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ATP REGULATES MUSCARINE-SENSITIVE POTASSIUM CURRENT IN DISSOCIATED BULL-FROG PRIMARY AFFERENT NEURONES

BY T. TOKIMASA AND T. AKASU

From the Department of Physiology, Kurume University School of Medicine, 67 Asahi-machi, Kurume 830, Japan

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SUMMARY

1. Bull-frog dorsal root ganglion cells in primary culture were voltage clamped in the whole-cell configuration. The pipette solution contained ATP (5 mM).

2. Step depolarizations (5–70 mV, 0·1–1 s) from a holding potential close to the resting potential (range, −64 to −79 mV) evoked a non-inactivating potassium current with properties indistinguishable from those which have been reported for the M-current of bull-frog sympathetic neurones.

3. An unhydrolysable ATP analogue APP(NH)P (5 mM), substitute with ATP in the pipette solution, did not support the M-current activation.

4. Bath application of ATP (30 nM–30 μM) reduced the amplitude of the M-current in a concentration-dependent manner, ≃ 50% inhibition of the current occurring with 1 μM-ATP. The main effect of ATP was to reduce the maximum M-conductance without changing the activation and deactivation kinetics of the M-current.

5. Essentially the same results were obtained with ADP (0·1–30 μM) and α, β-methylene-ATP (10–30 μM). AMP (10–100 μM) and adenosine (10–30 μM) were without effect on the M-current.

6. The ATP-induced inhibition of the M-current was *irreversible* when an unhydrolysable GTP analogue GTP-γ-S (10–30 μM) was present in the pipette solution. ATP (3 μM) reduced the amplitude of the M-current only by about 10% when GDP-β-S (100 μM) was present in the pipette solution. Pre-treatment of the cells with pertussis toxin (IAP; 500 ng ml^{-1}) for 24 h at 24 °C did not prevent the ATP-induced M-current inhibition.

7. Phorbol 12-myristate 13-acetate (PMA; 1–3 μM) reduced the amplitude of the M-current to about 50%. A reduction in the M-current amplitude by PMA (3 μM) and ATP (10 μM) was attenuated when staurosporine (200 nM) was present in the pipette solution. Forskolin (10 μM) was without effect on the M-current.

8. It is concluded that ATP acting at P_2 receptors, associated with an IAP-insensitive GTP-binding protein, inhibits the M-current in amphibian primary afferent neurones.

INTRODUCTION

Ample evidence has been accumulated to suggest the possible transmitter actions of ATP in both the central and peripheral nervous system (Phillis & Wu, 1981; Stone, 1981; Akasu, Hirai & Koketsu, 1983*b*; McAfee & Henon, 1985). Evidence for a sensory transmitter role of ATP has been provided by Holton & Holton (1954). They have reported that ATP is released from the peripheral terminals of sensory fibres and have suggested that release of ATP might also occur from central sensory terminals.

Two distinct excitatory responses to exogenously applied ATP have been reported in vertebrate primary afferent neurones (see Higashi, 1986). One is a rapid membrane depolarization of mammalian dorsal root ganglion cells (Jahr & Jessell, 1983; Krishtal, Marchenko & Pidoplichko, 1983). The depolarization results from an increase in a cation conductance showing an equal permeability for sodium and potassium ions (Krishtal *et al.* 1983). The other is a sustained membrane depolarization of amphibian dorsal root ganglion cells, which has been attributed to a decrease in a potassium conductance (Morita, Katayama, Koketsu & Akasu, 1984). However, neither the properties of the potassium conductance near the resting membrane potential nor the identity of a particular set of potassium conductances affected by ATP has yet been studied systemically (Ishizuka, Hattori, Akaike, 1984; Ito & Maeno, 1986). We therefore aimed to characterize more fully than before the properties of a potassium current regulated by ATP in dissociated bull-frog dorsal root ganglion cells. The results show that ATP inhibits preferentially a voltage-dependent potassium current which has been known as the M-current of bull-frog sympathetic neurones (Brown & Adams, 1980).

METHODS

All the experiments in the present study were carried out at 22–24 °C. The statistics are expressed as mean ± S.E. of the means for the cells tested. The pH of the superfusate was adjusted to 7·2.

Tissue culture. Small (200–250 g) bull-frogs (*Rana catesbeiana*) were used. After decapitation and pithing, dorsal root ganglia were rapidly isolated and minced with forceps into small pieces. These fragments were incubated with gentle pipetting or stirring at room temperature (22–24 °C) for 15–30 min in a Ringer solution containing the digestive enzymes trypsin (Sigma type XI; 2·5 mg ml^{-1}) and collagenase (Sigma type 1A; 0·5 mg ml^{-1}). The Ringer solution had the following composition (mM): NaCl, 112, KCl, 2; $CaCl_2$, 1·8; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 4; and tris(hydroxymethyl)aminomethane (Tris), 1. The enzymatic digestion was repeated up to six times. After each digestion, the dissociated cells were collected by centrifugation (80 *g* for 4–5 min) and stored for 2–10 days at 4 °C in Leibovitz's L-15 medium (GIBCO 320-1415); 10–20 % fetal bovine serum (GIBCO 200-6140 AG) was added and then diluted to 80 % with water in 35 mm culture dishes (Falcon 3001).

Whole-cell recordings. The cultured cells were re-suspended in the Ringer solution about 2 h before the electrophysiological experiments. The cells were then pipetted into the recording chamber (1·5 ml total volume) and continuously superfused (1–3 ml min^{-1}) with Ringer solution after they had settled to the bottom of the chamber. Pipettes for the whole-cell clamp had a tip resistance of 2–6 MΩ when filled with a solution having the following composition (mM): KCl, 100; $MgCl_2$, 4; adenosine 5′-triphosphate disodium (Na_2ATP), 5; ethyleneglycol-bis(β-aminoethyl-ether)*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA), 1; and HEPES (sodium salt), 2·5 (pH adjusted with KOH to 7·0). The electrode tip was coated with the silicone elastomer Sylgard (Dow Corning Corporation). The membrane patch was ruptured by increasing the negative pressure in the pipette

after the tight-seal ($\geqq 1$ GΩ) between the pipette and the cell membrane was established. A sample-and-hold/voltage-clamp amplifier (Axoclamp 2A) was used at the switching frequency of 10–17 kHz. The head-stage current gain of the amplifier was 0·1. This indicated that clamping currents as large as ±20 nA could be applied to the cells.

The output of the amplifier was monitored using a digital oscilloscope equipped with a disc recorder (Nicolet 4094 and XF-44) and another digital oscilloscope equipped with a chart recorder (Nihon Kohden VC-11 and RJG-4124). The frequency response of the chart recorder ranged from DC to 100 Hz ($\geqq -3$ dB). All the data were stored in a videocassette recorder (Sony KS-609 equipped with EP-61) for off-line analysis. The output of the Nicolet XF-44 was displayed using an *X–Y* plotter (Hewlett–Packard ColorPro). The head-stage of the amplifier was continuously monitored with a digital memory scope (Toshiba DM-1562A).

Cell classification. The mean diameter of the cells was measured as previously described for cultured bull-frog sympathetic neurones (Tokimasa & Akasu, 1990*a*, *b*). Large spheroidal cells having a mean diameter greater than 50 μm were selected for the whole-cell recordings. These cells were characterized by a prominent hyperpolarization-activated cationic inward rectifier (H-current; Mayer & Westbrook, 1983), which was selectively and reversibly blocked by adding caesium (1–2 mM) to the Ringer solution (Fig. 1*A–C*). Furthermore, the action potential of these large cells was significantly shorter lasting than that observed for small spheroidal cells having a mean diameter of less than 25 μm (typically lasting longer than 6 ms in ten cells). These observations suggest that the cells used in the present study could correspond to A-cells which have been reported for intact bull-frog primary afferent ganglia (Morita & Katayama, 1984, 1987, 1989*b*; Holz, Shefner & Anderson, 1985, 1986*b*; Higashi, 1986).

Isolation of the M-current. Attempts were made to isolate the M-current from other membrane currents in a Ringer solution containing tetrodotoxin (TTX; 3 μM), apamin (30 nM), tetraethylammonium (TEA; 30 mM), 4-aminopyridine (4-AP; 1 mM) and caesium (1 mM). This is the 'standard' solution. The concentration of NaCl was reduced to 80 mM in order to maintain the isosmolarity of the superfusate. In some experiments, the concentration of TEA in the standard solution was further elevated from 30 to 90 mM by equimolar substitution with NaCl (See Figs 9*C* and 10*A*, *B*). Tetrodotoxin (3 μM) was used to block an inward sodium current (Kostyuk, Veselovsky & Tsyndrenko, 1981*b*; Ishizuka *et al.* 1984; Ikeda, Schofield & Weight, 1986). TEA (30–90 mM) was used to block a classical delayed rectifier potassium current (Hodgkin & Huxley, 1952; Kostyuk, Veselovsky, Fedulova & Tsyndrenko, 1981*a*; Ito & Maeno, 1986; Penner, Petersen, Pierau & Dreyer, 1986). 4-Aminopyridine (1 mM) was used to reduce the amplitude of the transient A-current according to previously published data in bull-frog and rat dorsal root ganglion cells (Ito & Maeno, 1986; Mayer & Sugiyama, 1988). Apamin (30 nM) was used to block a calcium-activated potassium current (Morita & Katayama, 1989*a*). Caesium (1 mM) was used to block a hyperpolarization-activated cation current (H-current of Mayer & Westbrook, 1983, and Q-current of Stansfeld, Marsh, Parcej, Dolly & Brown, 1987).

H-current. The steady-state activation curve of the H-current was examined since properties of the current have not been characterized in bull-frog DRG neurones (see Morita & Katayama, 1989*b*). For this purpose, the superfusate was changed from the normal Ringer solution to a nominally calcium-free, potassium-rich (5 mM) Ringer solution containing TTX (3 μM), magnesium (10 mM), cobalt (2 mM), barium (2 mM), 4-AP (1 mM) and TEA (20 mM) (see Tokimasa & Akasu, 1990*a*). The concentration of sodium was decreased from 112 to 80 mM. The main effect of this solution was to eliminate the M-current by 2 mM-barium and to increase the H-current amplitude by 5 mM-potassium. The latter effect masked a decrease in the H-current amplitude by decreasing the sodium concentration from 112 to 80 mM (Tokimasa & Akasu, 1990*b*).

