by cAMP binding to the CNBD C helix, which is transmitted to the pore region by the C-linker. Indeed, chimera studies have shown the C-linker is important in determining the different depolarizing effects of cAMP between h channel subunits.[36]

While regulation of h channels by cAMP is an important factor in determining I_h gating both in vitro and in vivo, it is far from the only factor. Expression of individual h channel subunits in heterologous cells yields expression of functional I_h, yet these currents do not completely recapitulate native I_h gating parameters that are highly variable in many cell types.[4] One explanation is the formation of heteromeric channels by different h channel subunits. h channels are tetramers, and can exist as homotetramers or heterotetramers, which have been described in both in vitro and in vivo systems.[30,37–39] An example of this phenomenon is the comparison of I_h recorded in oocytes expressing HCN1 or HCN2 in comparison to hippocampal area CA1 pyramidal neurons, which are known to express both HCN1 and HCN2.[37,40] Oocytes coexpressing HCN1 and HCN2 subunits demonstrate I_h with voltage activation and cAMP sensitivity intermediate to that of these channel subunits expressed alone.[37] These properties however were not recapitulated by merely summing the properties of HCN1 and HCN2 homotetramers, arguing against an I_h composed of distinct populations of HCN1 and HCN2 homomers. Rather, the properties of these presumed HCN1/HCN2 heteromeric channels resemble I_h recorded from CA1 pyramidal neurons, implying that functional heteromerization occurs in vivo. Biochemical and functional heteromerization appears to be possible between all h channel subunits with the exception of HCN2 and HCN3.[39] Thus, heteromerization represents a distinct mechanism by which channels can alter their biophysical properties, with almost endless diversity depending on differential expression levels and presumably altered stoichiometries of heteromeric channels.

Joining cAMP, additional small molecules and ions can have distinct effects on h channel gating, including phosphoinositides and protons. As described earlier, much research in the

h channel field has been devoted to reconciling differences in heterologously expressed I_h from I_h in vivo. Excised patches or long-term dialyzed whole-cell recordings from transfected heterologous cells or cells naturally expressing h channels demonstrate a "run-down" phenomenon, whereby voltage activation is shifted significantly in the hyperpolarizing direction over time, a difference that cannot be wholly accounted for by changes in cAMP levels.[41] One mechanism attributed to run-down of other types of channels, including K_v,[42] K_{ir},[43] and P/Q-type voltage-gated Ca^{2+} channels[44,45] is the loss of phosphatidylinositol 4,5-bisphosphate (PIP_2), a membrane phospholipid. Experiments in oocytes expressing h channel subunits (as well as dopaminergic midbrain neurons and embryonic cardiomyoctes) have demonstrated that PIP_2 and other phospholipids with a negatively charged head-group shift the V_{50} of I_h produced by HCN1, HCN2 and HCN4 by ~20 mV in the depolarizing direction in a quasi-cAMP-independent mechanism.[41,46,47] Specifically, while PIP_2 shifts V_{50} in the depolarizing direction even in the presence of saturating levels of cAMP, the overall magnitude of the effect is less than when observed in the absence of cAMP.[41] It is thought that PIP_2 is an allosteric regulator of h channels at a site distinct from the CNBD, because its depolarizing effect remains in HCN subunit constructs lacking the CNBD, and that there may be downstream convergence of the biophysical mechanisms altering the gating of I_h.

Additional research however has found that PIP_2 is not the only molecule from the large group of lipid signaling molecules that regulates h channel gating. This possibility was suggested by the evidence that multiple receptors positively coupled to phospholipase C (PLC) *enhanced* gating of I_h, a finding opposite to that predicted if PIP_2 was the only molecule of importance within this cascade.[41,46–48] Fogle et al. sought to pharmacologically isolate the role of diacylglycerol (DAG) signaling on h channel function by using the DAG mimetic 4β-phorbol 12-myristate 13-acetate (4βPMA), an agonist of the C-1 DAG/4β-phorbol-binding site.[48] Incubation of Xenopus oocytes expressing HCN1 and HCN2 cDNA with 4βPMA led to both a depolarizing shift in V_{50} as well as a dose-dependent 3–5-fold decline in maximal current amplitude. Multiple proteins contain C-1 binding sites, with the classical examples being the protein kinase C (PKC) conventional and novel isoforms, cPKC and nPKC, respectively. PKC activation can potentially alter intracellular cAMP and proton concentrations, both second messengers known to affect h channel biophysics.[48] However, the actions of 4βPMA were separated from effects on cAMP/H^+ concentration by the demonstration that 4βPMA evoked the same current modulation on h channels rendered insensitive to cAMP and proton concentration by point mutations, implying that activation of C-1 site-containing proteins operates independently of cAMP/H^+ concentration. Experiments with cell-free excised inside-out patches revealed that 4βPMA does not interact directly with h channels but rather requires an intact intracellular environment, thus implying a signal transduction cascade downstream of the actions of 4βPMA. Further experimentation revealed that the actions of 4βPMA on h channel gating are directly manifest through phosphatidic acid (PA) and arachidonic acid (AA) generated specifically through two PKC-signaling pathways.[48] Interestingly, while the voltage gating changes by 4βPMA are specific to h channels, studies using KAT1 demonstrate that the observed maximal current decrease by 4βPMA is not specific for h channels. The mechanism of

action of PA and AA on h channels was demonstrated to most likely be due to specific channel-lipid interactions at the conserved core region.[48]

Similar to some other VGICs, h channels are sensitive to both intracellular and extracellular proton concentrations. In TC neurons of the rat ventrobasal complex, compared with control recordings performed at pH = 7.1 alkaline pipette solutions (pH = 7.5) elicited a 4–5 mV shift in V_{50} in the depolarizing direction while more acidic pipette solutions (pH = 6.7) brought about a small but Significant 2–3 mV hyperpolarizing shift in V_{50}.[49] These changes were also independent of cAMP concentration. Such modification of voltage gating of h channels by protons is controlled by a solitary histidine residue located at the boundary between the S4 helix and S4-S5 linker (H321 in HCN2) that is completely conserved amongst all four h channels sub-units.[50] Although TC neuron V_{50} does not appear to be sensitive to changes in extracellular pH, I_h in cells of the vallate papilla was modified by extracellular pH, suggesting these currents are important in the signal transduction of sour taste.[51] specifically, decreased extracellular pH induced the opposite effect on I_h as decreased intracellular pH, namely a shift of V_{50} to more depolarized potentials. The ability of protons to modify the voltage gating of h channels is similarly observed with other ion channels, including N-type Ca^{2+} channels and TRPV1.[52,53] Repetitive firing in TC neurons leads to intracellular acidification as measured by an intracellular pH-sensitive indicator, which alters the properties of I_h from before firing to after, thus representing a dynamic, activity-dependent mechanism of I_h regulation.[49] In addition, modification of intracellular pH may play a role in the mechanism of action of the anti-epileptic drug acetazolamide, a carbonic anhydrase inhibitor used for treatment of absence epilepsy. Extracellular acetazolamide induced an intracellular alkalinization of ~ 0.19 pH units, which led to a 5–7 mV depolarization of the I_h V_{50}.[54] Such enhancement of I_h most likely inhibits firing of rebound Ca^{2+} bursts and helps bring about the termination of oscillatory activity seen in absence epilepsy.

Phosphorylation is a central mechanism for modifying both the function and trafficking of ligand-gated and voltage gated ion channels, and a growing amount of evidence exists to support modification of h channel voltage gating by phosphorylation via different kinases. A hint of the importance of the tyrosine kinase Src for modulation of h channels stemmed from the use of the SH3 domain as bait in a yeast two-hybrid screen that ultimately identified HCN1.[55] Multiple subsequent studies went on to find that tyrosine phosphorylation by Src alters channel properties of I_h in rabbit SAN, typically by increasing I_f conductance rather than altering voltage gating (see next section) .[56–60] However, when expressed in Xenopus oocytes, the V_{50} of HCN2 was negatively shifted by genistein, a tyrosine kinase inhibitor, implying that tyrosine kinase activity leads to a depolarization of HCN2 gating. Similarly, in rat ventricular myocytes, which express mostly HCN2 subunits, application of genistein also shifted the midpoint of activation by 14 mV in the hyperpolarizing direction.[57] Furthermore, coexpression of HCN4 with a constitutively active form of Src in HEK293 cells depolarized the half-maximal voltage of activation by ~13 mV, an affect likely mediated by phosphorylation at tyrosine 531.[56,61] In addition to regulation of I_h by tyrosine kinase activity, Huang et al. recently hypothesized that altered activity of tyrosine phosphatases may also modify I_h . They found that the receptor-like protein-tyrosine phosphatase-a (RPTPα) dephosphorylates HCN2 tyrosines in HEK293 cells, which correlates with

decreased surface expression of HCN2 and decreased I_h .[62] From there, they went on to show that non-specific phosphatase inhibition using phenylarsine oxide or sodium orthovanadate shifted the threshold of activation of I_h in adult rat ventricular myocytes to significantly depolarized potentials.

