4848 Journal of Physiology (1996), 492.1, pp.97-106

Modulation of the hyperpolarization-activated current (I_h) by cyclic nucleotides in guinea-pig primary afferent neurons

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1. Whole-cell patch-clamp recordings were made from dissociated guinea-pig nodose and trigeminal ganglion neurons in culture to study second messenger mechanisms of the hyperpolarization-activated current (I_h) modulation.
2. Prostaglandin E_2 (PGE_2) and forskolin modulate I_h in primary afferents by shifting the activation curve in the depolarizing direction and increasing the maximum amplitude.
3. The cAMP analogues, RP-cAMP-S (an inhibitor of protein kinase A (PKA)) and SP-cAMP-S (an activator of PKA), both shifted the activation curve of I_h to more depolarized potentials and occluded the effects of forskolin. These results suggest that I_h is modulated by a direct action of the cAMP analogues.
4. Superfusion of other cyclic nucleotide analogues (8-Br-cAMP, 8-(4-chlorophenylthio)-cAMP and 8-Br-cGMP) mimicked the actions of forskolin and PGE_2, but dibutyryl cGMP, 5′-AMP and adenosine had no effect on I_h. 8-Br-cAMP and 8-Br-cGMP had similar concentration response profiles, suggesting that I_h has little nucleotide selectivity.
5. The inhibitor peptide (PKI), the catalytic subunit of PKA (C subunit) and phosphatase inhibitors (microcystin and okadaic acid) had no effect on forskolin modulation of I_h.
6. These results indicate that I_h is regulated by cyclic nucleotides in sensory neurons. Positive regulation of I_h by prostaglandins produced during inflammation may lead to depolarization and facilitation of repetitive activity, and thus contribute to sensitization to painful stimuli.

I_h is a hyperpolarization-activated non-selective cation current that has been described in the pacemaker cells of the heart (Yanagihara & Irisawa, 1980; DiFrancesco, Ferroni, Mazzanti & Tromba, 1986), in smooth muscle (Benham, Bolton, Denbigh & Lang, 1987), and in neurons (Mayer & Westbrook, 1983; Tokimasa & Akasu, 1990; Kamondi & Reiner, 1991). I_h plays a role in the generation of spontaneous action potentials (McCormick & Pape, 1990*a*; DiFrancesco, 1991; Noble, Denyer, Brown & DiFrancesco, 1992), and modulation of I_h results in the regulation of firing frequencies (DiFrancesco, Ducouret & Robinson, 1989; Denyer & Brown, 1990; McCormick & Pape, 1990*b*; Banks, Pearce & Smith, 1993). Activation of adenylyl cyclase in the heart causes a shift of the voltage dependence of I_h to more depolarized potentials (DiFrancesco *et al.* 1986), whereas inhibition of adenylyl cyclase shifts the voltage dependence to more hyperpolarized potentials (DiFrancesco & Tromba, 1988; Chang & Cohen, 1992). The second messenger pathway leading to modulation of I_h involves regulation of adenylyl cyclase but the mechanism is not completely understood. Protein kinase inhibitors shifted I_h activation to more hyperpolarized potentials and blocked the effects of adenylyl cyclase activation in Purkinje cells of the heart (Chang, Cohen, DiFrancesco, Rosen & Tromba, 1991) and sympathetic neurons (Tokimasa & Akasu, 1990), implicating cAMP-dependent protein kinase A (PKA) in tonic and receptor-mediated regulation of I_h. However, other second messenger mechanisms have also been proposed. Activated G protein α-subunits (G_s, G_o and G_i) mimicked the effects of noradrenaline and acetylcholine when applied to inside-out patches from SA node cells, suggesting that modulation may occur through a direct action of G proteins (Yatani, Okabe, Codina, Birnbaumer & Brown, 1990). Alternatively, direct application of cAMP and cAMP analogues augmented I_h in SA node myocytes (DiFrancesco & Tortora, 1991) and increased the probability of opening in single-channel recordings of I_h (DiFrancesco & Mangoni, 1994).

Prostaglandins are substances produced by the inflammatory cascade that produce hyperalgesia (Taiwo, Bjerknes, Goetzl & Levine, 1989). PGE_2 increases cAMP levels in cultured primary afferent neurons (Hingtgen, Waite & Vasko, 1995) and has also been shown to produce hyperalgesia and increase excitability of primary afferent neurons

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via a cAMP-dependent mechanism (Taiwo & Levine, 1991; Cui & Nicol, 1995). Since primary afferents have been shown to express I_h (Mayer & Westbrook, 1983), regulation of I_h by cAMP may play an important role in PGE_2-induced excitation of primary afferents. The purpose of this study was to examine the effects of PGE_2 on I_h and determine the mechanism by which cAMP modulates I_h in primary afferent neurons.

METHODS

Culture

Adult guinea-pigs were anaesthesized with 4% halothane in air and killed by opening the chest, severing the major blood vessels entering and leaving the heart, and allowing the blood to drain away. Nodose and trigeminal ganglia were dissected and washed. The culture technique was adopted from Beech, Bernheim, Mathie & Hille (1991). Briefly, the ganglia were minced and dissociated for 10 min in 2 ml of 20 units ml^{-1} papain (with 0·4 mg ml^{-1} cysteine) dissolved in Hanks' balanced salt solution (no divalent cations). They were placed into a second solution of dispase (25 mg ml^{-1}) and collagenase (730 units ml^{-1}) for 25 min. After dissociation, the enzymes were inhibited by washing twice with growth medium (10 ml minimum essential medium containing 10% fetal calf serum, 50 ng ml^{-1} nerve growth factor, 5000 Units ml^{-1} penicillin and 5000 μg ml^{-1} streptomycin) and triturated through a glass pipette flamed to approximately 300 μm. Neurons were plated onto coverslips coated with 0·1 mg ml^{-1} polylysine and 40 μg ml^{-1} laminin, and the medium was replaced every 2 days. Recordings were made between 2 and 7 days after plating.

