Activity-Dependent Regulation of HCN Pacemaker Channels by Cyclic AMP: Signaling through Dynamic Allosteric Coupling

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Summary

Signal transduction in neurons is a dynamic process, generally thought to be driven by transient changes in the concentration of second messengers. Here we describe a novel regulatory mechanism in which the dynamics of signaling through cyclic AMP are mediated by activity-dependent changes in the affinity of the hyperpolarization-activated, cation nonselective (HCN) channels for cAMP, rather than by changes in cAMP concentration. Due to the allosteric coupling of channel opening and ligand binding, changes in cellular electrical activity that alter the opening of the HCN channels modify the binding of static, basal levels of cAMP. These changes in ligand binding produce long-lasting changes in channel function which can contribute to the regulation of rhythmic firing patterns.

Introduction

Recent experiments have enhanced our view of signaling through the classic second messenger cAMP. The identification of local protein signaling complexes (Bhalla and Iyengar, 1999; Davare et al., 2001; Marx et al., 2002; Michel and Scott, 2002) and subcellular compartments (Karpen and Rich, 2001; Zaccolo and Pozzan, 2002) has shown how a ubiquitous messenger can achieve spatial specificity. Our understanding of cAMP signaling has been broadened to include novel targets other than PKA, including the cyclic nucleotide-gated (CNG) (Zagotta and Siegelbaum, 1996) and hyperpolarization-activated HCN channels (Gauss et al., 1998; Ludwig et al., 1998; Santoro et al., 1998). However, one prevalent assumption, since the pioneering studies of Sutherland and colleagues (Robison et al., 1971), is that the dynamics of information transfer through the cAMP signaling cascade are driven by transient changes in the concentration of cAMP, generally as a result of changes in the concentration of an external ligand. Here we show how constant, nanomolar levels of basal cAMP give rise to a slow kinetic component in the voltage gating of the HCN channels through the allosteric coupling of channel opening to cAMP binding. Moreover, this process imparts a long-lasting, activity-dependent memory to HCN channels that underlies slow integrative changes in neuronal electrical activity.

The HCN channels are encoded by a family of four related genes, HCN1–4, which are members of the voltage-gated channel superfamily, with a membrane topology similar to a voltage-gated K+ channel subunit (Santoro and Tibbs, 1998; Kaupp and Seifert, 2001). However, unlike most voltage-gated channels, the HCN channels are activated by hyperpolarization rather than depolarization. Additionally, the four HCN members all contain a conserved, 120 amino acid cyclic nucleotide binding domain (CNBD) in their cytoplasmic carboxy terminus. The direct binding of cAMP to the CNBD (DiFrancesco and Tortora, 1991) facilitates the activation of the HCN channels by removing an inhibitory influence of the CNBD on voltage gating (Wainger et al., 2001), shifting the voltage dependence of HCN gating to more positive potentials and speeding the rate of channel activation. This action of cAMP contributes to the increase in heart rate by autonomic signaling (DiFrancesco, 1993) and the alteration of neuronal firing patterns by modulatory synaptic inputs (Pape, 1996).

Previous studies showed that the modulation by cyclic nucleotides of steady-state gating of both the voltage-independent cyclic-nucleotide-gated channels (Goulding et al., 1994; Tibbs et al., 1997) and the voltage-gated HCN channels (DiFrancesco, 1999) could be explained by a cyclic allosteric model (Monod et al., 1965). According to this model, channel opening is allosterically coupled to a conformational change in the CNBD that enhances the affinity of the channel for ligand. The enhanced interaction of ligand with the open channel provides coupling energy that stabilizes the ligand-bound channel in the open state. For the hyperpolarization-activated HCN channels, the enhanced gating is manifest as a shift in the activation curve toward more positive potentials. Although the cyclic allosteric model provides a plausible mechanism for ligand gating, there have been few direct tests of the central hypothesis of the model that channel opening is allosterically coupled to enhanced ligand binding.