Inward calcium current. An inward calcium current was recorded in a nominally sodium-free Ringer solution of the following composition (mM): choline chloride, 105; KCl, 2; $BaCl_2$, 2; TEA chloride, 5; $CsCl_2$, 2; and HEPES, 4 (pH was adjusted with HCl to 7·2). TTX (300 nM) was present in this solution throughout. The pipettes were filled with a solution of the following composition (mM): $CsCl_2$, 96; TEA chloride, 5; EGTA, 2; HEPES (sodium salt), 2·5; and ATP (disodium salt), 5 (pH was adjusted with CsOH to 6·9).

Drugs. Drugs used were adenosine, adenosine 5′-triphosphate (disodium salt or magnesium salt), 5′-adenylylimidodiphosphate (tetralithium salt), α,β-methyleneadenosine 5′-triphosphate (lithium salt), adenosine 5′-diphosphate (sodium salt), adenosine 5′-monophosphate (sodium salt), guanosine-5′-*O*-(3-thiotriphosphate) (tetralithium salt), guanosine-5′-*O*-(2-thiodiphosphate) (trilithium salt), 2-chloroadenosine, forskolin, (±)-muscarine chloride, apamin, 4-aminopyridine and

phorbol 12-myristate 13-acetate (all from Sigma). Other drugs used were tetrodotoxin (Sankyo), tetraethylammonium chloride (Tokyo Kasei), staurosporine (Calbiochem) and N^6-cyclohexyladenosine (Research Biochemicals, Inc.). Phorbol 12-myristate 13-acetate was dissolved with dimethyl sulphoxide (Wako Pure Chemicals; 3·3 mg ml^{-1}) and stored at −20 °C. Staurosporine was also dissolved with dimethyl sulphoxide (0·1 mg ml^{-1}) and stored at −20 °C.

RESULTS

The resting membrane potential of the cultured bull-frog dorsal root ganglion (DRG) cells was -73 ± 2 mV (range, −64 to −79 mV) about 5 min after rupturing the membrane patch (Fig. 1) ($n = 100$; A-cells). The action potential evoked by brief (8–20 ms) depolarizing current pulses had a peak amplitude of 103 ± 3 mV ($n = 30$). Tetrodotoxin (3 μM) was present in Ringer solution throughout the experiments in the following sections.

Rectification near the resting potential

Step hyperpolarizations from near resting potential activated a time-dependent inward current, the H-current (Fig. 1*B* and *C*; Mayer & Westbrook, 1983). This induced inward rectification in the steady-state current–voltage (*I–V*) curve (Fig. 1*A*). The H-current was blocked by external caesium ions (Fig. 1*B*), which thereby induced an outward current at the resting potential, and a membrane hyperpolarization in unclamped cells (Fig. 1*D*), and suppressed the inward rectification (Fig. 1*A*). The H-current was augmented in a potassium-rich (5 mM) Ringer solution. This phenomenon is compatible with previous observations for the H-current of cultured rat DRG neurones (Mayer & Westbrook, 1983) and was due presumably to an increased permeability for sodium and potassium ions when the driving force was decreased in the potassium-rich solution (see Noble, 1962, 1965; McAllister & Noble, 1966).

The cells were clamped at −50 mV in a nominally calcium-free Ringer solution (see Methods) and the H-current was activated during hyperpolarizing step commands (5–80 mV, 2–10 s). The amplitude of the deactivating inward tail upon stepping back to the holding potential of −50 mV was measured. The peak amplitude of inward tail (I) at a given potential was normalized with respect to its maximum value (I_{max}) which was obtained between −120 and −130 mV. I/I_{max} was plotted as a function of voltage (V). The activation curve could be described (least-squares method) by the Boltzmann equation,

$$I/I_{max} = [1 + \exp((V - A)/k)]^{-1}, \quad (1)$$

where A and k denote the half-activation voltage and the slope factor (Mayer & Westbrook, 1983; Tokimasa & Akasu, 1990*b*). In six cells, A and k were -92 ± 2 mV and $7{\cdot}4 \pm 1$ mV, respectively. This implies that about 5% of the H-conductance is activated at −70 mV (see Discussion)

Step depolarizations (5–20 mV, 1–3 s) from the holding potential close to the resting potential evoked a slowly developing outward current during the depolarizations followed by an outward tail current after the termination of the step commands. The amplitude of the outward current measured at the end of the step commands was progressively increased as the commanding levels were made more positive leading to outward rectification on an *I–V* curve. The rectification was clearly depressed when the potassium concentration in the superfusate was elevated from 2 to 5 mM.

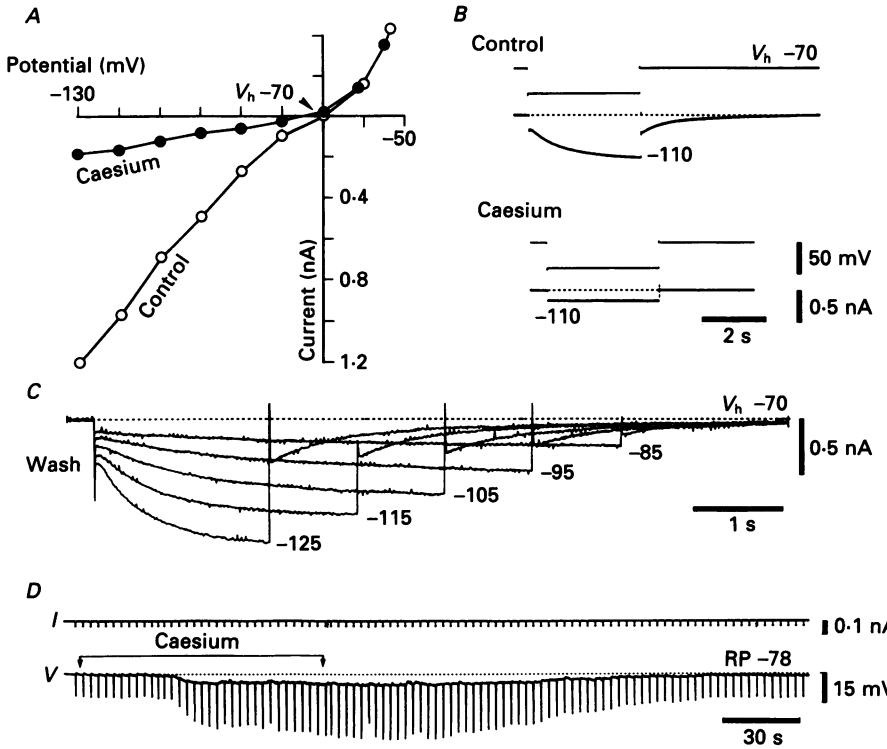

Fig. 1. H-current in cultured bull-frog DRG neurones. Results in *A–C* were obtained from a single cell. *A*, the steady-state *I–V* curve measured at the end of prolonged (2–10 s) depolarizing or hyperpolarizing step commands from a holding potential (V_h) close to the resting membrane potential of −70 mV. ○ and ● denote the *I–V* curve in control solution and in the presence of caesium (2 mM), respectively. *B*, caesium (2 mM)-induced blockade of the H-current at −110 mV. *C*, the control H-current was regained after washout of caesium. H-currents at −85, −95, −105, −115 and −125 mV are superimposed. *D*, results were obtained from another cell under the current-clamp mode. Caesium (2 mM) was added to the Ringer solution for a period indicated by the arrows. Downward deflections on the voltage trace (*V*) are hyperpolarizing electrotonic potentials evoked by current pulses of fixed amplitude (*I*). The resting potential (RP) was −78 mV. TTX (3 μM) was present in the superfusate in *A–D*.

Identification of the M-current in DRG neurones

The slowly developing outward current near the resting potential was reminiscent of the M-current of bull-frog sympathetic neurones (Brown & Adams, 1980). Hence, attempts were made first to learn whether the M-current occurs in bull-frog DRG neurones. For this purpose, the superfusate was changed from the Ringer solution to a solution containing TEA (30 mM), caesium (1 mM), 4-AP (1 mM) and apamin (30 nM) (see Methods). This is the 'standard' solution used in the present study.

A family of outward currents present in the standard solution is shown in Fig. 2*A*. In this particular example, step depolarizations from the holding potential of −80 mV to −62, −54 and −46 mV evoked an outward current during the step commands followed by an outward tail current after the termination of the commands. At −93 mV, the H-current was not seen because of the presence of

