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AUTORECEPTOR-MEDIATED PURINERGIC AND CHOLINERGIC INHIBITION OF MOTOR NERVE TERMINAL CALCIUM CURRENTS IN THE RAT

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SUMMARY

1. After blocking K^+ currents with 10 mM-tetraethylammonium (TEA) or TEA plus 250 μM-3,4-diaminopyridine (3,4-DAP), motor nerve terminal Ca^{2+} currents were recorded using focal extracellular electrodes. Two transmitters released from the terminal, ATP and acetylcholine (ACh), were then applied, and the effects on the nerve terminal Ca^{2+} current were measured.

2. ATP (50 μM) reduced the Ca^{2+} current by 34%, but this action is prevented when hydrolysis to adenosine is blocked by α,β-methyladenosine 5′-diphosphate (200 μM). Thus, inhibition by ATP presumably occurs subsequent to ATP hydrolysis to adenosine.

3. Adenosine (50 μM) inhibited the terminal Ca^{2+} current by 29%. This was mimicked by the adenosine analogue L-phenylisopropyl adenosine (L-PIA) and blocked by theophylline (100 μM), which antagonizes adenosine receptors at micromolar concentrations.

4. ACh (100 μM) or the anticholinesterase methane sulphonyl fluoride (MSF; 1 mM) also depressed the terminal Ca^{2+} current. This response was mimicked by muscarine (100 μM) and antagonized by atropine (100 μM) or pirenzipine (4 μM), which is generally specific for M_1 receptors.

5. Addition of Ba^{2+}, which blocks adenosine-mediated K^+ currents, had no effect on the inhibitory effects of either adenosine or ACh; similarly, neither adenosine nor ACh in the bath affected K^+ current records obtained after blocking all inward currents with 10 mM-Co^{2+} and focal application of tetrodotoxin.

6. Incubation of the muscle for 4 h in pertussis toxin (10^{-5} g ml^{-1}) eliminated both adenosine- and ACh-induced inhibition of the terminal Ca^{2+} current. This result indicates the possible involvement of a G protein in the transduction of the feedback pathway.

7. Neither cyclic AMP analogues, the adenylate cyclase activator forskolin (10 μM), the phorbol ester phorbol 12-myristate 13-acetate (PMA; 3 μM) nor the diacylglycerol analogue 1,2-oleoylacetylglycerol (OAG; 3 μM) had any effect on adenosine- or ACh-induced depression of the terminal Ca^{2+} current. Therefore,

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pathways involving these particular second messengers are most probably not involved.

8. The effects of adenosine and ACh are non-additive.

9. These results indicate that ATP and ACh, which are released during exocytosis, may inhibit their own release through attenuation of the terminal Ca^{2+} current via autoreceptors coupled to a G protein.

INTRODUCTION

In response to depolarization by an action potential, calcium inward currents flow through voltage-dependent channels in presynaptic nerve terminals (Llinás, Steinberg & Walton, 1976; Brigant & Mallart, 1982; Penner & Dreyer, 1986; Hamilton & Smith, 1987). The importance of Ca^{2+} in synaptic transmission combined with the lower concentration of intracellular free Ca^{2+} suggests the presence of very tight regulatory control of these currents. Many control pathways associated with the flux of Ca^{2+} through voltage-sensitive Ca^{2+} channels have indeed been identified. For example, acetylcholine (ACh) inhibits Ca^{2+} currents in cardiac (Siegelbaum & Tsien, 1983), sympathetic (Wanke, Ferroni, Malgaroli, Ambrosini, Pozzan & Meldolesi, 1987), and hippocampal (Toselli & Lux, 1989) cells. Similarly, adenosine depresses Ca^{2+} currents in dorsal root ganglion (Dolphin, Forda & Scott, 1986; Gross, Macdonald & Ryan-Jastrow, 1989), and hippocampal (Schubert, Heinemann & Kolb, 1986) cells as well as synaptosomal preparations (Wu, Phillis & Thierry, 1982).