Here we provide qualitative and quantitative evidence that supports the notion of allosteric coupling by testing a previously unexplored prediction of the cyclic allosteric model: low concentrations of cAMP should induce a novel, slow kinetic component of channel opening in response to hyperpolarizing voltage steps. This slow phase of opening is predicted to result from the selective binding of low concentrations of cAMP to the high-affinity, open state of the channel, causing a slow conversion of channels from the ligand-free, closed state to the ligand-bound, open state during prolonged hyperpolarizations. We have confirmed this prediction and have shown that the properties of the slow component of activation are in quantitative agreement with the cyclic allosteric model. Moreover, we found that a slow kinetic component of HCN channel activation in resting cells results from the allosteric conversion due to binding of nanomolar levels of basal cAMP. Finally, the enhanced binding of cAMP to the open state endows HCN chan-
channels with an activity-dependent memory that leads to long-lasting changes in cellular excitability, which are likely to contribute to the generation of slow rhythmic firing patterns in certain regions of the brain.

Results

A Slow Phase of HCN Channel Opening Induced by Low Concentrations of cAMP

We have focused our studies on HCN2, an isoform expressed widely in brain and heart that shows a robust response to cAMP (Ludwig et al., 1998; Santoro et al., 2000; Chen et al., 2001). As noted previously, a high concentration of cAMP (≥10 μM) accelerates the rate at which the hyperpolarization-activated current (Ih) turns on during negative voltage steps (Figure 1A) and shifts the voltage dependence of activation to more positive potentials (Figure 1B). We now show that a low concentration of cAMP induces a novel, slow phase of channel activation (Figure 1A). In response to a hyperpolarization in the presence of 10 nM cAMP, the early time course of Ih activation is identical to that observed in the absence of cAMP. After a few hundred milliseconds, however, the activation time courses diverge, with the low concentration of cAMP inducing an additional slow increase in Ih magnitude.

As mentioned above (see Introduction), these results are consistent with the predictions of the cyclic allosteric model (Figure 1C). Thus, in the absence of cAMP, unliganded closed channels (C) enter the open state (O) in response to a hyperpolarizing voltage step through the nonfacilitated voltage-gated reaction pathway (C → O). At high concentrations, cAMP will bind to the low-affinity, closed state of the channel prior to the hyperpolarization. As a result, during a hyperpolarization, these cAMP-bound channels will open rapidly and completely through the facilitated voltage-gated reaction pathway (AC → AO). In contrast, in the presence of concentrations of cAMP that are too low to bind to the closed, low-affinity state of the channel, the unliganded channels will initially open in response to a hyperpolarization by the nonfacilitated voltage-gated reaction pathway (C → O). This explains why the time courses of Ih activation in the absence of cAMP and in the presence of a low concentration of cAMP overlap initially. However, once unliganded channels open in the presence of cAMP, they will tend to bind ligand due to the enhanced affinity of the open state (A + O → AO). This depletes the concentration of channels in the open, unliganded state, causing a reequilibration of channels from the closed, unliganded state to the open, liganded state, and produces the slow phase of channel opening (C → A + O → AO).

Kinetic Analysis of Ih Confirms Predictions of Cyclic Allosteric Model

To test more thoroughly the predictions of the cyclic allosteric model, we examined activation kinetics over a range of voltages for a range of cAMP concentrations. In the absence of cAMP, HCN2 channels in cell-free patches activate upon hyperpolarization with a time course that is well fit by a single exponential function (after an initial delay) (see Experimental Procedures), presumably reflecting the kinetics of the nonfacilitated, voltage-gated reaction for unliganded channels (Figure 2A). As the membrane is hyperpolarized to increasingly negative voltages, the exponential time constant of activation decreases from ~3 to ~1 s (Figures 2A and 3A). Attempts to fit the data with two exponential functions either failed to converge or did not yield a substantial improvement in the goodness-of-fit over a single exponential function (determined by χ²/ν values, Figure 2D; see Experimental Procedures). We thus conclude that HCN2 currents are adequately described by a single exponential function.

In contrast to the single exponential kinetics of HCN2 in the absence of cAMP, two exponential components
Figure 2. Effect of cAMP on HCN2 Current Kinetics in Cell-free Inside-Out Patches

(A1, B1, and C1) Hyperpolarization-activated currents in response to voltage steps from −85 to −145 mV in 10 mV increments for variable durations (4.5–15 s) with indicated [cAMP] in the bath solution. Holding potential was −40 mV.

(A2, B2, and C2) Bottom, currents recorded at −135 mV (open circles) with superimposed single exponential fit (dark line); top, residuals of difference between the data and the fitted curve, with zero residual difference indicated by the dotted lines.

