

# Regulation of the mechanosensitive cation channels by ATP and cAMP in leech neurons

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## Abstract

Single-channel recordings were used to study the modulation of stretch-activated channels (SACs) by intracellular adenosine nucleotides in identified leech neurons. These channels exhibited two activity modes, spike-like (SL) and multiconductance (MC), displaying different polymodal activation. In the absence of mechanical stimulation, internal perfusion of excised patches with ATP induced robust and reversible activation of the MC but not of the SL mode. The ATP effect on channel activity was dose-dependent within a range of 1  $\mu$ M–1 mM and was induced at different values of intracellular pH and  $\text{Ca}^{2+}$ . The non-hydrolyzable ATP analog AMP-PNP, ATP without  $\text{Mg}^{2+}$  or ADP also effectively enhanced MC activity. Adenosine mimicked the effect of its nucleotides. At negative membrane potentials, both ATP and adenosine activated the channel. Moreover, ATP but not adenosine induced a flickering block. Addition of cAMP during maximal ATP activation completely and reversibly inhibited the channel, with activation and deactivation times of minutes. However, cAMP alone only induced a weak and rapid channel activation, without inhibitory effects. The expression of these channels in the growth cones of leech neurons, their permeability to  $\text{Ca}^{2+}$  and their sensitivity to intracellular cAMP are consistent with a role in the  $\text{Ca}^{2+}$  oscillations associated with cell growth.

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## 1. Introduction

Large conductance stretch-sensitive ion channels have been identified in the leech [1]. Their pharmacological properties are similar to those of typical mechanogated channels of vertebrate hair cells [2]. They are completely and reversibly blocked by extracellular  $\text{Gd}^{3+}$ , amiloride induces a flickering voltage-dependent block and gentamicin produces a complete voltage-dependent block. Both amiloride and gentamicin block the channel at negative membrane potentials [3]. These channels are expressed by cell bodies and growth cones of mechanosensory neurons, as well as by other neurons not involved in sensory mechano-transduction. Channel activation was induced by perfusion with hypotonic solutions in the cell-attached configuration [1] or by applying rapid pulses of negative pressure to the recording pipette in the inside-out configuration [4]. Analysis of channel ion

selectivity has shown that these stretch-sensitive ion channels admit cations and exhibit a substantial calcium permeability.

The leech mechanosensitive channels exhibited two activity modes differing in kinetics and single-channel subconductances. The first, called spike-like mode (SL), was mainly displayed in membrane patches excised from freshly desheathed quiescent cell bodies, while the second, called multiconductance mode (MC), was commonly found in cultured cell bodies and in growth cones [5], where the channels also displayed high mechanosusceptibility [6].

Although the molecular nature of these ion channels has not been determined, their functional properties indicate that they belong to the family of transient receptor potential (TRP) channels. The polymodal activation of leech mechanosensitive channels has been recently described [4]. Different stimuli such as membrane stretch, depolarization, intracellular acidification and elevations of intracellular calcium differently affect the two activity modes.

Since basic properties, such as mechanosensitivity, weak voltage dependence, main conductance value, outward rectification and pharmacology, were shared by the two activity modes, these

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probably belong to the same channel inserted in different molecular membrane complexes or to two subtypes of TRP channels.

As regards the physiological role of these channels, the activation of the SL mode by micromolar concentrations of intracellular calcium make it capable of responding to elevations in the free intracellular calcium of the order of magnitude of that induced by release from the internal stores. We have recently found that the intracellular calcium of leech neurons was induced to increase by hypotonic cell swelling. While the major component of this response did not require extracellular calcium, a component of the calcium response was sensitive to blockers of stretch-sensitive cation channels. Accordingly, it has been suggested that SL channels have a role in the amplification of mechanically-induced calcium signals in neurons [4].

The MC channels are expressed in high density in the growth cone membrane of leech neurons grown in culture [5]. On the other hand, addition to the culture medium of gentamicin, a non-specific blocker of these cation channels, which does not affect voltage-dependent currents in the leech neurons, increased the neurite extension in culture [3]. Thus, a role for these channels in cell growth has been proposed. In this study, a further characterization of leech mechanosensitive channels was performed, especially of the effects of intracellular adenosine nucleotides.