Since ACh and ATP, which is quickly hydrolysed to adenosine, are co-released at the neuromuscular junction (Silinsky, 1975), the question arises whether they might also inhibit Ca^{2+} currents in the motor nerve terminals. In this context, cholinergic autoregulation at motor nerve terminals has been debated for years. *d*-Tubocurarine and α-bungarotoxin, which block postsynaptic nicotinic ACh receptors, have been reported to increase ACh release (Miledi, Molenaar & Polak, 1978; Bierkamper, Aizenman & Millington, 1986). It is thus inferred that ACh inhibits its own release, perhaps by depressing inward Ca^{2+} currents. However, the effect is weak (Auerbach & Betz, 1971), and the presence of presynaptic nicotinic receptors has been challenged (Jones & Salpeter, 1983). Adenosine, too, has been shown to suppress neuromuscular transmission (Silinsky, 1984; Ribeiro & Sebastião, 1987), and there is evidence for specific adenosine receptors on motor nerve terminals (Silinsky, 1980).

In this study, regulation of motor nerve terminal Ca^{2+} currents by ACh and adenosine was addressed directly. The currents were recorded directly, and the effects of exogenous adenosine and ACh on these currents were assessed. Both purinergic and cholinergic compounds were found to inhibit the motor nerve terminal Ca^{2+} current. A G protein may be involved in this modulation, but protein kinase A and protein kinase C do not appear to play a role.

METHODS

Experimental preparation

All experiments were performed on the motor nerve terminals innervating the extensor digitorum longus (EDL) muscle of adult rats. Rats were anaesthetized with chloral hydrate (2·8 mmol kg^{-1}, I.P.), and the EDL muscle along with its branch of the common peroneal nerve were

carefully dissected avoiding any damage to superficial fibres and their associated nerve terminals. Care was also taken in dissecting a clean, sufficient length ($\geqslant$2 cm) of the nerve trunk for external stimulation with a suction electrode.

Following dissection, the nerve–EDL preparation was pinned out at resting length in a small chamber containing 5 ml of a modified Ringer solution comprising the following constituents (mM): NaCl, 137; KCl, 5·0; $CaCl_2$, 2·0; $MgCl_2$, 1·0; NaH_2PO_4, 1; $NaHCO_3$, 24; glucose, 11 (pH 7·3). In experiments utilizing Cd^{2+} the bicarbonate buffering system was replaced with a HEPES (10 mM) system to avoid precipitation of $CdCO_3$. A reservoir of this bathing solution was maintained at room temperature (22 °C) and aerated with 95% O_2–5% CO_2; this solution circulated over the preparation at 10 ml min^{-1}. Oxygen saturation was maintained at > 80% (608 Torr).

Visualization

In order to visualize the motor nerve terminals for electrode placement, the EDL preparation was incubated in a 10–20 μM solution of the fluorescent dye 4-(4-diethylaminostyryl)-*N*-methylpyridinium iodide (4-di$_2$ASP, Molecular Probes, Eugene, OR, USA) for 2 min. The dye was then rinsed away, and the preparation was recirculated with the bathing solution.

This dye is an intracellular label taken up by the unmyelinated terminal branches in the endplate region. Following incubation with the 4-di$_2$ASP, nerve terminals at the neuromuscular junction can be viewed with epifluorescence at 400× magnification, thus allowing placement of a recording electrode near the end of a nerve terminal. Extended viewing with the fluorescent light source will cause bleaching of the dye. However, 4-di$_2$ASP can be reapplied if a suitable recording is not achieved prior to this. Prolonged exposure of the preparation to the dye does not affect transmission. It did not cause any obvious change in either spontaneous or evoked endplate responses or in the sensitivity of the endplate potential to extracellular Ca^{2+} concentration.

Electrophysiological recordings

Recording electrodes were fashioned from capillary glass (Boralex, Rochester Scientific, Rochester, NY, USA) via a double-pull procedure on a vertical electrode puller (PP-83, Narishige, Tokyo, Japan). They were then polished using a heat forge to yield tip resistances generally in the range of 0·3–1·0 MΩ and tip inner diameters of the order of 2·5–3·5 μm. These tip diameters were generally similar to nerve terminal diameters (1–3 μm).

Focal extracellular recordings from the nerve terminal were obtained using a current amplifier (Model 8900, Dagan Corp., Minneapolis, MN, USA) with virtual ground and tip series- and seal-resistance compensation. Seal resistances were generally 1–3 MΩ. Current recordings were monitored on an oscilloscope and stored on FM tape for subsequent sampling (1·7–25 kHz, 256 samples per sweep) and digitization on a PDP 11/23 computer.