(B3 and C3) Bottom, same currents as above with superimposed double exponential fits; individual fast (red dashed line) and slow (green solid line) exponential components are shown, along with steady-state current (black dotted line); top, residuals of difference between data and fit.

(D) The difference between $\chi^2$, for single exponential fit and $\chi^2$, for double exponential fit for current at −135 mV. Bars represent SEM. Number of patches given above bars.

are needed to describe the time course of activation in the presence of low concentrations of cAMP (Figure 2B).

Thus, fits with a single exponential function show large residuals, and the goodness-of-fit is noticeably enhanced by the addition of a second exponential component (Figure 2D). Moreover, the parameters of the two exponential components are highly reproducible among different patches (see small standard errors in Figure 3). The relatively fast kinetic component has time constants similar to those of the single exponential component for HCN2 channel activation in the absence of cAMP (Figure 3A). Presumably this component represents the direct activation of unliganded, closed channels (C → O). The second, slow exponential component has time constants that are much slower than those in the absence of cAMP. These time constants decrease from ~15 to ~5 s with increasingly negative hyperpolarizations (Figure 3B). Presumably, the slow exponential component represents the slow reequilibration of channels from the unliganded, closed state to the ligand-bound, open state.

At low [cAMP], the relative amplitude of the slow exponential component is large for weak hyperpolarizing steps near the threshold for $I_{h}$ activation, accounting for up to 80% of the current amplitude. As the voltage step is made more negative, the relative amplitude of the slow component diminishes, accounting for less than 20% of the current amplitude during strong hyperpolarizing steps where activation is maximal (Figure 3C). With increasing concentrations of cAMP, the relative amplitude of the slow component decreases, and the fast and slow time constants accelerate (Figures 3A–3C). At very high concentrations of cAMP, the slow component makes only a minor contribution to the total current amplitude (Figures 2C and 3C).

The voltage and cAMP concentration dependence of the relative amplitudes of the fast and slow exponential components are in good agreement with the cyclic allo-
Figure 3. Comparisons of HCN2 Activation Kinetics in Inside-Out Patches and Intact Oocytes with Native Ih of Thalamocortical Relay Neurons (TRN)

(A) Voltage dependence of the fast exponential time constant ($\tau_f$) for currents recorded in the presence of indicated [cAMP] (µM) and of the single exponential time constant for currents measured in the absence of cAMP.

(B) Voltage dependence of the slow exponential time constant ($\tau_s$) for patches exposed to indicated [cAMP].

(C) Relative amplitude of the fast exponential component, $A_f/(A_f + A_s)$, as a function of voltage. Each point represents an average of 3–13 patches. Lower x axis shows the actual test voltage during recordings; upper x axis shows the corresponding voltage in intact cells, where $V_{1/2}$ is shifted by approximately +35 mV to compensate for voltage shift upon patch excision (Chen et al., 2001).

(D–F) Kinetic analysis of HCN2 currents in intact oocytes recorded with two-microelectrode voltage clamp and Ih recorded in whole-cell patch clamp from native TRNs. Currents were fit by one or two exponential functions (see Figure 5). Data for HCN2 and HCN2/R591E are averaged from five to nine cells. Data for TRN (recorded at 34°C) are replotted from Santoro et al. (2000) after conversion to expected rates at room temperature using a Q10 of 4.

(D) Voltage dependence of the fast exponential time constant ($\tau_f$) for HCN2 and TRN and single exponential time constant of HCN2/R591E point mutant. (E) Slow exponential time constant ($\tau_s$) for HCN2 and TRN. HCN2/R591E channels did not display a noticeable slow component (see Figure 5D). (F) Relative amplitude of the fast component for HCN2 and TRN. Bars indicate SEM.

The cyclic allosteric model also provides a good quantitative fit to HCN2 activation kinetics, giving us the first estimates for the rate constants of the cAMP binding reaction (Figure 4). We used a nonlinear routine to fit the activation time course for currents elicited at subsaturating cAMP concentrations upon hyperpolarization to four different voltages (see Experimental Procedures) (Figures 1C, 4A, and 4B). Based on fits from three separate experiments, we obtain an average forward rate constant for cAMP binding to the open state ($k_{fo}$) of $3.1 \times 10^6$ M$^{-1}$·s$^{-1}$ and an average dissociation rate constant for unbinding from the open state ($k_{bo}$) of 0.045 s$^{-1}$ (closed state ligand binding kinetics have little influence on Ih kinetics in these experiments and so are not well determined). The fits also yield cAMP equilibrium dissociation constants for binding to the open state ($K_C$) and closed state ($K_C$) of $\sim$15 nM and 1.2 µM, respectively.

