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M-currents in frog sympathetic ganglion cells: manipulation of membrane phosphorylation

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1 The inward current and the M-current (I_M) suppression produced when muscarine is applied to frog sympathetic ganglion cells was recorded by means of the whole-cell patch-clamp technique. The holding potential was −30 mV and $[K^+]_o$ was 6 mM.

2 The steady-state I_M was maintained for at least 20 min when the patch pipette contained neither adenosine 5'-triphosphate (ATP) nor adenosine 3':5'-cyclic monophosphate (cyclic AMP). Inclusion of these substances or the ATP antagonst, β,γ-methyleneadenosine 5'-triphosphate (β,γ-MethATP; 1 or 2 nM) (failed to alter the rate of I_M 'run down'. By contrast, inclusion of adenosine-5'-O-(3-thiotriphosphate) (ATP-γ-S, 1 or 2 mM) resulted in a 60% reduction of the current within 18 min.

3 Despite the inability of ATP-γ-S to maintain steady-state I_M, it had no effect on the ability of muscarine (2–100 μM) to suppress a constant fraction of the available current. ATP-γ-S and β,γ-MethATP increased the rise time and duration of the response to muscarine.

4 Inclusion of a phosphatase inhibitor, diphosphoglyceric acid (DPG, 1–2.5 mM) or alkaline phosphatase (100 μg ml^{-1}) failed to affect the amplitude of muscarinic responses.

5 These results question the role of the phosphorylation and/or dephosphorylation reactions in the transduction mechanism for muscarine-induced I_M suppression but are consistent with the possibility that M-channels are 'directly coupled' via G-protein to the muscarinic receptor.

Keywords: Adenosine nucleotides; potassium channel; muscarinic receptor; autonomic ganglia; M-current; adenosine-5'-O- (3-thiotriphosphate); protein phosphatase; β,γ-methyleneadenosine 5'-triphosphate; protein kinase; diphosphoglyceric acid

Introduction

Muscarine-induced depolarization of B-cells in amphibian paravertebral sympathetic ganglia results, in part, from the suppression of a voltage-dependent, non-inactivating K^+ current, called the M-current (I_M; Brown & Adams, 1980; Adams *et al.*, 1982a,b; Akasu *et al.*, 1984; Selyanko *et al.*, 1990). Despite extensive investigation, the transduction mechanism which underlies M-channel closure following receptor activation remains to be elucidated (Adams *et al.*, 1986; Hille, 1989; see also Owen *et al.*, 1990). Although experiments with non-hydrolysable guanosine 5'-triphosphate (GTP) analogues support the involvement of a (pertussis toxin-insensitive) G-protein (Pfaffinger, 1988; see also Brown *et al.*, 1989), experiments designed to test the role of known cytosolic second messengers have yielded negative or equivocal results. For example, the involvement of cyclic nucleotides seems unlikely (Busis *et al.*, 1978; Weight *et al.*, 1978; Adams *et al.*, 1982b; Selyanko *et al.*, 1990) and although suppression of I_M can be mimicked by the application of protein kinase C (PKC) activators, e.g. phorbol esters (Brown & Adams, 1987; Pfaffinger *et al.*, 1988; Bosma & Hille, 1989; Selyanko *et al.*, 1990), results with PKC inhibitors (Bosma & Hille, 1989; Selyanko *et al.*, 1990) provide evidence against involvement of the diacyl glycerol/protein kinase C mechanism. In addition, it had been demonstrated that the agonist-induced reduction of I_M is unlikely to be mediated by inositol trisphosphate (Pfaffinger *et al.*, 1988; Hille, 1989; Selyanko *et al.*, 1990; see also Brown *et al.*, 1989) and the possible involvement of changes in intracellular Ca^{2+} concentration remain to be clarified (Kirkwood *et al.*, 1991; Beech *et al.*, 1991; Marrion *et al.*, 1991). The involvement of arachidonic acid metabolites as second messengers for I_M suppression is also unlikely (Hille, 1989; Yu *et al.*, 1991).