Serine/threonine pathways have also been implicated in h channel modulation. A study by Poolos et al. demonstrated that p38 mitogen-activated protein kinase (p38 MAPK), but not other members of the MAPK family, can also regulate the voltage gating of I_h in hippocampal neurons.[63] Pharmacological inhibition of p38 MAPK leads to a ~25 mV hyperpolarizing shift of V_{50}, while anisomycin, a p38 MAPK activator, depolarizes I_h V_{50} by ~11 mV. Whether p38 acts through direct channel subunit phosphorylation or other mechanisms is as yet unknown.

A curious mechanism for regulation of the voltage dependence of I_h may be proteolysis of channel subunits. Knockout studies have identified important roles for both the HCN2 and HCN4 subunits in mouse heart, with I_f being significantly reduced in myocardium upon knockout of either of these subunits.[22,64,65] Ye et al. performed immunoprecipitation studies in order to determine whether HCN2 and HCN4 form heteromeric channels in adult mouse heart and found that although the size of HCN4 protein was as expected, only a truncated, ~60 kDa form of HCN2 (HCN2ΔC) was expressed.[66] HCN2ΔC lacks the C-terminal CNBD, and associates with HCN4 in both heart and heterologous cells upon coexpression. Interestingly, when expressed alone, HCN2ΔC does not express functional I_h, however, when coexpressed with HCN4, HCN2ΔC shifts the V_{50} of the resulting current slightly in the depolarized direction when compared to HCN4 V_{50} alone.

New data is also emerging to suggest that h channels are modulated by peptides. Orexin A, a peptide that promotes wakefulness, has been implicated in the regulation of h channels in layer V pyramidal neurons of the mouse prelimbic (PL) cortex.[67] In PL cortical slices, application of orexin A increases cellular excitability. In exploring mechanisms for enhanced excitability, orexin A was found to significantly inhibit I_h amplitude in these neurons at almost all hyperpolarized voltages tested, and the V_{50} was shifted in the hyperpolarized direction by ~8 mV. PKC inhibitors attenuated the action of orexin A on I_h amplitude, suggesting that orexin A may act through a PKC-mediated mechanism to inhibit I_h, similar to the peptide neurotensin[68] and the neurotransmitter serotonin.[69] Thus, likely through second messengers, orexin A may contribute to arousal by modulation of h channels in the prefrontal cortex.[67] Additional peptides, such as cortistatin, also influence I_h, and will be discussed at a later time.

Finally, interacting proteins of h channels have emerged as an important group of modulators of h channel voltage gating. While the most basic characteristics of VGICs are intrinsically governed by their pore-forming α-subunits, association with additional proteins can have a profound effect on almost all of the most important properties of VGICs, including voltage gating, kinetics, surface expression, channel conductance and localization. Binding partners for VGICs are most often classified as scaffolding proteins, adaptor proteins, chaperones or auxiliary subunits, with some proteins occupying more than one category. Scaffolding proteins provide a single site for aggregation of multiple proteins that

bind to the scaffold through a variety of interaction motifs and create a functional complex.[70] Postsynaptic density protein-95 (PSD-95) serves as an example of a classical scaffolding protein at the postsynaptic density, where it links *N*-methyl D-aspartate (NMDA) receptors with downstream signal transduction molecules. Adaptor proteins can be thought of as a large subset of scaffolding proteins that bind proteins through diverse protein-protein interaction domains, linking together two or more proteins that would not have ordinarily bound to each other yet oftentimes participate in a signal transduction cascade.[70] Chaperone proteins are highly conserved between species and temporarily interact with their binding partners to promote proper protein folding or trafficking, but are not part of the functional protein complex. Common examples include the heat shock proteins, which may be upregulated during periods of cellular stress and in disease.[71] Finally, auxiliary subunits (also referred to as β-subunits) bind to their partner OC-subunit in a stable complex. Auxiliary subunits may be either cytoplasmic or transmembrane, and oftentimes their expression leads to profound changes in channel biophysics, surface trafficking or protein stability. For example, the K_v channel-interacting protein (KChIP) family binds to K_V4 channels, promotes surface expression, and alters the kinetics of associated α-subunits.[72] Auxiliary subunits have been identified for almost all VGICs,[72–75] and mutations in many of these subunits lead to hereditary disorders in excitable cells of both the brain and heart. Examples include familial epilepsy ($Na_V\beta1$,[76]), periodic paralysis (KCNE3/ MiRP2,[77]), and familial long QT syndromes (LQTS) (KCNE1/ MinK[78,79] and KCNE2/MiRP1,[80,81]).

Although VGICs differ markedly in their ion selectivity and voltage gating, the general structure of the pore-forming a-subunits follows a small number of common rules.[75] Thus, although h channels differ in some respects from the voltage-gated potassium (K_V) channels, cyclic nucleotide-gated (CNG) channels, and transient receptor potential (TRP) channels, their overall structure is similar in that they are each tetramers of subunits. Indeed, the similarity between h channels and CNG channels, which contain a cyclic nucleotide binding (CNB) site, was exploited during initial efforts to clone the channel genes underlying I_h.[9–11, 82] Phylogenetic analysis of the four cloned HCN genes revealed that they form a new subfamily within the potassium channel superfamily, but that they are closely related to the ether-à-go-go (EAG)-related channels, CNG channels and KAT1 channels, all of which contain a CNBD.[82]

Because of shared structural motifs, it is not altogether surprising that an auxiliary subunit or group of auxiliary subunits interacts with more than one member of the K^+ channel superfamily, which is indeed the case for the MinK-related peptide (MiRP) family. MiRPs are encoded by *KCNE* genes, of which there are five known members, *KCNE1–5*, with *KCNE1* also known as MinK (minimal K^+ channel protein). The gene products of *KCNE1–5* are single transmembrane proteins with small intra- and extracellular domains, and MiRP family members have been reported to interact with $K_V1–4$, human ether-à-go-go related gene (hERG) channels, KCNQ, and HCN channels.[75,83,84] KCNE2 (MiRP1) has been demonstrated to interact with HCN1, HCN2 and HCN4 subunits, but its role in h channel voltage gating is controversial. Coexpression of HCN1, HCN2 or HCN4 with KCNE2 and subsequent evaluation of V_{50} in oocytes, HEK293 cells, or Chinese hamster ovary (CHO)

cells failed to demonstrate any significant change in voltage gating.[85–88] Furthermore, no change in V_{50} was found after adenoviral overexpression of HCN2 and KCNE2 in cultured neonatal rat ventricular myocytes.[89] However, Decher and colleagues found that coexpression of HCN4 with KCNE2, but not KCNE1, 3 or 4, could hyperpolarize I_h V_{50} on the order of ~24 to ~14 mV for oocytes and CHO cells, respectively.[90] The underlying reason for these discrepancies is unknown.

In an attempt to identify proteins required for functional expression of the channel underlying the low-threshold non-inactivating K^+ current $I_{K(ni)}$, KCR1 was isolated by suppression cloning.[91] KCR1 contains 12 transmembrane domains and is expressed in both brain (cerebrum and cerebellum) and a variety of peripheral tissues. Antisense experiments in oocytes injected with cerebellar poly(A)$^+$ RNA demonstrated that KCR1 is required for fully functional $I_{K(ni)}$, and coexpression of KCR1 with a rat homologue of ether-à-go-go (r-EAG) in both oocytes and COS-7 showed an increase in channel activation that appears to be mediated by a direct protein-protein interaction.[91] KCR1 also interacts with the human ether-à-go-go-related gene (HERG).[92] KCR1, like KCNE2, binds to multiple members of the voltage gated K^+ channel superfamily and has recently been found to interact with HCN2.[93] Coexpression of KCR1 with HCN2 in CHO cells hyperpolarized the V_{50} by ~8 mV.[93] KCR1 is expressed in multiple regions of rat and pig heart, and adenoviral infection of both neonatal and adult ventriculocytes with KCR1 also significantly hyperpolarized V_{50} of endogenous I_f. The opposite effects were found when siRNA was used to knockdown levels of KCR1 in neonatal myocytes.[93] The role of KCR1 in vivo with respect to h channels is difficult to answer since it is also expressed in many tissues not known to express h channels, and therefore may interact with a multitude of additional proteins.

