Regulation of M-Type Potassium Current by Intracellular Nucleotide Phosphates

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The effects of intracellular application of various concentrations of adenine nucleoside phosphates and nucleotide analogs on the M-type K current (I_M) of single neurons isolated from sympathetic ganglia were studied. With 1 mM MgATP intracellularly I_M decreased to 25% of its initial level 39 min after the start of whole-cell recording. In the absence of ATP the current decreased more rapidly. Addition of glucose and pyruvate extracellularly was equivalent to adding 1 mM MgATP intracellularly. AMP-PNP, a nonhydrolyzable ATP analog, at a concentration of 1 or 3 mM was unable to maintain I_M in the absence of ATP. When ATP and AMP-PNP were combined in the pipette, however, the maintenance of I_M was prolonged. A series of nucleotides and analogs have been combined with ATP to test for their ability to maintain I_M and to alter calcineurin phosphatase activity. There was a positive correlation between the ability of a nucleotide to prevent the rundown of I_M and its ability to inhibit calcineurin phosphatase activity. These findings show that the amplitude of I_M is dually regulated by cellular levels of adenine nucleotide diphosphates and triphosphates. A hydrolyzable form of ATP is necessary to maintain the M current. The maintenance of I_M is further enhanced by the simultaneous presence of ADP or other adenine nucleotides that alter calcineurin activity, but not by higher concentrations of ATP alone. These results are consistent with regulation of I_M by phosphorylation events that maintain I_M and dephosphorylation events that lead to current rundown.

Key words: ATP; M-current; sympathetic neuron; potassium current; calcineurin; nucleotide phosphates; phosphorylation; phosphatase

The M-current (I_M) is a voltage- and time-dependent potassium current that has been observed in a variety of neuronal cell types. I_M was first recorded from sympathetic neurons (Brown and Adams, 1980) and has subsequently been observed in recordings from brain (Halliwell and Adams, 1982; Halliwell, 1986), spinal cord (Murase et al., 1986), and smooth muscle (Sims et al., 1985). I_M is activated at membrane potentials positive to -60 mV and consists of an outward K current that does not inactivate. Compounds that act as agonists at gonadotropin-releasing hormone receptors, muscarinic receptors, substance P receptors, or purinergic receptors inhibit I_M. The precise signal transduction mechanism by which these compounds decrease I_M is not known, but the data clearly suggest that the inhibition of I_M by these receptors follows activation of guanine nucleotide-binding proteins (Pfaffinger, 1988; Brown et al., 1989; Lopez and Adams, 1989; Simmons and Mather, 1992).

Intracellular levels of ATP have been suggested to be involved in the control of I_M; however, the nature of this regulation remains unclear. There are conflicting findings with regard to the role of intracellular ATP in modulating I_M. Pfaffinger (1988) found that when I_M was recorded with electrodes containing a solution that lacked ATP, I_M declined much more rapidly than if the solution suffusing the cell contained ATP. When ATP was replaced by AMP-PNP, the M-current was lost. Tokimasa and Akasu (1990) found that I_M in dorsal root ganglion cells disap-

peared or was reduced to 20% of its initial value after 15 min when ATP was replaced by AMP-PNP in the whole-cell recording electrode. Our results (Simmons et al., 1990), however, showed that when intracellular ATP was replaced by AMP-PNP, the current was maintained at 84% of the initial value after 13 min. Chen and Smith (1992) also found that I_M was not affected when ATP was left out of the pipette solution and that AMP-PCP did not affect the rundown of I_M.

Direct comparisons of previous studies on I_M are difficult, because different laboratories have used different solutions, both intracellularly and extracellularly. Of particular concern are the concentrations of $MgCl_2$, ATP, and GTP added to pipette solutions and the presence or absence of metabolic substrates such as glucose and pyruvate in the extracellular solutions and in the pipette. Here, we examine the role of intracellular ATP, a variety of other nucleotide phosphate analogs, and extracellular pyruvate and glucose in the maintenance of I_M in single neurons.