Basal Levels of cAMP in Intact Cells Induce the Slow Allosteric Conversion

The high affinity of the open channel for cAMP suggests that basal levels of cAMP in intact cells may be sufficient to produce the slow allosteric conversion during prolonged hyperpolarization. Indeed, when we examine the kinetics of HCN2 activation in intact oocytes using a two-microelectrode voltage clamp, we observe a fast...
of the unliganded channels (C ↔ O). In contrast, the opening equilibrium at the end of prolonged hyperpolarizations, which enable channels to enter the ligand-bound open state, will be determined by the more positive voltage dependence of the ligand-bound channels (AC → AO). HCN2/R591E channels, which do not bind cAMP, show a much faster equilibration of their $V_{1/2}$ values, reflecting only the (relatively rapid) kinetics of the activation reaction for unliganded channels (Figure 5F).

In neurons, the kinetics of $I_h$ channels are also consistent with the induction of the slow allosteric conversion by basal levels of cAMP. Thus, in thalamocortical relay neurons (TRN), which show a high level of expression of HCN2 (Santoro et al., 2000), the time course of $I_h$ activation exhibits both a fast and a slow exponential component (Figures 3D–3F), whose voltage-dependent characteristics resemble the behavior of HCN2 in intact oocytes and in cell-free patches with low concentrations of cAMP. By comparing the activation kinetics of $I_h$ in intact oocytes or thalamocortical relay neurons with the kinetics of $I_h$ in cell-free patches (Figure 3), we estimate that basal levels of cAMP are \(~ 10–40 \text{ nM}\) in both types of cells.

**Slow Allosteric Conversion by Basal Levels of cAMP Imparts an Activity-Dependent Memory to $I_h$**

The slow rate of dissociation of cAMP from the open state (\(~ 0.045 \text{ s}^{-1}\)) predicts that $I_h$ should display a significant activity-dependent memory. Thus, in an intact neuron, a prolonged burst of hyperpolarizing inhibitory post-synaptic potentials (IPSPs) should result in the opening of HCN channels, followed by the binding of basal cAMP to the open channel, leading to both fast and slow phases of activation. Once the burst of IPSPs terminates, the channels should persist in the open, ligand-bound state for a prolonged period due to the slow off-rate of cAMP (and the fact that the cAMP-bound channels will tend to remain open at typical neuronal resting potentials of \(~ 70 \text{ mV}\)) and generate a prolonged $I_h$ tail current.

We have tested this prediction for HCN2 channels in intact oocytes using a train of hyperpolarizing voltage clamp pulses to mimic a burst of IPSPs (Luthi and McCormick, 1998a) (Figure 6A). This protocol does indeed generate a slow increase in $I_h$ during the hyperpolarizing burst that is followed by a prolonged tail of channel opening, in which $I_h$ requires tens of seconds to decay back to baseline. Moreover, we find that these slow kinetics are due to the slow binding and unbinding of cAMP, and not to the inherent voltage-gated kinetics of the channel, because HCN2/R591E channels, which cannot bind cAMP, fail to display the prolonged tail of channel opening (Figure 6B).

**Predicted Effects of Basal Levels of cAMP in a Model Thalamocortical Relay Neuron**

In intact neurons, the slow tail of $I_h$ activation should generate a prolonged afterdepolarization following a burst of IPSPs. To evaluate the likely characteristics of this effect, we combined the cyclic allosteric model of $I_h$ with a computer model of the electrophysiological properties of thalamic relay neurons (McCormick and
Huguenard, 1992; Destexhe et al., 1996). We first studied how cAMP alters the kinetics of membrane potential responses to constant current injection. In the absence of cAMP, negative current steps cause a membrane hyperpolarization followed by a rapid, partial depolarization or “sag,” reflecting activation of the inward I_h, (Figure 7A). At the end of the current step, a brief rebound afterdepolarization occurs before the membrane potential returns to rest, reflecting the relatively rapid kinetics of I_h deactivation (O → C).

