Effect of intracellular dialysis of ATP on the hyperpolarization-activated cation current in rat dorsal root ganglion neurons

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Abstract

The mechanism of the effect of intracellular ATP on the hyperpolarization-activated non-selective cation current ($I_h$) in rat dorsal root ganglion neurons was investigated using a whole-cell voltage-clamp technique. Under voltage-clamp conditions, $I_h$ was activated by hyperpolarizing pulses raised to a voltage of between −70 and −130 mV. The activation curve of $I_h$ in rat dorsal root ganglion (DRG) neurons shifted by about 15 mV in the positive direction with an intracellular solution containing 1 mM cAMP. When ATP (2 mM) was applied intracellularly, the half-maximal activation voltage ($V_{\text{half}}$) of $I_h$ shifted from −97.4 ± 1.9 mV to −86.8 ± 1.6 mV, resulting in an increase in the current amplitude of $I_h$ by the pulse to between −80 and −90 mV. In the presence of an adenylate cyclase inhibitor, SQ-22536 (100 µM), the intracellular dialysis of ATP also produced a shift in the voltage-dependence of $I_h$ in rat DRG neurons, indicating that the effect of ATP was not caused by cAMP converted by adenylate cyclase. Intracellular dialysis of a non-hydrolysable ATP analog, AMP-PNP or ATP-γ-S, also produced a positive shift in the voltage-dependence of $I_h$ activation, suggesting that the effect of ATP results from its direct action on the channel protein. These results indicate that cytosolic ATP directly regulates the voltage dependence of $I_h$ activation as an intracellular modulating factor.
Introduction

A hyperpolarization-activated non-selective cation current ($I_h$) has been observed in various neurons of the central and peripheral nervous systems (Halliwell and Adams 1982; Lamas 1998; Tokimasa and Akasu 1990) and in cardiac tissues (where it was named $I_f$, Brown et al. 1979; DiFrancesco 1981ab, 1993). The channel underlying $I_h$ is activated by membrane hyperpolarization and slowly depolarizes the membrane potential. Therefore, this current has been proposed to control neuronal excitability and its functional role and modulating mechanism have been studied by many investigators (Pape 1996).

It has been shown that intracellular cAMP caused the depolarizing shift in the voltage dependence of $I_h$ (DiFrancesco and Tromba 1988; Ingram and Williams 1996; Pedarzani and Storm 1995; Raes et al. 1997). In recent years, five different genes encoding $I_h$ channels have been isolated from mouse brain (mHCN1-3, Ludwig et al. 1998), rabbit heart sinoatrial node (HAC4, Ishii et al. 1999) and a sea urchin testis (spHCN) cDNA library (Gauss et al. 1998). The HCN family contains a cyclic nucleotide binding domain (CNBD) in its cytoplasmic C-terminus that is highly homologous to that of cAMP- and cGMP-dependent protein kinases (Biel et al. 1999). The heterologously expressed HCN channels were shown to be regulated by the direct effect of cytosolic cAMP on its CNBD (Ludwig et al. 1998; Santoro et al. 1998). The regulation of $I_h$ or $I_f$ by the direct action of cAMP was also reported in guinea-pig primary afferent neurons (Ingram and Williams 1996), rat hippocampal pyramidal cells (Pedarzani and Storm 1995) and rabbit sino-atrial node cells (DiFrancesco and Tortora 1991). In contrast, cAMP was reported to indirectly control the $I_h$ through the phosphorylation by cAMP-dependent protein kinase (PKA) in bull-frog sympathetic neurons and canine cardiac Purkinje fibers (Chang et al. 1991; Tokimasa and Akasu 1990). The presence of $I_h$ and its modulation by cAMP have been described in the growth cone and soma of neonatal rat dorsal root ganglion (DRG) neurons (Wang et al. 1997). In addition, it has been reported that rat DRG neurons express high levels of HCN1 and HCN2 and relatively low levels of HCN3 and HCN4 (Chaplan et al. 2003). However, in rat DRG neurons, the...
modulating pathway for $I_h$ and the possibility that other intracellular modulating factors affect $I_h$ channel activity have not been studied yet. It was reported that ATP applied intracellularly in the presence of a saturating concentration of cAMP, which could fully activate PKA and $I_h$, acted as a substrate for the phosphorylation by PKA and caused a further positive shift of the $I_h$ activation curve in addition to a direct effect by cAMP in mouse DRG neurons. However, no paper has investigated whether cytosolic ATP itself can affect $I_h$ activation. In the present study, therefore, we investigated the effect of intracellular dialysis of cAMP and ATP on the voltage dependence of $I_h$ in rat DRG neurons to confirm whether intracellular ATP itself acts as a modulating factor for $I_h$.