MATERIALS AND METHODS

Cell isolation. Bullfrogs (*Rana catesbeiana*) were obtained from Charles D. Sullivan Co. (Nashville, TN) and handled according to national and institutional guidelines with the approval of the Institutional Animal Use and Care Committee. To obtain sympathetic neurons, a frog was chilled in ice for 1 hr, rapidly decapitated, and double-pithed. Sympathetic ganglia were dissected and incubated for 90 min in dissociation medium with collagenase A (1 mg/ml) and trypsin (0.5 mg/ml), for 90 min in dissociation medium with collagenase B (0.75 mg/ml), and then stored in growth medium at 4°C for up to 3 d. On the day of the experiment, one or two ganglia at a time were triturated 25–50 times with a long-shanked glass Pasteur pipette to free single cells from the ganglia. The cells were placed in a Petri dish on the stage of an inverted microscope and continuously perfused with extracellular solution.
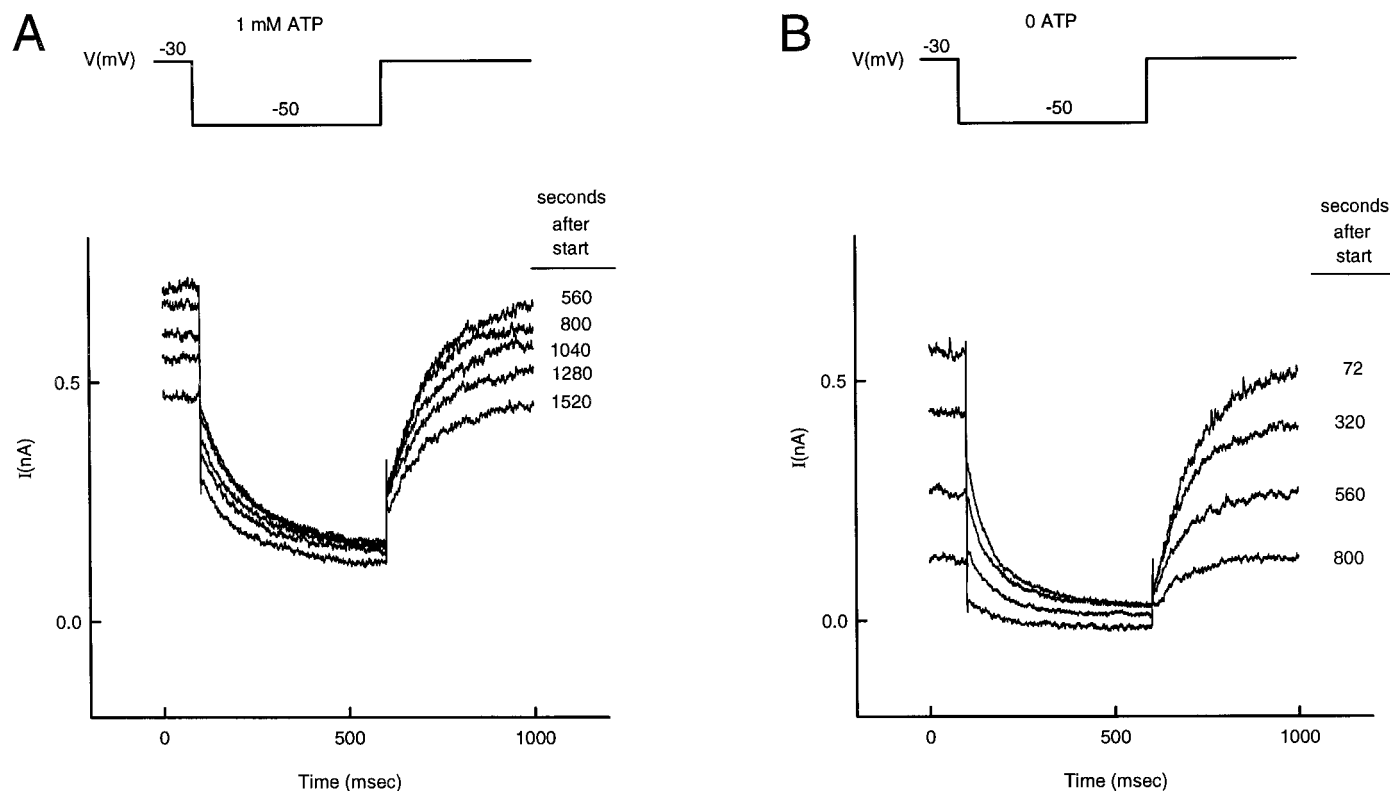
Whole-cell recordings. All experiments were conducted at room temperature, ~20°C. Whole-cell recordings (Hamill et al., 1981) were made

Figure 1. I_M relaxations and time-dependent changes in I_M during a whole-cell recording in the presence and absence of ATP. *A, B,* The *top panels* illustrate the voltage-clamp protocol, whereas the *bottom panels* show the resultant current tracings. *A,* Tracings of I_M at various times after beginning a whole-cell recording with 1 mM MgATP in the pipette. *B,* Tracings of I_M without ATP in the pipette. Note that I_M decreases more rapidly with time in *B* than in *A*.

with electrodes with resistances of 0.25–1 MΩ when filled with intracellular solution. The extracellular solution was controlled by a single-cell superfusion system as described previously (Simmons and Mather, 1991). In selected experiments, intracellular solutions were changed by perfusing the interior of the whole-cell recording electrode as described by Fischmeister and Hartzell (1987). The recordings were filtered at 1 kHz and stored on magnetic tape.

Measurement of I_M. The membrane potential was maintained at a holding potential of −30 mV. Every 8 sec the voltage was stepped to −50 mV for 500 msec followed by return to −30 mV. After a voltage step from −30 to −50 mV, the outward current decreases because of the time- and voltage-dependent closing of M-channels (Fig. 1) (Brown and Adams, 1980). After returning to −30 from −50 mV, the outward current returns exponentially (Fig. 1). The amplitude of this exponential relaxation of current is termed $I_M(−30)$ and provides a measure of I_M that is independent of changes in other currents or "leak" currents (Simmons et al., 1994). Instantaneous and steady-state current amplitudes at −30 and −50 mV were determined by computer.