Scaffolding proteins can also influence voltage gating of h channels in vitro. For example, in heterologous cells, expression of filamin A with HCN1 significantly hyperpolarized the V_{50} of I_h and slowed both activation and deactivation kinetics.[94] The filamin gene family in mammals consists of *FLNA*, *FLNB* and *FLNC*, which code for the three filamin proteins, filamin A, B and C, respectively. Filamin A is a large cytoskeletal protein capable of binding actin, and contains 24 immunoglobin-like repeats and an actin-binding site at the N-terminus.[95,96] It has been reported to interact with multiple ligand- and voltage-gated ion channels, including the dopamine receptors D_2 and D_3,[97] $K_V4.2$,[98] and $K_{ir}2.1$.[99] Similarly to KCNE2, in addition to binding to voltage-gated potassium channels, filamin A also binds the HCN1 C-terminus via its final two Ig-like repeats.[94] Filamin A did not interact with HCN2 or HCN4, and bound a 22 amino acid (amino acids 694–715) region distal to the HCN1 CNBD.[94]

A number of recent papers have elevated the tetratricopeptide-containing Rab8b–interacting protein (TRIP8b) toward the top of the list for researchers interested in how auxiliary subunits and binding proteins may affect the surface expression, subcellular localization, gating and kinetics of h channels in neurons. Two independent groups seeking answers to different questions are credited with identifying the TRIP8b protein. Chen et al. were interested in further understanding the function of Rab8b, a small GTP binding protein of the large Rab family of proteins involved in vesicle trafficking and identified TRIP8b as a Rab8b binding partner through a yeast two-hybrid (Y2 h) library screen of rat brain

cDNA.[100] TRIP8b is ~70 kDa in size, and contains six TPR motifs to form a TPR domain, a common protein-protein interaction domain. Specific antisera revealed that TRIP8b was highly enriched in the brain and overexpression of TRIP8b, similarly to overexpression of Rab8b, was found to increase cAMP-induced secretion of ACTH in ATt20 cells, implicating TRIP8b and Rab8b in a secretory pathway for forward vesicle trafficking.[100]

TRIP8b is closely related to the peroxisomal import receptor protein Pex5p, exhibiting significant identity within the C-terminal TPR domains. Pex5p binds to proteins containing a peroxisomal-targeting signal 1 (PTS1), a tripeptide located at the C-terminus of proteins destined for peroxisomal import.[101] In a screen for proteins related to Pex5p Amery et al. discovered Pex5p–related protein (Pex5Rp, their nomenclature for TRIP8b) via in silico and in vitro approaches.[102] Although Pex5Rp bound PTS1 in in vitro assays, it did not appear to act as a peroxisomal import protein, as it was unable to bind to Pex14p or Pex12p, and expression of Pex5Rp in Pex5p–deficient mouse fibroblasts did not rescue the peroxisomal import defect. In agreement with the findings of Chen et al. in rodent brain, Pex5Rp was also highly enriched in human brain, but mRNA was also found in much lower abundance in the pancreas, testis and pituitary gland.[100,102] Taken together, these two initial reports on TRIP8b/Pex5Rp demonstrated the protein to be a brain-enriched cytoplasmic protein able to bind PTS1 and function in vesicle trafficking, but that TRIP8b/Pex5Rp is not sufficient for peroxisomal protein import.

A Y2 h screen of a mouse brain cDNA library aimed at the identification of new h channel interacting proteins identified TRIP8b as a binding partner of all four h channel isoforms.[103] Similar to the binding mechanism with PTS1-containing peptides, the binding of TRIP8b to the h channel subunits required the presence of the C-terminal tripeptide, which in the case of HCN1, 2 and 4 ends -SNL, and for HCN3, -ANM. Immunohistochemistry for TRIP8b and HCN1 demonstrated exquisite costaining in distal dendrites of CA1 and layer V neocortical pyramidal neurons, implying that TRIP8b's association with at least HCN1 may play a functional role in vivo, an implication strengthened by the finding that TRIP8b enrichment in the distal dendrites of layer V neurons in the cortex was abolished in the $HCN1^{-/-}$ mouse. Given the association between TRIP8b and HCN1 in regions of the mouse brain known to possess high levels of h channels and I_h,[15,40,104,105] it was surprising that Santoro et al. found coexpression of HCN1 or HCN2 with TRIP8b led to dramatic downregulation of I_h and surface-associated h channel protein in oocytes as well as in cultured hippocampal neurons, with the decrease in I_h dependent upon the presence of the h channel C-terminal tripeptide.[103] These effects on I_h and h channel protein seemed to be mediated at least in part by trafficking to an $EEA1^+$ endosomal compartment.

The functional findings of TRIP8b on I_h in vitro were difficult to rectify given the in vivo immunohistochemical and biochemical findings. Recently, our group and others have proposed alternative splicing of the TRIP8b N-terminus as a potential explanation for this paradox, and have found at least nine different isoforms in vivo,[106,107] which encompass isoforms identified in the Santoro et al. 2004 study as well as those found by Chen et al.[100,103] Alternative splicing in nervous tissue is a common mechanism to generate diversity,[108] and has been described before in both ion channels[109–111] and ion channel binding and scaffolding proteins.[112, 113] TRIP8b splicing appears to be regulated on a

developmental basis,[106] and different isoforms increase or decrease I_h and h channel surface expression in oocytes, HEK cells and cultured hippocampal neurons,[106,107,114] discussed later in this review. Furthermore, via independent methodologies, Lewis et al. and Santoro et al. arrived at similar conclusions regarding the major two TRIP8b splice isoforms expressed in the brain. All splice isoforms of TRIP8b similarly hyperpolarized the V_{50} of I_h, indicating this action is independent of the variable N-terminus. The shift in voltage gating of I_h induced by TRIP8b appears to be mediated by antagonism of cAMP's effects on I_h,[107,114] thereby hyperpolarizing V_{50}. These voltage gating changes may be mediated through a novel interaction between TRIP8b and h channels different from the previously described PTS1-like interaction. Specifically, the gating effect of TRIP8b on I_h requires the TRIP8b N-terminal core[114] which was found to be required for TRIP8b binding to the HCN1 CNBD and represents a likely mechanism by which TRIP8b alters the voltage gating of I_h.[106]

Channel kinetics

(see Table 3) Just as different h channel subunits have distinctive voltage gating properties, so do they have differing kinetic properties. HCN1 has the fastest channel activation, with HCN4 displaying the slowest activation, while HCN2 and HCN3 lie somewhere in between. In vivo, kinetics of activation and deactivation vary markedly by cell type, likely a function of the exact h channel subunits expressed as well as regulatory factors, some of which are discussed herein.[1,4,82] The specific time constant for kinetics of activation of HCN1 and HCN2 are a function of test potential, and HCN2 activates roughly 15-times slower than HCN1. While application of cAMP does speed kinetics, this is attributable to changes in V_{50}, with HCN1 still activating faster than HCN2 in the presence of cAMP, even though HCN2 V_{50} is more significantly modified by cAMP than HCN1.[35] Indeed, as discussed above for V_{50}, deletion of the CNBD of HCN1 and HCN2, thought to relieve inhibition on channel opening, altered channel kinetics to a degree qualitatively similarly as when the channel is exposed to cAMP. Channel deactivation is also slowed by the presence of cAMP. Therefore, cAMP serves to promote both quicker activation and slower deactivation of I_h.

Similarly to properties of channel gating, kinetics of I_h differ in a cell-specific manner, and are rarely recapitulated by expression of a single h channel subunit, intimating a role for heterotetrameric channels in vivo. When coexpressed in oocytes, the activation kinetics of I_h from HCN1 and HCN2 lie somewhere intermediate between those produced by either subunit alone and could not be reproduced by an algebraic sum of two hypothetical populations of homotetramers, demonstrating that heteromerization is a mechanism by which I_h kinetics may be regulated.[37]

Although PIP_2 can significantly affect channel gating, its effects on activation kinetics are less clear-cut. Exposure to PIP_2 or analogues significantly slowed opening of HCN2 in oocytes at −135 mV, but not at −125 or −110 mV, and slowed the speed of deactivation at −40 mV.[41] PIP_2-induced changes in kinetics occur via mechanisms that are both convergent and unique to kinetic changes by cAMP.