**Materials & Methods**

**Cell culture**

Rat dorsal root ganglion (DRG) neurons were isolated from adult male Sprague-Dawley rats (7-12 week-old), using procedures that have been reported previously (Kim et al. 1980; Yong et al. 1988). Briefly, rats were killed by cervical dislocation and ganglia were dissected from the full length of the vertebral column. The ganglia were incubated at 37°C first in calcium-magnesium free phosphate-buffered saline (CMF-PBS) containing collagenase type IV (500 U/ml, Worthington Biochemicals) and DNAse I (0.12 µg/ml, Sigma Chemical) for 2h and then in CMF-PBS containing trypsin (0.25% w/v) for 15 min. After the trypsin digestion, cells were gently agitated with a silicon-coated Pasteur pipette and centrifuged to remove trypsin.

The isolated cells were suspended in Dulbecco's modified Eagle's Medium (DMEM, GibcoBRL) and cultured on coverslips coated with poly-D-lysine. The cells were kept at 37°C in a humidified atmosphere of 95% air and 5% CO₂ and cultured for a week until use in each experiment. The DMEM was supplemented with 10% fetal bovine serum (ICN Biochemicals), 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, and 10 µM cytosine arabinoside. The culture medium was changed every 2 days.
Whole-cell recording

Whole-cell recordings were made at room temperature. Heat-polished glass electrodes with 2-5 MΩ tip resistance were used. The cultured DRG neurons have neurites. We used the round shape neurons to minimize a space-clamp problem in the whole-cell recordings.

The bath solution contained (mM): 140 NaCl; 6 KCl; 1.2 MgCl₂; 2.5 CaCl₂; 10 D-glucose; 10 HEPES (pH = 7.2 adjusted with NaOH).

The pipette solution contained (mM): 140 K-methansulfonate (KMeSO₄); 2 MgCl₂; 1.1 EGTA; 10 HEPES (pH = 7.2 adjusted with KOH).

In a number of experiments, ATP, cAMP, ATP-γ-S and AMP-PNP were added to the pipette solution and BaCl₂, 4-aminopyridine (4-AP), tetraethylammonium (TEA), tetrodotoxin (TTX) and SQ-22536 (adenylate cyclase inhibitor, Sigma Chemical) were applied to the bath solution. The liquid junction potential (approximately 3.3 mV) between the pipette solution and the bath solution was not corrected. Neurons were continuously superfused with the bath solution at a flow rate of 1 ml/min throughout the experiments.

Whole-cell currents were measured with a patch-clamp amplifier (CEZ-2400, Nihon Koden) and sampled using an analog/digital converter (MacLab, AD Instruments). When $I_h$ was recorded, a sampling frequency of 0.4 kHz was used and data were stored in a personal computer (Macintosh, Apple). The built-in 4 pole bessel filter was used at the frequency higher than 0.2 kHz if needed. Activation curves were fitted with a Boltzman function using the Igor Pro program (Wave Metrics). Cell capacitance was determined by integrating the area under a capacity transient current elicited by a −10 mV voltage step from the holding potential and the amplitude of a current was normalized to the cell membrane capacitance. A series resistance (Rs) was also calculated from the capacity transient and the cell capacitance (9.9 ± 0.6 MΩ, n = 121). A potential error caused by Rs was corrected for each recording when the activation curves of $I_h$ were determined.

Data are presented as means ± standard errors of the mean (SEM). The significance of differences between the means of two experiments was assessed with Student's $t$-test. The level of significance chosen was 0.05.
Drugs

The following drugs were dissolved in the pipette solution to the desired concentration: adenosine-5′-triphosphate (ATP, Sigma Chemical), adenocine-3′-5′-cyclicmonophosphate (cAMP, Sigma Chemical), 5′-adenylimidodiphosphate (AMP-PNP, Sigma Chemical), adenosine-5′-(3′-thiotriphosphate) (ATP-γ-S, Sigma Chemical). Forskolin (Wako Pure Chemicals) was dissolved in a dimethyl sulfoxide stock solution (10 mM). SQ-22536 was dissolved in a distilled water stock solution (100 mM). The stock solutions were stored at –20°C and thawed immediately prior to use and diluted with the external solutions to desired final concentrations.