Quantification of the rundown of I_M. To quantify the time-dependent changes in the amplitude of I_M during an experiment, the current was measured every 8 sec, and the amplitude of $I_M(−30)$ was plotted versus time. The initial current is defined as the maximum current reached after obtaining the whole-cell configuration. The rundown of I_M under various experimental conditions is expressed as the time it took for the amplitude of I_M to decrease to 25% of this initial current value, $T(25\%)$. If the current did not reach 25% of the initial value after 1 hr, the experiment was halted, and the $T(25\%)$ for that cell assigned a value of 60 min.

Solutions for electrophysiology. Composition of solutions is in mM unless otherwise noted. Extracellular solution: NaCl 128, KCl 2.4, CaCl$_2$ 1.8, MgCl$_2$ 1.8, HEPES 10, and TTX 0.0003, pH 7.4 (with NaOH). Dissociation medium: NaCl 98, KCl 2.4, NaH$_2$PO$_4$ 0.6, MgCl$_2$ 1.8, sodium pyruvate 5, creatine 5.7, glucose 5, M199 10 ml/l, penicillin 100 U/ml, streptomycin 100 μg/ml, and HEPES 20, pH 7.4 (with NaOH). Growth medium: NaCl 118, KCl 2.4, creatine 5.7, glucose 5, sodium pyruvate 5, 100× MEM vitamins 10 ml/l, penicillin 100 U/ml, streptomycin 100 μg/ml, 50× MEM essential amino acids 20 ml/L, 100× MEM

nonessential amino acids 10 ml/l, bovine serum albumin 1 mg/ml, and HEPES 20, pH 7.4 (with NaOH). Intracellular (or recording electrode) solution: KCl 120, BAPTA 1, and HEPES 10, pH 6.8 (with KOH), plus MgCl$_2$ and nucleotides.

The amounts of MgCl$_2$ and nucleotides to be added to the pipette solutions were determined by a computer program developed by Robert E. Godt (Medical College of Georgia, Augusta, GA) (Godt and Lindley, 1982) with modifications by H. Criss Hartzell (Emory University, Atlanta, GA). Calculations were made to give a free [Mg] of 0.5 mM and the desired concentrations of MgATP. This program takes into account the stability constants of the various components in a manner similar to that described in Fabiato (1988). In addition to accounting for the concentrations of the various constituents, temperature, pH, and ionic strength are also considered in the calculations. The stability constants for ATP and ADP analogs were taken to be the same as for ATP and ADP, respectively.

Most chemicals were obtained from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Proteolytic enzymes, nucleotides, and leupeptin were obtained from Boehringer Mannheim (Indianapolis, IN).

Measurement of calcineurin activity. Calcineurin phosphatase activity was assayed by measuring the release of *para*-nitrophenol from *para*-nitrophenyl phosphate spectrophotometrically at 405 nm (Pallen and Wang, 1983) in a Vmax kinetic microplate reader from Molecular Devices Corp. (Menlo Park, CA). The assay was performed at 30°C in a 200 μl reaction volume containing 2 μg of purified bovine brain calcineurin (Upstate Biotechnologies, Lake Placid, NY) and 500 nM purified bovine brain calmodulin (Sigma) in buffer consisting of (in mM): 120 KCl, 10 HEPES, 2 dithiothreitol, and 0.35 ATP, pH 8.1. Varying amounts of MgCl$_2$ and adenine nucleotides were added according to the method described above to give buffers containing (in mM): 0.3 ADP, 3 ADP, 1 AMP-PNP, 3 AMP-PNP, or 3 sodium pyrophosphate. Blank wells were prepared containing (in mM) 120 KCl, 0.8 MgCl$_2$, and 10 HEPES, pH 8.1. To monitor background activity, control wells were prepared that contained buffer solutions without calcineurin and calmodulin. The microtiter plate was equilibrated for 5 min at 30°C in a precision convection oven. The reaction was initiated by the addition of 20 mM
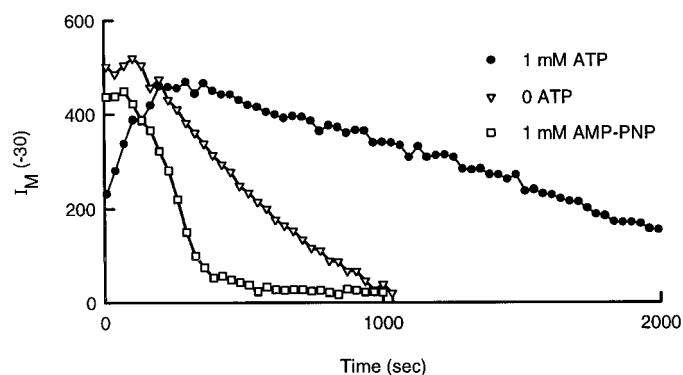
Figure 2. Plot of I_M amplitude as a function of time with and without intracellular ATP and AMP-PNP. *Filled circles*, ATP was included in the pipette solution to provide 1 mM MgATP. *Open triangles*, No ATP was added to the pipette solution. *Open squares*, AMP-PNP was included in the pipette solution to provide 1 mM MgAMP-PNP.