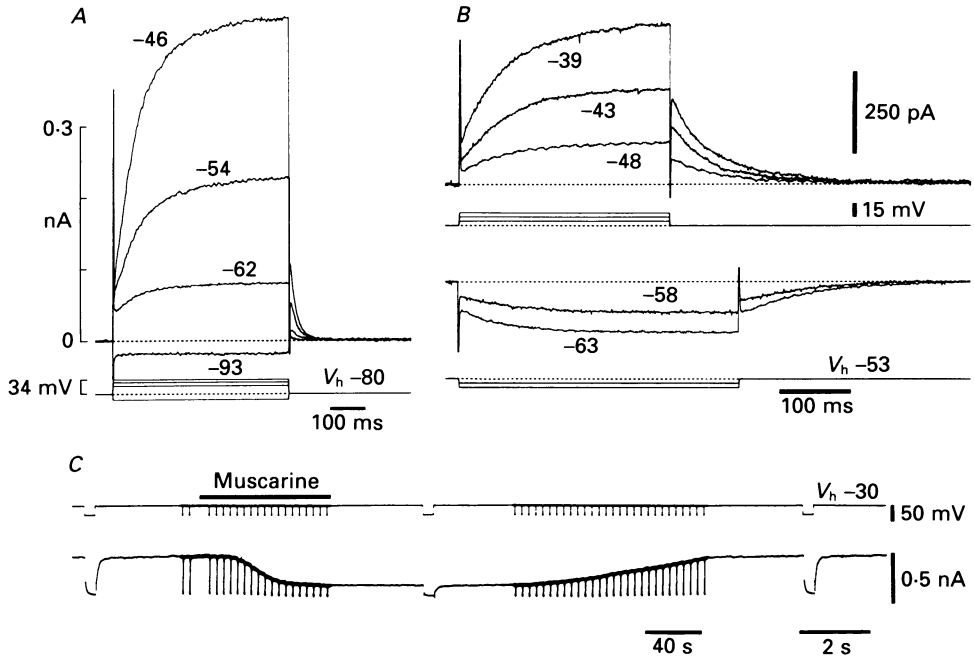

Fig. 2. The sensory M-current present in the standard solution. Results in *A–C* were obtained from three different cells which were superfused with the 'standard' solution for the M-current (see Methods for its composition). *A*, the cell was depolarized or hyperpolarized from a holding potential (V_h) of −80 mV. Command potentials are indicated beside each trace (mV). *B*, the cell was depolarized or hyperpolarized from V_h −53 mV. At −58 and −63 mV, deactivating M-current produced inward relaxations on the current trace followed by outward currents arising from the reactivation of the M-current when the potential was returned to −53 mV. *C*, bath application of muscarine (1 μM) reversibly and selectively blocked the M-current. Hyperpolarizing step commands were from −30 to −60 mV. On three occasions, the chart speed was increased to demonstrate the M-current on a faster time scale.

caesium (1 mM) in the standard solution (Fig. 2*A*). Step hyperpolarizations (5–40 mV, 0·2–1 s) from depolarized holding potentials with respect to rest (e.g. −53 mV in Fig. 2*B* and −30 mV in Fig. 2*C*) caused an inward relaxation on the current trace followed by an outward current after the termination of the commands. An ohmic current upon stepping back from −63 to −53 mV was of smaller amplitude than that at the onset of the step command indicating that the inward relaxation during the hyperpolarization was due to a deactivating outward current instead of an activating inward current and that the subsequent outward current was due to the reactivation of the current at −53 mV. Bath application of muscarine (0·1–3 μM) produced an inward shift of the holding current at −30 mV which had the same amplitude as that of the decrease in the outward current relaxation ($n = 6$; Fig. 2*C*). Taken together, it is strongly suggested that the M-current may also occur in bull-frog DRG neurones.

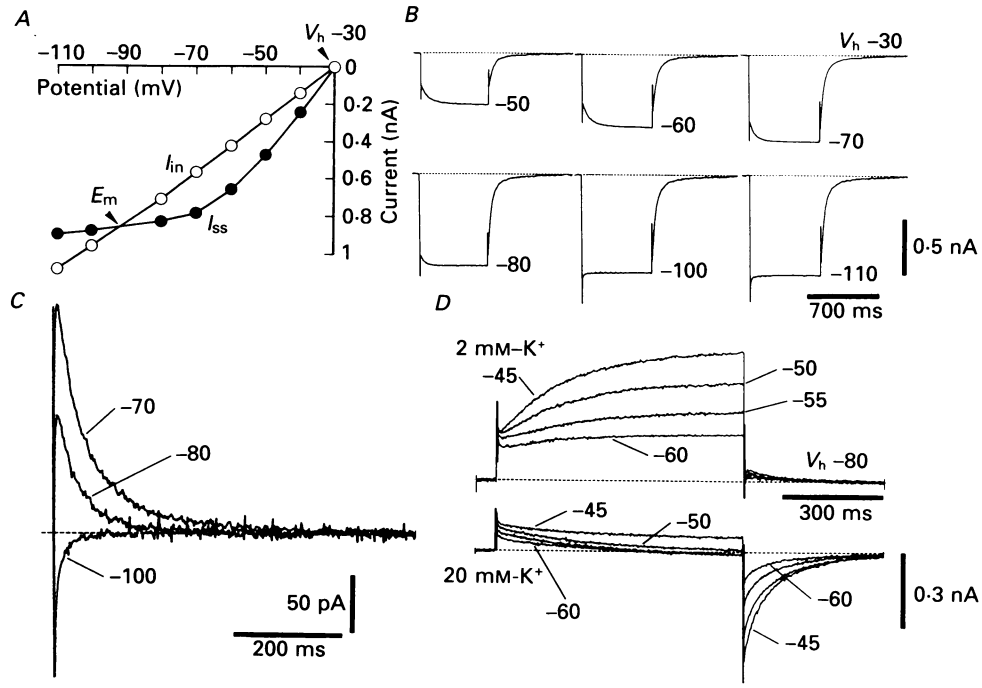

Fig. 3. Reversal potential of the M-current. Results in *A–C* and *D* were obtained from two different cells superfused with the 'standard' solution. *A*, the 'instantaneous' *I–V* curve (I_{in}) and the steady-state *I–V* curve (I_{ss}) intersected at −92·5 mV (an arrow) indicating the reversal potential of the M-current (E_M). The holding potential (V_h) was −30 mV. Sample recordings plotted in *A* are shown in *B*. *C*, three current traces represent deactivating M-current at −70, −80 and −100 mV shown in *B*. Current traces are superimposed on the steady-state level attained at each command potential indicated by the dashed line. *D*, activating M-current at −45, −50, −55 and −60 mV followed by the deactivation of the current at −80 mV with the potassium concentration in the superfusate at 2 mM (upper traces) and 20 mM (lower traces).

Voltage dependence of the sensory M-current

Reversal potential

The cells were clamped at −30 mV and subjected to step hyperpolarizations (5–80 mV, 0·5–1 s). The 'instantaneous' and steady-state current–voltage (*I–V*) curves (I_{in} and I_{ss} in Fig. 3*A*, respectively), measured respectively at the onset and the end of the hyperpolarizing step commands, intersected at $-93{\cdot}8 \pm 2{\cdot}3$ mV ($n = 19$) indicating the reversal potential of the M-current (E_M; Fig. 3*A–C*; Adams, Brown & Constanti, 1982*a*). E_M shifted to -54 ± 2 mV ($n = 3$) and -36 ± 2 mV ($n = 8$) with the potassium concentration in the standard solution at 10 and 20 mM, respectively. Figure 3*D* shows typical recordings of the activating M-current between −45 and −60 mV followed by the deactivating M-current at −80 mV in a 2 mM-potassium solution (upper traces) and in a 20 mM-potassium solution (lower traces).

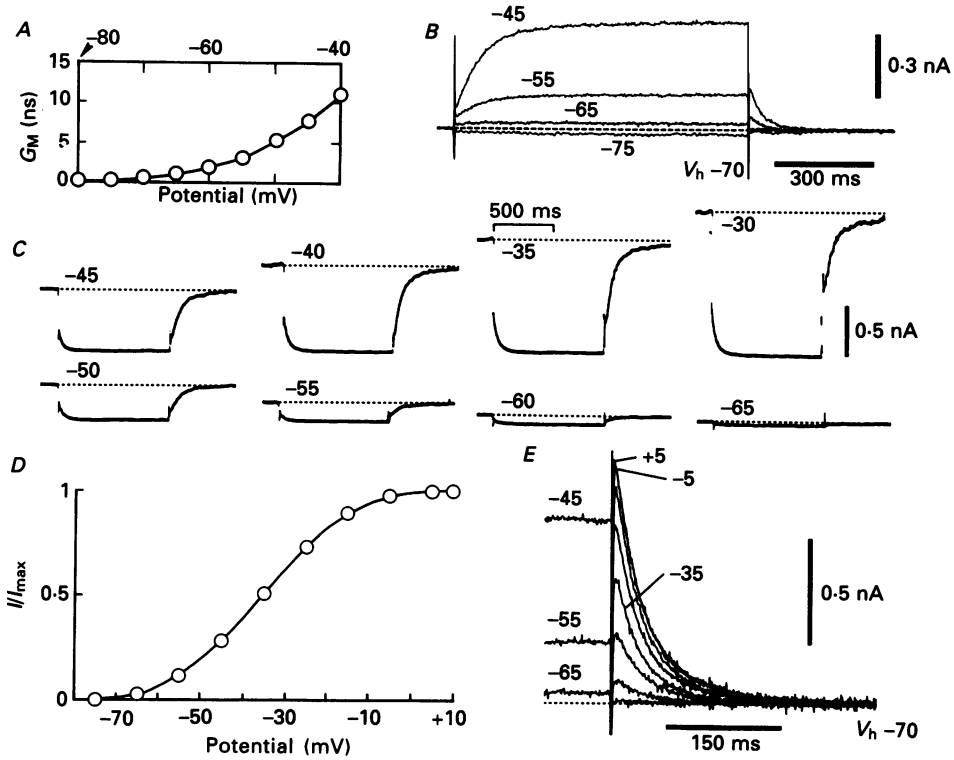

Fig. 4. The steady-state activation curve of the M-current. *A*, G_M was calculated by subtracting the leak conductance measured at the end of the command pulses from the M-conductance plus the leak conductance measured at the onset of the step pulses (see Adams *et al.* 1982*a*). Each circle denotes the mean value obtained from fifteen cells. The S.E. of mean was less than the size of each circle. Sample recordings plotted in *A* are shown in *B* and *C*. *B*, the cell was polarized from the holding potential (V_h) of −70 mV to −65, −55, −45 and −75 mV. *C*, results from the same cell as that in *B*. The cell was hyperpolarized to −70 mV from eight different holding levels indicated beside each trace (mV). *D*, the steady-state activation curve obtained from six cells (see text for details). The curve was drawn according to the Boltzmann equation (eqn (2)). Sample recordings plotted in *D* are shown in *E*. In *E*, deactivating tail currents upon repolarizations from +5, −5, −15, −25, −35, −45, −55 and −65 mV to V_h −70 mV are superimposed. The maximum tail current was obtained on stepping back to −70 mV from +5 and −5 mV.