Result

Properties of hyperpolarization-activated cation current ($I_h$) in rat DRG neurons

Rat dorsal root ganglion (DRG) neurons with diameters ranging from 20 to 50 µm (average 28.3 ± 1.0 µm, n = 53) were used for the experiments. The mean cell capacitance was 110.2 ± 5.7 pF (n = 110). Under conventional whole-cell voltage-clamp conditions, rat DRG neurons were held at –60 mV and the potentials raised step-wise to between –70 and –130 mV (Figure 1A). Hyperpolarizing voltage pulses elicited slowly activating inward currents without inactivation ($I_h$).

Figure 1B shows the current voltage relationships of $I_h$. The open circles indicate the amplitudes of the initial currents measured at the beginning of hyperpolarizing pulses (instantaneous current). The closed circles indicate the steady state I-V relationship measured at the end of 2 s pulses (steady
state current). The net amplitudes of $I_h$ activated by hyperpolarizing voltage pulses were determined by measuring the difference in the instantaneous and steady state currents achieved at the beginning and the end of the pulse, respectively. This current began to be activated at $-70$ mV and almost fully activated at $-130$ mV. The reversal potential was evaluated by determining the intersection of the instantaneous I-V relationships stepping up from $-60$ mV and from $-130$ mV (Mayer and Westbrook 1983). The reversal potential was $-20.3 \pm 5.0$ mV ($n = 10$). This value was consistent with the report that $I_h$ channel in various neuronal cells passes both $Na^+$ and $K^+$ (Pape 1996). The permeability ratio of $I_h$ channel in rat DRG neurons for $Na^+$ and $K^+$ ($P_{Na}/P_K$) determined with the Goldman constant-field equation was 0.40.

$I_h$ and the current through HCN clone channels have been reported to be almost abolished by extracellular Cs$^+$ and only partially inhibited by extracellular Ba$^{2+}$ (Ludwig et al. 1998; Pape 1996). In mHCN2 channels expressed in human embryonic kidney (HEK) 293 cells, the effects of TEA and 4-aminopyridine (4-AP) on $I_h$ were also examined (Ludwig et al. 1998). The pharmacological profile of $I_h$ in cultured rat DRG neurons was investigated to compare with that of $I_h$ in other tissues. The magnitude of the inhibition was estimated by measuring the amplitudes of $I_h$ in the presence and absence of the blocking agents. Extracellular applications of 2 mM Cs$^+$, 2 mM Ba$^{2+}$ and 20 mM TEA reduced the amplitude of $I_h$ by $79.2 \pm 6.4\%$ ($n = 6$), $34.5 \pm 7.1\%$ ($n = 8$) and $19.2 \pm 8.6\%$ ($n = 7$), respectively (Figure 1C). However, this current was insensitive to 1 mM 4-AP.

**Effect of cyclic AMP and ATP on the voltage dependence of $I_h$**

It is well known that intracellular cAMP produces a positive shift in the activation curve of $I_h$ in DRG neurons and other neuronal cells (Cardenas et al. 1999; Raes et al. 1997; Pape 1996). Furthermore, it was reported that cAMP induced a positive shift in HEK293 cells with mHCN2 channels (Ludwig et al. 1998).

The effects of intracellular dialysis of nucleotides on $I_h$ in rat DRG neurons
were examined. \(\text{BaCl}_2\) (0.5 mM), 4-AP (2 mM), TEA (3 mM) and TTX (0.5 µM) were added to the extracellular solution to minimize contamination from voltage-gated \(\text{Na}^+\) and \(\text{K}^+\) currents. We used cAMP at a concentration of 1 mM, which produced the maximal effect in heterologously expressed HCN channels (Ludwig et al. 1998; Santoro et al. 1998; Ishii et al. 1999).