Figure 3. Extracellular metabolic substrates rescue I_M from the rundown produced by intracellular AMP-PNP. With no glucose and pyruvate in the extracellular solution and AMP-PNP in the pipette (*open triangles*), I_M decreased in a few hundred seconds. When glucose and pyruvate (5 mM each) were added to the extracellular medium with AMP-PNP in the pipette, rundown was slowed (*filled circles*).

para-nitrophenyl phosphate (Sigma). After incubation at 30° for 60 min, the absorbance values for each well were measured and the background value subtracted to obtain the final optical density values.

Data analysis and statistics. Data are expressed as mean ± SD. Tests for differences among experimental groups were determined by one-way or two-way ANOVA as appropriate followed by Student–Newman–Keuls test for multiple comparisons.
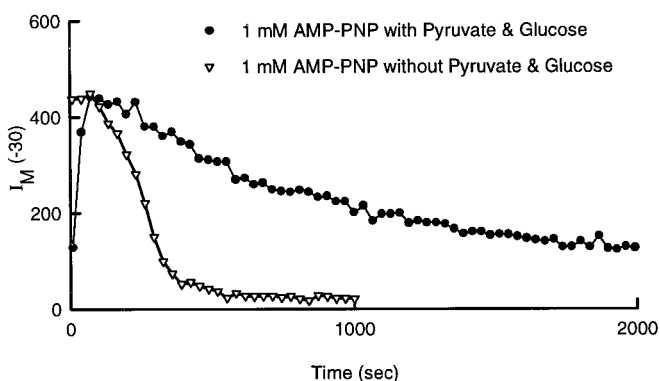
RESULTS

Dependence of I_M on intracellular ATP

Typical cellular levels of MgATP are in the range of 1 mM. Previous studies of I_M have typically used solutions in the recording electrode that would yield approximately this amount of MgATP. The time-dependent changes in I_M observed throughout a recording period with this concentration of MgATP (1 mM) or without ATP in the recording pipette are shown in Figure 1. In this experiment, when the electrode contained 1 mM MgATP, the current ran down 43%, from a peak of 460 to 265 pA in the first 25 min after the start of whole-cell recording (Fig. 1*A*). When ATP was omitted from the pipette solution, I_M declined more rapidly (Fig. 1*B*), decreasing 81% from 520 to 100 pA within ~13 min. The amplitude of $I_M(-30)$ for the duration of these two experiments is plotted as a function of time in Figure 2. This graph shows that when ATP (1 mM) was included in the pipette (Fig. 2, *filled circles*), the maintenance of I_M was prolonged compared with the amplitude of I_M when ATP was omitted from the pipette (Fig. 2, *open triangles*). In 12 cells tested with 1 mM MgATP in the pipette, the time it took to decline to 25% of the initial current level, the $T(25\%)$, was 39.0 ± 19.0 min. In 10 cells with no ATP added, the $T(25\%)$ was 12.8 ± 3.5 min.

Maintenance of I_M requires ATP hydrolysis

There are two obvious mechanisms by which ATP may be used to prevent rundown of I_M: ATP could serve as a phosphate donor in a phosphorylation reaction that maintains the M-current, or the energy from ATP hydrolysis could be required to maintain I_M. Either of these mechanisms would require the availability of a hydrolyzable form of ATP. To determine whether hydrolyzable ATP was required to maintain I_M, the ATP in the pipette was replaced by AMP-PNP, a nonhydrolyzable ATP analog. I_M disappeared more rapidly when the electrode contained MgAMP-PNP at a concentration of 1 mM (Fig. 2, *open squares*) than when it contained MgATP (1 mM) (Fig. 2, *filled circles*). The average $T(25\%)$ with 1 mM MgAMP-PNP in the cell was 8.9 ± 5.3 min

($n = 6$). Increasing the intracellular concentration of AMP-PNP to 3 mM resulted in a rapid rundown of the current with an average $T(25\%)$ of 7.6 ± 3.5 min ($n = 20$).

Extracellular glucose and pyruvate are equivalent to intracellular ATP

The finding that AMP-PNP resulted in a rapid disappearance of I_M was in contrast to our earlier finding that I_M was maintained at 84% of control after 13 min when AMP-PNP replaced ATP in the pipette (Simmons et al., 1990). In the previous study, 5 mM pyruvate and 5 mM glucose had been included in the extracellular solution, whereas in the present study glucose and pyruvate were omitted. Thus, it was possible that in our earlier studies the cells were utilizing the available pyruvate and glucose to synthesize enough ATP to maintain I_M in the presence of intracellular AMP-PNP. To test this possibility, glucose and pyruvate were added to the bath solution, and the effect of AMP-PNP was reexamined. When glucose and pyruvate (5 mM each) were present, the decrease in I_M observed with 1 mM AMP-PNP in the cell was slowed from a $T(25\%)$ of 8.9 ± 5.3 min ($n = 6$) to a $T(25\%)$ of 21.2 ± 14.7 min ($n = 7$) (Fig. 3).