In addition to investigating modulation of voltage dependence by both intracellular and extracellular pH, Munsch and Pape, as well as Zong and colleagues investigated the effect of pH on kinetics of I_h activation.[49,50,54] Increases in pipette pH evoked decreased time

constants of activation. This change was substantial; for example, the τ of activation when tested at −93 mV for a pipette pH of 7.5 is half that at a pH of 6.7. As expected, the regulation of I_h activation kinetics, like V_{50}, is dependent on histidine residue 321, and although not explicitly tested, it is presumed that the modulation of activation kinetics by intracellular protons is independent of cAMP-induced changes in activation kinetics.[50] Interestingly, the effect of extracellular protons on activation kinetics is opposite to that of intracellular protons. Examination of the τ of activation of I_h from heterologously expressed HCN4 revealed a drastic increase in speed of activation with decreased extracellular pH. This response was much more exaggerated for HCN4 than for HCN1, and the dramatic change in HCN4 activation kinetics at low pH may explain this channel's role in transduction of sour stimuli.[51]

Studies directed toward understanding the role of Src kinase in modulation of I_h have also found that tyrosine phosphorylation may alter kinetics in addition to voltage gating. Using genistein, Yu et al. found that this non-specific tyrosine kinase inhibitor differentially alters activation kinetics in heterologously expressed h channels whereby it slows the τ of HCN2 and HCN4 at some membrane potentials, but not HCN1. Furthermore, genistein slowed the τ of activation of cultured ventricular myocytes.[57] Such studies however were unable to demarcate between a direct effect on h channels or a process upstream of activation kinetics. One explanation for the actions of genistein may be by inhibition of Src kinase, which was later found to directly modulate both the activation and deactivation kinetics of I_h as well as bind to the C-terminus of h channels.[56,60] Specifically, inhibition of Src kinase slowed both activation and deactivation of heterologously expressed HCN2 in HEK293 cells, and this effect on activation kinetics persisted qualitatively in the presence of 1 mM cAMP.[60] These effects on both activation and deactivation kinetics appear to be mediated by direct phosphorylation of tyrosine 476 (Y476).

Furthermore, a Y476 mutant was no longer sensitive to kinetics changes by genistein. Similarly, mutation of the corresponding tyrosine of HCN4, Y554, also led to insensitivity of kinetics by inhibition of Src. Finally, inhibition of Src in cultured cells from heart or dorsal root ganglion (DRG) caused significant slowing of activation kinetics, solidifying Src as a physiologically important regulator of h channel kinetics.[60] A recent report has demonstrated that inhibition of tyrosine phosphatase activity speeds activation kinetics of I_h in adult ventricular myocytes, providing further evidence for tyrosine phosphorylation as a modifier of h channel kinetics.[62] Finally, it is worth noting that not all phosphorylation mechanisms that induce changes in channel voltage gating produce significant alterations in channel kinetics, as blockade of p38 MAPK did not significantly change kinetics of I_h activation in pyramidal neurons despite its potent ability to shift V_{50}.[63]

The addition of truncated HCN2, HCN2ΔC, along with full length HCN4, generates an I_h with activation kinetics different from those observed with expression of HCN2 or HCN4 alone.[66] Interestingly, HCN2ΔC significantly speeds the π of activation to values very similar to that observed in endogenous heart, providing further evidence that this post-translational processing of HCN2 may be important for processes in vivo.[66]

Finally, just as seen with voltage gating, interacting proteins may also alter the kinetics of I_h, although oftentimes by poorly understood mechanisms. KCNE2 has been found to have an effect on kinetics of I_h in multiple studies, but similar to its effects on gating, results from different groups do not always agree, implying sensitivity to experimental conditions. In oocytes, cooexpression of KCNE2 with HCN1 or HCN2 increased the speed of activation with no change in the time constant of deactivation.[85] This study reaches different conclusions than multiple other reports, including that of Altomare et al. which found no effect of KCNE2 on HCN4 or an HCN4-1 concatameric construct in HEK293 cells,[87] or of Decher et al. who found that rather than speed activation, coexpression of KCNE2 with HCN4 in CHO cells and oocytes significantly slowed the time constant of activation.[90] Furthermore, adenoviral expression of KCNE2 with HCN2 in cultured neonatal rat ventricular myocytes demonstrated increased activation kinetics of I_h.[89]

The effects of TRIP8b on I_h kinetics have been recently elucidated.[107] All isoforms inhibit h channel opening by both slowing the kinetics of activation and speeding the kinetics of deactivation, an effect most likely mediated by the central core of TRIP8b, similar to the mechanism affecting activation gating. These findings were observed in both oocytes and cultured hippocampal neurons.

Finally, the scaffolding protein filamin A, like TRIP8b, slowed I_h activation kinetics when overexpressed with HCN1, but also slowed the deactivation kinetics.[94] Yeast two-hybrid results indicated that HCN1, but not HCN2 or HCN4, interacted with filamin A. As expected, coexpression of HCN4 with filamin A did not change channel kinetics, implying that the kinetic action of filamin A is specific for HCN1. Much work remains to be done in order to elucidate what role filamin A may play in vivo with respect to HCN1. In vitro evidence demonstrates effects of filamin A on both HCN1 membrane clustering as well is its biophysical properties,[94] but whether this effect occurs in neurons is yet unknown.

I_h amplitude via changes in channel conductance and surface expression

(see Table 4) The overall effect of a VGIC is determined not only by voltage activation, whose action is in turn governed by voltage gating as well as kinetics, but also by the number of channels expressed on the membrane surface their conductance. Thus, even under voltage conditions highly favorable for channel activation, if few channels are properly expressed on a cell surface or their conductance is very low, minimal current and little effect on membrane potential may be observed. We have previously described how a diverse range of molecules can affect essentially all voltage related characteristics of h channels and I_h. Here, we shift focus to concentrate on how this group of regulators alters h channel conductance and surface expression, properties critical for completing the picture of h channel regulation.

cAMP has been discussed previously and has effects on I_h voltage gating and kinetics. Interestingly, cAMP has been reported to have a small effect on the maximal conductance of I_h in CA1 pyramidal neurons, as addition of 8-bromoadenosine 3'5'-cyclic monophosphate (8-Br-cAMP) led to a 6% increase of I_h recorded at −140 mV.[115]

The hyperpolarizing action on I_h V_{50} by the peptide orexin A was previously discussed. In addition, orexin A suppressed I_h inward currents in PL pyramidal neurons as well as reduced voltage sag in response to hyperpolarizing current injection. These effects were at least partially mediated by orexin receptor 1 (OXR1) as blockade with the specific OXR1 antagonist SB334867 partly eliminated the effects of orexin A on I_h currents.[67] Orexin A is not the only peptide found to modify I_h. Cortistatin (CST) is a brain-specific peptide similar to somatostatin (SST), and has sleep modifying properties. CST and SST bind to similar receptors, and therefore produce similar effects, but CST can also produce physiological effects distinct from those of SST, implying divergent actions.[116] For example, CST induces slow-wave sleep while SST promotes rapid eye movement sleep. Because sleep is intimately associated with rhythmic network activity, Schweitzer et al. sought to determine postsynaptic actions of CST and SST on CA1 hippocampal neurons, and examine where the two peptides diverged in attempt to uncover CST-specific effects.[117] Both peptides enhanced I_M and $I_{K(L)}$; however, CST but not SST enhanced I_h conductance by ~35% in a CST concentration-de-pendent manner that was independent of cAMP. The I_h blocker ZD7288 blocked this effect, and CST did not shift the I_h voltage activation curve. CST joins muscarinic receptor agonists, gabapentin, serotonin, and a κ-opioid receptor agonist as modulators of hippocampal I_h independent of cAMP, an interesting finding given that many modulators of I_h act by altering cAMP levels.[6,117] Schweitzer et al. hypothesized that CST may act by intracellular calcium release, although this hypothesis awaits substantiation with additional evidence.

Thyroid hormone (TH) has important cardiac chronotropic effects, yet the mechanism of action of TH on heart rhythmicity is still not completely understood. Hyperthyroidism leads to tachycardia and a tendency to develop arrhythmias, while a hypothyroid state oftentimes leads to bradycardia and prolonged cardiac repolarization. I_f is one of multiple currents that contributes to pacemaker activity in many regions of the heart, including the SAN and Purkinje fibers. In neonatal atrial myocytes, 3,3',5-triiodo-L-thyronine (T_3) Significantly increased the beating rate in a time- and dose-dependent manner.[118] Interestingly however, in experiments designed to test whether T_3 enhanced I_f, only about half of the cells tested exhibited I_f, regardless of treatment with T_3 When only the cells with discernible I_f were examined, treatment with T_3, led to an ~4-fold increase in I_f current density. These data agree with studies conducted in adult rabbit SAN that found ~2-fold increases in I_f maximal conductance after treatment with T_3 without changes in voltage activation.[119] Interestingly however, application of 1 mM Cs^+, which blocks I_f, did not change the basal rate of beating, both with and without T_3 treatment, suggesting that I_f is not important for pacemaker activity in neonatal atrium. T_3 acts via two sets of nuclear thyroid hormone receptors (TRs), TRα1, TRβ1, TRβ2 and TRβ3, which are encoded by two genes. In heart, TRα1 is the major expressed isoform, with TRβ1 being the other expressed iso-form.[120] Gassanov et al. explored the role of these two TR's on I_f activity by adenoviral-mediated gene delivery in spontaneously beating neonatal rat cardiomyoctes.[120] Overexpression of TRα1 led to increased spontaneous beating rate compared to control, and $TR\alpha1^+$ cells treated with T_3 demonstrated increased beating rate compared to control cells treated with T_3. Interestingly, TRα1-overexpression significantly sped up the diastolic depolarization velocity. Conversely, cells overexpressing TRβ1 exhibited decreased spontaneous beating rate that

was unaffected by T_3 treatment. Additionally, TRβ1-expressing cells demonstrated decreased diastolic depolarization velocity. I_f current density was significantly increased in cells overexpressing TRα1 with or without treatment with T_3, with no change in V_{50} or kinetics. TRβ1 cells trended toward decreased I_f current density and no change with T_3 treatment. Mechanistically, these differential changes in I_f with TRα1 or TRβ1 overexpression are potentially due to changes in HCN2 and/or HCN4 gene transcription and protein expression.[120]