Steady-state activation curve

The amplitude of the reactivating M-current at the termination of hyperpolarizing step commands (5 mV, 0·5–1 s) was zero when the holding potential was −71·5 ± 1·5 mV (n = 15; range, −66 to −74 mV; Fig. 4*B*). This potential was defined as the bottom of the activation curve. The cells were held between −30 and −65 mV and hyperpolarized to the bottom of the activation curve (e.g. −70 mV in Fig. 4*C*). The ohmic current at the onset of step hyperpolarizations denotes a sum of the steady-state M-conductance (G_M) and the leak conductance. The leak conductance, calculated separately by measuring the amplitude of the ohmic current upon stepping back to holding potentials less negative than −45 mV, was subtracted from

that sum. G_M was thus estimated as $14{\cdot}9 \pm 2{\cdot}1$ nS at -35 mV ($n = 15$; range 9–23 nS).

The cells were clamped at the bottom of the activation curve and the amplitude of the outward tail of the M-current after the termination of prolonged (2 s) depolarizing command steps was measured (Fig. 4*E*). The maximum amplitude of the tail current (I_{max}) was obtained between -5 and $+5$ mV (occasionally near $+10$ mV), indicating the top of the activation curve (Fig. 4*D*). The peak amplitude of the tail current relative to its maximum value (I/I_{max}) was plotted as a function of membrane potential (V) in order to obtain the steady-state activation curve of the M-current. The curve could be described (least-squares method) by the Boltzmann equation,

$$I/I_{max} = [1 + \exp((A - V)/k)]^{-1}, \quad (2)$$

where A and k denote the half-activation voltage and the slope factor, respectively. In six cells tested, A and k were $-34{\cdot}6 \pm 1{\cdot}2$ mV and $10{\cdot}1 \pm 0{\cdot}2$ mV, respectively (Fig. 4*D*).

Instantaneous I–V curve for the steady-state M-conductance

The cells were polarized between -35 and -120 mV with and without applying depolarizing pre-pulses, for 1 s to -30 mV, from the bottom of the activation curve (e.g. -70 mV). A sum of the amplitude of the deactivating M-current measured with the pre-pulses and the activating M-current measured without the pre-pulses gave the measure of an instantaneous I–V relation for the current flow through the M-channel being fully opened at -30 mV. There was no M-current at the holding potential and therefore the amplitude of the deactivating M-current was measured between -70 and -120 mV upon stepping back from -30 mV to those potentials. The I–V curve thus obtained was linearly related to the membrane potential and had a slope of $19{\cdot}3 \pm 3{\cdot}5$ nS ($n = 3$; range, 13–26 nS). This implied that the unitary M-conductance may lack an intrinsic rectification. The I–V curve intersected with the voltage axis at $-92{\cdot}9 \pm 1{\cdot}9$ mV ($n = 3$) indicating the E_M.

Time course of activation and deactivation

The currents activated at various potentials, between -60 and $+5$ mV, all had the same deactivation time constant (τ_{off}) when their tails were measured at any one voltage (e.g. -80 mV in Fig. 2*A*, -53 mV in Fig. 2*B* and -70 mV in Fig. 4*B*, *C* and *E*). Similarly, the onset time constant (τ_{on}) for any one potential was the same irrespective of the pre-activation voltage (e.g. -53 mV in Fig. 2*B* and -30 mV in Fig. 3*B*). τ_{on} and τ_{off} were independent of the potassium concentration (Figs 3*D* and 5*A*; Adams *et al.* 1982*a*). The reciprocal time constants for τ_{off} (τ_{off}^{-1}, s^{-1}) and τ_{on} (τ_{on}^{-1}, s^{-1}) are summarized in Fig. 5 ($n = 5$–15 for 2 mM-potassium and $n = 4$ for 20 mM-potassium). As can be predicted from the steady-state activation curve shown in Fig. 4*D*, the reciprocal time constants showed a Boltzmann relation: τ_{on}^{-1} and τ_{off}^{-1} were about 8 s^{-1} at the half-activation voltage of -35 mV (Adams *et al.* 1982*a*).

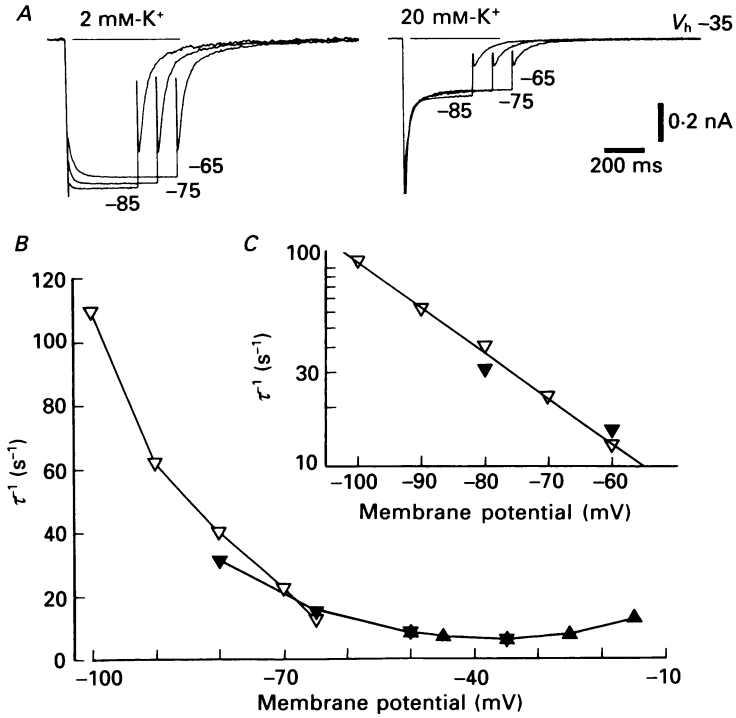

Fig. 5. Voltage dependence of the time course of the M-current. *A*, the cell was hyperpolarized from a holding potential (V_h) of −35 mV to −65, −75 and −85 mV for 550, 450 and 350 ms, respectively. The time course of the reactivating inward tail currents upon stepping back to −35 mV was the same irrespective of the potassium concentration in the superfusate (2 or 20 mM). The peak amplitude of the deactivating M-current at −75 and −85 mV in a 20 mM-potassium solution was off-scale. *B*, τ_{on}^{-1} (▽ and ▼) and τ_{off}^{-1} (▲) expressed as a function of membrane potential. Filled symbols represent data (means for five to fifteen cells) in the superfusate containing 2 mM-potassium; open symbols are for 20 mM-potassium (means for four cells). *C*, a semilogarithmic plot for data shown in *B* between −60 and −100 mV. The straight line denotes a regression line (least-squares method) having a slope of e-fold per 20 mV.

Divalent cations

The M-current was eliminated when barium (1–3 mM) was added to the standard solution (Fig. 6*A* and *B*; Adams, Brown & Constanti, 1982*b*). In the presence of barium (1–3 mM), step depolarizations (20–40 mV, 100–500 ms) from holding potentials of between −45 and −35 mV evoked an inward calcium current (Fig. 6*C*; Ishizuka *et al.* 1984; Fox, Nowycky & Tsien, 1987).

Consistent with the previously published data in bull-frog sympathetic neurones (Adams *et al.* 1982*b*), the amplitude of the M-current was clearly reduced when divalent cations such as cobalt (0·1–1 mM) were added to the standard solution (Fig. 7*A–C*). As much as 80% depression of the current was observed with 1 mM-cobalt ($n = 3$). Essentially the same results were obtained with manganese (0·1–1 mM; $n = 3$), cadmium (0·1–1 mM; $n = 4$) and nickel (0·1–1 mM; $n = 3$). The rank order of potency was $Cd^{2+} > Co^{2+} > Ni^{2+} \simeq Mn^{2+}$ (0·3 mM for each cation). Any divalent cation caused a downward shift in the steady-state activation curve of the

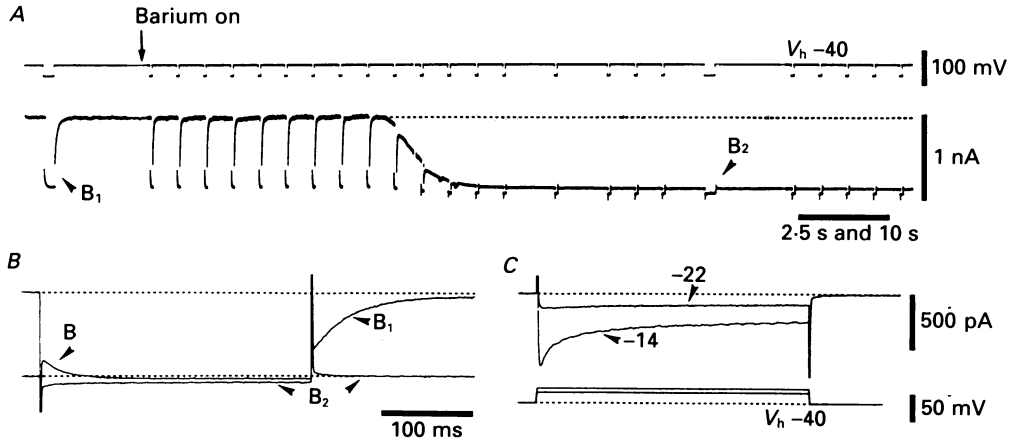

Fig. 6. Sensitivity of the M-current to barium. *A*, the cell was clamped at a holding potential (V_h) of −40 mV and hyperpolarized to −70 mV for 300 ms every 3 s before and after barium (2 mM) was added to the 'standard' solution. On two occasions, the chart speed was increased to demonstrate the M-current on a faster time base (B_1 and B_2). B_1 and B_2 are superimposed in *B*. *C*, the calcium/barium current at −22 and −14 mV. V_h was −40 mV.