Recordings

Whole-cell recordings were made at 37 °C with glass electrodes pulled and fire-polished to obtain a 2–4 MΩ pipette resistance. Access resistances ranged from 3 to 6 MΩ and the recordings could often be maintained for approximately 60 min. Both capacitance (10–40 pF) and series resistance (2–5 MΩ; 80%) compensation were used. Control medium contained (mM): NaCl, 146; $MgCl_2$, 1·2; KCl, 5; $CaCl_2$, 2·5; Hepes, 5; and dextrose, 30; pH 7·3. The control internal pipette solution contained (mM): caesium gluconate, 140; NaCl, 10; Hepes, 10; EGTA, 1; free Ca^{2+}, 30 nM; Na_2ATP, 2 and NaGTP, 0·25. Ca^{2+} experiments were done with internal solutions buffered with BAPTA (20 mM). $CaCl_2$ (17 μM) and $CaCl_2$ (9·1 mM) were added to the BAPTA internal solution to obtain a pCa10 and pCa7, respectively. Forskolin, PGE_2 (Sigma), okadaic acid (Biomol, Plymouth Meeting, PA, USA), and microcystin-LR (Gibco BRL, Gaithersburg, MD, USA) were dissolved into dimethyl sulphoxide stock solutions. Adenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (RP-cAMP-S), adenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer (SP-cAMP-S), 8-bromo-adenosine-3′,5′-cyclic monophosphate (8-Br-cAMP), and 8-bromo-guanosine-3′,5′-cyclic monophosphate (8-Br-cGMP) (BioLog, La Jolla, CA, USA) were dissolved in water. CPT-cAMP (8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate), N^2,2′-O-dibutyrylguanosine 3′,5′-cyclic monophosphate (dBcGMP), adenosine 5′-monophosphate (5′-AMP) (Sigma), and adenosine (Boehringer-Mannheim, Indianapolis, IN, USA) were dissolved directly into the extracellular solution to their final concentrations. The PKA inhibitor peptide fragment (5–24) (PKI) and PKA catalytic subunit (C subunit) were generous gifts from the laboratory of John Scott, Vollum Institute, Portland, OR, USA. RP-cAMP-S, SP-cAMP-S, PKI, C subunit, okadaic acid and microcystin were added to the internal pipette solution while all other substances were perfused in the external solution.

Whole-cell patch-clamp recordings of I_h from nodose and trigeminal ganglion neurons in culture were made. Neurons were held at −40 mV (positive to I_h activation) and stepped to hyperpolarizing potentials until steady-state currents were attained. Drugs were perfused or added to the internal solution. Two experimental protocols were used: a current–voltage (I–V) protocol and a two-step time protocol. Activation plots were made from tail current amplitudes measured at −60 mV evoked by prepulses to a number of hyperpolarized potentials using the I–V protocol. The tail currents were normalized to the maximum control amplitude ($I/I_{max(control)}$). The second experimental protocol, the two-step time protocol, used a prepulse from V_{hold} = −40 to −70 mV to activate I_h and a step back to −60 mV to elicit the tail current repeated every 30 s to determine the time course of I_h modulation by various drugs. Holding currents at −40 mV and tail currents evoked at −60 mV were plotted *versus* time. Results from drug applications were expressed as the percentage change from control.

Data analysis

Data were collected via an Axopatch 1-D amplifier and filtered at 2 kHz with a Bessel filter. Currents were digitized and recorded with pCLAMP software and analysed using Axograph (Axon Instruments, Inc.) and Kaleidograph software. Activation curves were fitted with a Boltzmann function using a least-squares algorithm to estimate the half-maximal voltage of activation ($V_{1/2}$), maximum amplitude and slope values (Kaleidograph, Synergy Software). Descriptive statistics used were the mean ± S.E.M. Student's paired t tests were used to determine statistical significance of the effects of forskolin and PGE_2 on control parameters. One-way ANOVAs were used to compare results between cells with control, C subunit, PKI, phosphatase inhibitors, RP-cAMP-S, or SP-cAMP-S in the internal pipette solution. Dunnett's *post hoc* comparison test was used to determine the statistical significance of comparisons between control and other internal pipette solutions. A repeated ANOVA was used to determine if Boltzmann curves from cells recorded with control, RP-cAMP-S or SP-cAMP-S were significantly different. A two-way ANOVA was used to analyse the statistical difference of results from cells recorded with different Ca^{2+} internal solutions and treated with control, forskolin or PGE_2 external solutions. Scheffe's *post hoc* test was used to compare all possible combinations of means from the repeated and two-way ANOVAs. $P < 0·05$ was taken to indicate statistical significance in all tests.

RESULTS

Recordings were made from approximately 212 neurons. Once the whole-cell recording was established, a slow decline in the outward holding current at V_{hold} = −40 mV was apparent during the first 5 min. This was probably due to the block of K^+ currents resulting from the diffusion of Cs^+ into the cell. I_h generally activated between −50 and −70 mV and reached maximal amplitude at −100 to −110 mV (tail currents at −60 mV overlapped at these potentials). External Cs^+ (2 mM), an I_h blocker, blocked all of the time-dependent inward current elicited with steps to hyperpolarized potentials.