When either one of these energy substrates alone was added to the extracellular perfusate, the decrease of I_M with time was intermediate between that seen with both present and that seen with neither present. With 5 mM glucose the $T(25\%)$ was 11.3 ± 5.2 min ($n = 7$), and with 5 mM pyruvate the $T(25\%)$ was 11.7 ± 5.9 min ($n = 6$). With pyruvate and 2-deoxy-D-glucose the $T(25\%)$ was similar to the result seen with pyruvate alone (11.2 ± 3.4 min; $n = 13$).

Concentration–effect curve for ATP

To determine the dependence of I_M on the concentration of intracellular ATP, electrodes were filled with solutions containing different amounts of MgATP. The $T(25\%)$ with various [MgATP] in the pipette is plotted in Figure 4 (*open circles*). The maintenance of I_M was prolonged as [MgATP] was increased from 0 to 1 mM. With a further increase of [MgATP] to 3 mM, however, the decrease of I_M with time was unchanged.

A combination of intracellular AMP-PNP with intracellular ATP slows the rundown of I_M

In contrast to the findings with AMP-PNP alone, when AMP-PNP and ATP were combined in the pipette a different story
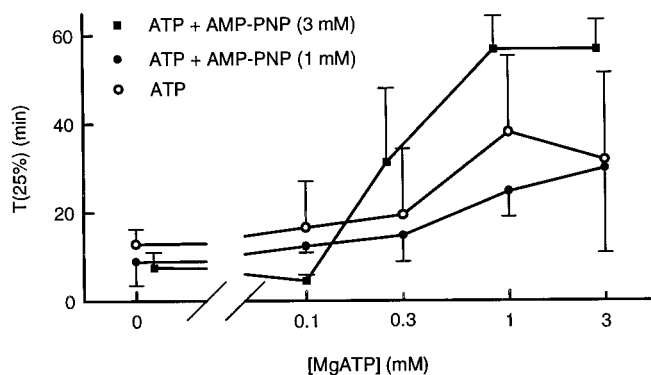
Figure 4. Dose–response curves for the effects of ATP with various concentrations of AMP-PNP. *Open circles*, ATP alone in the pipette; $n = $ 9–12 for each point. *Filled circles*, ATP plus 1 mM AMP-PNP in the pipette; $n = 4$–8 for each point. *Filled squares*, ATP plus 3 mM AMP-PNP in the pipette; $n = 5$–20 for each point. Two-way ANOVA showed that the effect of ATP was significant ($p < 0.0001$), the effect of AMP-PNP was significant ($p < 0.0002$), and there was a significant interaction between the effects of ATP and AMP-PNP ($p < 0.0001$).

Figure 5. Reversal of rundown of I_M produced by intracellular AMP-PNP by intracellular perfusion with ATP. A whole-cell recording from a single sympathetic neuron was inititated at time 0 with an electrode containing 3 mM MgAMP-PNP and 0 ATP. As typically observed with AMP-PNP, the current ran down. At the time indicated by the *diamond*, the solution perfusing the whole-cell recording electrode was changed to one containing 3 mM AMP-PNP plus 1 mM ATP. Addition of ATP intracellularly halted and partially reversed the rundown produced by AMP-PNP alone.

emerged. In Figure 4 the *filled symbols* show the concentration-effect curve for MgATP in the presence of a constant concentration of 1 mM MgAMP-PNP (*filled circles*) or 3 mM MgAMP-PNP (*filled squares*).

The concentration–effect curve for ATP exhibited a downward shift in the presence of 1 mM MgAMP-PNP. At a concentration of 3 mM, however, MgAMP-PNP in the simultaneous presence of MgATP at concentrations of ≥0.3 mM resulted in a sustained maintenance of I_M. With 1 mM MgATP and 3 mM MgAMP-PNP, there was essentially no rundown of the current during a 1 hr recording period. This compares to a rundown to 25% in 39 min with 1 mM MgATP alone or in 7.6 min with 3 mM MgAMP-PNP alone.
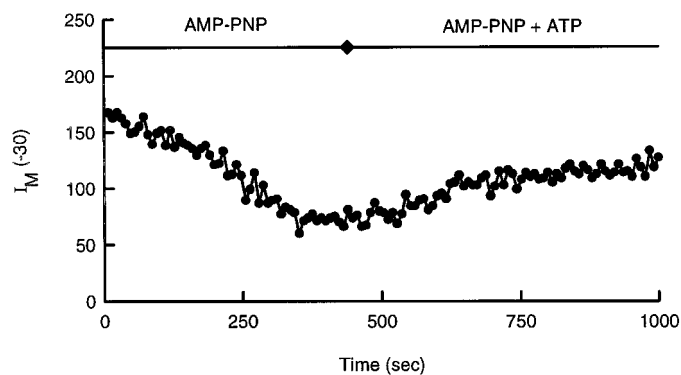
The rapid rundown observed with 3 mM AMP-PNP could be reversed by intracellular perfusion of 1 mM ATP along with 3 mM AMP-PNP. This experiment was repeated in five cells. In all cases the rundown normally observed with 3 mM AMP-PNP was halted when ATP was perfused intracellulary. In four of the five cells the current rundown was not only halted but was also reversed, as illustrated in Figure 5.