## 2. Materials and methods

### 2.1. Preparation

The experiments were performed on adult specimens of *Hirudo medicinalis* L., obtained from a commercial supplier (Ricarimpex, Eysines France). Segmental ganglia were removed from the leech central nervous system in standard leech saline (composition in mM: NaCl 115, KCl 4, CaCl<sub>2</sub> 1.8, Tris-maleate buffer 10, glucose 10, pH 7.4). Identified Anterior Pagoda (AP) motor neurons and mechanosensory neurons were selected in this study. The capsule enveloping the ganglion was opened with microscissors to expose single cell bodies for patching.

### 2.2. Electrophysiology

The patch-clamp technique [7] was used in the inside-out configuration. Single channel currents were recorded from naked cell bodies in desheathed ganglia. Patch electrodes, pulled in two stages from 1.5-mm o.d. glass capillary tubes 7087 (Blaubrand, Wertheim, Germany), had resistances of 5–7 MΩ, a “bubble number” [8] of about 4 and were coated with Sigmacote (Sigma, Milan, Italy). ‘Gentle’ sealing protocol [9] was routinely carried out. In most recordings we obtained a tight seal by simply releasing a positive pressure of about 10 mm Hg, without applying negative pressure. Currents were recorded with a patch-clamp amplifier (RK 300 Biologic, Claix, France or Axopatch 1D, Axon Instruments, Foster City, CA, USA), by low pass filtering at 1 kHz with a 4-pole Chebyshev filter. Data were displayed on an oscilloscope and stored on the hard disk of a PC for off-line analysis as Axoscope (Axon Instruments, Foster City, CA, USA) files, after AD conversion at 5 kHz with a Digidata 1200 interface (Axon Instruments, Foster City, CA, USA). Analysis was carried out using both pClamp software (Axon Instruments, Foster City, CA, USA) and software developed for the purpose in Visual Basic. For channel identification, stretch-activation was induced by applying rapid negative pressure stimuli to the membrane patches, using a two-electrovalve system, similar to that described by Hurwitz and Segal [10].

### 2.3. Solutions

In most recordings, symmetrical solutions of pipette and bath contained (in mM): 155 NaCl, 1 MgCl<sub>2</sub>, 10 HEPES (N-(2-hydroxyethyl) piperazine-N'-2 ethansulphonic acid) NaOH, 5 KCl, 1 CaCl<sub>2</sub>, pH 7.2, glucose 10. Cell-free patches were tested with solutions of various composition applied by a rapid solution changer. Solutions containing a calcium concentration of 0.5 μM were obtained using 2 mM EGTA (ethyleneglycol-bis[β-aminoethylether]N,N-tetraacetic acid) and following instructions of the MaxC software by C. Patton (Stanford University). ATP, AMP-PNP (adenosine 5'-(β,γ-imido)-triphosphate), ADP, cyclic AMP and adenosine were purchased from Sigma.

### 2.4. Data processing and statistical analysis

Transitions between open and closed states were detected using a half-amplitude threshold criterion and a minimum event width of 0.2 ms. The mean patch current was computed by dividing the total ionic charge transported by the duration of analyzed data. The mean frequency of events was obtained by