Experiments on rat cultured sympathetic ganglion cells with single-channel recording techniques (Owen *et al.*, 1990) have also failed to resolve the question of whether cytosolic second messengers are involved. Had it been possible to record a response in the cell-attached mode when muscarine was applied outside the pipette, this would have been consistent with the involvement of cytosolic second messengers in I_M suppression. If a response had been recorded in an outside-out patch, this would be consistent with 'direct G-protein coupling' as has been suggested for atrial muscarinic receptors (Pfaffinger *et al.*, 1985). Unfortunately, neither type of response has hitherto been reported (Owen *et al.*, 1990).

Many of the effects of the above second messengers are exerted via activation of protein kinases which phosphorylate membrane proteins, such as ion channels (Levitan, 1985 but see also DiFrancesco & Tortora, 1991). The present study was therefore designed to examine the role of phosphorylation and dephosphorylation processes in the transduction mechanism which underlies muscarine-induced I_M suppression. A preliminary report of some of these data has appeared (Zidichouski *et al.*, 1990).

Methods

Medium size leopard frogs (*Rana pipiens* <8 cm 'nose to tail') were purchased from a biological supply house and stored in running water at room temperature (20°C). Each frog was killed by pithing and the VIth to Xth paravertebral sympathetic ganglia removed and dissociated with trypsin and collagenase as described by Selyanko *et al.* (1990). Dissociated cells were left to adhere to the bottom of plastic petri dishes for about 80 min before electrophysiological analysis. Dissociated neurones were observed under a Nikon 'Diaphot' microscope and all experiments were carried out at room temperature (20°C). Some experiments were carried out on small

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bullfrogs (*Rana catesbiana* < 10 cm 'nose to tail') when *Rana pipiens* was unavailable.

Since detailed methods for whole-cell patch-clamp recording from amphibian autonomic neurones have been published (Selyanko *et al.*, 1990), only a brief description will be given here. Recordings were made with an Axopatch 1B amplifier, a Labmaster interface and an IBM-XT computer running 'Pclamp' software (Axon Instruments, Foster City, CA, U.S.A.). Data were stored for off-line analysis on a removable hard disk system and permanent records obtained from an *x-y* plotter. On-line records were obtained with a d.c. rectilinear pen recorder (Gould-Brush 2400; pen rise time < 8 ms). The corner frequency of the filter on the Axopatch amplifier was set to 200 Hz for voltage-ramp experiments and to 500 Hz for voltage-jumps. Current was zeroed at resting membrane potential (r.m.p.) and the holding potential was set to −30 mV. An estimate of the cell size was obtained from the input capacitance (C_{in}) and experiments were only done on the 'large' cells (C_{in} > 30 pF) which exhibited inward current responses to muscarine (at −30 mV with $[K^+]_o$ = 6 mM). Since the currents to be recorded were < 0.4 nA, no corrections were made for the voltage-drop across the series resistance which was always < 10 MΩ (i.e. the maximum voltage error due to series resistance was < 4 mV). Whole-cell M-channel conductance (G_M) was examined using a 5 s ramp command from the holding potential of −30 mV to −110 mV (16 mV s^{-1} see Figure 1). The high conductance part of the resulting *I*-V relationship (i.e. above −75 mV) represents current through M-channels plus leak current (Selyanko *et al.*, 1990). Total G_M at −30 mV was estimated after digitally-subtracting the leak current predicted by the slope between −75 and −90 mV. In order to document the mean change in I_M which occurred with time, I_M in each cell was measured at arbitrary time intervals and mean values for a series of cells at 3 min intervals estimated by extrapolation.

The physiological salt solution contained (mM): NaCl 113, KCl 6, $MgCl_2$ 2, $CaCl_2$ 2, HEPES/NaOH (pH 7.2) 5 and D-glucose 10. Patch pipettes (10–20 MΩ) were pulled from borosilicate glass and coated with Sylgard elastomer. The solution used to fill the pipettes contained (mM): KCl 110, NaCl 10,