These results suggest that there are dual effects of adenine nucleotides on I_M. Maintenance of I_M requires a hydrolyzable form of ATP, because a nonhydrolyzable analog alone is insufficient to maintain I_M. In the presence of ATP, I_M is further regulated by the simultaneous presence of nonhydrolyzable adenine nucleotides.
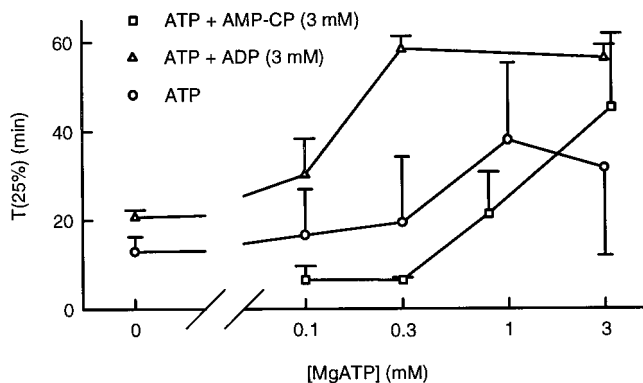
To see whether other nucleotides or analogs might be more effective than AMP-PNP in producing this effect, we have tested the effects of a variety of other nucleotide analogs on I_M.

Effects of adenine nucleoside diphosphate analogs on the rundown of I_M

Concentration–effect curves for MgATP have been constructed in the presence of a constant concentration of ADP or AMP-CP (3 mM). In these experiments, Mg and ATP were added at concentrations calculated to provide 0.5 mM free Mg and the desired amount of MgATP. Of the 3 mM added ADP or AMP-CP, 1 mM is predicted to be free ion, 0.8 mM complexed to Mg ions, and the remainder complexed to other metals. Figure 6 shows a comparison of the effects of ATP alone (*open circles*) and in the presence of 3 mM ADP (*open triangles*) or 3 mM AMP-CP
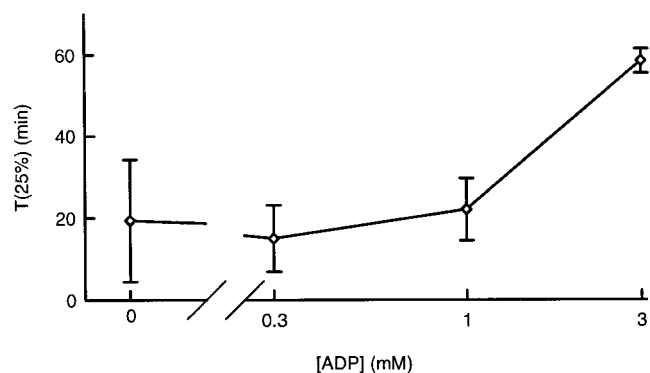

Figure 6. Dose-response curves for the effects of ATP in the presence of ADP and an ADP analog. *Open circles*, ATP alone in the pipette; $n = $ 9–12 for each point. *Open triangles*, ATP plus 3 mM ADP in the pipette; $n = 3$–4 for each point. *Open squares*, ATP plus 3 mM AMP-CP in the pipette; $n = 3$–5 for each point. Two-way ANOVA for the effects of ATP and ADP showed that the effect of ATP was significant ($p < 0.0001$), the effect of ADP was significant ($p < 0.001$), and the significance of the interaction between ATP and ADP was borderline ($p = 0.054$). Analysis of the effects of ATP and AMP-CP showed that the effect of ATP was significant ($p < 0.0002$), the effect of AMP-CP was not significant ($p > 0.05$), and the significance of the interaction between ATP and AMP-CP was borderline ($p = 0.072$).

(*open squares*). The ability of ATP to slow the rundown of I_M was enhanced in the simultaneous presence of ADP. In contrast to this, the ability of ATP to slow the rundown of I_M was inhibited by 3 mM AMP-CP for ATP concentrations of ≤1 mM but was enhanced when ATP was present at a concentration of 3 mM. A concentration–effect curve for ADP in the presence of 0.3 mM ATP is shown in Figure 7.

From the dose–response curves shown, it is apparent that the ability of compounds to alter the rundown of I_M is most easily observed with 0.3 mM MgATP in the pipette. With 0.3 mM MgATP, the $T(25\%)$ for AMP-CP (3 mM) was 6.4 min, for AMP-PNP (1 mM) was 14.7 min, for MgATP alone was 19.4 min, for AMP-PNP (3 mM) was 31.2 min, and for ADP (3 mM) was 58.3 min. The actions of a variety of other analogs on I_M rundown in the presence of 0.3 mM MgATP have also been tested. The

Figure 7. Dose–response curve for various concentrations of ADP to maintain I_M in the presence of a constant concentration of 0.3 mM MgATP; $n = 11$ at 0 ADP and 4 at the other points. The effect of ADP on the $T(25\%)$ was statistically significant ($p < 0.0002$).