After attaining a recording site, the nerve was stimulated at 0·5 Hz in the presence of *d*-tubocurarine (4 μM) or α-bungarotoxin (100 nM) to block the postsynaptic currents. The K^+ channel blocker tetraethylammonium (TEA; 10 mM) was added to expose the underlying Ca^{2+} current (cf. Brigant & Mallart, 1982; Penner & Dreyer, 1986; Lindgren & Moore, 1989). Sample records illustrating the inward current exposed by TEA are shown in Fig. 1. In these and all other illustrated current records, the average of fifty individual traces are shown. The current is dependent on extracellular Ca^{2+} levels (Fig. 1*B*), carried by Ba^{2+} (Fig. 1*B*), and blocked by the inorganic calcium channel blockers Cd^{2+}, Ni^{2+} and Co^{2+} (Fig. 1*C*). Although a minor fraction (estimated to be less than 22%) may be carried by Na^+, the primary component of the exposed inward current is thus carried by Ca^{2+}. Addition of 3,4-DAP (250 μM) plus TEA blocked further K^+ channels, exposing a much longer (> 70 ms) inward Ca^{2+} current (Penner & Dreyer, 1986); the current durations seen in different records with 3,4-DAP plus TEA present may vary because the current duration becomes progressively shorter as stimulation frequency increases. Similar results, though, were obtained with either TEA or TEA plus 3,4-DAP present.

The magnitude of currents recorded extracellularly depends on the location of the electrode with respect to the terminal. Electrode movement during an experiment affects the amplitude of both the positive and negative components in the current records. If there was any evidence of electrode movement during the course of an experiment manifest as a change in the positive Na^+ capacitative spike amplitude (e.g. Fig. 1*A*), records from that site were excluded from further analysis. Once a stable recording site was obtained, it could often be held for as long as 90 min.