In the presence of 40 nM cAMP, the sag and rebound contain an additional slow phase. The slow phase of the sag reflects the slow component of activation of I_h in low concentrations of cAMP (due to accumulation of channels in the cAMP-bound open state: C → A + O → AO). The slow, persistent phase of the afterdepolarization reflects the persistent tail of I_h activation (due to the slow rate of cAMP dissociation from open channels: AO → O → C). The amplitude and duration of the rebound depolarization increase with the duration of the current step, reflecting increased accumulation of channels in the cAMP-bound, open state (Figures 7A and 7B).

By contrast, in the presence of saturating cAMP, the sag and rebound depolarizations are surprisingly small and rapid, with a duration similar to those observed in the absence of cAMP (Figure 7A). This is because, at high concentrations, cAMP will bind to the closed state of the channel. Moreover, because cAMP-bound channels can open at the resting potential due to their facilitated gating, most I_h channels will exist in the cAMP-bound open state (AO) prior to the hyperpolarizing current pulse. As a result, relatively few closed I_h channels are available to activate during the hyperpolarization pulse (or shut upon termination of the current step), causing a reduction in the size of the depolarizing sag (and subsequent rebound afterdepolarization). In addition, the slow phase of both the sag and rebound depolarization are eliminated because the kinetics are dominated by the facilitated voltage-gating reaction of channels in the cAMP-bound state (AC ↔ AO). These results thus demonstrate the importance of the dynamic allosteric conversion, rather than cAMP per se, for the slow kinetic processes.

The cyclic allosteric model also predicts that a transient increase in cAMP can have long-lasting effects on channel gating that are dependent on the electrical activity of a cell. At membrane potentials where HCN channels are partially open, a brief increase in cAMP concentration leads to a prolonged enhancement of channel activation that persists even in the absence of cAMP (Figure 7B). This is because, at high concentrations, cAMP will bind to the closed state of the channel. Moreover, because cAMP-bound channels can open at the resting potential due to their facilitated gating, most I_h channels will exist in the cAMP-bound open state (AO) prior to the hyperpolarizing current pulse. As a result, relatively few closed I_h channels are available to activate during the hyperpolarization pulse (or shut upon termination of the current step), causing a reduction in the size of the depolarizing sag (and subsequent rebound afterdepolarization). In addition, the slow phase of both the sag and rebound depolarization are eliminated because the kinetics are dominated by the facilitated voltage-gating reaction of channels in the cAMP-bound state (AC ↔ AO). These results thus demonstrate the importance of the dynamic allosteric conversion, rather than cAMP per se, for the slow kinetic processes.

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for slow, activity-dependent processes have been less thoroughly studied. Here we show how low levels of cAMP induce a very slow component of HCN channel activation due to the allosteric coupling of channel opening to enhanced ligand binding. Moreover, the very slow rate of cAMP dissociation endows $I_\text{h}$ with an activity-dependent memory that generates a persistent phase of channel opening that lasts for tens of seconds. Such slow modulatory processes, when layered onto the faster kinetics of HCN channel voltage gating, impart a dynamic range to $I_\text{h}$ kinetics that can span more than two orders of magnitude. This helps explain how HCN channels underlie both relatively fast rates of spiking at 5–10 Hz and slower periodic oscillations that wax and wane over many seconds (Pape, 1996; Luthi and McCormick, 1998a, 1998b, 1999).

The importance of $I_\text{h}$ for neuronal rhythmic activity has been extensively investigated in the thalamus, where it contributes to spindling, a form of synchronized network activity during slow wave sleep, consisting of seconds-long bursts of high frequency oscillations (6–14 Hz) separated by periods of quiescence lasting 5–10 s (Luthi and McCormick, 1998a, 1998b). The long silent period is due to a cumulative build-up of $I_\text{h}$ in thalamocortical relay neurons during a burst of hyperpolarizing IPSPs from the inhibitory thalamic reticular neurons, generating a prolonged afterdepolarization that terminates the burst of activity (Steriade et al., 1993; Bal et al., 1995).

In addition to the physiological implications for neuronal rhythmic activity, our results also suggest a novel mechanism for dynamic signaling through second messenger cascades. To date, most studies have focused on the importance of transient changes in second messenger levels, due to the rapid deactivation of $I_\text{h}$ at positive voltages (AO → AC), which leads to the rapid dissociation of cAMP from the low-affinity, closed state of the channel (AC → C → A). This regulation of $I_\text{h}$ due to interaction of transient changes in cAMP with membrane voltage is likely to contribute to prolonged voltage changes that occur in thalamocortical neurons during spindling, a form of rhythmic activity found during slow wave sleep (Luthi and McCormick, 1998a, 1998b, 1999).