Run-down of I_h was noted in most cells and was associated with both a decrease in maximum amplitude and a shift of the activation curve to more hyperpolarized potentials. The time course and extent of the run-down was extremely variable between cells. In an attempt to understand the underlying mechanism of run-down, the changes in $V_{1/2}$, slope and maximal amplitude values as estimated from Boltzmann fits were calculated over a 10 min period (15 min time point minus the 5 min time point). The $V_{1/2}$ shifted in the hyperpolarizing direction by 5 ± 1 mV and the maximum amplitude decreased by 10 ± 3% in cells recorded with control internal solution ($n = 5$). The negative shift of $V_{1/2}$ was not significantly different in cells with any of the cAMP pathway modulators applied to the internal solution (Dunnett's test, $P > 0.05$). However, the negative shift in maximum amplitude associated with run-down was significantly greater in cells recorded with okadaic acid (1 μM) in the internal pipette solution (39 ± 11%; $n = 3$) compared with controls (Dunnett's test, $P < 0.05$). Thus, the $V_{1/2}$ and maximum amplitude variables may be regulated by different mechanisms. Forskolin and PGE_2 were applied 15 min after the onset of recording so that internal Cs^+ would be equilibrated and the amount of run-down could be assessed. After wash-out of forskolin and PGE_2, I_h often over-recovered so that activation curves of each cell were more hyperpolarized than in controls. However, despite run-down, responses to both PGE_2 and forskolin could be elicited repeatedly (see Fig. 1).

Prostaglandin E_2 and forskolin shifted the voltage dependence and increased the amplitude of I_h

PGE_2 (1 μM) shifted the voltage dependence of activation of I_h to more depolarized potentials (4 ± 1 mV; $n = 5$) and increased the maximum amplitude of I_h (18 ± 5%; $n = 5$; Fig. 1*A*) in five out of thirteen cells tested with control internal solution. Thus, only a subpopulation of these neurons respond to PGE_2, suggesting that not all cells express prostaglandin receptors. Forskolin (10 μM) shifted the voltage dependence of I_h in all cells tested under control conditions (5 ± 1 mV; $n = 6$) and increased the amplitude of I_h (21 ± 5%; $n = 6$). There were no differences in slope values during application of forskolin or PGE_2. Figure 1*B* shows the effects of both repeated forskolin applications and a PGE_2 application on the I_h current evoked with the two-step protocol (a step from −40 to −70 and back to −60 mV every 30 s). Although the amplitude of I_h runs down over the course of the experiment, forskolin and PGE_2 responses could be repeated. External Cs^+ completely blocked all of the inward current, indicating the isolation of I_h in the presence of internal caesium gluconate.

Role of phosphorylation by protein kinase A (PKA)

Forskolin and PGE_2 increase the levels of cAMP in primary afferent neurons, suggesting that the modulation of I_h may involve the cAMP second messenger pathway. In order to test the role of PKA, the cAMP analogues RP-cAMP-S and SP-cAMP-S were used in the internal pipette solution. RP-cAMP-S inhibits PKA, while SP-cAMP-S activates the

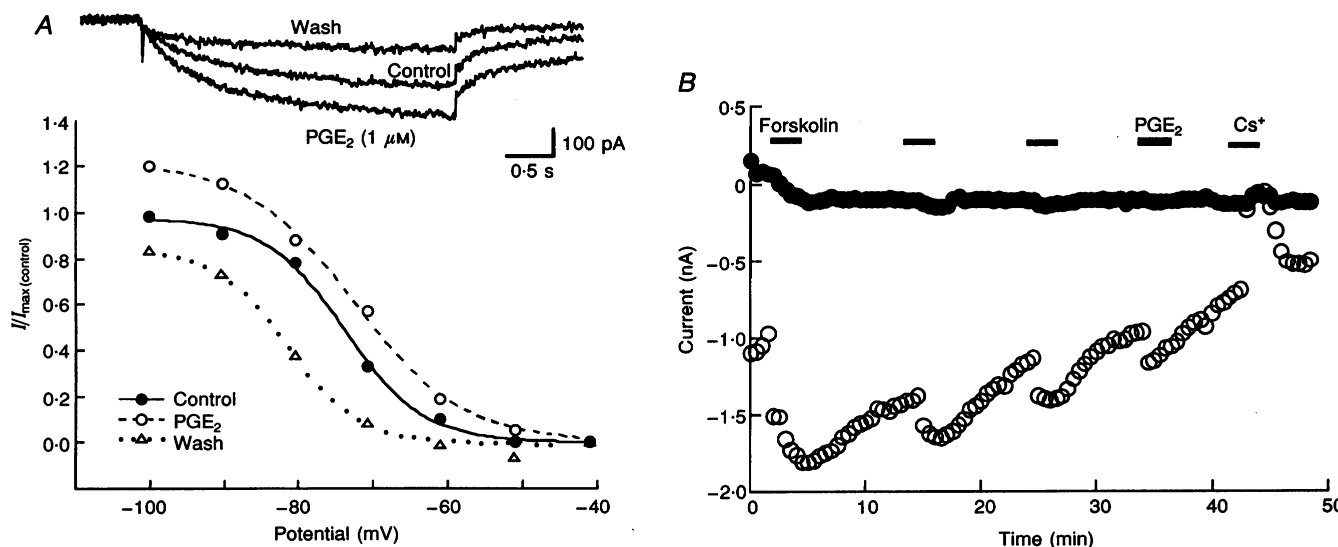

Figure 1. PGE_2 and forskolin shift the voltage dependence and increase the amplitude of I_h

A, superfusion of PGE_2 (1 μM) increases the maximum amplitude and shifts the voltage dependence of I_h ($V_{1/2}$) to more depolarized potentials. Representative traces of a step from −40 to −70 mV are depicted in inset. *B*, change in I_h over time with two-step protocol during applications of forskolin (10 μM) and PGE_2 (1 μM). Steps were made from $V_{hold} = -40$ mV (●) to −70 mV and tail currents were measured at −60 mV (○). The inward current was entirely I_h since external Cs^+ effectively blocked all of this current. Both forskolin and PGE_2 augment I_h. The amount of run-down during the recording period is highly variable between cells.