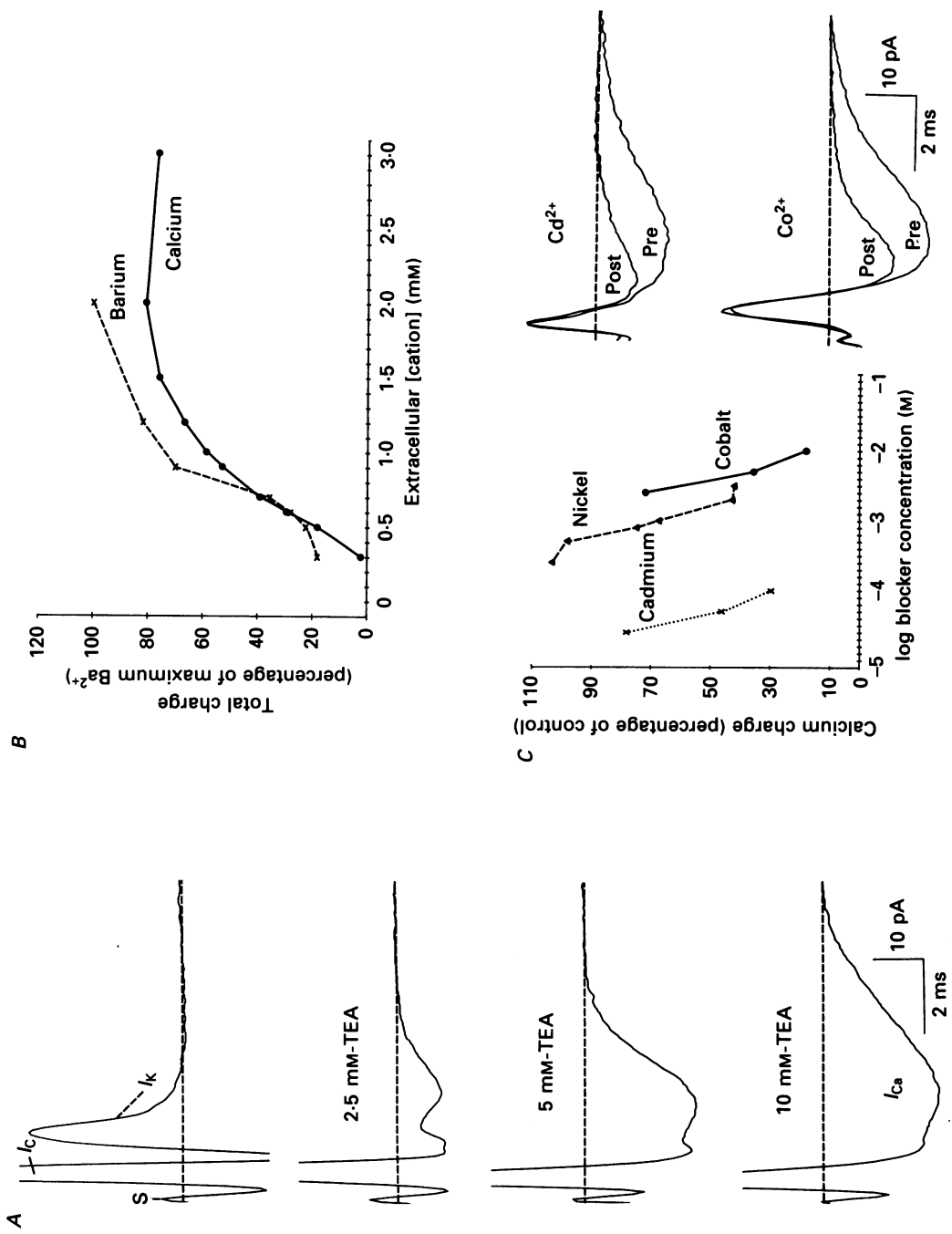

Fig. 1. For legend see facing page.

The total charge carried by the current, sometimes referred to as the total current, was determined by integrating the inward current over its duration.

Chemicals

All compounds were obtained from Sigma Chemical Co., St Louis, MO, USA, except 8-(4-chlorophenylthio)-cyclic adenosine monophosphate which was acquired from Boehringer Mannheim (Indianapolis, IN, USA) and methane sulphonyl fluoride (MSF) which was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA).

Statistics

Routine two-sided t tests were used to determine the statistical significance.

RESULTS

Purinergic inhibition

ATP

ATP reduced the total Ca^{2+} current in the motor nerve terminal (Fig. 2*A*). The effect was completely reversible within < 10 min after exchanging to ATP-free bath solution. The total charge carried by the current was decreased by 17–43% in the presence of 50 μM-ATP; the average reduction at this concentration was 34% (Fig. 2*C*). ATP (1 mM) reduced the total current by 75%, the maximum decrease attainable.

Inhibition by ATP proved to require hydrolysis to adenosine however. In the presence of 200 mM-α,β-methyladenosine 5′-diphosphate, which blocks hydrolysis of adenine nucleotides to adenosine in this preparation (Smith, 1991) presumably by inhibiting the enzyme ecto-5′-nucleotidase (Burger & Lowenstein, 1970), reduction of the Ca^{2+} current by 100 μM-ATP is also substantially blocked (Fig. 2*B* and *C*). (α,β-Methyladenosine 5′-diphosphate, however, does not block Ca^{2+} current inhibition by adenosine; Fig. 3*E*.) Furthermore, the non-hydrolysable ATP analogue, α,β-methyladenosine 5′-triphosphate (100 μM), did not produce a decrease in the total Ca^{2+} current (Fig. 2*C*). Thus, the ability of ATP to reduce motor nerve terminal Ca^{2+} current is dependent upon the hydrolysis of ATP to adenosine.

Adenosine

Adenosine also decreased the prejunctional Ca^{2+} current (Fig. 3*A*). As in the case of ATP, the effect was completely reversible and was concentration dependent. Adenosine (50 μM) decreased the total current by 15–40%; the average decrease was

Fig. 1. Currents in response to nerve stimulation. *A*, the magnitude and time course of the stimulus artifact (S) depended on the location of the stimulus electrode relative to the recording site. It was followed by an initial outward component (I_C) due to capacitative discharge of the terminal membrane by invading Na^+ currents. The K^+ outward current (I_K) is progressively depressed by increasing the TEA concentration. An inward current (I_{Ca}) is unmasked as the TEA concentration increases. *B*, the magnitude of this exposed inward current is sensitive to extracellular Ca^{2+} or Ba^{2+} concentrations. 'Total charge' was determined by integrating the current records. Ba^{2+} carried the largest current, and its maximum value was used as a reference in this figure. *C*, inorganic cations inhibited this inward current. The 'calcium charge' was calculated by integrating the current records. Representative records are shown on the right, prior to (pre) and following (post) addition of Cd^{2+} (100 μM) and Co^{2+} (10 mM).