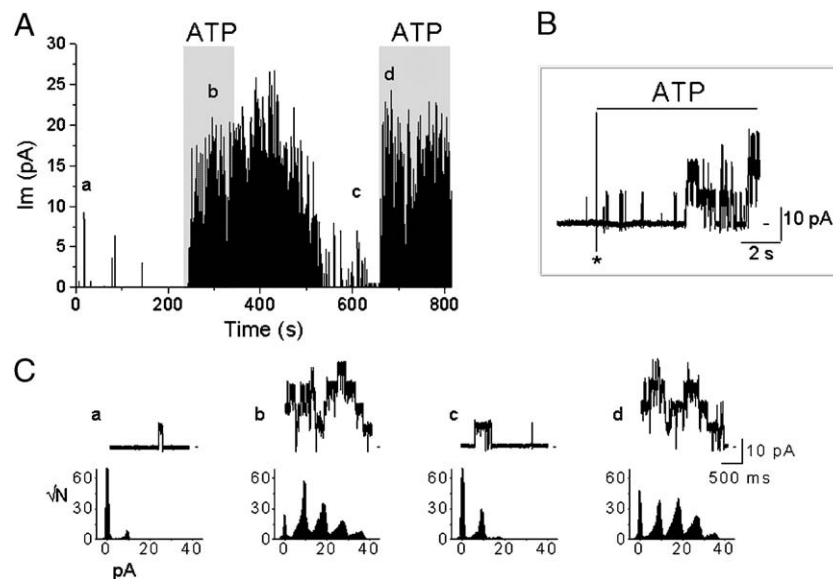


Fig. 1. Reversible activation of MC channel by ATP. The cytoplasmic side of a membrane patch containing four channels was perfused twice by a solution containing 1 mM MgATP. The membrane potential was held at +80 mV. Columns in the plot A represent the mean patch current calculated from 1-s-long consecutive data segments. Trace B illustrates the transition between c and d, on a rapid time scale, to estimate the activation delay (the asterisk marks the opening of the electrovalve in the solution changer). The current traces in C are samples of activity at the times marked a, b, c and d in plot A. The baseline current with all channels closed is indicated by the tick on the right of each record. The corresponding all-points histograms are displayed below each trace.

calculating the ratio between the number of opening transitions and the total record time. The number of channels in each membrane patch was determined by the maximum number of channels open simultaneously, observed in data segments longer than 2 min. Only recordings with a single channel were used to study channel kinetics. Mean channel open and closed times were determined by the arithmetic means of dwell time data.

Mathematical transformations and statistical analysis were performed using Origin (Microcal) and Sigma Stat (Jandel Scientific). Results are expressed as means  $\pm$  standard error of means (S.E.M.) and compared by paired *t*-test, taking as significant *P*-values of less than 5%.

### 3. Results

As previously reported, membrane patches just excised from leech neurons in symmetrical  $\text{Na}^+$  solutions and held at a depolarizing membrane potential displayed occasional brief openings. In the absence of mechanical stimulation, a progres-

sive activation spontaneously developed within several minutes, reaching values of open probability that varied from patch to patch. Whatever activity was maintained at a depolarizing membrane potential, it robustly increased by perfusion of the internal side of the patch with MgATP (16/18 patches studied). The plot in Fig. 1A illustrates the effects of two consecutive stimulations on the mean current of a membrane patch containing four channels in MC mode. The activation delay consistently lasted a few seconds, as shown in Fig. 1B, whereas deactivation following the withdrawal of MgATP from the perfusing solution was typically longer. The upmodulation of the channel activity by MgATP was absent in SL mode (nine out of nine cases studied).

MC channels upregulated by ATP maintain their voltage-dependence as well as their mechano-sensitivity, as shown in Fig. 2. In an inside-out membrane patch containing two MC