Discussion

Although cyclic allosteric models have been used previously to explain channel modulation (Bean, 1989; Zaghotta and Siegelbaum, 1996), their dynamic implications...
Figure 7. The Physiological Implications of the Cyclic Allosteric Gating Scheme Are Demonstrated in a Computational Model of a TRN

(A) Calculated membrane potential responses to a series of −100 pA current steps (top traces) of increasing duration (0.125, 1, and 4 s) with indicated concentrations of intracellular cAMP. Slow components of the sag and rebound afterdepolarization (arrows) were only observed at the intermediate concentration of cAMP. Calculated resting potentials were −77 mV in 0 cAMP, −74 mV in 40 nM cAMP, and −68 mV in 100 µM cAMP. Similar differences in the sag and rebound were observed when the resting potential was adjusted to the same level in each condition by injection of a constant current (data not shown). (Note: an apparent increase in input resistance with increasing cAMP results from estimating input resistance in current clamp at membrane potentials where voltage-gated channels are active. The apparent increase in input resistance is not found in instantaneous voltage clamp currents during steps from −70 to −75 mV. Input resistances of 159, 143, and 102 MΩ were obtained with 0, 0.04, and 100 µM cAMP, respectively.)

(B) Occupancy of open states during a 4 s hyperpolarizing (−100 pA) current step in 40 nM cAMP. A slow transition from the open, unbound state (O, blue line) to the open, cAMP-bound state (AO, red line) causes the slow sag in membrane potential (black traces, bottom). After the pulse ends, channels exit AO very slowly, causing the prolonged rebound depolarization (the channels eventually return to state C via state O). Resting membrane potential (−74 mV) is indicated by the dotted line.

(C) Brief (1 s) pulses of increasing concentrations of cAMP (10–250 nM) cause a prolonged (>20 s) depolarization.

(D) The depolarization (bottom, solid black trace) in response to a 250 nM cAMP pulse (upper, solid black trace) is due to an accumulation of channels in the open, cAMP-bound state (AO, solid red lines). A brief depolarizing current step (500 pA for 1 s; upper, black dashed trace) causes a rapid closure of the channels that curtails the depolarization (bottom, black dashed trace). All dashed lines indicate the altered response after the depolarizing step.

al., 2000), and the G protein-regulated, voltage-gated Ca²⁺ channels (Bean, 1989).

Although the simple version of the cyclic allosteric model we have used in this analysis captures a number of key features of how cAMP binding regulates Iₜ gating, it clearly is an oversimplification. In particular, it does not account for the sigmoidal activation kinetics upon hyperpolarization, in either the absence or the presence of cAMP. Recently, Altomare et al. (2001) modeled the kinetics of Iₜ activation in the absence of cyclic AMP using a multistate, allosteric activation scheme consistent with the presumed tetrameric structure of the channels. In this model, each individual subunit independently undergoes a voltage-dependent gating transition between a resting and an active state. Each activation reaction enhances the energetics of a single concerted,
 allosteric conformational change that opens the channel through a cooperative interaction among all four subunits. Our treatment essentially combines these multiple voltage-gating steps into a single, first-order gating reaction (G → O). Insofar as voltage-gating is rapid relative to cyclic nucleotide binding, a condition that is likely to be met for low concentrations of cAMP, this simplification should not significantly alter the conclusions of our study.

A second simplification concerns the number of cyclic nucleotides that bind to modulate channel gating. At present, there is no direct data concerning the cAMP stoichiometry of Ih channel gating. For the related CNG channels, each channel has been shown to bind up to four molecules of cyclic nucleotide, although binding of fewer than four ligands is sufficient to enhance channel gating (Ruiz and Karpen, 1997; Liu et al., 1998). In the absence of detailed empirical data for HCN channels, we have assumed that there is only a single cyclic nucleotide binding event. Although the presence of multiple ligand binding events would clearly affect the quantitative estimates for rate constants of ligand binding and unbinding, they would not alter our qualitative conclusions that channel gating is allosterically coupled to enhanced ligand binding, and that the enhanced binding to the open state persists for many seconds.