29% (Fig. 3*E*), and maximum reduction of about 45% was obtained in the presence of 100 μM-adenosine. The adenosine analogue L-phenylisopropyl adenosine (L-PIA) also reduced the Ca^{2+} current (Fig. 3*E*). L-PIA (10 μM) decreased the total current by 35%, indicating that it may be somewhat more potent than adenosine.

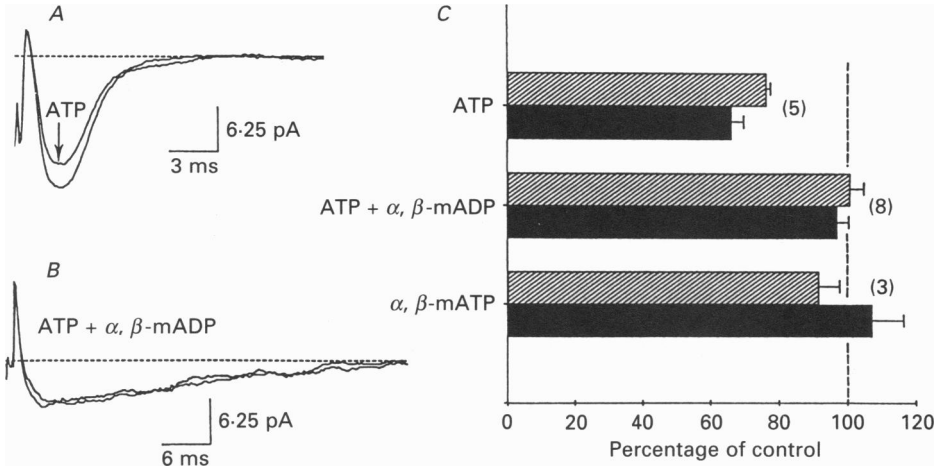

Fig. 2. Effect of extracellular ATP on motor nerve terminal Ca^{2+} current. *A*, extracellular ATP (50 μM) decreases the Ca^{2+} current. K^+ current was blocked by TEA (10 mM) in this example. *B*, α,β-methyladenosine 5′-diphosphate (200 μM) prevents the effect of ATP on the inward Ca^{2+} current. K^+ currents were blocked by TEA (10 mM) plus 3,4-DAP (250 μM) in this example. *C*, average (±standard error of the mean) values of the peak Ca^{2+} current and total charge following addition of ATP (50 μM), ATP (50 μM) plus α,β-methyladenosine 5′-diphosphate (α,β-mADP, 200 μM), and α,β-methyladenosine 5′-triphosphate (α,β-mATP, 100 μM). The number of experiments (each from a different animal) is given in parentheses. The data are presented relative to control Ca^{2+} currents. ▨, peak calcium current; ■, total calcium charge.

The presence of adenosine receptors was further tested by first adding adenosine to reduce the Ca^{2+} current and then adding theophylline, which is known to block adenosine receptors at micromolar concentrations (Phillis & Wu, 1981). After adding 50 μM-adenosine to decrease the total current, 100 μM-theophylline was found to increase the adenosine-depressed Ca^{2+} current. In the example shown in Fig. 3*D*, adenosine (50 μM) decreased the total Ca^{2+} current by 27%; subsequent addition of theophylline (100 μM) increased the current by 44%. Thus, at these concentrations the inhibitory effects of adenosine are completely blocked by theophylline. Furthermore, in control experiments conducted in the absence of adenosine, addition of 100 μM-theophylline results in an average (±standard error of the mean) increase of 20±3% ($n = 3$) in total Ca^{2+} currents during nerve stimulation at 2 Hz.