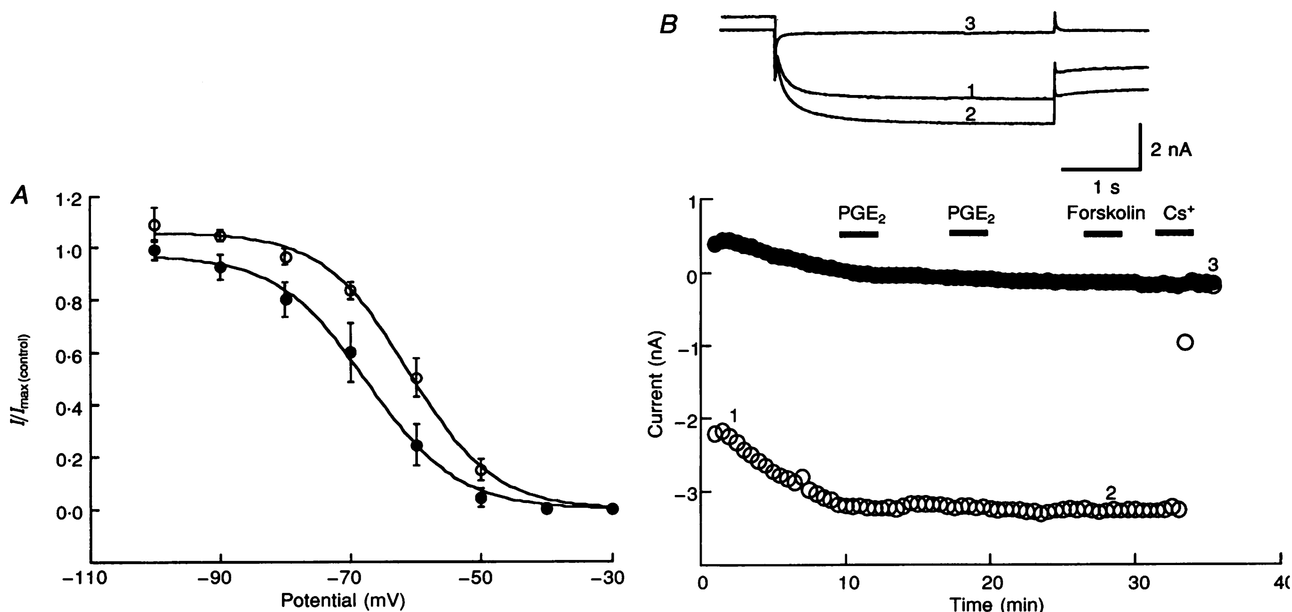

Figure 2. RP-cAMP-S modulates I_h and occludes forskolin and PGE_2 effects

A, averaged activation curves for I_h in the presence (○, $n = 4$) and absence (●, $n = 4$) of RP-cAMP-S (1 mM) are significantly different over the potential range. *B*, steps from $V_{hold} = -30$ mV (●) to −70 mV and back to −60 mV (○) were made every 30 s after break-in to the whole-cell mode. Representative traces are indicated by numbers to emphasize several points: the baseline shifts in the first 5 min, there is no effect of PGE_2 (1 μM) or forskolin (10 μM) and, as Cs^+ (2 mM) blocks the entire inward current, there is no indication that RP-cAMP-S activates another inward current.

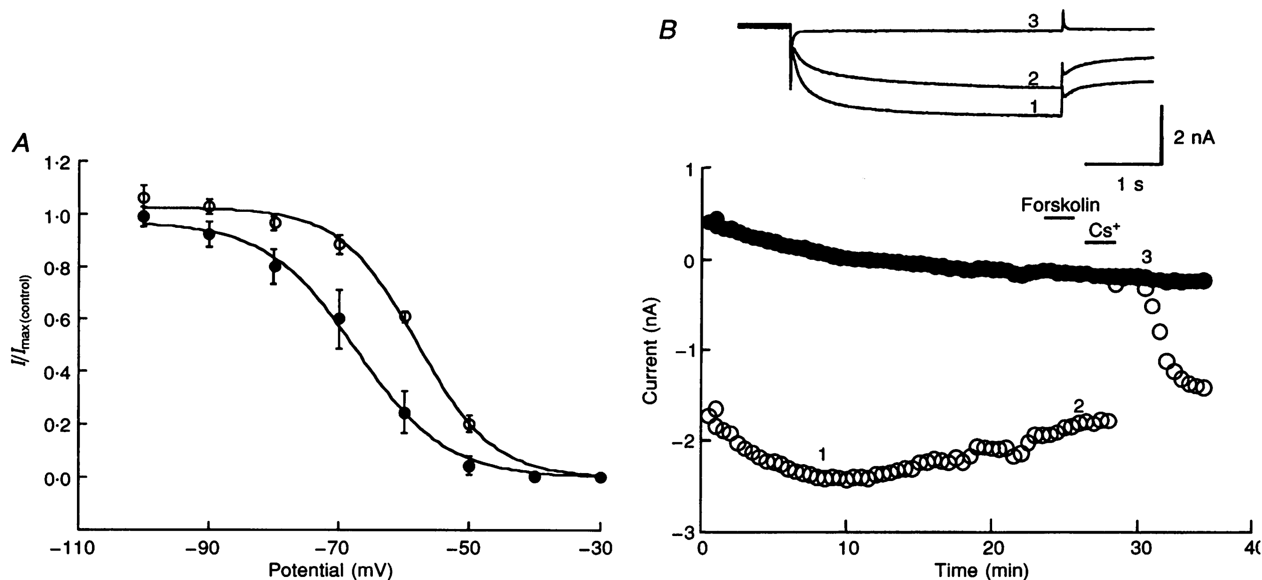

Figure 3. SP-cAMP-S modulates and occludes forskolin modulation of I_h

A, averaged activation curves for I_h in the presence (○, $n = 4$) and absence (●, $n = 4$) of SP-cAMP-S (1 mM) showing the significant shift of activation 5 min after breaking into whole-cell mode. *B*, a recording with internal SP-cAMP-S (1 mM). Measurements were taken from tail currents at −50 mV (○) elicited by prepulses from $V_{hold} = -30$ mV (●) to −60 mV. The forskolin (10 μM) response is occluded and Cs^+ blocks all of the inward current. Representative traces are taken from numbered time points. This cell showed run-down during the recording. Run-down was not seen in all cells with SP-cAMP-S and was also seen in some cells with RP-cAMP-S.