I_f/I_h amplitude is sensitive to extracellular chloride concentration.[121,122] In myocytes from rabbit SAN, replacement of extracellular chloride with larger anions such as aspartate decreased I_f amplitude, and was found to be a function of extracellular chloride concentration.[123] This change in amplitude was independent of a change in voltage gating, implying that extracellular chloride regulates I_f conductance, and extracellular chloride concentration also did not affect channel kinetics. The effects of chloride substitution were still observed in excised patch conformation. The intriguing feature of this system is that although I_f amplitude is highly sensitive to extracellular chloride concentration, I_f is completely impermeable to chloride anions. Frace et al. suggest that I_f sensitivity to external chloride concentration is due to chloride acting as a “positive charge screen” that is required for cation permeability.[123] Wahl-Schott et al. sought to further understand the mechanism behind this phenomenon and found that cloned HCN1, HCN2 and HCN4 channels had differing sensitivities to extracellular chloride, with HCN1 channels still passing ~50% of normal current even in the absence of chloride, while HCN2 and HCN4 current was almost entirely eliminated under the same conditions.[124] By exploiting the different chloride sensitivities of the three h channels and construction of multiple chimeras, Wahl-Schott et al. determined that chloride sensitivity is determined by a single arginine residue in the channel pore region that is present in HCN2 and HCN4 but is replaced by an alanine residue in HCN1.[124] This finding sheds further light on chloride’s mechanism of action, and the authors hypothesized that chloride binding to this arginine, which is located very close to the pore opening, may induce a conformational change permitting passage of cations. A regulatory role for intracellular chloride has also been found. I_f is comprised of a fast component that activates immediately upon hyperpolarization as well as the more commonly described slow component that reaches steady state.[86] This instantaneous current is suppressed by high intracellular chloride, but the steady state current is insensitive to intracellular chloride concentration.[125]

We have previously discussed the major role phosphorylation plays in regulating the biophysical properties of I_h. In addition to altering voltage gating and kinetics, phosphorylation can also alter channel conductance. Inhibition of endogenous tyrosine kinases with either genistein or herbimycin A in rabbit SAN myocytes significantly reduced I_f conductance.[59] This inhibition was via a cAMP-independent signaling pathway. To support this finding, treatment of rabbit SAN myocytes with epidermal growth factor led to increased I_f that was blocked by genistein.[58] This effect was presumed to be via tyrosine phosphorylation by the EGF receptor tyrosine kinase. Interestingly, in Xenopus oocytes, inhibition of tyrosine kinases by genistein selectively inhibited I_f for HCN2 and HCN4 but not for HCN1.[57] In rat ventricular myocytes, HCN2 is the most highly expressed h channel isoform. Accordingly, Yu et al. found that treatment of isolated rat ventricular myoctes with

genistein significantly reduced I_f current density.[57] Evidence for phosphorylation of h channels was reported recently by Huang et al. who found that RPTPα-enhanced tyrosine dephosphorylation led to a decrease or abolition of HCN2-mediated I_h in HEK293 cells that correlated with dephosphorylation of HCN2 protein.[62] Furthermore, dephoshorylation of HCN2 by RPTPα led to a significant decrease in surface expression of channel protein as evidenced by immunocytochemistry.

As reviewed earlier, protein binding partners for VGICs are typically classified as scaffolds, adaptors, chaperones or auxiliary subunits. While these proteins can influence biophysical properties, they are also often involved in regulating the trafficking of their VGIC binding partners. h channels have numerous interacting proteins, some of which have been found to affect channel trafficking to and from the cell surface. KCR1 when coexpressed with HCN2 in CHO cells dramatically decreases I_h current density. Consistent with these in vitro findings, native I_h amplitude was either down or upregulated when native KCR1 was adenovirally overexpressed or knocked down by siRNA in neonatal rat cardiomyocytes, respectively.[93] KCR1 may be an important regulatory molecule of I_f in vivo, especially given the finding that overexpression or decreased expression has the functional consequences of altering spontaneous beating of neonatal cardiomyoctes.

KCNE2 has previously been discussed as an h channel interacting molecule that appears to have differing biophysical effects depending on experimental system. In Xenopus oocytes, coexpression of KCNE2 with HCN1 or HCN2 led to a 3- or 5-fold increase in I_h conductance, respectively, while coexpression with KCNE1 did not enhance I_h.[85] Additionally, protein expression of KCNE2 and HCN1 was increased when coexpressed versus individual expression. In contrast, CHO cells coexpressing KCNE2 and HCN2 exhibited *decreased* rather than increased I_h.[86] This study also identified an instantaneous inward current, $I_{inst(HCN2)}$ upon HCN2 overexpression that is modulated by cAMP, requires surface expression of HCN2, and whose amplitude correlates with I_h. KCNE2 expression increased $I_{inst(HCN2)}$, demonstrating that KCNE2 has opposite effects on two different currents presumably provided by the same a-subunit. In HEK293 cells, KCNE2 did not affect the current amplitude, kinetics, or voltage gating of HCN4 I_h, or of I_h resultant from a concatameric HCN4-1 con-struct.[87] In rabbit SAN, HCN1, HCN4 and KCNE2 are highly expressed,[85,126–128] thus these results suggest the physiological role of KCNE2 at least in rabbit SAN is limited. However, another study similar in design to that of Altomare et al. found that in Xenopus oocytes and CHO cells KCNE2 had a profound effect on human HCN4, enhancing current amplitude, hyperpolarizing the V_{50}, and slowing the activation kinetics.[90] This study also found direct biochemical interaction between h channels and KCNE2. Finally, a recent study reexamined the effects of KCNE2 on the h channel subunits most highly expressed in the heart, HCN1, HCN2 and HCN4, and found that KCNE2 significantly increased current densities and sped activation kinetics of all subunits without an effect on channel voltage gating in CHO cells, in partial agreement with previous studies.[88] However, a novel finding of the study was that KCNE2 differentially regulated the mean open time of single h channels depending on subunit identity while at the same time increasing single channel amplitude and conductance. The evidence for functional in vitro interaction of KCNE2 with h channels is thus highly variable and greatly dependent

upon both the h channel isoform studied and the system in which it is studied. While the heterologous data is confusing, there is even less information concerning the role of KCNE2 as an h channel β-subunits in vivo. To address this, Qu and colleagues used adenoviral overexpression of HCN2 and KCNE2 in cultured neonatal rat ventricular myocytes and found that KCNE2 coexpression led to a significant increase in maximal I_h conductance and activation kinetics with no change in V_{50}.[89] Furthermore, coimmunoprecipitation experiments provided some evidence for a protein complex between HCN2 and KCNE2. Taken together, this is the first and to our knowledge only series of experiments that provide evidence for the role of KCNE2 as an h channel β-subunit in primary cells.

A growing body of evidence implicates I_f dysfunction in the pathophysiology of heart disease,[20,129,130] and misregulation may stem from auxiliary subunit derangement rather than from the pore-forming subunit itself. Numerous mutations in the *KCNE* gene family have been reported to lead to LQTS resulting from impaired repolarization, and mutations in the *KCNE2* gene specifically have also been found to cause LQTS.[80,84] However, whether pathological mutations or altered expression of *KCNE2* might effect I_f and lead to cardiac disorders remains unknown. Ventricular and atrial HCN2 and HCN4 mRNA and protein is upregulated in patients with heart failure. However, this remodeling of I_f by altered h channel expression is not accompanied by changes in either KCNE2 mRNA or protein level.[131] Furthermore, an experimental canine model of atrial tachyarrhythmia demonstrated that 7-day atrial tachypacing significantly decreased SAN HCN2 and HCN4 mRNA levels and decreased I_f current densities, yet again no change in KCNE2 mRNA was observed.[132] Interestingly, mRNA for MinK was also significantly reduced. Thus, direct evidence for KCNE2 alterations causing human disease specifically through disruption of I_f is lacking. The high promiscuity of KCNE2 in vitro clouds a clear understanding of its role in vivo. Indeed, KCNE2 has been shown to interact with hERG, KCNQ1–3, K_V3.1, K_V3.2, K_V4.2, K_V4.3, and as discussed above, h channels.[84] Mice with a targeted deletion of *kcne2* have been useful to understand the protein's function in processes as diverse as gastric acid secretion,[133] ventricular repolarization,[134] and synthesis of thyroid hormone.[135] Future research will need to examine I_h/I_f in these knockout animals to determine if *kcne2* is indeed required for proper I_h expression in vivo.