Table 1. Effects of various nucleotides and nucleotide analogs on run down of I_M

	$T (25\%)$ (min)		
Condition	Mean	SD	n
3 mM ADP + 0.3 mM ATP	58.3	2.9	4
3 mM sodium pyrophosphate + 0.3 mM ATP	51.7	17.6	4
3 mM UDP + 0.3 mM ATP	32.4	16.7	3
3 mM AMP-PNP + 0.3 mM ATP	31.2	16.8	5
3 mM ADP + 0.3 mM ATP, 0 Mg	26.2	10.0	3
3 mM AMP + 0.3 mM ATP	24.7	19.4	4
0.3 mM ATP	19.4	14.9	11
3 mM CDP + 0.3 mM ATP	19.0	8.7	4
3 mM sodium pyrophosphate + 0 ATP	14.9	5.0	4
1 mM AMP-PNP + 0.3 mM ATP	14.7	5.8	8
3 mM AMP-PCP + 0.3 mM ATP	14.1	2.8	6
3 mM IDP + 0.3 mM ATP	13.9	5.6	4
3 mM GDP + 0.3 mM ATP	9.8	2.5	3
3 mM cAMP + 0.3 mM ATP	6.6	4.2	4
3 mM AMP-CP + 0.3 mM ATP	6.4	0.6	3

$T(25\%)$ for these compounds is shown in Table 1. The data are listed in order of decreasing $T(25\%)$.

Effects of adenine nucleotides and analogs on other electrophysiological properties

In addition to measuring I_M, several other parameters have been measured to examine the specificity of the effects with respect to I_M. For statistical comparisons, t tests have been made between the data obtained under the various experimental conditions to the data obtained with 0.3 mM ATP. When recording I_M a run-up of current is sometimes observed during the first 30–60 sec of recording (Figs. 2, 3). The time from obtaining whole-cell recording to reaching this initial peak current was not altered by the different nucleotides. Nor was the amplitude of I_M at this initial time point affected. The leak current at this time and at the $T(25\%)$ were also measured and did not differ from the values obtained with 0.3 mM ATP.

Calcineurin activity

It has been shown previously that the activity of the calcium- and calmodulin-dependent phosphatase 2B calcineurin modulates I_M in neurons from rat sympathetic ganglia (Marrion, 1996). It has

Table 2. Effects of various nucleotides and nucleotide analogs on calcineurin phosphatase activity

	OD	
Condition	Mean	SD
3 mM sodium pyrophosphate + 0.3 mM ATP	0.02*	0.03
3 mM AMP-PNP + 0.3 mM ATP	0.23*	0.10
3 mM ADP + 0.3 mM ATP	0.30*	0.09
0.3 mM ATP	0.54	0.17
0.3 mM ADP + 0.3 mM ATP	0.60	0.35
1 mM AMP-PNP + 0.3 mM ATP	0.95*	0.27

A one-way ANOVA showed significant effects of the various solutions on calcineurin activity ($p < 0.001$). Results grouped within the ruled area are not significantly different from one another.
* indicates significantly different from 0.3 mM ATP.
$n = 12$ for each point.
The assay was repeated twice with six wells under each condition.

also been shown that phosphates modulate calcineurin activity (King and Huang, 1984). To test the hypothesis that the effects of intracellular nucleotide phosphates on I_M may be accounted for by the effects of these nucleotides on calcineurin activity, the effects of concentrations of selected nucleotides that were shown to alter I_M above have been tested on calcineurin phosphatase activity. The results of the calcineurin phosphatase assays are shown in Table 2.

Effects of a calcineurin inhibitor

Cyclosporin A is an inhibitor of calcineurin activity (Liu et al., 1991). When applied intracellularly to single neurons with 0.3 mM ATP, cyclosporin A at a concentration of 500 nM increased the $T(25\%)$ from 19.4 ± 14.9 to 30.5 ± 10.7 and to 41.9 ± 15.8 min when added at a concentration of 1 μM. Cyclosporin A (500 nM) had no effect when applied outside the cell [$T(25\%) = 9.1 \pm 1.8$, $n = 2$].

DISCUSSION

The results clearly show that nucleotide phosphates play a critical role in the regulation of I_M. Furthermore, there are at least two ways by which adenine nucleotides can modulate I_M. First, hydrolyzable ATP must be available for I_M to be maintained. Second, above and beyond this requirement for a basal level of ATP, other nucleotide phosphates and analogs act to further modulate the level of I_M. These results are consistent with regulation of I_M by kinases and phosphatases.

Several previous studies have suggested that I_M is regulated by phosphorylation catalyzed by myosin light chain kinase. Application of myosin light chain kinase inhibitors decreases I_M, whereas intracellular perfusion of catalytic subunits of myosin light chain kinase enhances I_M (Akasu et al., 1993; Tokimasa et al., 1995). The result presented here, that ATP is required to prevent the rundown of I_M, extends these findings to suggest that myosin light chain kinase-catalyzed phosphorylation events not only alter the amplitude of I_M but may be required for the maintenance of I_M.