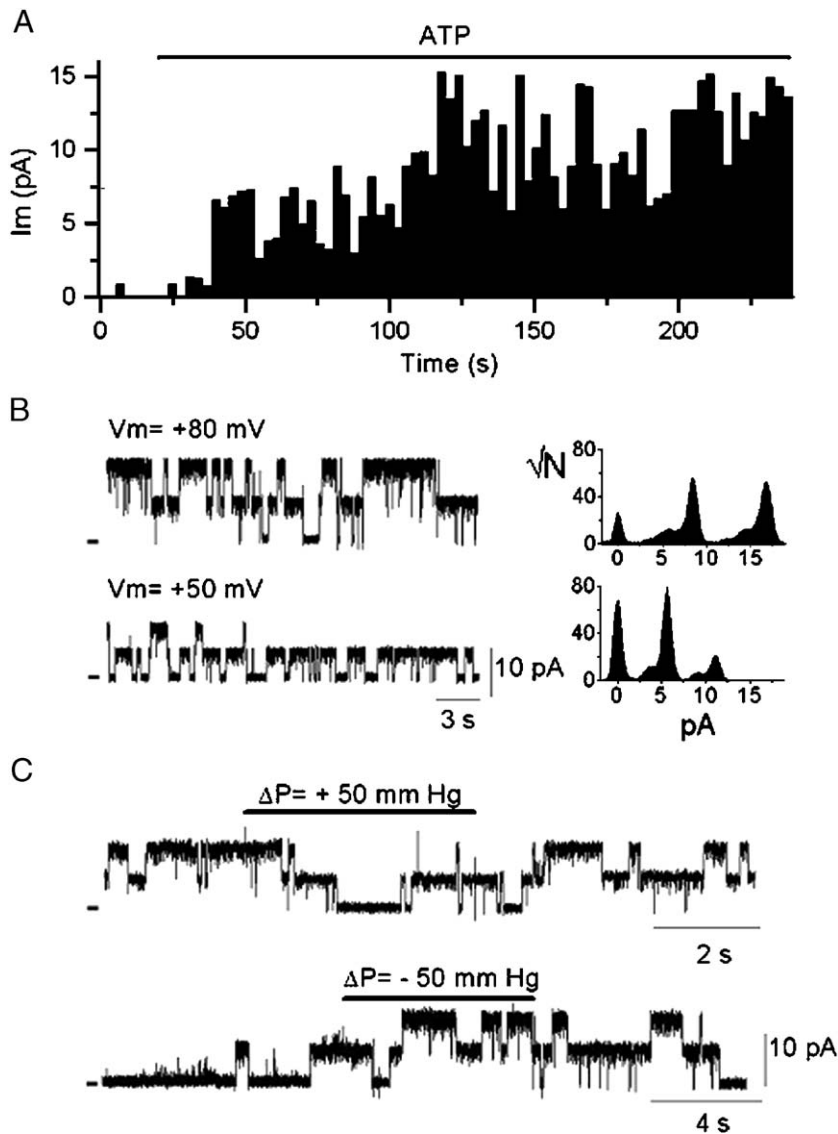


Fig. 2. Voltage-dependence and mechano-sensitivity of MC channels upregulated by ATP. (A) Plot representing the mean patch current calculated from 3-s-long consecutive segments at the beginning of perfusion with a solution containing 1 mM MgATP. (B) Samples of channel activity and corresponding all points histograms at two holding membrane potentials, in the presence of MgATP. (C) Responses to the application of fast positive (upper trace) and negative (lower trace) pressure pulses, in the presence of MgATP. The baseline current with both channels closed is indicated by the tick on the left of each record. Filtering 1 kHz.

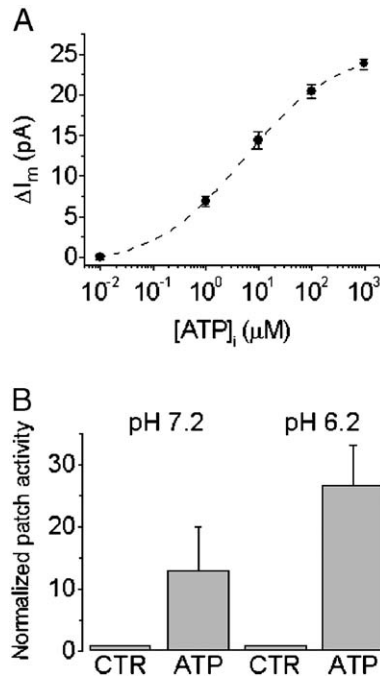


Fig. 3. Features of channel activation by ATP. (A) Dose–response curve obtained from the same membrane patch containing six MC channels, repeatedly stimulated by perfusion with ATP at different concentrations. (B) Plot showing the effects of 1 mM ATP on membrane patches maintained at a  $pH_i$  of 7.2 ( $n = 6$ ) and 6.2 ( $n = 5$ ). Both differences were statistically significant ( $P < 0.05$ , paired  $t$ -test).

channels, the activity was first upregulated by perfusion with MgATP (Fig. 2A), then the holding membrane potential was changed from +80 mV to +50 mV and a reduction of NPo from 1.39 to 0.58 was observed (Fig. 2B). The corresponding all points histograms presented well-defined peaks, indicating a main level corresponding to about 115 pS of conductance and a sublevel of about 80 pS. The two channels also exhibited an outward rectification (ratio 0.7) which is typical of MCs. In the presence of MgATP, the application of a fast positive pressure pulse induced a clear-cut reduction of channel activity (Fig. 2C, upper trace), while the application of a negative pressure pulse produced an activity increase.

The activating action of ATP was dose-dependent, as shown in Fig. 3A. The threshold concentration was micromolar while the saturating effect was attained with millimolar doses. The ATP-induced activation was also observed at lower intracellular pH, a condition previously reported to stimulate channel activity [4]. Fig. 3B illustrates the values of the mean current measured in the presence and in the absence of ATP 1 mM at pH of 7.2 and 6.2. At both values of  $pH_i$  the increase in activity was significant. Control experiments indicated that MgATP was capable of increasing channel mean current regardless of the concentration of calcium bathing the internal side of the patches (data not shown).