A detailed model based on the multistate activation kinetics of Ih, combined with future experimental findings on cAMP stoichiometry will, no doubt, be useful in refining our quantitative picture of HCN channel gating and modulation. However, our present experimental results and analysis provide a clear indication of the important role that the allosteric coupling of channel gating to ligand binding plays in both the action of cAMP to enhance channel activation (Ruiz and Karpen, 1997; Liu et al., 1998). In the absence of detailed empirical data for HCN channels, we have assumed that there is only a single cyclic nucleotide binding event. Although the presence of multiple ligand binding events would clearly affect the quantitative estimates for rate constants of ligand binding and unbinding, they would not alter our qualitative conclusions that channel gating is allosterically coupled to enhanced ligand binding, and that the enhanced binding to the open state persists for many seconds.

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Experimental Procedures

Electrophysiological recordings

Cell-free inside-out patches were obtained 3–6 days after cRNA injection as previously reported (Wang et al., 2001). The pipette solution contained 107 mM KCl, 5 mM NaCl, 10 mM HEPES, 1 mM MgCl₂, and 1 mM EGTA (pH 7.3). The (internal) bath solution contained 104 mM K-Aspartate, 3 mM KCl, 5 mM NaCl, 10 mM HEPES, 1 mM MgCl₂, and 1 mM EGTA (pH 7.3). The holding potential was −40 mV. Hyperpolarizing voltage steps of variable length (4–15 s) were used. Two microelectrode voltage-clamp recordings were performed 1–2 days after cRNA injection as previously reported (Chen et al., 2001). The recordings were obtained with oocytes bathed in a high KCl solution containing 96 mM KCl, 2 mM NaCl, 10 mM HEPES, and 2 mM MgCl₂ (pH 7.5). Microelectrodes were filled with 3 M KCl and had resistances of 0.5–2 MΩ. Holding potential was −30 mV. Analysis was done using Pulsefit (HEKA) and IgorPro (WaveMetrics). Linear leak current was not subtracted. All recordings were obtained at room temperature (22–25°C).

Ih Current Analysis

Channel Kinetics

Activation curves were fit with either a single exponential \((I - I_{h0} + A\exp[-(V - V_{1/2})/s])\) or a double exponential function \((I - I_{h0} + A\exp[-(V - V_{1/2})/s] + B\exp[-(V - V_{2/2})/s])\) after an initial delay (Wang et al., 2001). \(I_{h0}\) represents the steady-state current, and \(s\) represents the time constant. Goodness-of-fit was evaluated using \(\chi^2 = \Sigma x^2 - x^2/k\), where \(x\) is the experimental current value at a given time point and \(k\) is the fitted value. For a quantitative analysis of the adequacy of single versus double exponential fits of the time course of \(I_{h}\) activation in the absence and presence of cAMP, we calculated the difference between \(\chi^2\) for a single exponential fit and \(\chi^2\) for a double exponential fit. To compare \(\chi^2\) values at different pulse lengths, these values were divided by the number of points per current record (a constant sampling rate of 1 kHz used for all pulse lengths). In experiments in the absence of cAMP or with saturating cAMP, where channel kinetics are relatively rapid, we used a variable pulse length protocol (DiFrancesco, 1997) that allowed channel activation to approach steady-state values at each voltage but minimized the length of the hyperpolarization to prevent membrane damage during hyperpolarization. In subsaturating cAMP where channel kinetics are slow, we used a 15 s pulse to allow activation to approach steady state. To ensure that the use of different pulse lengths did not influence the exponential fitting procedure, we compared fits using 15 s pulses to fits using shorter pulses (6–10 s) in a subset of experiments (in the absence of cAMP or the presence of saturating cAMP). We observed no difference in the time constants of activation or in the quality of single versus double exponential fits in these experiments. After normalization, there was no significant difference in \(\chi^2\) values for fits using different pulse lengths, justifying the use of the variable pulse protocol.

Steady-State Activation

Activation curves were determined from the amplitudes of tail currents measured on return to −40 mV after hyperpolarizing steps to different test voltages as previously described (Wang et al., 2001). Tail currents were fit with a modified Boltzmann equation: \(I - I_{h0} + A(1 + \exp[-(V - V_{1/2})/s])\), where \(I_{h0}\) is the offset caused by a nonzero holding current, \(A\) is the maximal tail current amplitude, \(V\) is voltage during the hyperpolarizing test pulse (in mV), \(V_{1/2}\) is the midpoint activation voltage, and \(s\) is the slope of the relation (in mV). Tail current amplitudes from individual experiments were normalized by subtracting \(A_{0}\) and dividing by \(A_{m}\). The normalized data were averaged among the different experiments and refit by the Boltzmann equation with \(A_{0}\) set to 0 and \(A_{m}\) set to 1.