enzyme. In Fig. 2*A*, cells with internal RP-cAMP-S (1 mM) have activation curves that are shifted to significantly depolarized potentials (-60 ± 0.4 mV; $n = 4$) over control (-71 ± 1 mV; $n = 4$) 5 min after the whole-cell recording was established. The continuous two-step protocol with prepulses to -70 mV showed that the augmentation of I_h by PGE_2 and forskolin was occluded in these cells ($n = 5$; Fig. 2*B*). Application of Cs^+ (2 mM) to the external solution completely blocked the inward current, suggesting that RP-cAMP-S had augmented I_h.

As with RP-cAMP-S, the cells with internal SP-cAMP-S (1 mM) had significantly depolarized activation curves compared with cells with control internal solution (-59 ± 0.3 mV; $n = 4$; Fig. 3*A*). Similarly, forskolin no longer augmented I_h in cells with internal SP-cAMP-S (Fig. 3*B*), and external Cs^+ blocked the entire inward current elicited by the hyperpolarizing step. Since forskolin always augmented I_h in control cells, even in the presence of marked run-down, the absence of forskolin responses during perfusion with RP-cAMP-S and SP-cAMP-S was due to occlusion.

The concentration dependence of RP-cAMP-S and SP-cAMP-S modulation was studied to determine if lower concentrations could differentiate the effects of these analogues on I_h. Only the highest concentration of RP–cAMP-S (1 mM; $n = 4$) and SP-cAMP-S (1 mM; $n = 5$) shifted the activation curves of I_h to significantly more depolarized potentials. However, concentrations of analogues above 10 μM significantly occluded the forskolin-induced shift of I_h activation (Fig. 4). Lower concentrations had no effect. Although RP-cAMP-S inhibits and SP-cAMP-S activates PKA, they had similar effects on I_h at all concentrations, suggesting that modulation of I_h occurs through a direct action of cyclic nucleotides. Therefore, PKA is probably not involved in the tonic or forskolin-mediated modulation of I_h.

Cyclic nucleotides modulate I_h in primary afferents

Cyclic nucleotide-gated channels in other sensory neurons can be selective for cAMP or cGMP or non-selective. The ability of cAMP and cGMP analogues to affect I_h was investigated to determine the cyclic nucleotide selectivity for I_h. Figure 5 shows that 8-Br-cAMP and 8-Br-cGMP (100 μM and 1 mM) shifted the voltage dependence of I_h to more depolarized potentials but had little effect at 10 μM. There was also an increase in maximum amplitude of the I_h activation curve in the presence of 8-Br-cAMP (1 mM; 12 ± 6%; $n = 5$) and 8-Br-cGMP (1 mM; 12 ± 4%; $n = 11$). The slope values did not change consistently in the presence of the analogues. The effects of 8-Br-cAMP and 8-Br-cGMP were long lasting. It often took twice as long to wash out 8-Br-cAMP and 8-Br-cGMP (1 mM) than to wash out forskolin and only rarely did a second application of cAMP or cGMP analogues have any effect, suggesting that the effects of these analogues are very slow to reverse. In fact, after a 10 min wash-out period of 1 mM 8-Br-cAMP or 8-Br-cGMP, forskolin had no effect. Forskolin was effective, however, 10 min after washing out lower concentrations of 8-Br-cAMP and 8-Br-cGMP (10 and 100 μM). The results of perfusion of other substances are included in Fig. 5*C*. CPT-cAMP (1 mM; $n = 4$) shifted the voltage dependence of activation to more depolarized potentials, but dBcGMP (1 mM; $n = 5$), 5′-AMP (1 mM; $n = 5$) and adenosine (1 mM; $n = 4$) did not.

Effects of other PKA modulators

To further test the possibility that PKA is involved in the tonic or receptor-mediated modulation of I_h, PKI (20 μM), C subunit (1·5 μM), microcystin (2 μM) and okadaic acid (1 μM) were applied via the internal solution and compared to cells with internal RP-cAMP-S and SP-cAMP-S. Only RP-cAMP-S ($n = 5$) and SP-cAMP-S ($n = 6$) significantly shifted the I_h activation curve to more depolarized potentials than control cells ($n = 5$) after the 5 min equilibration period (Dunnett's test, $P < 0.05$). After

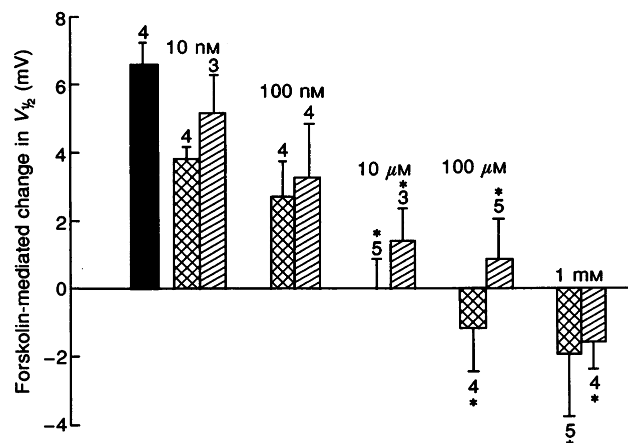

Figure 4. RP-cAMP-S and SP-cAMP-S have similar effects over a large range of concentrations

Forskolin shifts the voltage dependence ($V_{1/2}$) of I_h to depolarized potentials (positive direction) in control (■). SP-cAMP-S (▩) and RP-cAMP-S (▨) occlude the forskolin-mediated change in $V_{1/2}$ compared to control in a concentration-dependent manner. * Significant decrease in the forskolin effect from control.