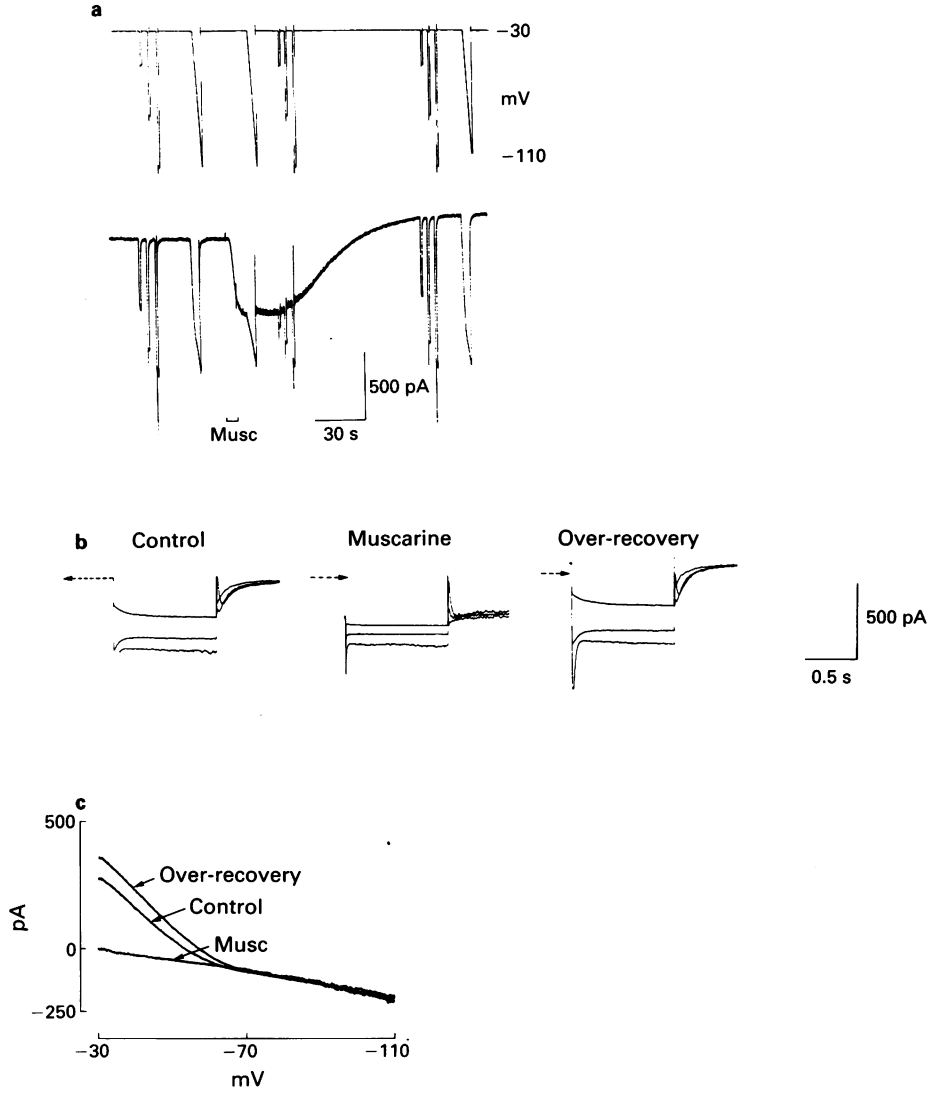

Figure 1 Response of a bullfrog sympathetic ganglion cell to muscarine (10 μM applied via U-tube), pipette contained 1 mM ATP (a) Upper record; voltage commands, comprising steps to −50, −80 and −110 mV or 5 s ramps to −110 mV, from the holding potential of −30 mV. Lower record; steady-state current response, muscarine produces an inward current associated with decreased membrane conductance followed by an outward current (over-recovery). (b) Current responses to voltage steps before, during and after response to muscarine shown on a faster time scale. Arrows represent control current level prior to the application of the drug. I_M relaxations are suppressed during response to muscarine and steady-state outward current is apparent during the 'over-recovery'. (c) Current responses to the ramp commands shown on a faster time scale. Note biphasic nature of the current prior to the application of muscarine and suppression followed by enhancement of conductance in the I_M range in response to muscarine. 500 pA/30 s calibration refers to records in (a) which were from a rectilinear pen recorder. 500 pA/0.5 s calibration refers to records in (b), which like the records in (c) were from an *x* − *y* plotter.