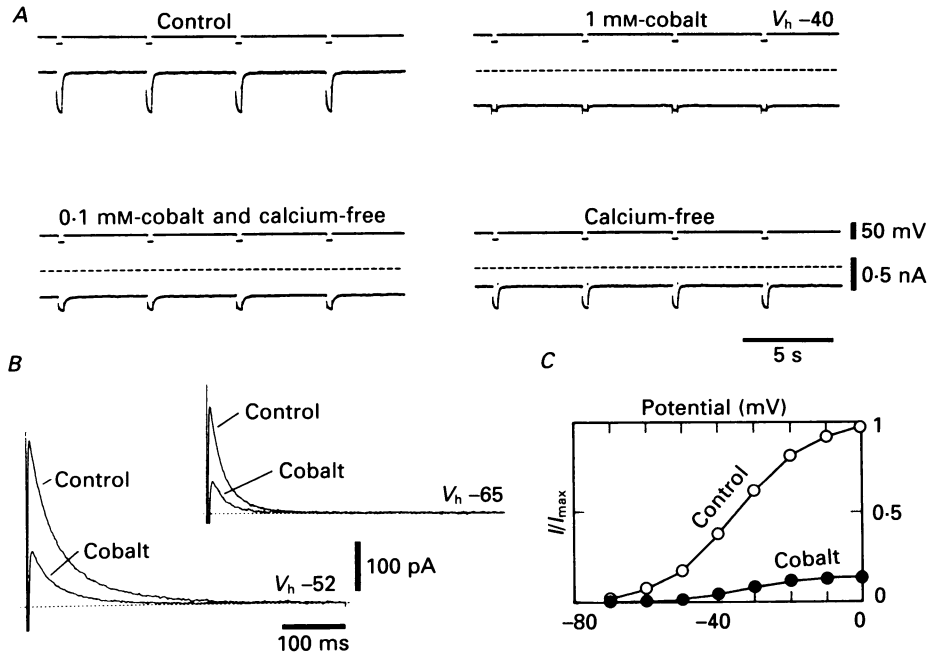

Fig. 7. Calcium dependence of the M-current. Results in *A*, *C* and *B* were obtained from two different cells. *A*, the cell was hyperpolarized from a holding potential (V_h) of −40 mV to −63 mV for 300 ms every 5 s. Traces from upper left to lower right are the M-current in control solution, in a solution containing cobalt (1 mM), in a nominally calcium-free solution containing magnesium (10 mM) and cobalt (0·1 mM) and in a nominally calcium-free solution containing magnesium (10 mM) and EGTA (30 μM). *B*, the deactivating M-current at V_h −52 and −65 mV in control and in the presence of cobalt (0·3 mM). *C*, the steady-state activation curve for the M-current in control (○) and in the presence of 1 mM-cobalt (●).

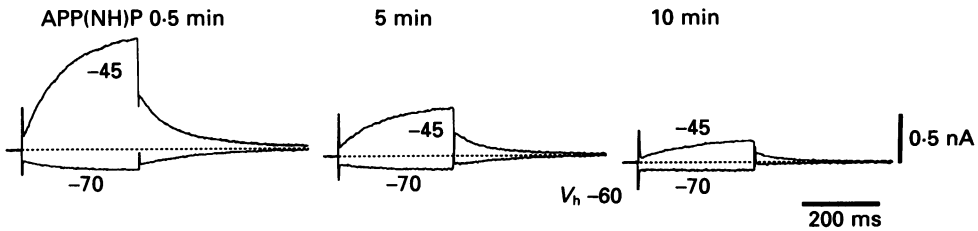

Fig. 8. Requirement of intracellular ATP for the M-current. Data were obtained from a single cell. The superfusate was changed from the normal Ringer solution to the 'standard' solution in the cell-attached configuration with the patch pipette containing APP(NH)P (5 mM) instead of ATP. At time zero, the membrane patch was ruptured. The holding potential (V_h) was −60 mV. Commanding levels were −45 and −70 mV. The 'run-down' of the M-current was judged by the disappearance of relaxations on the current trace and the inward shift of the holding current at −60 mV.

M-current without significantly affecting the reversal potential, the half-activation voltage and the time course of both activating and deactivating M-current ($n = 1$ for each cation; Fig. 7*B* and *C*). The M-current amplitude was also decreased to as much as 50% in a nominally calcium-free solution containing 4 mM-magnesium and 30 μM-EGTA ($n = 6$; Fig. 7*A*). These observations are consistent with a recent study on the M-current of cultured bull-frog sympathetic neurones (Tokimasa & Akasu, 1990*b*). Possible mechanisms of these phenomena were not studied in further detail in the present study.

Requirement of intracellular ATP for the M-current

The M-current showed only a moderate (≦ 15%) 'run-down' during the course of experiments for 2 h when the pipette solution contained ATP (5 mM, $n = 40$). In contrast, the M-current showed significantly greater and faster 'run-down' when ATP (5 mM) in the pipette was replaced by its unhydrolysable analogue 5-adenylylimidodiphosphate (APP(NH)P; Fig. 8). In three cells, the M-current disappeared 10–15 min after rupturing the membrane patch. In three other cells, the amplitude of the M-current reduced to about 20% of its respective control 15 min after the rupturing of the patch. These observations indicated that a hydrolysable form of ATP is required for the activation of the sensory M-current (see also Pfaffinger, 1988).

Bath application of ATP inhibits the M-current

Bath application of ATP (30 nM–30 μM) reduced the amplitude of the M-current (Fig. 9; see also Akasu, Hirai, & Koketsu, 1983 *a*). The M-current inhibition by ATP could be detected as soon as the drug-containing solution reached the recording chamber, as judged by an inward shift of the holding current at −30 mV (Fig. 9*A*). The maximum inhibition of the current was obtained about 0·5–1 min thereafter. The control M-current was regained within several minutes when the bath application of ATP was discontinued. The minimum effective concentration of ATP was between 30 and 100 nM (≃ 10% inhibition with 100 nM; $n = 6$) and approximately 50% inhibition was obtained with 1 μM-ATP ($n = 15$; Fig. 9*B*). The ATP-induced inward

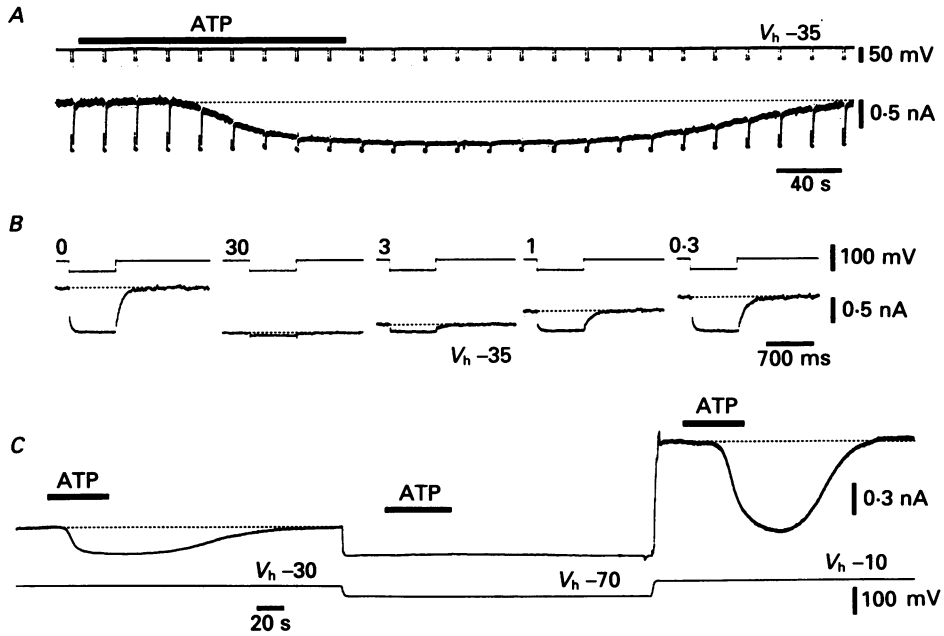

Fig. 9. Depression of the M-current by ATP. Results in *A–C* were obtained from two different cells. The concentration of TEA in the 'standard' solution was 30 mM in *A* and *B*, and 90 mM in *C*. *A*, ATP (3 μM) was added to the bath for a period of time indicated by the continuous line. The cell was hyperpolarized from the holding potential (V_h) of −35 mV to −75 mV. *B*, the concentration-dependent inhibition of the M-current in the same cell as that in *A*. The concentration of ATP (in μM) is indicated beside each current trace (0 μM in the control). Note that the amplitude of the inward shift of the holding current produced by 30 μM-ATP at −35 mV was greater than the decrease in the repolarizing tail current. *C*, voltage dependence of the ATP-induced inward shift of the holding current. The chart trace is continuous. At V_h −70 mV, ATP (3 μM) did not produce any inward shift of the holding current.

shift of the holding current simply disappeared when the cells were clamped at the bottom of the activation curve of the M-current ($n = 7$ with 3 μM-ATP) (e.g. −70 mV in Fig. 9*C*). However, in three of eight cells tested, ATP (30 μM) caused an inward shift of the holding current, the amplitude of which was greater than the peak amplitude of the repolarizing M-current (Fig. 9*B*). This implied that ATP, at higher concentrations, may affect some additional membrane conductances.

Depression of the maximum M-conductance

In five cells, ATP (1 μM) caused a downward shift in the steady-state activation curve without significantly affecting the half-activation voltage (Fig. 10*A* and *B*) or the activation–deactivation time course of the M-current (Fig. 10*C*). The reversal potential of the M-current, estimated with the methods described for Fig. 3*A*, was $-94{\cdot}1 \pm 1{\cdot}5$ mV and $-93{\cdot}0 \pm 0{\cdot}6$ mV in control and in the presence of ATP (1 μM), respectively, in three pairs of cells. These observations indicate that the main effect of ATP on the M-current was to reduce the maximum M-conductance.