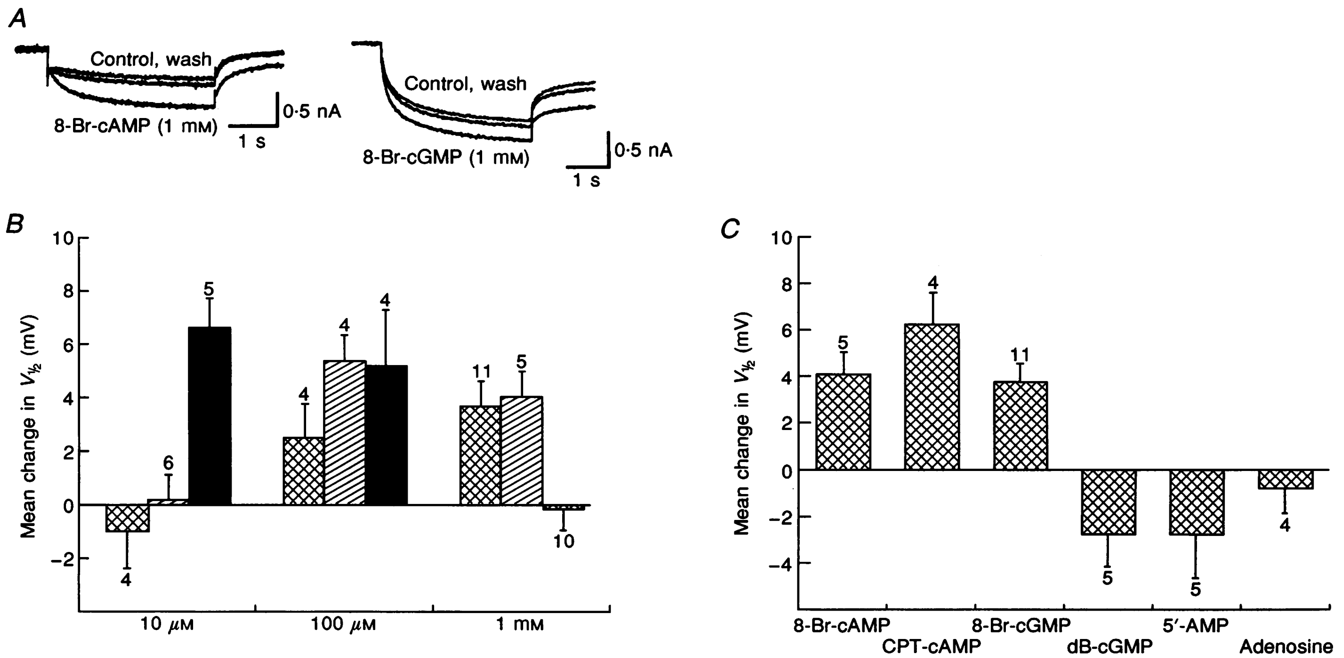

Figure 5. Cyclic nucleotides modulate I_h in primary afferent fibres

A, representative traces elicited by prepulses from −40 to −70 mV and tail currents at −60 mV from two different cells given 8-Br-cAMP (1 mM) or 8-Br-cGMP (1 mM). *B*, histogram showing the mean change in $V_{½}$ during perfusion of different concentrations of 8-Br-cAMP (▨) and 8-Br-cGMP (▩). ■, shift in the activation curve by forskolin (10 μM) applied after a 10 min wash-out period of the respective concentrations of analogues. *C*, histogram showing the mean change in $V_{½}$ during perfusion of several cAMP and cGMP analogues as well as 5′-AMP and adenosine. Data for the 8-Br-cAMP and 8-Br-cGMP (1 mM) are from the same cells as above. Numbers of cells tested with each substance are indicated.

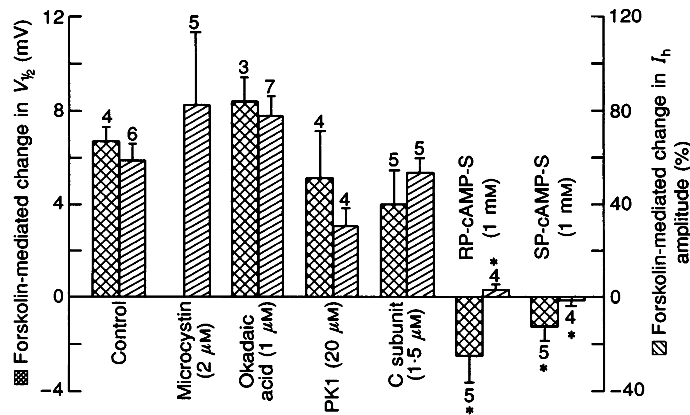

Figure 6. Effects of phosphorylation modulators on the forskolin-mediated change in $V_{½}$ (▩) and percentage change in I_h amplitude (▨)

Inhibitors of PKA (PKI and RP-cAMP-S), activators of PKA (C subunit and SP-cAMP-S), and phosphatase inhibitors (microcystin and okadaic acid) were applied via the internal solution. *I–V* protocols were run every 5 min and forskolin (10 μM) was applied after 15 min of recording. Forskolin was not effective in shifting the voltage dependence of I_h or the tail current amplitudes in cells with RP-cAMP-S or SP-cAMP-S in the internal solution. PKI, C subunit and the phosphatase inhibitors had no significant effect on either variable. Microcystin was not used in *I–V* protocol experiments.