$MgCl_2$ 2, $CaCl_2$ 0.4, EGTA 4.4, HEPES/KOH (pH 6.7) 5 and D-glucose 10. The pCa of this solution, measured with a Ca^{2+}-electrode, was about 7. The Na^+ salts of ATP (1 mM), β,γ-methyleneadenosine 5'-triphosphate (β,γ-MethATP), adenosine 3':5'-cyclic monophosphate (cyclic AMP) or the tetralithium salt of adenosine-5'-O-(3-thiotriphosphate) (ATP-γ-S; 1 or 2 mM) were included in the internal solution. It was assumed that all of these substances would readily enter the cytoplasm because application of ATP analogues via a patch pipette has already been shown to exert pharmacological effects on these neurones (Simmons *et al.*, 1990). Also, we have observed prolongation of agonist-induced responses when GTP-γ-S is applied by this route (Selyanko *et al.*, 1990). Robust and repeatable responses to muscarine were recorded without the inclusion of guanosine triphosphate (GTP) in the patch pipette.

Muscarine was applied either by pressure ejection, using a 'Picospritzer' (General Valve, Fairfield, NJ, USA) or using the U-tube technique (Krishtal & Pidoplitchko, 1980; Selyanko *et al.*, 1990). The fluid exchange time for the U-tube was about 0.2 s. The rise time of the muscarine-induced current was defined as the time taken from the onset of the response to the time when the response had reached 63% of its maximum amplitude. All chemicals and drugs were from Sigma, St. Louis, MO, U.S.A. except for ATP-γ-S which was from Boehringer, Mannheim, Germany. Data are expressed as mean ± s.e.mean and significance of differences were estimated by Student's two-tailed, unpaired t test.

Results

The steady-state inward current and the pronounced I_M suppression produced by 'U-tube' application of 10 μM muscarine are illustrated in Figure 1. Prior to the application of the drug, hyperpolarizing voltage commands from the holding potential of −30 mV produce inward relaxations which reflect I_M deactivation. At command potentials negative to the potassium equilibrium potential (−73 mV), I_M deactivation is accelerated and the relaxations are reversed in polarity (Adams *et al.*, 1982a). The current response to a ramp to −110 mV (I-V relationship) is biphasic and displays a region of increased conductance due to activation of M-conductance (g_M) at potentials positive to −75 mV (Selyanko *et al.*, 1990). Muscarine evokes a steady-state inward current which is associated with decreased membrane conductance. The amplitude of I_M relaxations are reduced and the high conductance region of the I-V relationship is almost completely eliminated. I_M over-recovers following the removal of muscarine (cf. Pfaffinger, 1988). Application of 10 μM muscarine from the 'U-tube' almost completely eliminated I_M in all cells tested and since robust, submaximal responses could be elicited with 2 μM muscarine, this concentration was used for the majority of experiments in the present study. Since 'Picozpritzer' application was less efficient than 'U-tube' application, it was necessary to use 10 or occasionally 100 μM muscarine to produce robust, submaximal responses when this method of drug application was employed.

Dependence of I_M *on intracellular nucleotides*

Although previous work has suggested that I_M tends to 'run down' during whole-cell recording unless ATP or cyclic AMP is included in the patch pipette (Pfaffinger, 1988; Selyanko *et al.*, 1990), the current recorded in the present series of experiments was maintained for at least 20 min in the absence of adenosine nucleotides (Figure 2a and b). The effect of 1 mM ATP on the maintenance of the current was examined in 10 neurones and Figure 2c and d show that I_M was unattenuated during the first 18 min of recording in the presence of the nucleotide but that some slight 'run-down' of the current occurred after 24 min. I_M was also well-maintained when cyclic AMP (100 μM) or lower concentrations of ATP (100 μM) were included in the patch pipette (data not shown). Surprisingly, the ATP-antagonist, β,γ-MethATP (1 or 2 mM) failed to promote 'run-down' of the current (data from 9 cells, Figure 2e and f). By contrast, ATP-γ-S (1 or 2 mM), which is a substrate for protein kinase A and forms stable protein thiophosphates (Eckstein, 1985) was unable to maintain I_M and the current declined to 31.5 ± 7.5% (n = 8) of control in 21 min (Figure 2g and h).