The rapid activation suggested a mechanism that does not involve ATP hydrolysis. This estimate was further confirmed by the results of substituting MgATP with ATP (data not shown) or with the non-hydrolyzable ATP analog AMP-PNP. Fig. 4A–B shows that the effect of this analog is indistinguishable from that

of MgATP. In Fig. 5A, we can see that ADP was also effective at enhancing MC activity, confirming that the channel is activated by non-hydrolytic binding of adenosine nucleotides. Adenosine itself mimicked the effect of its nucleotides, as shown in Fig. 5B.

The action of ATP was then studied at negative membrane potentials. Even in this experimental condition, the nucleotide is capable of activating the channel. Fig. 6 shows stimulation at a membrane potential of –80 mV. An apparent extra-activation at the beginning of wash is evident. This may be explained by a blocking effect of ATP on the channel, at negative membrane potentials. As shown in Fig. 6B, the application of ATP induced a rapid increase of open probability and at the same time a flickering partial block. Accordingly, the net effect on the mean patch current was an increase, because the activation prevailed over single channel current reduction. During the initial phase of washing, the block was promptly removed, whereas deactivation took longer to develop, resulting in an apparent extra-activation. Adenosine is able to activate at negative membrane potentials, but is ineffective in modifying the single-channel conductance (data not shown), suggesting that the block is due to the negative charge associated with phosphate groups.

The analysis was extended to cAMP, which is a second messenger involved in growth cone dynamics. Perfusion of the cytoplasmic side of excised inside-out membrane patches with cAMP alone induced just a weak and rapid increase in the mean patch current (not shown), but interestingly, when the cyclic nucleotide was applied in the presence of MgATP it produced a complete inhibition of channel activity. As shown in Fig. 7, typically the effect took minutes to develop, as well as to be reversibly removed. The long latency suggests a mechanism involving an endogenous protein kinase. A remarkable finding is that cAMP is effective at completely inhibiting this activity even in the presence of 1 mM ATP, a dose which is saturating for channel activation.

#### 4. Discussion

The results show that mechanosensitive channels of leech neurons in the MC mode are quickly and powerfully activated by adenosine or its nucleotides, in excised membrane patches. In the presence of ATP, cAMP is capable to slowly overcome this activation to reach a complete channel inhibition. These findings highlight an effective metabolic control on this cationic membrane conductance. This modulation represents a mechanism of protection, suitable for avoiding cytotoxic effects due to an excessive activity of cation channels. This problem is specially relevant in the leech (which lacks a rigid skeleton) for stretch-sensitive channels admitting calcium.

Adenosine nucleotides are reported to directly affect many different ion channels, including mechanosensitive channels. Non-hydrolytic activation has been found for TREK-like channels of rat ventricular myocytes [11], TREK of adrenal cortical cells [12],  $K_{Ca,ATP}$  of chick ventricular myocytes [13], cation channels of frog renal tubules [14] and ENaCs expressed in NIH-3T3 [15]. Non-hydrolytic inhibition has been reported for ATP-sensitive  $K^+$  channels [16], cation mechanogated channels of the amphibian kidney [17], rat neuronal TRPV1 channels