15 min, activation curves for cells with C subunit-containing ($n = 5$) and okadaic acid-containing ($n = 3$) internal solutions were significantly hyperpolarized compared to control, but SP-cAMP-S was still depolarized (Dunnett's test, $P < 0.05$). These results suggest that PKA phosphorylation may be involved in the run-down process, but the mechanisms are not clear.

The effects of forskolin in the presence of these modulators were assessed by applying forskolin 15 min after the initiation of the recording in the same cells described above. Forskolin shifted the voltage dependence of I_h to more depolarized potentials in control, PKI-, C subunit- and okadaic acid-containing internal solutions but was not effective in cells with RP-cAMP-S and SP-cAMP-S in the internal solution. The results suggest that I_h was maximally shifted in the presence of the cAMP analogues (Fig. 6).

The two-step time protocol was used to determine if PKI, C subunit, okadaic acid or microcystin could modulate forskolin-induced I_h tail current amplitudes elicited by steps near the half-activation voltage (−70 mV) over time (Fig. 6). Again, the results were consistent with results from $I-V$ plots; RP-cAMP-S and SP-cAMP-S occlude the action of forskolin on I_h. PKI, C subunit and the phosphatase inhibitors did not significantly alter the response to forskolin.

Role of Ca^{2+} in PGE_2 response

In the heart, I_h has been shown to be stimulated by increased intracellular calcium. Experiments were performed with BAPTA (20 mM) to buffer the internal solution to pCa 10 and pCa 7 in order to test the possibility that I_h in primary afferents is also sensitive to intracellular calcium. There was no significant difference in the $V_{1/2}$ values between pCa 7 (-79 ± 2 mV; $n = 9$) and pCa 10 (-79 ± 2 mV; $n = 9$) after 5 min of recording, suggesting that there is no tonic regulation of I_h by calcium in primary afferents. Forskolin shifted the voltage dependence of I_h to the right by 3 ± 1 mV and increased the maximum amplitude by 6 ± 4% in high-Ca^{2+} (pCa 7) internal solution ($n = 5$) and 6 ± 2 mV and 15 ± 8% in low-Ca^{2+} (pCa 10) internal solution ($n = 6$). PGE_2 shifted the voltage dependence of I_h to the right by 5 ± 2 mV and increased the maximum amplitude by 30 ± 17% in high-Ca^{2+} (pCa 7) internal solution ($n = 4$) and 6 ± 2 mV and 2 ± 6% in low-Ca^{2+} (pCa 10) internal solution ($n = 4$). None of these changes were significantly different between high-Ca^{2+}, low-Ca^{2+} or control internal solutions (Scheffe's test, $P > 0.05$). There was also no difference in activation parameters during run-down (15 min minus 5 min) between different Ca^{2+} conditions. Therefore, calcium does not seem to be involved in either tonic regulation of I_h or in the ability of forskolin or PGE_2 to stimulate I_h.

DISCUSSION

Cell type

Forskolin mimics the PGE_2-induced shift in I_h activation in trigeminal and nodose ganglion primary afferents. PGE_2 modulation occurred in a subpopulation of medium- to large-sized neurons in the nodose ganglion and medium-sized cells in the trigeminal ganglion. Therefore, primary afferent neurons are probably heterogeneous with respect to expression of prostaglandin receptors coupled to activation of adenylyl cyclase. Primary afferent neurons have already been shown to be heterogeneous with respect to expression of I_h. Small-diameter neurons (C fibres) in the dorsal root ganglion do not express I_h (Tokimasa, Shiraishi & Akasu, 1990), an observation confirmed in the nodose and trigeminal ganglia in these studies. In addition, neurons that expressed I_h had short-duration action potentials that are indicative of Aδ-type cells (Scroggs, Todorovic, Anderson & Fox, 1994). The nodose ganglion consists primarily of neurons with C fibres and Aδ-fibres, so I_h modulation probably occurs in Aδ-fibre neurons.

I_h type

PGE_2 and forskolin increase cAMP in primary afferent neurons. Other neurotransmitter receptors coupled to G proteins which stimulate adenylyl cyclase shift the activation curve of I_h to more depolarized potentials in the heart (DiFrancesco *et al.* 1986), bull-frog sympathetic neurons (Tokimasa & Akasu, 1990) and central neurons (Bobker & Williams, 1989; McCormick & Pape, 1990*b*; Banks *et al.* 1993). Neurotransmitter receptors coupled to inhibitory G proteins that decrease cAMP levels in cells, such as muscarinic M2 receptors in the heart (DiFrancesco & Tromba, 1988; DiFrancesco *et al.* 1989; Chang & Cohen, 1992) and μ-opioids in primary afferent neurons (Ingram & Williams, 1994), shift the voltage dependence of I_h in the hyperpolarizing direction. Although I_h has been described in many cell types, there are some qualitative differences that suggest that I_h may be modulated differently in different tissues. I_h is a non-selective cation current that activates with hyperpolarizing voltage steps and is blocked by external Cs^+, but the activation range, amplitude, and modulation of I_h vary in different preparations. β-Adrenergic agonists and forskolin (DiFrancesco *et al.* 1986) shift the activation curve of I_h without an increase in maximum amplitude in SA node myocytes, but PGE_2 and forskolin actually increase both of these parameters in primary afferent neurons. These results are consistent with the observations of Tokimasa & Akasu (1990) in sympathetic neurons. In addition, PKA phosphorylation (Chang *et al.* 1991) has been proposed as the mechanism of I_h regulation in heart Purkinje fibres, but results refuting the role of PKA in SA node myocytes (Yatani *et al.* 1990; DiFrancesco & Tortora, 1991) suggest that second

messenger modulation of I_h is not the same in all cells. In light of these conflicting observations, it is important to understand the mechanism by which cAMP modulates I_h in primary afferent neurons.

Phosphorylation or direct action of cAMP?