Effects of intracellular nucleotides on the response to muscarine

Despite the differing abilities of the various adenosine nucleotides to support steady-state I_M, the ability of muscarine to suppress a constant fraction of the available current was independent of nucleotide content. For example, 2 μM muscarine produced 60.1 ± 10.9% suppression of the available I_M in neurones studied after 3 min with intracellularly applied ATP-γ-S (n = 5). After 18 min, the available I_M had decreased to 38.0 ± 6.0% of its control level (n = 8) but muscarine still produced about the same fraction suppression (64.4 ± 7.2% n = 5, $P > 0.7$) of the remaining I_M. These data are illustrated in Figure 2g and the effects of other nucleotides on the response to muscarine are shown in Figure 2c and e. The rise time of the muscarine response in neurones filled with ATP-γ-S was about 50% greater than in neurones filled with cyclic AMP. The duration of the response was increased even for relatively short periods of recording (i.e. < 15 min) and little or no recovery of the muscarine-evoked inward current was observed in 9 out of 23 cells tested. There was also a progressive increase in duration of successive muscarine responses during recording with intracellularly-applied ATP-γ-S and the amplitude of successive responses decreased with time. These data are summarized in Table 1 and a typical experiment is illustrated in Figure 3.

The rise time of muscarine responses increased about 3 fold when the pipette contained β,γ-MethATP rather than cyclic AMP. The presence of this ATP antagonist also increased response duration by about 50% but failed to exhibit any significant effect on response amplitude (Table 1).

Effect of the phosphatase inhibitor, diphosphoglyceric acid

There is evidence that at least in heart, muscarinic agonists can activate protein phosphatases (Ahmad *et al.*, 1989). If this mechanism were involved in muscarine-induced I_M suppression in frog ganglia, such that channel closure would require dephosphorylation (cf. Pfaffinger, 1988), the response should be blocked by phosphatase inhibitors such as diphosphoglyceric acid (DPG; Downes *et al.*, 1982). If, on the other hand, M-channel closure involved phosphorylation, the action of a protein phosphatase might be required for the termination of the response. If this were the case, irreversible responses to muscarine would be expected in the presence of DPG. However, when DPG (1–2.5 mM plus 100 μM cyclic AMP) was added to the intracellular solution, muscarine responses exhibited normal rise times and amplitudes. Although the responses were always reversible, their duration was increased by about 50% (Table 1).

As a final test for the involvement of phosphorylation and/or dephosphorylation reactions, alkaline phosphatase (100 μg ml^{-1} plus 100 μM cyclic AMP or 1 mM ATP) was included in the patch pipette. This enzyme, which should dephosphorylate membrane proteins, failed to alter amplitude of responses to muscarine for periods of up to 83 min. The average duration of the response was increased by about 20% (Table 1).