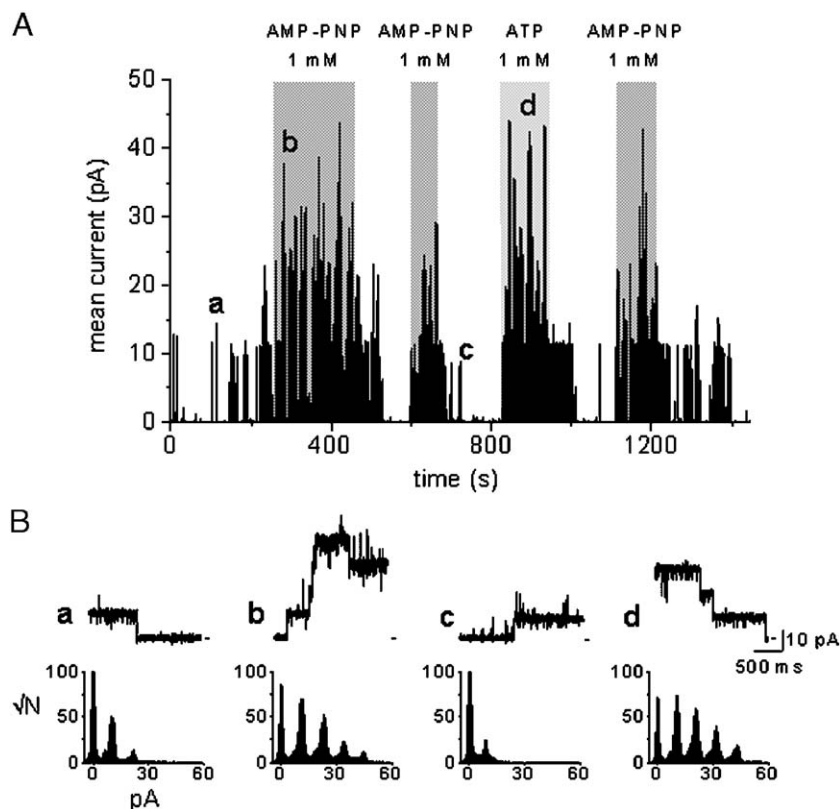


Fig. 4. Reversible effects of ATP and its non-hydrolyzable analog AMP-PNP on the same membrane patch containing four MC channels. (A) Each column in the plot represents the mean patch current calculated from a consecutive 1-s-long data segment. The membrane potential was held at +80 mV. (B) Current traces are samples of activity at the times marked a, b, c and d in the plot. The closed-channel current is indicated by the mark on the right of each recording. The corresponding all-points histograms are displayed under each trace.

[18] and TRPM4 channels expressed in HEK-293 cells [19]. First, it is remarkable that leech cation channels are quickly upmodulated by micromolar doses of ATP and secondly, that adenosine nucleotides, which are normally present in neurons at least at millimolar concentration [20,21], are capable of maintaining a powerful channel activation. The physiological meaning of this upmodulation cannot be easily explained without taking into account the effect of cAMP.

Although the molecular mechanism of cAMP inhibition has not been further investigated, the need for MgATP and the long latency make the involvement of an endogenous PKA likely.

Considering the consistent occurrence of this effect in the inside-out configuration, it is conceivable that its target is the channel itself or a closely associated molecule.

The picture that emerges from these results is that adenosine and related nucleotides provide a rapid activating bias, whereas cAMP is capable of a slow but strong inhibition of the channels. Apart from other factors affecting channel activity, one may expect that oscillations of cAMP concentration on a timescale of minutes would induce the channel activity and the associated calcium influx to oscillate. It is worth noting that interplay between intracellular cAMP and free calcium has been recently

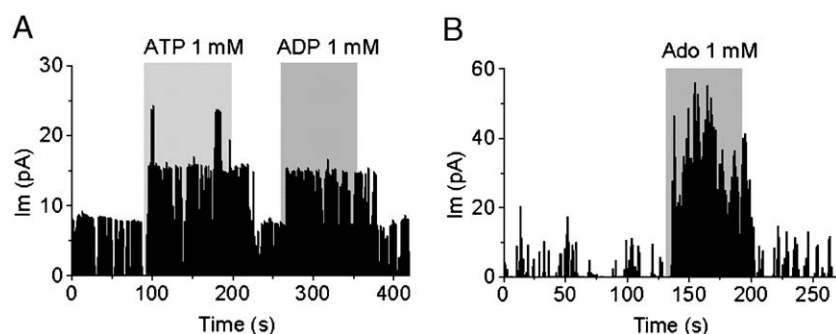


Fig. 5. Plots showing that a reversible channel activation can be induced by ATP as well as by ADP or adenosine (Ado). The membrane potential was maintained at +80 mV. Columns in the plots represent the mean patch current calculated from consecutive 1-s-long data segments. Three MC channels in A and six in B were contained in the membrane patches.