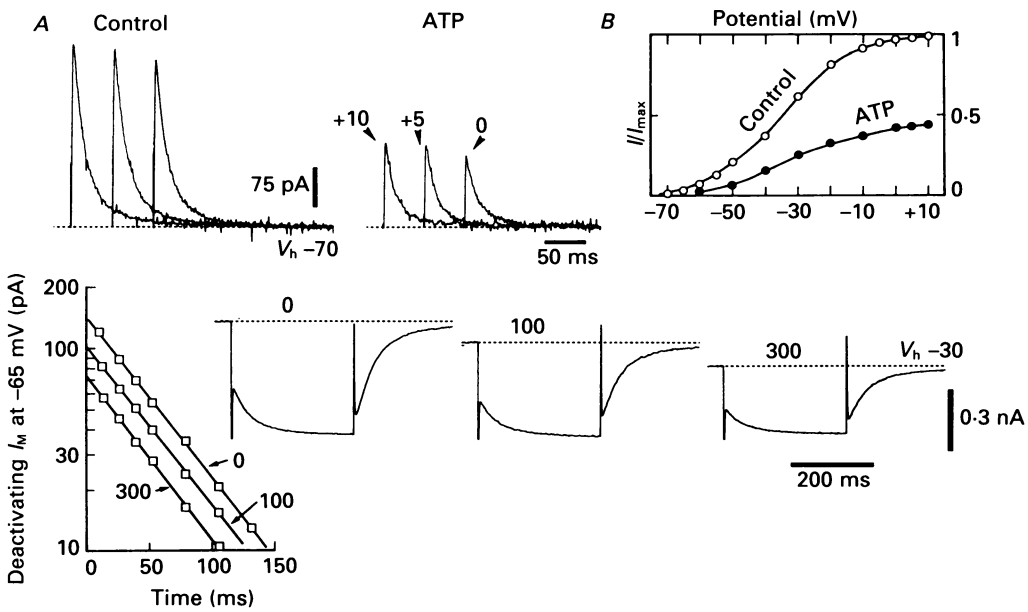

Fig. 10. Depression of the maximum M-conductance by ATP. Results in *A* and *C* were obtained from two different cells. *A*, the deactivating M-currents upon stepping back to the holding potential (V_h) of −70 mV from +10, +5 and 0 mV are superimposed. Traces on the left denote the control tail currents, and those in the presence of ATP (1 μM) are shown on the right. *B*, the steady-state activation curve of the M-current in control (○) and in the presence of ATP (1 μM; ●). Results were obtained from three different cells. Ordinate denotes the amplitude of the outward tail current (*I*) relative to its maximum value (I_{max}). Abscissa denotes the membrane potential. Each circle represents the mean value obtained from three different cells. V_h was −70 mV. 90 mM-TEA was present throughout the experiments in *A* and *B*. *C*, deactivating time course of the M-current at −65 mV in control and in the presence of ATP (100 and 300 nM). Corresponding M-current is shown on the right. The concentration of ATP is indicated beside each line and trace in nM. 30 mM-TEA was present in *C*.

Receptor subtypes

External APP(NH)P (30 μM) was ineffective on the M-current ($n = 4$; Fig. 11*A*). α,β-Methyleneadenosine 5′-trisphosphate (α,β-Met-ATP; 30 μM) was approximately one-tenth as potent as ATP (3 μM; $n = 3$). ADP (0·1–30 μM) was as effective as ATP ($n = 10$; Fig. 11*A*) whereas AMP (10–100 μM) failed to depress the M-current ($n = 6$). Adenosine (10–30 μM; $n = 3$) and 2-chloroadenosine (10–30 μM; $n = 3$) did not affect the M-current (Fig. 11*B*).

Coupling between ATP receptors and the M-current

Pertussis toxin-insensitive GTP-binding protein

In all seven cells tested, ATP-induced inhibition of the M-current was *irreversible* when an unhydrolysable GTP analogue, guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S; Jakobs, Gehring, Gaugler, Pfeuffer & Schultz, 1983; 10–30 μM), was present in the pipette solution (Fig. 12). The holding current at −30 mV showed an inward shift irreversibly even when the bath application of ATP (1–3 μM) was discontinued.

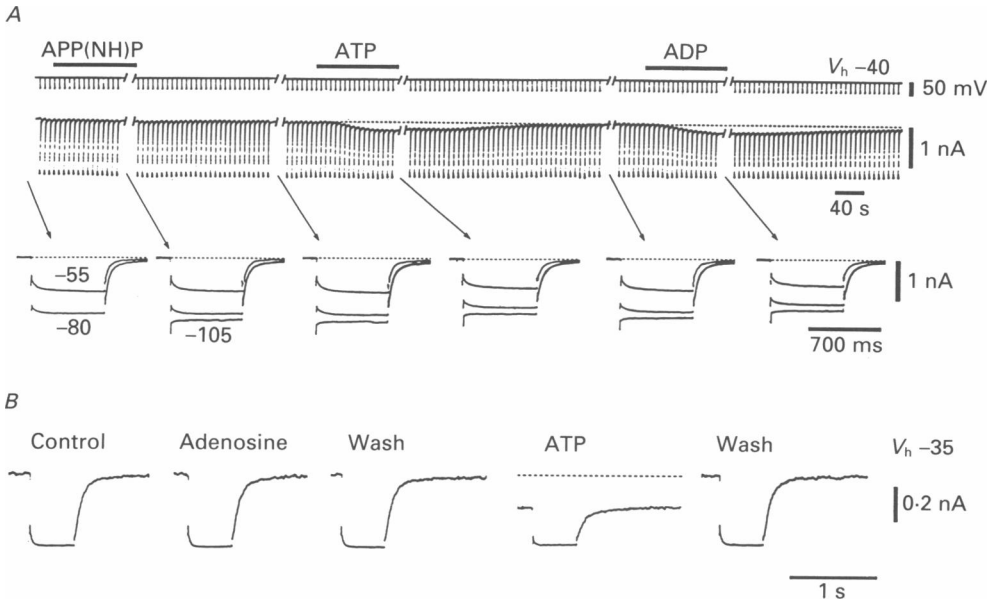

Fig. 11. Agonists for the ATP receptor. Results in *A* and *B* were obtained from two different cells. *A*, APP(NH)P (30 μM) failed to block the M-current whereas ATP (100 nM) and ADP (100 nM) clearly inhibited the M-current. The holding potential (V_h) was −40 mV. The M-currents at command potentials of −55, −80 and −105 mV are shown. *B*, the cell was hyperpolarized from V_h −35 to −70 mV. Adenosine (10 μM) failed to block the M-current while ATP (1 μM) approximately halved the M-current amplitude.

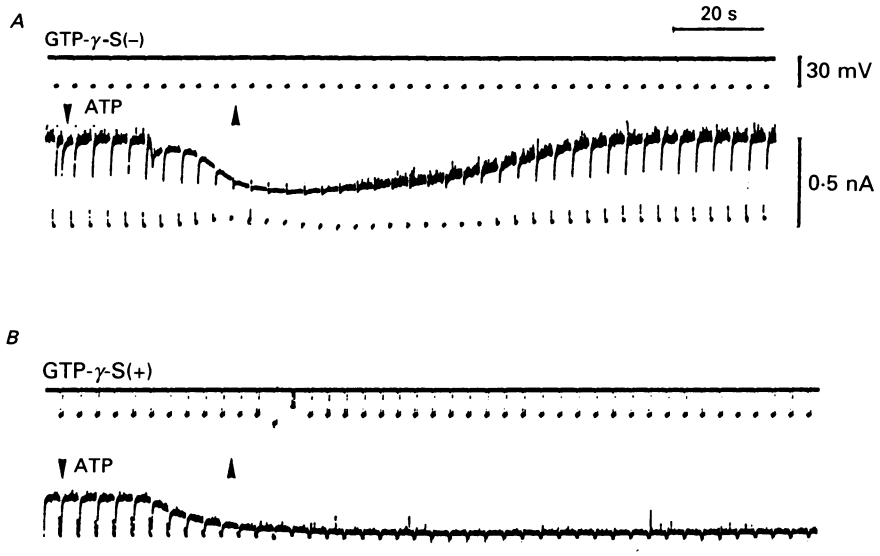

Fig. 12. Involvement of a GTP-binding protein between P_2 receptors and the M-current. Results in *A* and *B* were obtained from two different cells. The holding potential was −30 mV and the cells were hyperpolarized to −60 mV during the step commands. The pipette solution contained GTP-γ-S (30 μM) in *B*. ATP (3 μM) was added to the 'standard' extracellular solution between the two arrows in *A* and *B*. ATP caused the irreversible inhibition of the M-current only when GTP-γ-S was present in the pipette solution.

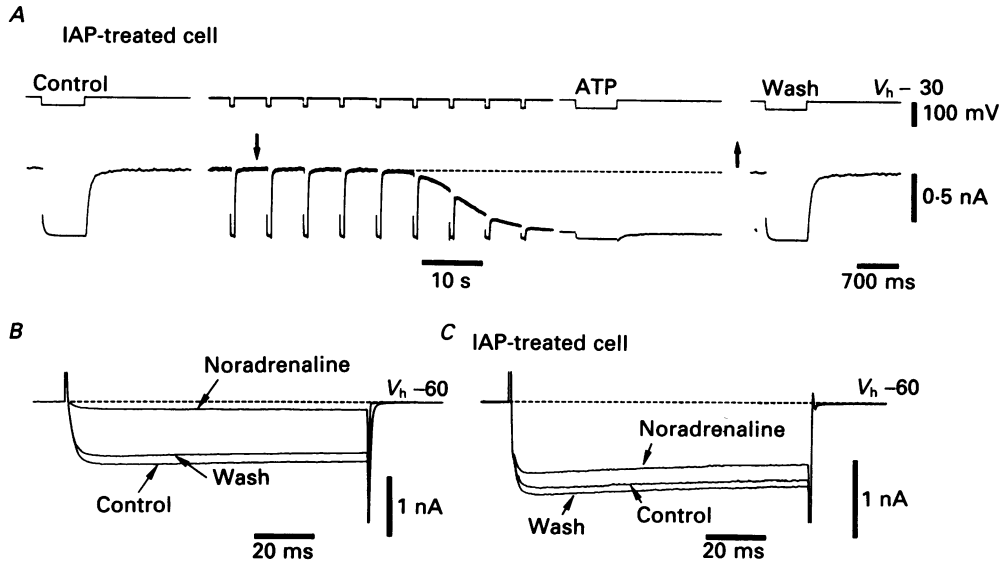

Fig. 13. IAP-insensitive GTP-binding protein involved in P_2 receptors. Results in *A–C* were obtained from three different cells. *A*, the cell was hyperpolarized from a holding potential (V_h) of −30 to −65 mV. ATP (10 μM) still inhibited the M-current to about 11% even when the cell was pre-treated with IAP (500 ng ml^{-1}) for 24 h. *B* and *C*, the cells were depolarized from V_h −60 to −20 mV (*B*) and −15 mV (*C*) for 100 ms. *B*, noradrenaline (50 μM) decreased the peak amplitude of the inward barium current at −20 mV to about 12%. This is the control experiment for the pre-treatment with IAP. *C*, the cell was pre-treated with IAP (500 ng ml^{-1}) for 9 h at 24 °C. Noradrenaline (50 μM) now decreased the peak amplitude of the barium current at −15 mV only to about 85%.