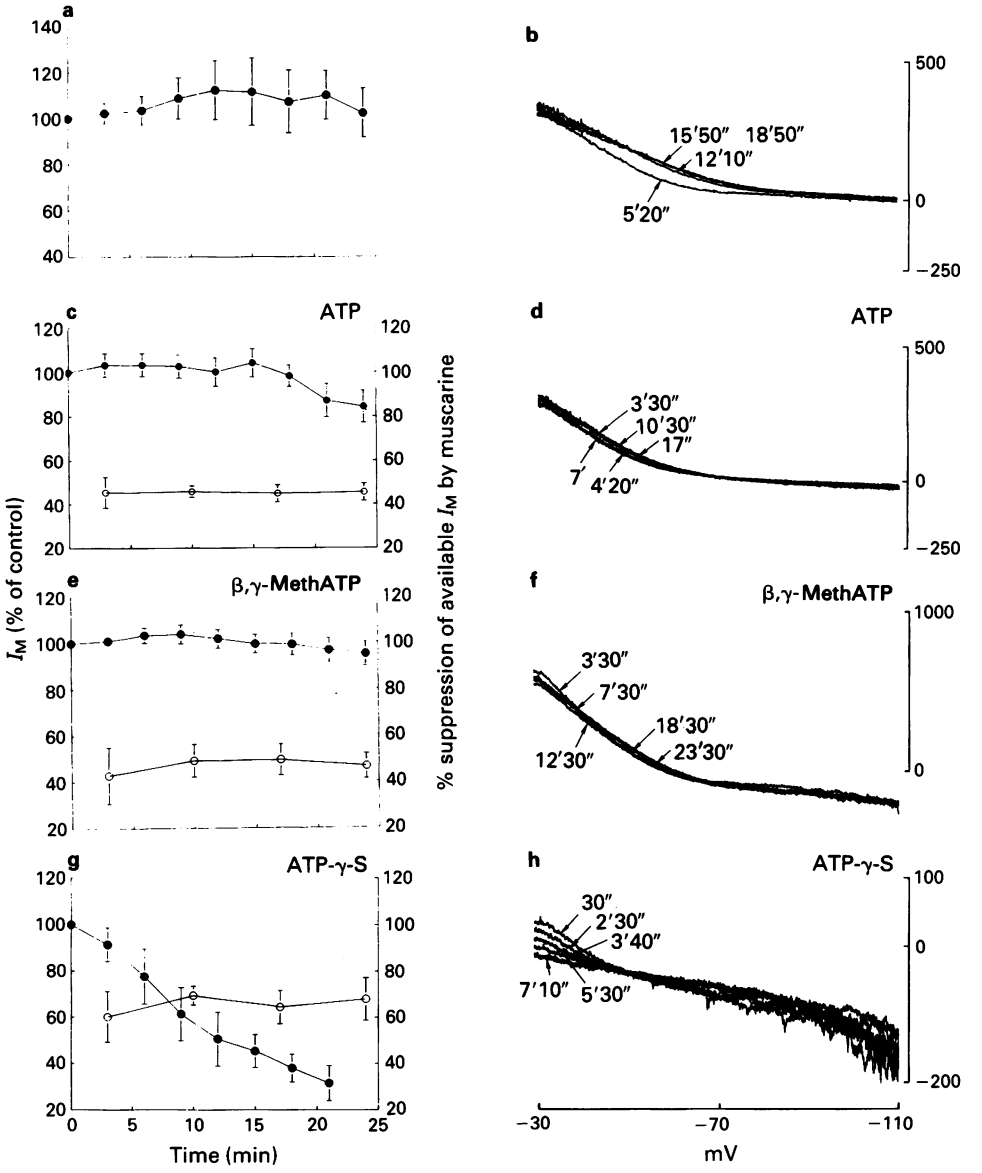

Figure 2 Effects of pipette content on maintenance of I_M and on the ability of muscarine to suppress I_M (Except where otherwise indicated, all data are from *Rana pipiens* cells). (a) Persistence of I_M when no adenosine nucleotides were included in the pipette (data averaged from 7–9 cells; not all cells were studied for the full 25 min period). (b) Original data records to show persistence of I_M. (c) (●) Persistence of I_M when 1 mM ATP was included in the patch-pipette (○). Percentage suppression of the available I_M by muscarine (10 μM, applied from Picospritzer) (data from 10 cells). (d) Original data records of I_M recorded with 1 mM ATP in the patch pipette. (e) (●) Persistence of I_M when 1 or 2 mM β,γ-methyleneadenosine 5'-triphosphate (β,γ-MethATP) was included in the patch-pipette (○). Percentage suppression of the available I_M by muscarine (100 μM, applied from Picospritzer) (Data from 9 cells). (d) Original data records of persistance of I_M in a bullfrog neurone with 2 mM β,γ-MethATP in the patch pipette. (g) (●) Lack of persistence of I_M when adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S, 1 or 2 mM) was included in the patch-pipette (○). Percentage suppression of the available I_M by muscarine 2 μM, applied from U-tube (Data from 5–8 cells) (h). Original data records of the effect of 2 mM ATP-γ-S on persistance of I_M. Current responses shown in (b), (d), (f) and (h) are evoked from 5 s hyperpolarizing ramp commands as shown in Figure 1. These data are x-y plots of digitally-stored data. Percentage suppression of I_M in (a), (c), (e) and (g) is not the same because different concentrations of muscarine and different methods of application were used in each series of experiments.

Discussion

If a phosphorylation process were involved in the suppression of I_M by muscarine, ATP antagonists such as β,γ-MethATP would be expected to block the response. If a dephosphorylation process were involved (cf. Pfaffinger, 1988), the re-opening of the channels during recovery from muscarine would require phosphorylation so that β,γ-MethATP would be expected to promote irreversible responses. However, responses recorded with intracellular β,γ-MethATP were neither irreversible nor were they any smaller than those recorded with cyclic AMP. Although ATP-γ-S promoted I_M run-down, muscarine still caused the same fractional depression of the remaining current even after 20 min. These results, as well as the lack of blockade by DPG and alkaline phosphatase argue strongly against involvement of phosphorylation and/or dephosphorylation mechanisms in the transduction process for muscarinic responses. This conclusion is supported by previously published data on the lack of effect of kinase inhibitors such as 1-(5-isoquinolinyl-sulphonyl)-2-methyl piperazine (H-7), staurosporine and gold sodium thiomalate (Bosma & Hille, 1989; Selyanko *et al.*, 1990).