Potassium currents are not activated

Calcium current reduction by adenosine could be secondary to adenosine-mediated activation of a K^+ conductance. Although TEA and 3,4-DAP appear to block all motor nerve terminal K^+ currents (Saint, Quastel & Guan, 1987; Tabti, Bourret & Mallart, 1989) this possibility was further tested by adding Ba^{2+} (1 mM) to the bath,

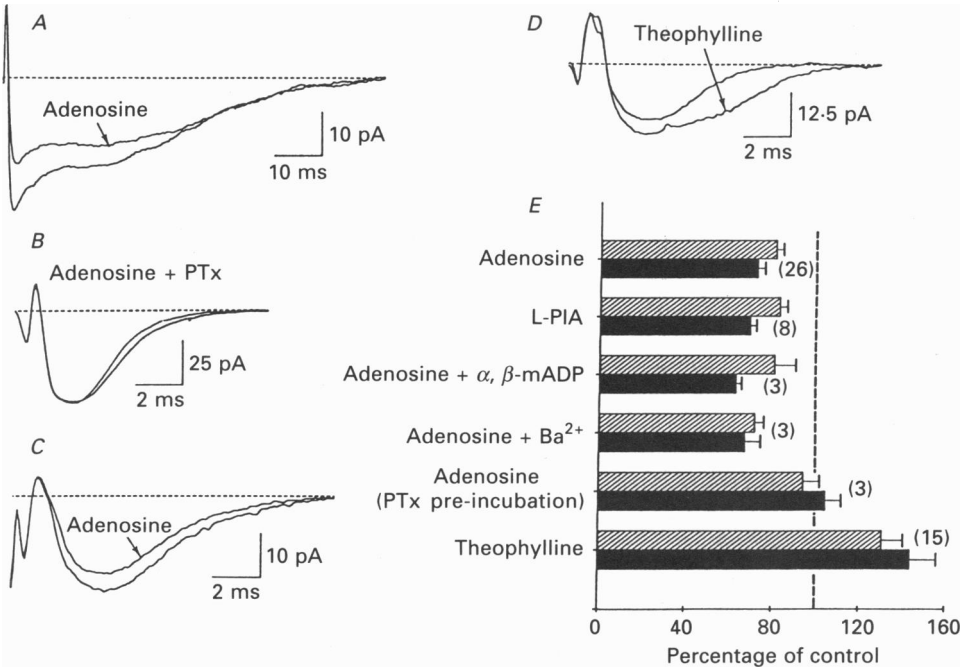

Fig. 3. Effect of extracellular adenosine on the nerve terminal Ca^{2+} current. *A*, extracellular adenosine (50 μM) decreases the Ca^{2+} current. K^+ currents were blocked with 10 mM-TEA plus 250 μM-3,4-DAP in this example. *B*, adenosine does not inhibit Ca^{2+} currents in cells that have been pre-treated for 4 h with pertussis toxin (PTx; 10 μg ml^{-1}); records obtained from PTx-treated cells before and after the addition of adenosine (50 μM) are shown. K^+ currents were blocked with 10 mM-TEA. *C*, in corresponding control experiments, cells remained sensitive to adenosine after 4 h incubation in normal saline not containing PTx. Records were obtained before and after addition of 50 μM-adenosine. K^+ currents were blocked with 10 mM-TEA. *D*, inhibition by adenosine is reversed by 100 μM-theophylline. The upper record was obtained after inhibition by 50 μM-adenosine; addition of theophylline (lower record) then resulted in an increased current despite the continued presence of adenosine. (Irregularities in the theophylline record are artifacts.) K^+ currents were blocked by TEA (10 mM). *E*, average (±standard error of the mean) values of the peak current and total charge following addition of adenosine (50 μM), L-phenylisopropyl adenosine (L-PIA; 10 μM), adenosine plus α,β-methyladenosine 5′-diphosphate (α,β-mADP, 200 μM), adenosine plus Ba^{2+} (1–2 mM; 2 mM-Ca^{2+} was also in the saline), and adenosine following 4 h pre-incubation in pertussis toxin (10 μg ml^{-1}). These data are presented relative to control Ca^{2+} currents. Average changes in the peak current and total charge following the addition of theophylline (100 μM) to Ca^{2+} currents already inhibited by adenosine (50 μM) are also presented. In this case, the adenosine-depressed currents serve as the control; thus, theophylline is seen to reverse inhibition by adenosine. The number of experiments (each from a different animal) is given in parentheses. ▨, peak current; ■, total charge.

since Ba^{2+} has been shown to eliminate adenosine-activated K^+ currents (Trussell & Jackson, 1987). The addition of Ba^{2+} did not affect the adenosine-mediated inhibition of the Ca^{2+} current (Fig. 3*E*). Moreover, equimolar replacement of Ca^{2+} with Ba^{2+} did not change the inward current reduction caused by adenosine.