As previously mentioned, TRIP8b is subject to extensive alternative splicing at the molecule's N-terminus that does not participate in either of the channels presumed multiple interaction domains with h channels, and the N-terminus is also not required to elicit the V_{50} hyperpolarization in I_h, nor is it likely to play a role in kinetic changes brought about by TRIP8b.[106,107,114] However, the identity of the TRIP8b N-terminus appears to be critically important for regulating h channel surface trafficking. Our group, as well as Santoro et al. identified splice isoforms that either enhanced or decreased I_h current densities in either HEK293 cells or Xenopus oocytes when coexpressed with h channels.[103,106,107] Additional experiments using flow cytometry, biochemistry, and confocal microscopy suggested that this change in current density is due to a change in the number of h channels present on the membrane surface and not in channel conductance. This is consistent with findings both in cultured hippocampal neurons and in vivo in hippocampus that different isoforms of TRIP8b can dramatically rearrange the subcellular localization of h channels.[106,107] Furthermore, in

hippocampal neurons, overexpression of a TRIP8b isoform predicted to enhance h channel surface expression, or siRNA knockdown of TRIP8b, led to an increase or decrease of native I_h, respectively, demonstrating a role for TRIP8b in the trafficking of in vivo h channels.[106,107] How exactly does the TRIP8b N-terminus carry out this trafficking function? From research reports so far, the answer is not straightforward. Santoro et al. explored the role of consensus protein trafficking motifs variably present in different exons of the TRIP8b N-terminus.[107] Exon 2 contains a tyrosine-based trafficking motif (YXXØ), while exons 4, 5 and 6 contain dileucine-based ([DE]XXXL[LI]) trafficking motifs, both of which can interact with cellular protein sorting systems. Mutation of the YXXØ motif in exon 2 of TRIP8b(1b-2) eliminated its strong downregulating effects, suggesting that this motif is required for I_h downregulation. However, TRIP8b(1a) does not have such a motif, yet was still found in this study (but not in Lewis et al.[106]) to be a downregulating isoform. Mutation of the dileucine-based trafficking motif of TRIP8b(1a) in exon 5, but not in exon 6, abolished TRIP8b(1a) downregulation, which suggests that this dileucine motif is another motif important for downregulation. The story is not finished there however, since TRIP8b(1a-4), which contains dileucine motifs in exons 4, 5 and 6, is strongly upregulating for I_h. Thus, it remains to be seen whether the actions of individual TRIP8b isoforms can be distilled to the presence of specific sequences or if combinatorial effects of trafficking motifs are important. Interestingly, in vivo TRIP8b forms a molecular complex with HCN1 and AP-2, suggesting a potential mechanism for h channel endocytosis by some isoforms of TRIP8b.[107]

Filamin A has also been found to regulate h channel surface expression and I_h in vitro. The interaction of HCN1 with filamin A appears to cluster HCN1 protein on cell membranes and decrease I_h conductance in a melanoma cell line.[94] As noted above in discussing effects on gating and kinetics, the role of filamin A in h channel surface expression and clustering in neurons is yet to be explored.

h Channel Interacting Proteins with as Yet Unknown Functions on I_h

Tamalin/GRP1-associated scaffold protein (GRASP)

Tamalin, or GRP1-associated scaffold protein (GRASP), was identified by a yeast two-hybrid screen of a rat brain cDNA library using the class I PDZ-binding motif-containing C-terminal sequences of group 1 and group 2 metabotropic glutamate receptors (mGluRs) as bait.[136] It is the same gene product as GRASP, first reported as a retinoic acid-induced gene[137] isolated from P19 embryonal carcinoma cells. Tamalin contains multiple distinct protein regions, including a PDZ-domain, alanine-rich, proline-rich and glycine-rich regions, a leucine zipper sequence, four YXXL motifs, and a C-terminal PDZ-binding motif.[136] Ultimately it was found that tamalin increases intracellular trafficking and surface expression of group 1 mGluRs in COS-7 cells and neurites of cultured hippocampal neurons, and forms an in vivo complex with mGluR1a and cytohesin-2.[136]

Like group I and group II mGluRs, HCN1–4 contain potential PDZ-binding motifs at their C-termini,[138] prompting Kimura et al. to ask whether tamalin also binds to these h channel subunits. A yeast two-hybrid assay surprisingly demonstrated that tamalin binds to the final seven amino acids of HCN2, but not HCN1, 3 or 4, an interaction confirmed by

coimmunoprecipitation experiments with tagged full length HCN2 and tamalin in COS-7 cells.[139] Protein interaction studies revealed not surprisingly that HCN2 interacted with the N-terminal PDZ-domain of tamalin. However, this interaction is not completely straightforward, as deletion of the C-terminal 10 amino acids from HCN2 reduced, but did not completely eliminate HCN2 binding to tamalin. Binding was eliminated only after deletion of all HCN2 amino acids distal to the CNBD.[139] These data therefore implicate not only the PDZ-binding motif of HCN2 but also a region within the intracellular C-terminal region between the CNBD and the PDZ-binding motif as important for this protein-protein interaction.

The functional consequences of the interaction of tamalin with HCN2 remain to be elucidated. specifically, it is unknown whether tamalin binds directly to HCN2 in vivo, although tamalin binds to proteins known to bind HCN2 in vivo (see below). Recently a tamalin knockout mouse has been reported, demonstrating no differences in protein levels or localization of mGluR1a, mGluR2, mGluR5 and $GABA_{B2}$.[140] It is still unknown whether these mice have alterations in I_h in any region in which tamalin and HCN2 are expressed. Such experiments will be critical for tamalin to be established as a physiologically important interacting protein with HCN2.

Synaptic scaffolding molecule (S-SCAM) and Mint2 (X11-like)

In addition to binding HCN2, tamalin is known to form complexes with multiple other proteins, including synaptic scaffolding molecule (S-SCAM), Mint2 and PSD-95.[141] S-SCAM is a brain specific protein that contains a number of protein interaction motifs, specifically a guanylate kinase-like domain, two WW and five PDZ domains, and binds to a large number of functionally important yet diverse proteins, including HCN2.[139,142] Mint2 is also a brain specific protein that contains two PDZ-domains and a phosphotyrosine domain and interacts with amyloid precursor protein (APP).[143,144] Mint2 plays a role in APP processing, and mice with a genetic deletion of Mint2 demonstrate increased levels of β-secretase catalyzed C-terminal fragments of APP.[145] Recently, Mint2 has been found to play a role in the behavioral neurobiology underlying murine conflict resolution.[146] Mint2 also interacts with a number of other proteins involved with governing cellular properties including Munc-18 and HCN2.[139,144]

Thus, both S-SCAM and Mint2 have been demonstrated to bind to a large number of different proteins including voltageand ligand-gated ion channels and cell adhesion molecules, and both bind HCN2 from rat brain extract.[139] To rule out a possible indirect interaction of S-SCAM or Mint2 with HCN2 through protein complexes, co-immunoprecipitation experiments in transfected COS-7 cells were performed demonstrating that indeed HCN2 interacts directly with both S-SCAM and Mint2. S-SCAM, like tamalin, binds to HCN2 via a PDZ-domain, and binding to HCN2 is reduced by deletion of an HCN2 portion distal to the CNBD with abolition of binding following deletion of HCN2 CNBD and beyond.[139] Mint2 interacts with the region distal to the HCN2 CNBD via its munc18-interacting domain. Interestingly, Mint2 appeared to exert a protein stabilizing effect on HCN2, as coexpression of Mint2 with HCN2 increased HCN2 expression compared to HCN2 expressed alone or HCN2 coexpressed with a Mint2 mutant unable to bind HCN2.[139]

What is the physiological importance of the binding of S-SCAM and Mint2 to HCN2? First, it remains to be seen whether S-SCAM and Mint2 bind with any other HCN subunits. From a more hyperopic point of view, S-SCAM appears to be an important synaptic scaffolding molecule for many classes of proteins, including ion channels, but also neuronal cell adhesion molecules which may play important roles in poorly understood diseases such as autism. In neocortex and hippocampus, the predominant expression of HCN1 and HCN2 is along the plasma membrane of dendritic shafts, with less h channel immunoreactivity in the spine and none observed at asymmetric synapses.[40,105] S-SCAM is highly expressed in cortex and interacts strongly with various transmembrane AMPA receptor regulatory protein (TARP) isoforms.[147] Yet since HCN2 is not highly expressed in dendritic spines and absent at synapses of cortical pyramidal neurons, it is possible that the interaction of S-SCAM with HCN2 plays another unknown role. Knockout mice with genetic deletion of S-SCAMα (the longest of three alternatively spliced forms of S-SCAM) have been generated, and although they develop and are born live, die within 24 hours. These mice have profound abnormalities in neuronal spine morphology.[148] A more viable conditional knockout mouse model of S-SCAM might help explore the physiological importance of S-SCAM's association with HCN2.