Voltage pulses to various potentials followed by the pulse to \(-130\) mV, where \(I_h\) appeared to be fully activated, were applied to cells. To examine the voltage dependency of the \(I_h\) activation more closely, hyperpolarizing voltage steps were applied to rat DRG neurons from a holding potential of \(-30\) mV. This voltage protocol elicited \(I_h\) followed by tail currents (\(I_{\text{tail}}\)) as shown in Figure 2A. The peak amplitude of the tail current from \(-30\) mV was defined as \(I_{\text{tail max}}\) (Figure 2A bottom panel). The degree of the activation of \(I_h\) at the various voltages was determined from normalizing \(\Delta I_{\text{tail}}\) with \(I_{\text{tail max}}\). \(\Delta I_{\text{tail}}\) was defined as the difference between \(I_{\text{tail}}\) from various voltage steps and \(I_{\text{tail max}}\) (Figure 2A bottom panel). The data points were fitted with the Boltzmann function: \(\Delta I_{\text{tail}}/I_{\text{tail max}} = (A_{\text{max}} - A_{\text{min}})/\{1 + \exp [(Vm - V_{\text{half}})/k]\} + A_{\text{min}}\), where \(A_{\text{max}}\) is the maximal activation value, \(A_{\text{min}}\) is the minimal activation value, \(Vm\) is the test potential, \(V_{\text{half}}\) is the membrane potential for half-maximal activation and \(k\) is the slope factor. Under this condition, \(V_{\text{half}}\) and \(k\) with the control pipette solution were \(-97.4 \pm 1.9\) mV (\(n = 22\)) and \(7.4 \pm 0.5\) mV (\(n = 22\)). With the pipette solution containing 1 mM cAMP, the amplitude of \(I_h\) observed at between \(-80\) and \(-90\) mV increased (Figure 2B). \(V_{\text{half}}\) obtained from cAMP-dialyzed cells was \(-82.3 \pm 2.6\) mV (\(n = 11\), Figure 2A). This value of \(V_{\text{half}}\) was significantly different from that obtained with the control pipette solution (\(p < 0.001\)). The mean current density measured at \(-130\) mV was \(-2.3 \pm 0.4\) pA/pF (\(n = 21\)) under the control condition and \(-3.1 \pm 0.6\) pA/pF (\(n = 11\)) in the presence of cAMP. The peak amplitude of \(I_h\) was not influenced by cAMP (\(p = 0.68\)).

In mouse DRG neurons, it was reported that intracellular perfusion with solution containing ATP (5 mM) also induced a depolarizing shift of \(V_{\text{half}}\) of \(I_h\) (Raes et al. 1997). In rat DRG neurons, when 1 mM ATP was added to the pipette solution, \(V_{\text{half}}\) was \(-97.6 \pm 2.3\) mV (\(n = 10\)), which was not significantly different from that obtained in the absence of ATP (Figure 2C). When the concentration of ATP was raised to 2 mM, \(V_{\text{half}}\) was a more positive value \((-86.8 \pm 1.6\) mV, \(n = 19\), \(p < 0.001\)) than that measured with
the pipette solution containing 1 mM ATP or no nucleotides (Figure 2C). As shown in Figure 2B, in neurons dialyzed with 2 mM ATP, the current amplitude elicited by voltage steps to –90 mV increased. The amplitudes of maximum current density (–3.0 ± 0.4 pA/pF, n = 19) did not significantly change (p = 0.89). In rat DRG neurons, 2 mM ATP added to the intracellular solution produced a depolarizing shift of $V_{\text{half}}$ of $I_h$, almost equal to that produced by 1 mM cAMP. The $V_{\text{half}}$ of $I_h$ using the pipette solution containing both ATP (2 mM) and cAMP (1 mM) together was –86.0 ± 2.0 mV (n = 6), a value which was not significantly different from that obtained in the presence of ATP (2 mM, $p = 0.79$) or cAMP (1 mM, $p = 0.36$) alone. In addition in the presence of 5 mM ATP in the pipette solution, the $V_{\text{half}}$ (–88.2 ± 1.5 mV, n = 4) was not significantly different from that obtained in the presence 2 mM ATP ($p = 0.69$). Although the membrane permeable analog of cAMP (db-cAMP, 8-bromo cAMP, 8-cpt cAMP) was also tested at the concentration range of 1-5 mM, no analog added to the bath solutions had an effect on $I_h$ in the rat DRG neurons.