An additional experiment was performed to assess whether adenosine activated a

K^+ current. After obtaining a recording site, inward currents were blocked by addition of 10 mM-Co^{2+} and focal application of tetrodotoxin by pressure ejection. The amplitude of the K^+ current was then measured before and after addition of adenosine (50 μM) to the bath. Adenosine had no detectable effect on the magnitude of the K^+ current.

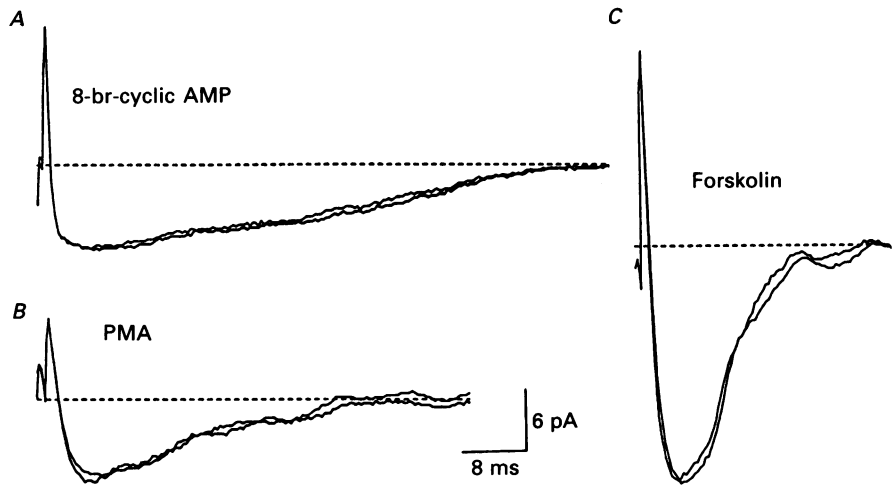

Fig. 4. Selected second messenger related compounds display no effect on the inward Ca^{2+} current. K^+ currents were blocked by TEA (10 mM) and 3,4-DAP (250 μM) in each case. *A*, the cyclic AMP analogue, 8-bromoadenosine 3′,5′-cyclic monophosphate (8-br-cyclic AMP; 10 μM), did not affect the Ca^{2+} current when applied following 50 μM-adenosine; records shown were obtained following inhibition by adenosine and then subsequent to addition of 8-br-cyclic AMP. *B*, the phorbol ester phorbol 12-myristate 13-acetate (PMA; 3 μM) had no influence on the terminal Ca^{2+} current; records shown were obtained before and after addition of PMA. *C*, the adenylate cyclase activator forskolin (10 μM) had no effect on the Ca^{2+} current; records obtained after Ca^{2+} current inhibition by adenosine and subsequent addition of forskolin are shown.

Sensitivity to pertussis toxin

Adenosine may inhibit the Ca^{2+} current via a G protein (Scott & Dolphin, 1987; Gross *et al.* 1989). Pertussis toxin (PTx) inactivates G_i and G_o through ADP ribosylation (Gilman, 1984). Thus, to test for G protein involvement, muscles were incubated in PTx (10^{-5} g ml^{-1}) for at least 4 h. This treatment with PTx prevented the adenosine-mediated reduction in nerve terminal Ca^{2+} currents (Fig. 3*B*). In contrast, terminals bathed 4 h in normal bath solution remained responsive to adenosine (Fig. 3*C*). These results indicate that G proteins may be involved.

Cyclic AMP and protein kinase C may not be involved

The inhibitory effects of adenosine could involve adenylate cyclase inactivation (reviewed in Dunlap , Holz & Rane, 1987). However, addition of the cyclic AMP analogues 8-bromoadenosine 3′,5′-cyclic monophosphate (10 μM; Fig. 4*A*) and 8(4-chlorphenylthio)-cyclic AMP (8(4-CPT)-cyclic AMP; 50 μM) or the activator of adenylate cyclase, forskolin (10 μM; Fig. 4*C*), was unable to reverse the observed reduction in the prejunctional Ca^{2+} current induced by adenosine (Table 1).