Three other observations which may be interpreted to support the involvement of a phosphorylation/dephosphorylation mechanism fail to provide unequivocal evidence for this hypothesis.

Firstly, ATP-γ-S causes progressive reduction and prolongation of responses. If I_M suppression required membrane

Table 1 Effect of pipette contents on muscarinic responses

Pipette content	*Rise time* (s)	*Duration* (s)	*Amplitude* (pA)
U-tube application (Rana pipiens) [Musc] = 2 μM			
Cyclic AMP (0.1 mM) (Control)	10.2 ± 0.8 (n = 26)	125.5 ± 6.3 (n = 26)	127.3 ± 9.3 (n = 26)
ATP-γ-S (<15 min; 1 or 2 mM)	15.8 ± 2.0 (n = 14)	Not determined.	131.1 ± 17.9 (n = 14)
	P < 0.001		P > 0.8
Diphosphoglyceric acid	9.4 ± 0.3 (n = 27)	173.3 ± 11.2 (n = 27)	124.4 ± 11.8 (n = 27)
(1–2.5 mM + 100 μM cyclic AMP)	0.1 > P > 0.05	P < 0.001	P > 0.8
U-tube application (Bullfrog) [Musc] = 2 μM			
Pipette content	*Rise time* (s)	*Duration* (s)	*Amplitude* (pA)
Cyclic AMP (0.1 mM) (Control)	3.7 ± 0.8 (n = 10)	133.8 ± 80 (n = 10)	304.0 ± 24.7 (n = 10)
β,γ-MethATP (1 or 2 mM)	14.2 ± 1.3 (n = 26)	198.1 ± 11.6 (n = 26)	243.1 ± 14.8 (n = 26)
	P < 0.001	P < 0.001	P < 0.05
Picospritzer application (Rana pipiens) [Musc] = 10 μM			
Pipette content	*Rise time* (s)	*Duration* (s)	*Amplitude* (pA)
ATP (1 mM) (Control)	6.5 ± 0.4 (n = 35)	94.3 ± 6.2 (n = 35)	168.6 ± 15.3 (n = 35)
Alkaline phosphatase (100 μg ml^{-1} +	5.9 ± 0.5 (n = 33)	113.0 ± 5.9 (n = 33)	134.2 ± 15.1 (n = 33)
0.1 mM cyclic AMP or 1 mM ATP).	P > 0.2	0.05 > P < 0.025	P > 0.1

Since different methods of drug application, drug concentration and species of frog were used, data are compared to the control situation in each group where the cells contained adenosine 3':5'-cyclic monophosphate (cyclic AMP) or ATP. It was not possible to determine the mean duration of responses recorded with intracellularly-applied adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S) because the response failed to recover in 7 out of the 20 *Rana pipiens* cells which were tested. All data were collected within the first 15 min of whole-cell recording. Musc = muscarine; β,γ-MethATP = β,γ-methyleneadenosine 5'-triphosphate.