In contrast, ATP (3 μM) and ADP (3 μM) reduced the amplitude of the M-current only by about 10% when guanosine-5′-*O*-(2-thiodiphosphate) (GDP-β-S; Eckstein, Cassel, Levkovitz, Lowe & Selinger, 1979; 100 μM) was present in the pipette solution ($n = 4$).

The cells were pre-treated with islet-activating protein (IAP; 500 ng ml^{-1}) for 24 h at room temperature (≃ 24 °C). In all six of these cells, the amplitude of the M-current was reduced to 53±3% and 11±1% of its respective control by 1 μM- and 10 μM-ATP (Fig. 13*A*).

IAP-sensitive G-protein mediating the noradrenaline-induced calcium current inhibition

The main purpose of the experiments described in this section was to demonstrate that a pre-treatment of the cells with IAP is, in fact, effective in preventing a receptor-mediated response caused by transmitters other than ATP in cultured bull-frog DRG neurones. For this purpose, we have chosen noradrenaline since previous studies in embryonic chicken DRG neurones have shown that noradrenaline inhibits an inward calcium current via an IAP-sensitive GTP-binding protein (Holz, Rane & Dunlap, 1986*a*; Rane & Dunlap, 1986; Hockberger, Toselli, Swandulla & Lux, 1989). Noradrenaline (50 μM) reduced the amplitude of the inward calcium channel current (barium ions as charge carriers; see Methods) between −20 and −10 mV to 13±3% ($n = 4$) in control experiments (Fig. 13*B*). After pre-incubation with IAP (500 ng ml^{-1}) at 24 °C for about 9 h, noradrenaline (50 μM) now decreased the barium current amplitude only to about 81±4% ($n = 4$; Fig. 13*C*).

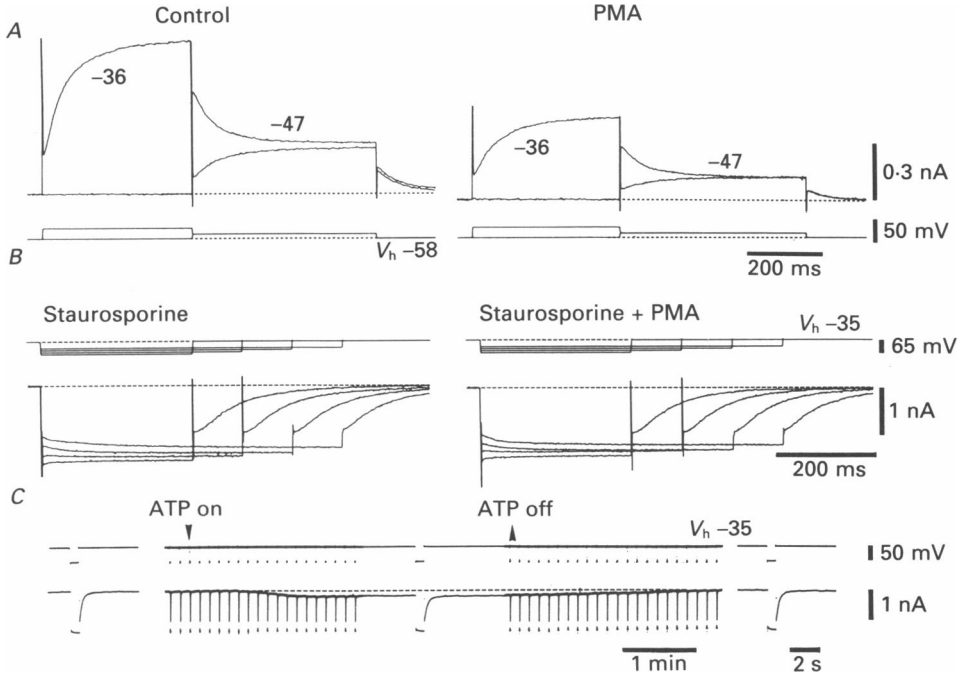

Fig. 14. Involvement of protein kinase-C. Results in *A–C* were obtained from three different cells. *A*, phorbol 12-myristate 13-acetate (PMA, 1 μM) reduced both the activating M-current at −47 and −36 mV and the deactivating M-current at −47 and the holding potential (V_h) of −58 mV. The steady-state M-current at −58 mV was also blocked as judged by the inward shift of the holding current at −58 mV. The control M-current was regained about 25 min after wash-out of the cell with PMA-free 'standard' solution (not shown here). *B* and *C*, results were obtained with a pipette solution containing staurosporine (200 nM). *B*, four current traces represent the M-current in response to step hyperpolarizations from V_h −35 mV to −70 (the longest pulse), −80, −90 and −100 mV (the shortest pulse). Current traces on the left denote the M-current approximately 30 min after the rupture of the membrane patch. In these conditions, PMA (3 μM) now decreased the M-current amplitude by less than 10%. *C*, similar experiment to that described in *B* but with 10 μM-ATP instead of PMA. The cell was hyperpolarized from V_h −35 to −85 mV. ATP was added to the superfusate between the two arrows. On three occasions, the chart speed was increased to observe the M-current on a faster time scale.

Protein kinase-C activator

In view of the proposal that protein kinase-C is a second messenger involved in controlling the M-current (Tsuji, Minota & Kuba, 1987; Brown, 1988*a*; Brown, Marrion & Smart, 1989), the effects of phorbol 12-myristate 13-acetate (PMA), an activator of the protein kinase-C (Castagna, Takai, Kaibuchi, Sano, Kikkawa & Nishizuka, 1982; Nishizuka, 1984), on the M-current were tested. PMA (1–3 μM) approximately halved the amplitude of the M-current ($n = 3$; Fig. 14*A*). The activation and deactivation time constants (τ_{on} and τ_{off}) of the M-current were not significantly affected by PMA (Fig. 14*A*).

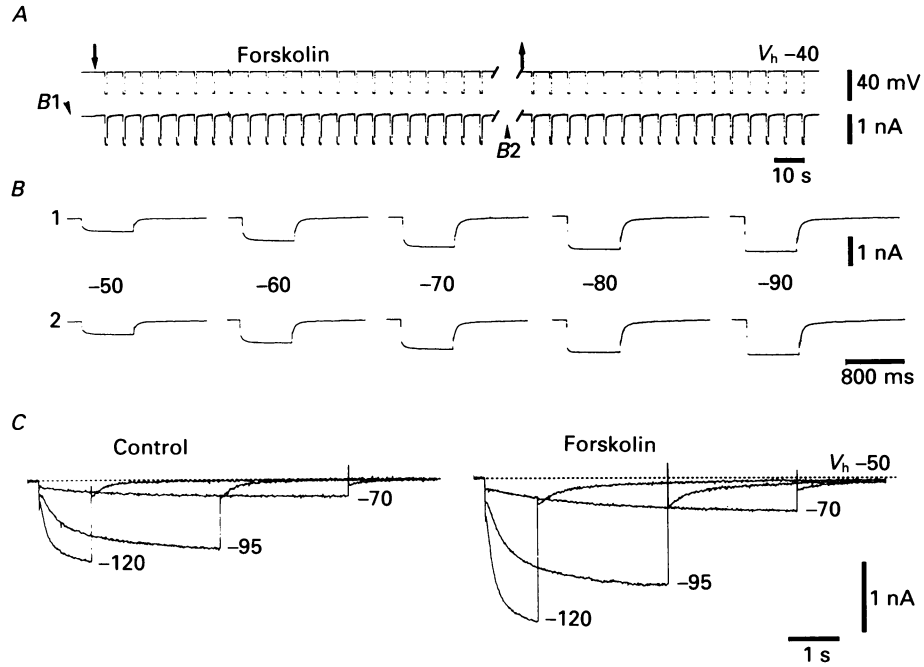

Fig. 15. Forskolin actions on M- and H-currents. *A*, the cell was held at V_h −40 mV and hyperpolarized during the step commands to −70 mV. Forskolin (10 μM) was added to the 'standard' solution for 10 min between the two arrows. The original chart during the final 8 min is not shown here (indicated by —//—). On two occasions (indicated by *B*1 and *B*2), the effect of forskolin of the *I–V* curve was tested. Sample recordings are shown in *B*. The *I–V* curve shown as *B*2 was taken from the last 40 s during the 10 min drug application. Forskolin did not have any significant effect on the M-current observed at five different commanding levels. *C*, results were obtained from another cell superfused with a nominally calcium-free solution (see Methods). The H-current was activated during step hyperpolarization from V_h −50 to −70, −95 and −120 mV (indicated beside each current trace). Note that the activation of the current was accelerated whereas the deactivation of the current was slowed down by 10 μM-forskolin.

Protein kinase-C inhibitor

Staurosporine, an inhibitor of protein kinase-C with an IC_{50} of 3 nM, and of other kinases at slightly higher concentrations (Tamaoki, Nomoto, Takahashi, Kato, Morimoto & Tomita, 1986), was used to test whether this protein kinase inhibitor attenuates the actions of PMA and ATP on the M-current. For this purpose, a pipette solution containing staurosporine (200 nM) was used and the cells were held for at least 20–30 min in the whole-cell configuration to allow the inhibitor to diffuse into the cytoplasm. Under these conditions, PMA (3 μM) decreased the M-current amplitude only by less than 10% in all five cells tested (Fig. 14*B*). Furthermore, 10 μM-ATP reduced the reactivating M-current amplitude, measured on stepping back from −85 mV to the holding potential of −40 mV, to only 85±3% when staurosporine (200 nM) was present in the pipette solution ($n = 3$, Fig. 14*C*) (13±2% in control experiments, $n = 6$).