**Effect of adenylate cyclase inhibitor on the ATP-induced positive shift in the voltage dependency of $I_h$**

The effect of intracellular cAMP on the voltage dependency of $I_h$ was explained to be caused by its direct action on the channel protein in some neuronal cells (Ingram and Williams 1996; Pedarzani and Storm 1995) and Xenopus oocytes and HEK 293 cells expressing the mHCN1 and mHCN2 channel respectively (Ishii et al. 1999; Ludwig et al. 1998). In order to investigate the modulating pathway of ATP in rat DRG neurons, we examined whether the activation of adenylate cyclase contributes to the ATP-induced positive shift of $V_{\text{half}}$ of $I_h$ in rat DRG neurons. An adenylate cyclase activator, forskolin (10 µM), significantly increased the amplitude of $I_h$ induced by hyperpolarizing voltage steps between -70 and -90 mV by 29.1 ± 9.0% (n = 6), suggesting that $I_h$ in rat DRG neurons could be regulated through the modulating pathway including adenylate cyclase. However this effect of forskolin was not observed in the presence of a membrane permeable adenylate cyclase inhibitor, SQ-22536 at 100 µM (Madison and Nicoll 1986; Goldsmith and Abrams 1992; Grupp et al. 1980). This result
indicated that SQ-22536 certainly inhibited the adenylate cyclase activity in rat DRG neurons under our experimental conditions. The effect of SQ-22536 on \(I_h\) activation was tested with the pipette solution containing 2 mM ATP. After the activation curve was determined using the normal external solution, SQ-22536 (100 µM) was applied to the extracellular solution. \(V_{half}\) was \(-90.4 \pm 1.7\) mV (n = 8) and \(-91.5 \pm 1.2\) mV (n = 8) before and after application of SQ-22536 (100 µM), respectively (Figure 3). These values were not significantly different from each other \((p = 0.58)\). The effect of ATP on the \(I_h\) activation was not influenced by the adenylate cyclase inhibitor. Another adenylate cyclase inhibitor, MDL 12,330A was also tested. MDL 12,330A almost abolished the \(I_h\) amplitude at \(-130\) mV with the pipette solution containing 2 mM ATP. However, a similar effect was observed regardless of the presence of ATP or cAMP. Therefore this result was probably caused not by an inhibitory effect on adenylate cyclase but by an unknown effect of this compound.

**Effects of non-hydrolysable ATP analogs on the voltage dependency of \(I_h\)**

In several neuronal tissues, it was reported that the phosphorylation process was involved in the modulation pathway of \(I_h\) by intracellular cAMP (Chang et al. 1991; Tokimasa and Akasu 1990). If cytosolic ATP shifted the \(V_{half}\) of \(I_h\) in rat DRG neurons without being converted to cAMP by adenylate cyclase and phosphorylation by PKA, a non-hydrolysable ATP analog would be expected to produce a similar effect on \(I_h\) activation. To test this hypothesis, we observed \(I_h\) with a pipette solution containing 2 mM AMP-PNP (Leventhal and Bertics 1991; Tokimasa 1995) or ATP-\(\gamma\)-S (Goody et al. 1972; Chrysogelos et al. 1983) and compared its voltage dependency in the presence and absence of these analogs. LiCl (6.4 mM) was added to the control pipette solution when the effect of AMP-PNP was tested because AMP-PNP was supplied in the form of a lithium salt (AMP-PNP: Li\(^+\)=1:3.2). The current density and the voltage dependency of \(I_h\) in rat DRG neurons were not influenced by LiCl. When DRG neurons were dialyzed with AMP-PNP (2 mM), the amplitude of the slow activating current at \(-90\) mV was greater than that with the control pipette solution (Figure 4A). The peak
current density was \(-2.1 \pm 0.5\) (n = 5) and \(-2.6 \pm 0.5\) pA/pF (n = 5) in the absence and presence of AMP-PNP (2 mM), respectively, and they were not significantly different (p = 0.45). \(V_{\text{half}}\) was \(-99.5 \pm 0.5\) mV (n = 10) with the control pipette solution, whereas the \(V_{\text{half}}\) in AMP-PNP-dialyzed cells was shifted to a more positive potential \((-89.6 \pm 1.0, n = 5, p < 0.01)\) (Figure 4B). Another non-hydrolysable ATP analog, ATP-\(\gamma\)-S (2 mM), also mimicked the effect of ATP. In ATP-\(\gamma\)-S-dialyzed cells, the \(V_{\text{half}}\) of \(I_h\) shifted toward positive potential \((-91.3 \pm 3.9\) mV, n = 5, \(p < 0.01\)) without changing the fully activated current density \((-3.4 \pm 1.7\) pA/pF in control vs \(-3.5 \pm 0.6\) pA/pF in the presence of ATP-\(\gamma\)-S, \(p = 0.98\)).