Mint2 is highly expressed in excitatory neurons of the hippocampus, although it is expressed throughout the brain.[149,150] In the hippocampus, Mint2 staining appears most strongly in cell bodies with weaker staining in dendrites. Mint2 does not appear to be expressed in presynaptic compartments or in the endoplasmic reticulum in cultured hippocampal neurons, but strongly colocalizes with TGN38, a marker of the trans-golgi network, a localization similar to that of APP. In HEK293 cells, expression of Mint2 increased the expression levels of cotransfected APP,[150] similarly to what was described for Mint2 and HCN2.[139] Thus, unlike S-SCAM, Mint2 may play a role in the early processing of h channels before trafficking from the cell body to dendrites. Analysis of Mint2-deficient mice may help narrow down the role of Mint2 on h channel function and/or localization.

Conclusions

In this review we have attempted to provide an overview of the major modulatory factors and interacting proteins of h channel subunits that affect h channel biophysical properties and surface expression (Table 5). These channels are members of a larger superfamily of voltage-gated ion channels, and many of the processes that influence HCN channels also influence other members of this superfamily. When evaluating studies on this topic, two questions should be kept in the mind of the reader. First, where in the signaling pathway are the regulatory mechanism(s) and h channel located? For example, does the modulator acts directly on the h channel, such as with cAMP or TRIP8b? Or is the h channel downstream of the effector, such as occurs with 4βPMA? This will help reveal how global signaling changes may affect h channels. Second, especially when evaluating new h channel interacting proteins, one must ask which of these interactions are of relevance in vivo? KCNE2 provides an excellent example for the requirement of in vivo work. Studies attempting to elucidate the role of KCNE2 in regulating I_h via interaction with HCN channel subunits have been hampered by great variability between model systems. On the other hand, a dearth of work has been published examining what role, if any, KCNE2 plays in

regulating I_h in the living animal, whether through pantissue or tissue-specific knockout animals, virally-expressed siRNA knockdown, or at the very least, cultured primary cells such as cardiomyocytes or neurons. Additional proteomic work such as performed by Zolles and colleagues for determination of TRIP8b interaction with h channels is also of great utility in exploring the role of a particular interacting partner in vivo. This type of work demonstrated that the overwhelming majority of h channels in the brain are associated with TRIP8b, contributing to the hypothesis that TRIP8b serves as a critically important auxiliary subunit in rodent brain.[114] As researchers continue to elucidate roles for h channels in both normal and pathological states of the nervous system and the heart, interest in the regulatory molecules and proteins of h channels will undoubtedly grow. Furthermore, just as mutations in regulatory subunits rather than pore-forming alpha subunits of K^+ channels have been found to cause pathologies in humans such as LQTS, there exists the chance that mutations in h channel regulatory subunits may exert a pathological function through either overt or subtle changes in I_h in multiple tissues. Because I_h is a subthreshold conductance in which small changes in amplitude, kinetics, or gating may be amplified to have large effects on overall circuits, a greater understanding of the mechanisms by which I_h is regulated through interacting ions, molecules and proteins is required to begin to design therapeutics to target I_h dysfunction.

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Abbreviations

HCN	hyperpolarization-activated cyclic nucleotide-gated
I_h	hyperpolarization-activated current
I_f	funny current
TRIP8b	tetratricopeptide repeat-containing Rab8b–interacting protein
VGIC	voltage-gated ion channel
SAN	sinoatrial node
I_q	queer current
RMP	resting membrane potential
EPSP	excitatory post-synaptic potential
TC	thalamocortical
I_t	low-threshold Ca^{2+} current
RTN	reticular thalamic nucleus
IPSP	inhibitory post-synaptic potential
TLE	temporal lobe epilepsy

cAMP	3'-5'-cyclic adenosine monophosphate
CNBD	cyclic nucleotide binding domain
cGMP	3'-5'-cyclic guanosine monophosphate
PKA	cAMP-dependent protein kinase (protein kinase A)
PIP_2	phosphatidylinositol 4,5-bisphosphate
DAG	diacylglycerol
4βPMA	4β-phorbol 12-myristate 13-acetate
PKC	protein kinase C
PA	phosphatidic acid
AA	arachidonic acid
TRPV1	transient receptor potential cation channel, subfamily V, member 1
RPTPα	receptor-like protein-tyrosine phosphatase-α
MAPK	mitogen-activated protein kinase
PL	prelimbic
PSD-95	postsynaptic density-95
NMDA	*N*-methyl D-aspartate
KChIP	K_v channel-interacting protein
CNG	cyclic nucleotide-gated
EAG	ether-á-go-go
MiRP	MinK-related peptide
MinK	minimal K^+ channel protein
hERG	human ether-à-go-go related gene
CHO	chinese hamster ovary
HEK	human embryonic kidney
KCR1	K^+-channel regulator protein 1
Y2 h	yeast two-hybrid
Pex5p	peroxin protein 5
OXR1	orexin receptor 1
CST	cortistatin
SST	somatostatin
TH	thyroid hormone
T_3	3,3',5-triiodo-L-thyronine

TR	thyroid hormone receptor
LQTS	long QT syndrome
mGluR	metabotropic glutamate receptor
PDZ	PSD-95/disc large/zonula occludens 1
S-SCAM	synaptic scaffolding molecule
TARP	transmembrane AMPA receptor regulatory protein
DIV	day in vitro
PTS1	peroxisomal targeting sequence 1
PLC	phospholipase C

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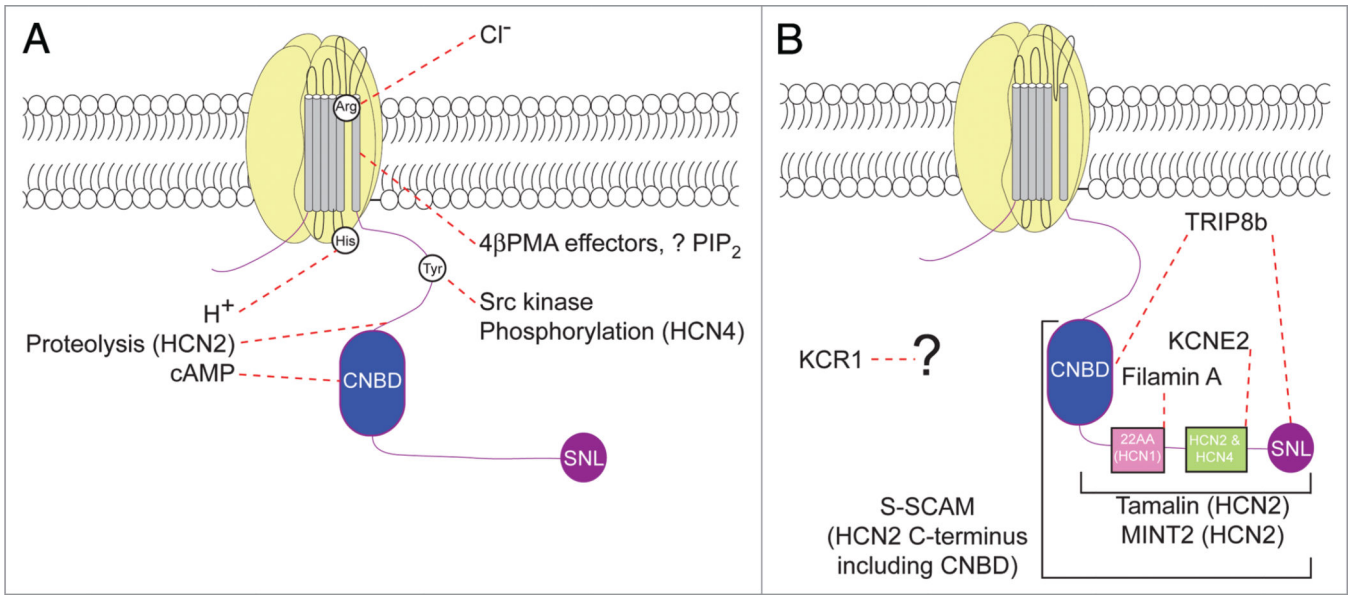

Figure 1.

Directly interacting small molecule and protein regulatory mechanisms of of I_h function. See text for details on binding sites. (A) Schematic depicting HCN channel tetrameric structure. One HCN channel subunit is shown in detail. Dashed red lines indicate known sites of interaction of small molecules. (B) Schematic depicting HCN channel and known interaction sites of HCN channel interacting proteins. CNBD, cyclic nucleotide binding domain; SNL-C-terminal tripeptide of HCN1, HCN2 and HCN4.