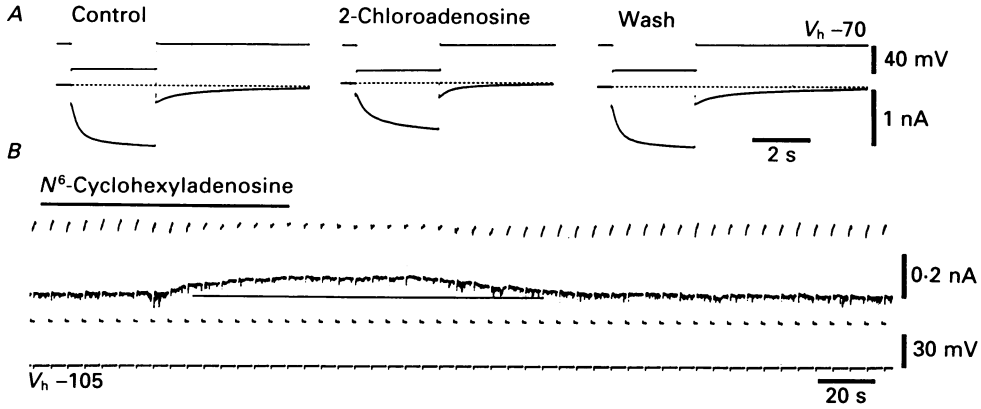

Fig. 16. Depression of the H-current mediated by A_1 receptor agonists. In *A* and *B*, the H-current was isolated from other membrane currents in a nominally calcium-free solution (see Methods). *A*, 2-chloroadenosine (1 μM) reduced the amplitude of the activating H-current at −105 mV and the deactivating H-current at a holding potential (V_h) of −70 mV. *B*, data were obtained from another cell. The cell was clamped at V_h −105 mV and depolarized to −65 mV during the step commands. N^6-Cyclohexyladenosine (10 nM) caused an outward shift of the holding current, which was associated with a decreased chord conductance between −65 and −105 mV.

Adenylate cyclase

Forskolin (10 μM), known to activate adenylate cyclase (Seamon & Daly, 1981), did not significantly affect the M-current ($n = 3$; Fig. 15*A* and *B*) while markedly increasing the amplitude of the H-current (Fig. 15*C*; see Tokimasa & Akasu, 1990*a*). This implies that cyclic AMP-dependent protein kinase is not a second messenger for the sensory M-current (but see Sims, Singer & Walsh, 1988).

A_1 agonists inhibit the H-current

In three cells, 2-chloroadenosine (0·3–1 μM) reversibly decreased the amplitude of the H-current (Mayer & Westbrook, 1983; Fig. 16*A*). Bath application of N^6-cyclohexyladenosine (10 nM), a selective agonist for A_1 purinoceptors (van Calker, Muller & Hamprecht, 1979; Daly, 1982), inhibited the inward H-current at −105 mV and thereby caused an outward shift of the holding current which was associated with a decreased membrane conductance ($n = 2$; Fig. 16*B*). The H-current was not significantly affected by ATP (10 μM) ($n = 5$).

The H-current inhibition by 2-chloroadenosine (1 μM) was associated with a hyperpolarizing shift in the half-activation voltage from -93 ± 2 to -100 ± 2 mV in three cells (paired *t* test, $P < 0{\cdot}05$). The activation of the H-current became faster with increased negativity of the command potential (e-fold speeding-up per $\simeq 25$ mV, $n = 3$). In contrast to the onset, the deactivation of the current became faster with increased positivity of the holding potential level (e-fold speeding-up per $\simeq 15$ mV, $n = 3$). The slowing-down in the activation time course and the speeding-up in the deactivation time course of the H-current by 2-chloroadenosine (Fig. 16*A*) strongly suggested that A_1 agonists may also produce a displacement in the voltage dependence of the activation–deactivation time courses of the current to a hyperpolarizing direction.

DISCUSSION

The results in the present study leave no doubt that the M-current is present in vertebrate primary afferent neurones and that it can be regulated by ATP.

Properties of the sensory M-current

Three lines of evidence in the present study have indicated that the voltage dependence of the sensory M-current was essentially the same as that which has been reported for the sympathetic M-current (Adams *et al.* 1982*a*). First, the current was non-inactivating. Second, the instantaneous *I–V* curve for the steady-state M-conductance was linearly related to the membrane potential, implying the unitary M-conductance may lack an intrinsic rectification. Finally, the sensory M-current had essentially the same activation and deactivation kinetics as that of the sympathetic M-current. The pharmacological profile of the sensory M-current is also compatible with that for the sympathetic M-current in terms of its sensitivity to muscarine and barium (Adams *et al.* 1982*b*).

The M-current may balance the H-current

Previous studies in cultured rat DRG neurones have speculated for some neurones that the resting potential may be determined by counterbalancing two voltage-dependent conductances (Mayer & Westbrook, 1983; Mayer, 1986). One is the H-current exerting the depolarizing influence and the other is a non-inactivating outward current exerting the hyperpolarizing influence. Although direct evidence is still lacking, the M-current has been thought to be one suitable candidate (Mayer, 1986; Brown, 1988*b*). We have shown that this may be the case in bull-frog DRG neurones for four reasons. First, the M-current co-existed with the H-current in a single cell (see Tokimasa & Akasu, 1990*a*). Second, the bottom of the activation curve of the M-current was −70 mV (Fig. 4). Third, approximately 5% of the H-conductance is activated at −70 mV. Finally, caesium (1–2 mM) consistently produced a membrane hyperpolarization associated with an increased cell input resistance (Fig. 1*D*). In this context it would be interesting to learn whether the M-current is still lacking in cultured rat DRG neurones even when the pipette solution contains ATP (Mayer, 1986).

ATP-induced depolarization in intact DRG neurones

The ATP-induced depolarization (or inward current) has been reported to reverse its polarity at potential more negative than −90 mV in intact DRG neurones (Morita *et al.* 1984). We have shown that 2-chloroadenosine and N^6-cyclohexyladenosine reduce the amplitude of the H-current (Fig. 16). This implies that the extremely high concentration of ATP (1 mM) used by Morita *et al.* (1984) may have activated concomitantly P_2 and A_1 receptors and thereby inhibited the M-current (between −70 and 0 mV) and the H-current (between −60 and −130 mV). The depression of the H-current may have looked like the ATP-induced hyperpolarization (or outward current such as that in Fig. 16*B*) at potentials negative to −90 mV.

Morita *et al.* (1984) have demonstrated that ATP causes an outward shift of the

holding current at −132 mV even in C-cells. Since the H-current was hardly detected in small spheroidal cells (most likely C-cells), it is quite possible to develop a schema where ATP inhibits additional membrane currents present at very negative potentials with respect to rest. The identity of such membrane currents in C-cells should be examined by further experiments.

Acetylcholine-induced depolarization in intact DRG neurones

Previous studies in intact bull-frog DRG neurones have demonstrated that acetylcholine (ACh) induces an atropine-sensitive membrane depolarization in C-type DRG neurones, which reverses its polarity to a hyperpolarization as the membrane is hyperpolarized to −100 mV or more (Morita & Katayama, 1984). Therefore, simultaneous inhibition of the M-current and the H-current by the muscarinic action of ACh cannot account for the presence of the reversal potential for the muscarinic response. Since all the experiments in the present study were carried out in A-cells (see Methods), further experiments should be carried out in C-cells to examine whether muscarine affects ionic conductances other than the M-current.

Receptor subtypes

The results presented here have generally confirmed that the ATP-induced decrease in the membrane conductance could be mimicked by ADP and α,β-Met-ATP but not by AMP and adenosine (Morita *et al.* 1984). This indicates that P_2 purinoceptors (Burnstock, 1978) are responsible for the M-current inhibition by ATP and ADP. It seems that a hydrolysable form of ATP may be necessary to activate P_2 receptors since an unhydrolysable ATP analogue APP(NH)P did not inhibit the M-current.

Coupling between P_2 receptors and the M-current

Recent studies in dissociated sympathetic neurones have demonstrated that the IAP-insensitive GTP-binding protein (G-protein; Gilman, 1987) is involved in the muscarinic receptor-mediated inhibition of the M-current (Pfaffinger, 1988; Brown *et al.* 1989). Our observations have suggested that the IAP-insensitive G-protein is also involved in the purinoceptor-mediated M-current inhibition.

Three lines of evidence in the present study have suggested that protein kinase-C rather than protein kinase-A could be involved in the signal transduction between P_2 purinoceptors and the M-current. First, a protein kinase-C activator PMA (1–3 μM) approximately halved the M-current amplitude and the PMA action on the M-current was significantly attenuated when an inhibitor of protein kinase-C, staurosporine (200 nM), was present in the pipettes. Second, the magnitude of the ATP-induced M-current inhibition was also attenuated by staurosporine (200 nM in the pipettes). Finally, an adenylate cyclase activator forskolin (10 μM) did not significantly affect the M-current while the drug clearly facilitated the H-current (Tokimasa & Akasu, 1990*a*). However, recent studies in bull-frog sympathetic neurones have proposed that protein kinase-C may not necessarily be a true second messenger for the muscarinic receptor and the peptidergic receptor-mediated

inhibition of the M-current (Pfaffinger, Leibowitz, Subers, Nathanson, Almers & Hille, 1988; Bosma & Hille, 1989). Hence, further experiments might be needed to reach conclusive evidence in favour of our hypothesis.

In conclusion, we propose that P_2 purinoceptor-mediated M-current inhibition is the most likely mechanism by which ATP produces a sustained membrane depolarization in intact amphibian primary afferent neurones. We also propose that protein kinase-C could be involved in signal transduction between ATP receptors and the M-channels.

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