**Discussion**

Intracellular cAMP induced a positive shift in the voltage dependence of \(I_h\) activation by a direct effect on the channel protein in the study with heterologously expressed HCN channels (Ludwig et al. 1998; Santoro et al. 1998). In addition, in many types of neuronal cells, it has been shown that cAMP causes a positive shift in the voltage dependence of \(I_h\) (McCormick and Pape 1990; Pape 1996; Raes 1997) and its effect was attributed to the direct action of cAMP against the channel protein (Pedarzani and Storm 1995). The direct effect of cAMP on \(I_f\) in cardiac tissue was also demonstrated in inside-out patches (DiFrancesco and Tortora 1991). On the other hand, it was reported that similar effects of cAMP on \(I_h\) in bull-frog sympathetic neurons (Tokimasa and Akasu 1990) and canine Purkinje fibers were explained by the phosphorylation through PKA activity (Chang et al. 1991). In rat DRG neurons, serotonin (5-HT) shifted the activation curve of \(I_h\) toward the positive potential by increasing the cAMP concentration and this effect was mimicked by forskolin (Cardenas et al. 1999).

In this study, we found that cytosolic ATP regulates the voltage dependency of \(I_h\) in rat dorsal root ganglion neurons. The application of ATP (2 mM) to the pipette solution caused the positive shift of the activation curve of \(I_h\).

The electrophysiological properties of \(I_h\) in rat DRG neurons almost agree with those of \(I_h\) in other neuronal cells and HCN channels (Pape 1996; Biel et
On the other hand, the pharmacological properties of $I_h$ in rat DRG neurons differ from those previously reported. The magnitude of the inhibition by extracellular $\text{Ba}^{2+}$ was rather greater than that reported in acutely isolated rat DRG neurons (Scroggs et al. 1994) and mHCN2 channels (Ludwig et al. 1998). It was shown that in mouse DRG neurons, TEA (25 mM) applied extracellularly failed to block $I_h$ (Mayer ML and Westbrook 1983). However, in cultured rat DRG neurons, $I_h$ was slightly but not significantly reduced by TEA. In this study, although intracellular application of cAMP shifted the $I_h$ activation curve toward a more depolarized voltage as compared with that in the absence of cAMP in rat DRG neurons, membrane permeable analogs of cAMP (db-cAMP, 8-bromo cAMP and 8-CPT cAMP) applied extracellularly were ineffective against $I_h$ activation in contrast with the report in neonatal rat DRG neurons (Wang et al. 1997) and in guinea-pig primary afferent neurons (Ingram and Williams 1996). The reason for this is unclear at present. In mouse DRG neurons, Raes et al. reported that 5 mM ATP added to a pipette solution containing 100 µM cAMP induced a 7 mV additional shift of $V_{\text{half}}$ (1997). This ‘additional’ shift was explained to be caused not by the direct action of ATP on the channel but by the action of PKA activated by cAMP. In rat DRG neurons, we could not demonstrate an additional positive shift of $V_{\text{half}}$ with the pipette solution containing 1 mM cAMP and 2 mM ATP. We do not have an explanation for the difference between our result and that in mouse DRG neurons. It might be related to the difference in the nucleotides concentrations.