Fitting of the Kinetics by the Allosteric Model

We used the model of Figure 1C to fit \(I_{h}\) activation kinetics during hyperpolarizations in the presence of intermediate concentrations of cAMP using the Levenberg-Marquardt nonlinear least squares algorithm of Igor Pro. The model contains four voltage-dependent rate constants for the two voltage-gating reactions and four voltage-independent rate constants for the cAMP binding reactions. Because of the cyclic nature of the model, the rate constant for cAMP dissociation from the closed state, \(k_{d}\), was calculated from the other seven rates. Values for the four voltage-dependent rates were partially constrained from experimental measurements of activation time constants in the absence of cAMP, \(\tau = 1/(k_{bc} + k_{bd})\), and of the major, fast time constant of activation in the presence of saturating cAMP, \(\tau = 1/(k_{fo} + k_{fc})\). Steady-state open probability for the unliganded state, \(P_{0} = k_{fo}/(k_{fo} + k_{fc})\), was a free parameter. Although \(P_{0}\) could have been constrained by our measured value of \(P_{0}\) in the absence of cAMP, a small progressive negative shift in \(V_{1/2}\) after patch excision (Chen et al., 2001) led to small shifts in \(P_{0}\) during the experiment. Fitted values of \(P_{0}\) were always within 25% of the actual measured values. Steady-state open probability for the ligand-bound state, \(P_{m} = k_{fo}/(k_{fo} + k_{fc})\), was calculated from the fitted value of \(P_{0}\) and the measured shift in \(V_{1/2}\) of the steady-state activation curve in saturating cAMP (−20 mV) (Wang et al., 2001). The forward association rate constant for cAMP binding to the closed and open channels, \(k_{c}\), and \(k_{e}\), as well as the rate constant for cAMP dissociation from the open state, \(k_{d}\), were free parameters. The time course of activation of \(I_{h}\) for a given set of rate constants was calculated using the Q-Matrix approach (Colquhoun and Hawkes, 1995).

Thalamocortical Neuron Simulations

Thalamocortical relay cells were simulated with a modified version of a previously described single compartment model (McCormick and Huguenard, 1992; Destexhe et al., 1996) using the program Neuron (Hines and Carnevale, 1997, 2000). To focus on the role of \(I_{h}\), low-threshold calcium currents were not included in the simulations. The kinetic description of \(I_{h}\) included in the model was based on the four state cyclic allosteric model (Figure 1C) using rate constants derived from fits to our experimental data. Transitions between open
and closed states (C → O and AC → AO) were governed by forward and backward rate constants (α, β, α', β') with a voltage dependence described by a two state Boltzmann distribution: \( r(V) = \frac{r_0}{1 + \exp \left( \frac{V - V_{1/2}}{k_B} \right)} \), where \( r_0 \) is the value of a given rate constant at voltage V. To account for the difference in \( V_{1/2} \) for \( I_h \) between inside-out patches from Xenopus oocytes and intact thalamocortical cells, the \( V_{1/2} \) values for each rate constant were increased by 48mV. The parameters used are given in Table 1. The ligand binding reaction between states O and AO was described by \( K_a = 3.086 \times 10^5 \) M \(^{-1} \) s \(^{-1} \) and \( K_c = 0.04465 \) s \(^{-1} \), based on the fit of the model in Figure 1C to the inside-out patch data. The closed-state dissociation constant, \( K_c \), was set equal to 80 times that of the open-state dissociation constant, \( K_a \), (according to \( \Delta V = -\ln(K_a/K_c) \)), where \( \Delta V \) is approximately 20mV. The corresponding on and off rates for cAMP binding to the closed state were reduced and increased, respectively, by \( \sqrt{80} \) relative to the corresponding rates for the open state. To adjust the model parameters, which were determined from experiments at \( -22^\circ C \), to the simulated temperature of 36°C, we assumed a \( Q_{10} \) of 4 for voltage-dependent transitions and 1.5 for ligand binding/unbinding.

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