Although earlier studies with protein kinase inhibitors (H-7, H-8) suggested that PKA phosphorylation was involved in the tonic modulation of I_h (Tokimasa & Akasu, 1990; Chang *et al.* 1991), the inhibitors were very non-selective. RP-cAMP-S and SP-cAMP-S were used in the present studies to determine if PKA was involved in the augmentation of I_h in primary afferent neurons because they are cAMP analogues that selectively inhibit and activate PKA, respectively. The surprising result was that both analogues augmented I_h and occluded stimulation of I_h by forskolin and PGE_2. There were no differences at any concentration of RP-cAMP-S or SP-cAMP-S that could be attributed to either inhibition or activation of PKA, supporting the hypothesis that I_h in primary afferents is directly regulated by cAMP. This observation was confirmed by studies using PKI, C subunit and phosphatase inhibitors (okadaic acid and microcystin) in that none of these substances shifted the activation curve of I_h to depolarized potentials or blocked the effects of forskolin. The results of the present study are consistent with results from single-channel and inside-out patch recordings from SA node cells showing direct modulation of I_h by cAMP (DiFrancesco & Tortora, 1991; DiFrancesco & Mangoni, 1994). Patches of primary afferent neurons with I_h have altered kinetics immediately after pulling the patch and run down very quickly (authors' unpublished observations). Therefore, RP-cAMP-S and SP-cAMP-S were the best tools to use in the present experiments. Although negative results with PKI and C subunit applied via the internal solution were a concern because there were no obvious positive controls, there are several reasons to believe that diffusion of these substances into the cell occurred. Electrode resistances were small (2–3 MΩ) and a 5 min equilibration period was more than sufficient to observe the effects of internal RP-cAMP-S and SP-cAMP-S on I_h. There was also a significant effect of C subunit on the rate of run-down, suggesting the possibility that PKA is involved in run-down or some other aspect of tonic maintenance of I_h.

Cyclic nucleotide-gated channels

In these studies, I_h was modulated by both cAMP and cGMP analogues. 8-Br-cGMP (1 mM) shifted the voltage dependence and increased maximum amplitude of I_h to the same extent as the cAMP analogues. dBcGMP was ineffective in these experiments, but dBcAMP was much less effective than CPT-cAMP or 8-Br-cAMP in augmenting I_h in a previous study (Ingram & Williams, 1994). Therefore, there may be some selectivity between analogues. Cyclic nucleotide-gated channels have been described from vertebrate retinal and olfactory sensory neurons (for review see Kaupp, 1991). The major difference between these channels is the selectivity for cyclic nucleotides. The retinal cyclic nucleotide-gated channel is approximately 30-fold more selective for cGMP (Kaupp *et al.* 1989; Goulding *et al.* 1992), and the olfactory cyclic nucleotide-gated channels are opened in the presence of micromolar concentrations of both cAMP and cGMP (Nakamura & Gold, 1987). The results of this present study suggest that I_h is regulated by 8-Br-cAMP and 8-Br-cGMP at similar concentrations and may be related to the olfactory cyclic nucleotide-gated channel. However, the cyclic nucleotide-gated channels are actually gated by cyclic nucleotides, while I_h seems to be gated by voltage and modulated by cyclic nucleotides. I_h has also been described in rod photoreceptors but modulation by cyclic nucleotides has not been addressed (Bader, Bertrand & Schwartz, 1982). Since photoreceptor responses to light are regulated by cGMP, it would be interesting to determine if I_h in these cells is also regulated by cGMP.

I_h in SA node cells has been shown to be sensitive to changes in internal Ca^{2+} (Hagiwara & Irisawa, 1989). There are prostaglandin receptor subtypes localized to dorsal root and trigeminal ganglion neurons that activate phospholipase C or adenylyl cyclase (Sugimoto *et al.* 1994). Thus, PGE_2 may be able to act on primary afferents through stimulation of internal Ca^{2+}. Our results indicate that internal Ca^{2+} concentrations buffered to the same concentrations used by Hagiwara & Irisawa (1989) had no effect on I_h activation parameters or run-down. There were also no significant effects of internal calcium on forskolin and PGE_2 responses, although there was a hint of a difference between high and low calcium concentrations in the ability of PGE_2 to increase the maximum amplitude. Thus, the main effects of PGE_2 in primary afferent neurons are probably via activation of a prostaglandin receptor subtype coupled to stimulation of adenylyl cyclase. This is consistent with recent studies that show that one of the prostaglandin receptor subtypes, EP_3, mediates peripheral hyperalgesia (Minami, Nishihara, Uda, Ito, Hyodo & Hayaishi, 1994) through an increase in cAMP (Khasar, Ouseph, Chou, Ho, Green & Levine, 1995).

Significance

PGE_2 increases excitation and sensitizes small-diameter primary afferent neurons (Handwerker, 1976; Schaible & Schmidt, 1988). Increases in cAMP are associated with pain and hyperalgesia (Taiwo *et al.* 1989), and activation of adenylyl cyclase is thought to be the mechanism by which prostaglandins released during the inflammatory response produce hyperalgesia (Ferreira & Nakamura, 1979). Modulation of I_h by cAMP leads to increased spontaneous firing of SA node cells of the heart (Brown & DiFrancesco, 1980; DiFrancesco, 1991; Noble *et al.* 1992) and central neurons (McCormick & Pape, 1990*b*). Therefore, since

forskolin and PGE_2 shift the voltage dependence and increase I_h amplitude through stimulation of adenylyl cyclase, augmentation of I_h may lead directly to sensitization and/or excitation of primary afferent neurons and increased pain.

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Acknowledgements

We thank Z. Hausken for helpful discussion, K. Drews for technical assistance, and N. Marrion and E. McCleskey for comments on this manuscript. This work was supported by NIH grants DA08163 and DA07262.

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Received 27 July 1995; accepted 30 October 1995.