In rat DRG neurons and other neuronal cells, an adenylate cyclase activator, forskolin, activates $I_h$, by shifting its activation curve to a more positive voltage (Cardenas et al. 1999; Ingram and Williams 1996; Tokimasa and Akasu 1990). In this study, extracellularly applied forskolin (10 µM) significantly increased the $I_h$ amplitude at –90 mV by about 30%. Therefore adenylate cyclase-dependent steps seem to be included in the modulation cascade of $I_h$ in rat DRG neurons. If the effect of ATP observed in rat DRG neurons resulted from the phosphorylation by PKA, cAMP would be produced from ATP by adenylate cyclase (Richards et al. 1981). However, this is not consistent with this study. The effect of ATP on $I_h$ in rat DRG neurons was not influenced by the adenylate cyclase inhibitor, SQ-22536, indicating that adenylate cyclase activity does not contribute to the
ATP-induced shift in the activation curve of $I_h$. Application of a non-hydrolysable ATP analog, ATP-$\gamma$-S (2 mM), to the pipette solution also shifted the $V_{half}$ in the positive direction, suggesting that ATP modulates the voltage dependency of $I_h$ in rat DRG neurons without conversion to cAMP. However, it is well known that ATP-$\gamma$-S can act as a substrate for some kinds of protein kinases and cause an irreversible protein thiophosphorylation (Eckstein 1985; Liou et al. 1999). Therefore, the possibility that the change in the phosphorylation balance caused by ATP-$\gamma$-S leads to a modulation of $I_h$ could not be eliminated. Moreover, the effect of ATP was also mimicked by another non-hydrolysable ATP analog, AMP-PNP. Thus, ATP, AMP-PNP and ATP-$\gamma$-S may act directly on the channel protein. If the direct action of ATP against the channel protein facilitated $I_h$ in rat DRG neurons, the free ATP concentration in the intracellular solution is important. Then, we calculated free ATP concentration with Maxchelator software (Stanford University, USA), which was discussed in detail by Bers et al. (1994). Parameters of temperature, pH and ionic strength, we used in this software, were 25°C, 7.2 and 0.16 N, respectively. The free ATP concentrations in the pipette solutions containing totally 1 mM and 2 mM ATP were 91.3 µM and 417 µM, respectively. The free ATP concentration is about 4 times higher in the pipette solution containing 2 mM ATP than that containing 1 mM ATP. This may cause the positive shift in the voltage dependence of $I_h$ when the concentration of ATP added to the pipette solution is increased. In adult guinea pig and chick embryo dorsal root ganglion neurons, the total intracellular ATP concentration was calculated as 1.7 mM and 1.5-2 mM, respectively (Fukuda et al. 1983; Schousboe et al. 1970). In rat DRG neurons, the total ATP concentration is presumably similar to that in guinea pig or chick DRG neurons. Because ATP at the concentration beyond 2 mM modulated the activation of $I_h$ in this study, it is highly probable that ATP contribute to regulating the voltage dependency of $I_h$ in intact DRG neurons. In many neurons, it has been reported that $I_h$ contributes to setting the resting membrane potential (Pape 1996). In rat DRG neurons, it was shown that the application of CsCl (2 mM), which could completely abolish $I_h$, hyperpolarized the membrane potential reversibly by approximately 5 mV, suggesting that $I_h$ played an indispensable role in setting membrane potential (Wang et al. 1997). Thus, it is possible that change in the voltage dependency of $I_h$ by ATP contributes to regulating the membrane potential. However, it is
still unknown what extracellular signals can regulate $I_h$ activation via change in the intracellular ATP concentration. For example, under the condition in which supply of ATP is stopped, such as hypoxia, the neuronal excitability might be influenced by the inhibition of $I_h$. Further experiments are necessary to clarify whether the regulation of $I_h$ by cytosolic ATP has physiological or pathophysiological role in the neuronal excitability.

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Figure Legends

**Figure 1** Properties of hyperpolarization-activated currents ($I_h$) in rat dorsal root ganglion (DRG) neurons.

Typical current responses (upper traces) to hyperpolarizing voltage steps (lower panel) in rat DRG neurons.

The neurons were hyperpolarized by the voltage steps to various potentials (–70 to –130 mV) from the holding potential at –60 mV.

**B** Current-voltage relationship of $I_h$.

Current density at the beginning (open circles, instantaneous current, n = 10) and the end (filled circles, steady state current, n = 10) were plotted against the membrane potentials. Symbols and vertical bars indicate mean values and SEM.

**C** Pharmacological property of $I_h$ in rat DRG neurons.

CsCl (2 mM), BaCl$_2$ (2 mM), TEA (20 mM) and 4-aminopyridine (4-AP, 1 mM,) were applied extracellularly to voltage clamped neurons. The left panel shows the typical records of $I_h$ evoked by hyperpolarizing pulse to –130 mV from holding potential of –60 mV before (open circles), during (filled circles) and after (open triangles) application of Cs$^+$, Ba$^{2+}$, 4-AP and TEA. In the right plot, columns and vertical bars indicate the mean and SEM of $I_h$ inhibition rates at –130 mV by Cs$^+$ (n = 6), Ba$^{2+}$ (n = 8), 4-AP (n = 7) and TEA (n = 6).