In addition to the positive regulation of I_M by myosin light chain kinase-catalyzed phosphorylation events, I_M has also been shown to be negatively regulated by phosphatase-catalyzed dephosphorylation events (Marrion, 1996). Marrion (1996) showed that I_M was decreased when a catalytically active form of calcineurin was applied intracellularly to rat sympathetic neurons. This suggests that I_M is regulated by the calcium and calmodulin-

dependent phosphatase type 2B, calcineurin, and that this phosphatase activity results in a decrease in the amplitude of I_M.

In the present study, several compounds have been shown to prevent the rundown of I_M. These compounds include ADP, PP_i, and cyclosporin A. The results of the phosphatase assay show that ADP and PP_i, at concentrations that prevent the rundown of I_M, are effective inhibitors of calcineurin phosphatase activity. Cyclosporin A is well established as a calcineurin inhibitor (Liu et al., 1991). These data support the previous findings that I_M is regulated by phosphatase activity (Marrion, 1996) and further illustrate that this phosphatase activity can be regulated by endogenous compounds such as ADP and PP_i. Pyrophosphate and ADP at a concentration of 3 mM significantly inhibited calcineurin phosphatase activity and slowed I_M rundown. A 0.3 mM concentration of ADP changed neither calcineurin activity nor the rundown of I_M.

This conclusion is further supported by the results obtained with AMP-PNP. The effect of AMP-PNP depended on both its concentration and on the concentration of ATP present. As intracellular ATP was increased, the maintenance of I_M was prolonged. When AMP-PNP at a concentration of 1 mM was added along with ATP, the ability of ATP to maintain I_M was decreased. When AMP-PNP at a concentration of 3 mM was added along with ATP at concentrations of ≥ 0.3 mM, however, the maintenance of I_M was enhanced. These results can be interpreted in accordance with the effects of AMP-PNP on calcineurin activity. At a concentration of 1 mM, AMP-PNP increased calcineurin activity and speeded I_M rundown. At 3 mM, AMP-PNP decreased calcineurin activity and slowed I_M rundown.

Although there is qualitative agreement between the effects of the various nucleotides in the calcineurin assays and their effects on I_M rundown, there are quantitative differences. For example, 3 mM ADP and 3 mM AMP-PNP have equivalent, less than PP_i, effects in the calcineurin assay. On I_M rundown, the effect of ADP is greater than that of PP_i, whereas the effect of AMP-PNP is less than PP_i. This difference might be accounted for by the different situations under which these two measurements were made. The bovine brain calcineurin *in vitro* is clearly under different conditions than the calcineurin in a bullfrog sympathetic ganglion cell undergoing whole-cell recording.

Importance of considering the role of pyruvate and glucose in physiological solutions on ionic currents

Many studies of ionic currents in single cells use whole-cell recordings. With this technique, it is generally assumed that the electrode contents effectively suffuse the cell and thereby control the cellular contents. Metabolic substrates such as glucose and pyruvate are routinely added to either the extracellular medium or to the pipette solution during such experiments. The data presented here demonstrate that these additives can have important modulatory effects on ionic currents. These effects may occur even when the pyruvate and glucose are only added extracellularly and recordings are made with large electrodes that would be expected to provide good control of intracellular constituents.

The ability of extracellularly applied pyruvate and glucose to substitute for intracellular ATP shows that enzymatically dissociated single neurons maintain active metabolic processes. The $T(25\%)$ in the presence of extracellular pyruvate and glucose and intracellular AMP-PNP was 21.2 min. A similar $T(25\%)$ of 24.6 min was obtained with 1 mM MgATP and 1 mM AMP-PNP. Thus,

adding 5 mM glucose and 5 mM pyruvate to the extracellular medium is equivalent to putting 1 mM MgATP in the pipette.

This can account for some of the discrepancies observed in previous work from different laboratories. Those studies that had reported I_M was maintained without ATP had 5 mM pyruvate and 5 mM glucose in the extracellular solution (Simmons et al., 1990) or 10 mM glucose in the extracellular solution and 10 mM glucose in the pipette solution (Chen and Smith, 1992). Those studies that had reported that I_M disappeared in the absence of ATP (Pfaffinger, 1988; Tokimasa and Akasu, 1990) did not add either pyruvate or glucose to their solutions.

These data also show that ATP is required for the maintenance of I_M as suggested previously (Pfaffinger 1988; Tokimasa and Akasu 1990). The finding that the M current was maintained in the presence of intracellular ATP, but not with AMP-PNP alone, supports previous work showing modulation of I_M by kinases and phosphatases (Akasu et al., 1993; Marrion, 1996).

In conclusion, the data presented here show that the levels of endogenous compounds such as ATP, ADP, and PP_i play an important role in modulating the level of I_M in single neurons. The present results, when taken in perspective with other studies on I_M, are consistent with a dual regulation of I_M by kinases and phosphatases. In the presence of ATP, myosin light chain kinase-catalyzed phosphorylation enhances I_M. Calcineurin-catalyzed dephosphorylation inhibits I_M. High levels of ADP or PP_i decrease the activity of calcineurin and enhance I_M. The ultimate result of an increased I_M is a decrease in neuronal excitability. Thus, the intracellular levels of adenine nucleotides provide an important mechanism for cellular regulation of neuronal excitability by influencing the level of I_M.

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