Table 1

Regulatory mechanisms hyperpolarizing I_h/I_f voltage gating

Modulatory mechanism or treatment	Experimental system	Approximate magnitude of V_{50} shift (mV)	Ref.
↓$[pH]_i$	Rat TC neurons	−2 to −3 (with pH decrease from 7.1 to 6.7)	49
	HEK293 cells expressing HCN2	−10 (with pH decrease from 7.4 to 6.0)	50
Genistein (tyrosine kinase Inhibition)	Rat ventricular myocytes	−14	57
Orexin A	Mouse prelimbic cortex layer V pyramidal neurons	−8	67
KCNE2	Coexpression with HCN4 in oocytes	−23	90
	Coexpression with HCN4 in CHO cells	−14	
KCR1	Coexpression with HCN2 in CHO cells	−8	93
	Viral overexpression in neonatal and adult ventriculocytes	−30 (single channel patch)	
Filamin A	Coexpression with HCN1 in melanoma cell line	−8	94
TRIP8b	Coexpression with HCN1 in HEK293T cells or oocytes	−7 to −10	106, 107
	Coexpresion with HCN2 in oocytes (inside-out patch + cAMP)	−10	114
	Viral overexpression of TRIP8b in 6 DIV organotypic hippocampal slice culture	−7	114

Table 2

Regulatory mechanisms depolarizing I_h/I_f voltage gating

Modulatory mechanism or treatment	Experimental system	Approximate magnitude of V_{50} shift (mV)	Ref.
cAMP	Numerous	Dependent on HCN channel subunit	Reviewed in 1–8
PIP_2 (or analogues)	Oocytes expressing various HCN channel subunits (Excised inside-out patches)	>+20	41, 45–47
	Wortmannin treatment of dopaminergic midbrain neurons or embryonic cardiomyoctes	+12 (neurons) +11 (cardiomyocytes) [inferred from wortmannin inhibition]	46
PA, AA	Oocytes expressing HCN2 (excised inside-out patches)	+6	48
$\uparrow[pH]_i$	Rat TC neurons	+4 to +5 (with pH increase from 7.1 to 7.5)	49, 54
	HEK293 cells expressing HCN2	+10 (with pH increase from 7.4 to 9.0)	50
$\downarrow[pH]_o$	FLP-IN-293 cells expressing HCN1	+9 (with pH decrease from 7.4 to 4.9)	51
Tyrosine phosphorylation	Coexpression of constitutively-active Src with HCN4 in HEK293 cells	+13	56, 61
	Phenylarsine oxide or sodium orthovanadate (non-specific tyrosine phosphatase inhibition) in adult rat ventricular myocytes	Large positive shift in I_f activation threshold	62
P38 MAPK	Treatment of hippocampal neurons with anisomycin (p38 MAPK activator)	+11	63
Proteolysis of HCN channel subunits	Coexpression of proteolyzed HCN2 (HCN2ΔC) with HCN4 in HEK293 cells	+4 compared to HCN4 alone	66

Table 3

Regulatory mechanisms altering I_h/I_f activation kinetics

Modulatory mechanism or treatment	Experimental system	Increased or decreased rate of activation	Ref.
cAMP	Numerous	Increased	1–8
PIP_2	Oocytes expressing HCN2 (inside-out patch)	Decreased at some but not all tested potentials	41, 46, 47
$[pH]_i$	Rat TC neurons or HEK293 cells expressing HCN2	Increased with ↑pH; decreased with ↓pH	49, 50, 54
$[pH]_o$	FLP-IN-293 cells expressing HCN1 or HCN4	Increased with ↓pH	51
Genistein (tyrosine kinase inhibition)	Oocytes expressing HCN1, 2 or 4	Decreased for HCN2 and 4, no effect for HCN1	57
	Rat ventricular myocytes	Decreased	
Src kinase	HEK293 cells expressing HCN2 treated with PP2 (Src inhibitor)	Decreased with PP2 treatment	60
Proteolysis of HCN channel subunits	Coexpression of proteolyzed HCN2 (HCN2ΔC) with HCN4 in HEK293 cells	Increased compared to HCN4 alone	66
KCNE2	Oocytes expressing HCN1 and HCN2	Increased	85
	Oocytes or CHO cells expressing HCN4	Decreased	90
	Adenoviral overexpression in cultured neonatal rat ventricular myocytes	Increased	89
	CHO cells expressing HCN1, 2, 4	Increased	88
TRIP8b	Oocytes expressing HCN1	Decreased	107
Filamin A	Coexpression with HCN1 in melanoma cell line	Decreased	94

Magnitude is not included here as it is typically dependent upon voltage step.

Table 4

Regulatory mechanisms altering I_h/I_f amplitude

Modulatory mechanism or treatment	Experimental system	Effect on I_h amplitude	Ref.
cAMP	Rat CA1 pyramidal neurons	Increased conductance	115
Orexin A	Mouse prelimbic cortical layer V pyramidal neurons	Decreased	67
Thyroid hormone (T_3)	Neonatal atrial myocytes Rabbit SAN cells	Increased current density and conductance	118–120
$[Cl^-]_o$	Rabbit SAN myocytes (whole cell and isolated patch)	Decreased with ↓$[Cl^-]_o$ (less effect on HCN1 than HCN2, 4)	121–124
$[Cl^-]_i$	HEK293 cells expressing HCN4	Decreased instantaneous current with ↑$[Cl^-]_I$, no effect on steady state current	125
Genistein (tyrosine kinase inhibition)	Rabbit SAN myocytes	Decreased I_f conductance	59
	Oocytes expressing HCN1, HCN2 or HCN4	Decreased for HCN2 and HCN4, no effect for HCN1	57
	Rat ventricular myoctes	Decreased	
RPTPα	HEK293 cells expressing HCN2	Decreased surface expression and current density	62
KCR1	CHO cells expressing HCN2	Decreased	93
	Adenoviral overexpression of KCR1 in neonatal rat cardiomyocytes	Decreased	
	siRNA knockdown of endogenous KCR1 in neonatal rat cardiomyocytes	Increased	
KCNE2	Oocytes expressing HCN1 or HCN2	Increased I_h conductance	85
	CHO cells expressing HCN2	Decreased steady state I_h, but Increased instantaneous I_h	86
	Oocytes and CHO cells expressing HCN4	Increased	90
	Adenoviral expression in neonatal rat ventricular myocytes	Increased conductance	89
	CHO cells expressing HCN1, 2, 4	Increased	88
TRIP8b	Oocytes or HEK293 cells expressing HCN1	Increased or Decreased surface expression depending on TRIP8b isoform	103, 106, 107, 114
	siRNA knockdown of all TRIP8b isoforms in cultured rat hippocampal neurons	Decreased	106
Filamin A	Coexpression with HCN1 in melanoma cell line	Decreased I_h conductance	94

Table 5

Holistic overview of I_h/I_f regulatory mechanisms

Modulatory mechanism or treatment	V_{50}	Rate of activation	Current amplitude	Refs.
cAMP	↑	↑	↑, NE	1–8, 115
PIP_2	↑	↓, NE (dependent on test-potential)	NE	41, 45–47
PA, AA	↑	NDI	Effect not specific for I_h	48
$[pH]_i$	↑ (↑$[pH]_i$) ↓ (↓$[pH]_i$)	↑ (↑$[pH]_i$) ↓ (↓$[pH]_i$)	NE	49, 50, 54
$[pH]_o$	↓ (↑$[pH]_o$) ↑ (↓$[pH]_o$)	↓ (↑$[pH]_o$) ↑ (↓$[pH]_o$)	NE	51, but see 50
Tyrosine phosphorylation	↑	↑ at some potentials	↑	56, 58, 61, 62
p38 MAPK	↑	NE	NE	63
HCN channel proteolysis	↑	↑	NDI	66
Genistein (tyrosine kinase inhibition)	↓ or NE (depends on HCN isoform)	↓ or NE	↓ or NE	57, 59
Orexin A	↓	NDI	↓	67
KCNE2	↓	↑↓	↑↓	85–90
KCR1	↓	↓	↓	93
Filamin A	↓	↓	↓	94
TRIP8b	↓	↓	↑↓	103, 106, 107, 114
Thyroid hormone (T_3)	NE	NE	↑	118–120
$[Cl^-]_i$	↑ with ↑ $[Cl^-]_i$	NE	↑ $I_{h(inst)}$ w/↑ $[Cl^-]_i$	125
$[Cl^-]_o$	NE	NE	↓ w/↓ $[Cl^-]_o$	121–124
RPTPα	NDI	NDI	↓	62

↑, increased/depolarized; ↓, decreased/hyperpolarized; ↑↓, both reported; NE, no effect; NDI, not directly investigated.