**Figure 2** Effects of cyclic nucleotides on the voltage dependence of $I_h$ in rat DRG neurons.

*Upper panel:* A two-step pulse protocol was used to determine the voltage dependency of $I_h$ in rat DRG neurons. Hyperpolarizing voltage steps to various voltages for 2 s, followed by voltage steps to –130 mV were applied from a holding potential of –30 mV. *Lower panel:* The typical tail current traces of $I_h$ elicited at –130 mV following hyperpolarizing pre-pulse (–30 mV and –90 mV). $I_{\text{tailmax}}$ is defined as the maximal amplitude of tail current evoked after the pre-pulse at –30 mV. $\Delta I_{\text{tail}}$ represent the difference between the tail current from various voltage
steps and $I_{\text{tailmax}}$.

B Typical records of $I_h$ evoked by the hyperpolarizing pulse to $-90$ mV followed by the pulse to $-130$ mV from the holding potential at $-30$ mV in DRG neurons dialyzed with pipette solutions containing no nucleotide (control), 1 mM cAMP, 1 mM ATP and 2 mM ATP. The bath solution contained BaCl$_2$ (0.5 mM), 4-AP (2 mM), TEA (3 mM) and TTX (0.5 µM).

C Voltage dependence of $I_h$ activation measured in the absence of any nucleotides (open circles, n = 22) and the presence of 1 mM cAMP (filled circles, n = 11), 1 mM ATP (filled triangle, n = 11), or 2 mM ATP (filled squares, n = 19). The degree of activation at each potential was measured by comparing current amplitude at the beginning and the end of the voltage step to $-130$ mV. Symbols and vertical bars indicate the mean values ± SEM of activation rates at each potential.

**Figure 3** Effect of adenylate cyclase inhibitor on the ATP-induced activation of $I_h$ in rat DRG neurons.

The activation curves of $I_h$ before (open circles, n = 8) and after (filled circles, n = 8) application of an adenylate cyclase inhibitor, SQ-22536 (100 µM) with the pipette solution containing 2 mM ATP. The bath solution contained BaCl$_2$ (0.5 mM), 4-AP (2 mM), TEA (3 mM) and TTX (0.5 µM). Symbols and vertical bars indicate the mean values ± SEM of activation rates at each potential.

**Figure 4** Effect of non-hydrolysable ATP analogs on the voltage dependency of $I_h$

A Typical records of $I_h$ evoked by the hyperpolarizing pulse to -90 mV followed by the pulse to $-130$ mV from the holding potential at $-30$ mV in DRG neurons dialyzed with a normal pipette solution containing no nucleotides (control) and solution with 2 mM ATP-γ-S or 2 mM AMP-PNP. The bath solution contained BaCl$_2$ (0.5 mM), 4-AP (2 mM), TEA (3 mM) and TTX (0.5 µM).

B Voltage dependence of $I_h$ activation in the absence of any nucleotides (open circles, n = 10) and the presence of 2 mM AMP-PNP (filled circles, n = 5) and 2 mM ATP-γ-S (open squares, n = 5). Symbols and vertical bars indicate the mean values ± SEM of activation rates at each potential.
FIGURE 1

A

instantaneous current
-60 mV
steady state current
-130 mV

100 pA
1 s

B

membrane potential (mV)
-140 -120 -100 -80 -60

0

current density (fA/PF)

C

Cs⁺

Ba²⁺

TEA

4-AP

50 pA
500 ms

50 pA
500 ms

100 pA
500 ms

inhibition (%)

0  20  40  60  80

Cs⁺  Ba²⁺  4-AP  TEA
FIGURE 3

![Graph showing the relationship between membrane potential (mV) and activation (ΔVmax/ΔVmax)](image-url)
FIGURE 4

A

control

ATP-γ-S

AMP-PNP

-30 mV

-90 mV

-130 mV

-90 mV

-130 mV

-90 mV

-130 mV

100 pA

200 pA

2 s

2 s

2 s

B

activation (ΔI/ΔI_{max})

membrane potential (mV)

-160

-140

-120

-100

-80

-60

-40

-20

0

1.0

0.8

0.6

0.4

0.2

0.0