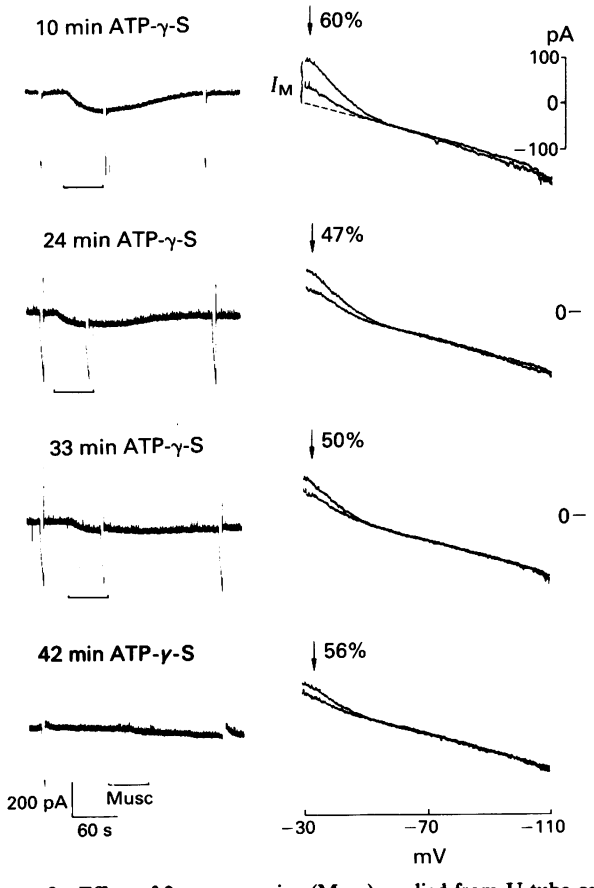

Figure 3 Effect of 2 μM muscarine (Musc) applied from U-tube onto a *Rana pipiens* sympathetic ganglion cell. Pipette contained adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S, 2 mM). I_M was assessed using 5 s hyperpolarizing ramp commands as in Figures 1 and 2. Note decrease in amplitude and elongation of responses with time. Percentages refer to the percentage depression of the available I_M produced by each test application of muscarine. Note that the percentage depression of the available current changes little with time. 200 pA/60 s calibration refers to left-hand series of traces which were from rectilinear pen recorder. −100/0/+100 pA calibration refers to all right hand traces which are from x-y plotter. Zero current levels are indicated at the extreme right of each trace.

phosphorylation and the recovery of the current following muscarine removal required dephosphorylation, a response recorded in the presence of ATP-γ-S would be expected to be irreversible. This is because ATP-γ-S forms non-hydrolysable protein thiophosphates (Eckstein, 1985). An alternative explanation for the effect of ATP-γ-S is that the thiophosphate moiety is transferred from ADP to GDP resulting in the formation of GTP-γ-S. This type of reaction has been reported to occur both in atria (Otero *et al.*, 1988) and in nodose ganglion cells (Gross *et al.*, 1990). GTP-γ-S would promote persistent G-protein activation (Pfaffinger, 1988; Elmslie *et al.*, 1990) which would explain the progressive prolongation and reduction of muscarine responses. Furthermore, the progressive suppression of I_M as more of the available pool of G-proteins became irreversibly activated would explain the observed run-down of I_M seen only with ATP-γ-S. Another explanation for prolongation of muscarine responses by ATP-γ-S is that it inhibits the desensitization process (Simmons *et al.*, 1990).

Secondly, the prolongation of responses by β,γ-MethATP may imply that recovery requires ATP-dependent phosphorylation so that I_M suppression may have involved dephosphorylation. An alternative explanation for this effect is that it reflects inhibition of the desensitization process in a fashion similar to ATP-γ-S (Simmons *et al.*, 1990).

Thirdly, the prolongation of responses by DPG may imply that I_M recovery requires dephosphorylation so that muscarine-induced M-channel closure would require phosphorylation. If this were the case, it is impossible to explain the inability of the ATP antagonist, β,γ-MethATP to block the response.

Since almost all of the present data support the view that muscarine-induced I_M suppression is independent of phosphorylation and/or dephosphorylation reactions, other possible transduction mechanisms for I_M suppression by muscarine must be considered. One possibility is that a novel or even a conventional second messenger, which exerts its effects independently of protein kinases, could be involved. In fact, it has recently been suggested that cyclic AMP may be able to directly gate ion channels (Di Francesco & Tortora, 1991). This substance is, however, unlikely to be involved in the transduction mechanism for agonist-induced I_M suppression because it fails to mimic the effect of muscarine when applied intracellularly or when membrane permeable ana-

logues are applied extracellularly (Busis *et al.*, 1978; Weight *et al.*, 1978; Adams *et al.*, 1982b; Selyanko *et al.*, 1990). Alternatively, the muscarinic receptor could be 'directly G-protein coupled' to the M-channel. Although it is impossible at present to decide between these possibilities, the latter mechanism is more attractive because several examples of 'direct G-protein coupling' of agonist-binding site to ion channel have now been documented (Pfaffinger *et al.*, 1985; Yatani *et al.*, 1987; North, 1989; Yatani *et al.*, 1990). Confirmation of this hypothesis will only be possible when muscarine-induced I_M suppression can be demonstrated in an outside-out patch (cf. Owen *et al.*, 1990).

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