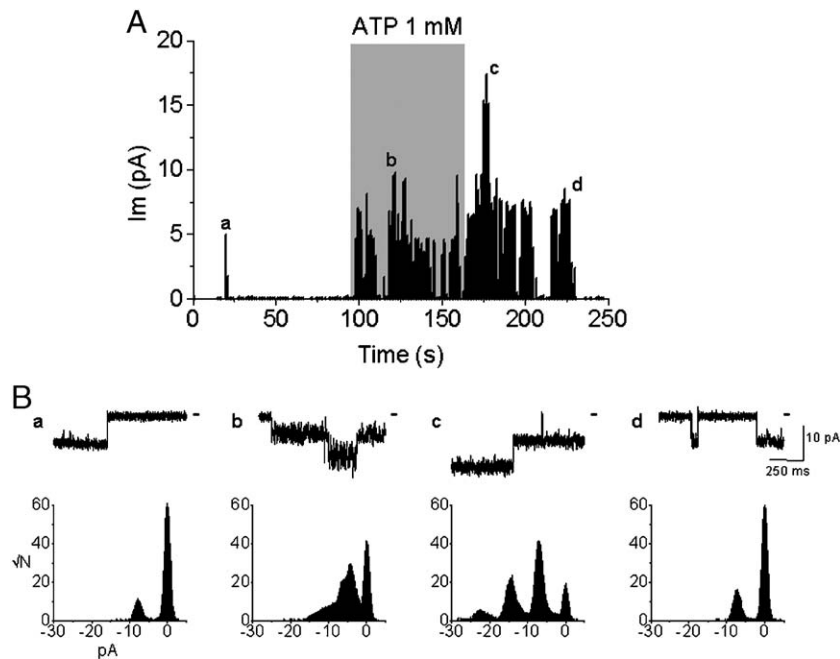


Fig. 6. Effect of perfusion with ATP of a membrane patch held at a negative membrane potential ( $-80$  mV). (A) Plot of the mean patch current measured at consecutive intervals of 1 s. (B) Current traces which illustrate the occurrence of a flickering channel block, during its activation.

pointed out as a general factor that regulates signalling by these messengers in different cells [22]. As far as the neurons are concerned, some cellular regions, such as the growth cones of advancing neurites, continuously sample the environment to detect chemical and physical cues, in order to regulate growth rate and direction [23]. Intracellular calcium oscillations have been reported to occur in association with growth cone dynamics, both *in vitro* and *in vivo*. These calcium transients are inversely related to the growth rate and probably represent a general signal regulating cell motility [24–26]. Intracellular calcium elevations are generated both by release from intracellular stores and by influx, through different types of ion channels. Recently, some members of the TRP channel family have been found to play an essential role in axon pathfinding [27–29]. Moreover, spontaneous slow oscillations of intracellular cAMP concentration have been measured and their dynamic interactions with calcium transients have been modeled. Experimentally induced changes in the cAMP transients modify the frequency of calcium oscillations; on the other hand, cAMP transients are generated only in the presence of specific patterns of calcium oscillations [30]. The notion that the levels of intracellular cyclic nucleotides have a pivotal role in determining growth cone behaviour has been established [31].

Among the various cues which affect growth cone navigation, physical interactions at the cell–substrate interface have been found to be relevant in modulating cell motion. Accordingly, it has been suggested that mechanosensitive ion channels may provide mechanical feedback as a function of substrate stiffness [32,33]. Some of the signalling pathways regulated by adhesion molecules are also involved in signal transduction activated by receptors for growth factors and by those for mitogenic neuropeptides [34,35]. Cellular responses to these stimuli include tyrosine phosphorylation [36], cytoplas-

mic changes in pH [37] and transient elevations of intracellular calcium [38]. There is a wide consensus that neurite and growth cones membranes undergo considerable changes in tension during their extension and retraction [39]. Stretch-sensitive cation channels could provide a local and contingent link between membrane tension and transmembrane  $\text{Ca}^{2+}$  fluxes directly, through a calcium inflow, and indirectly, by depolarization and consequent activation of voltage-gated calcium channels [40]. Thus, leech MC channels exhibit properties which are consistent with this function: they are expressed in clusters in the growth cone membrane and their blockage affects neurite outgrowth in culture [3], they are activated by mechanical stimuli and intracellular acidification [4] and are modulated by intracellular messengers involved in the control of growth cone dynamics.

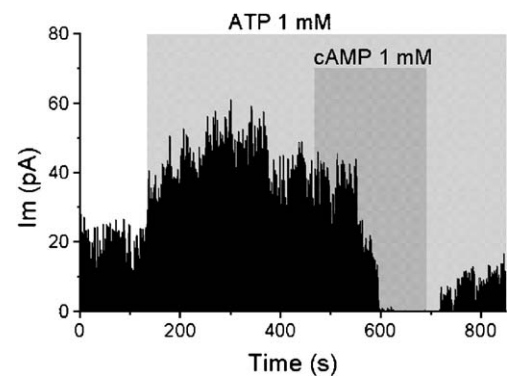


Fig. 7. Addition of cAMP during sustained activation by ATP produces a slow, complete and reversible channel inhibition. The membrane patch contained eight channels and was held at a potential of  $+80$  mV. The plot shows the mean patch current measured at consecutive intervals of 1 s.

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