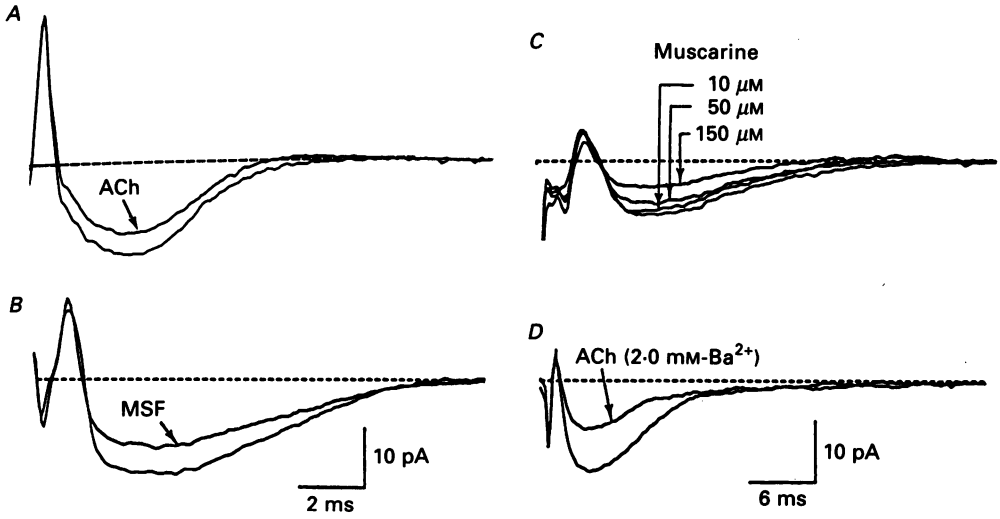

Fig. 5. Effects of various cholinergic agents on the nerve terminal Ca^{2+} current. *A*, ACh (100 μM) inhibited the Ca^{2+} current. K^+ currents were inhibited by TEA (10 mM). *B*, addition of 1 mM-methanesulphonyl fluoride (MSF) also inhibited the Ca^{2+} current; records shown were obtained before and after addition of MSF. K^+ currents were blocked by TEA (10 mM). *C*, muscarine (10–150 μM) also inhibited the Ca^{2+} currents; records were obtained before and after progressive addition of muscarine. The control record (unlabelled) is slightly offset for clarity; this is manifest as a negative shift in both the positive and negative components. K^+ currents were blocked by TEA (10 mM) and 3,4-DAP (250 μM). *D*, inhibition by ACh (100 μM) following equimolar replacement of Ca^{2+} by Ba^{2+}; records obtained in the presence of Ba^{2+} before and after addition of ACh are shown. K^+ currents were blocked by TEA (10 mM) and 3,4-DAP (250 μM).

TABLE 1. Effects of activators and analogues of second messenger pathways on terminal calcium currents

Protein kinase A			Protein kinase C	
	Total calcium charge (percentage of control)			Total calcium charge
Compound	Post adenosine	Post ACh	Compound	(percentage of control)
8-br-cyclic AMP (10 μM)	—	106±3	OAG (3 μM)	98±7 (n = 4)
8(4-CPT)-cyclic AMP (50 μM)	95±6 (n = 3)	103±18 (n = 3)	PMA (3 μM)	95±13 (n = 5)
Forskolin (10 μM)	101±2 (n = 3)	100±6 (n = 2)		

Percentage (±S.E.M.) changes in total calcium movement in response to some second messenger pathway agonists and analogues. In the case of protein kinase A compounds, values were obtained 12 min after steady-state Ca^{2+} current reduction was induced by adenosine (50 μM) or ACh (100 μM). Values in response to the protein kinase C compounds were obtained 2 min after recording normal Ca^{2+} currents. In all cases values were obtained from analysis of signal averaged records (thirty signals) recorded at 0·5 Hz. See text for full chemical names of analogues.

Furthermore, neither the protein kinase C-stimulating phorbol ester phorbol 12-myristate 13-acetate (PMA 3 μM; Fig. 4*B*) nor the diacylglycerol analogue 1,2-oleoylacetylglycerol (OAG, 3 μM) had any effect on control Ca^{2+} currents (Table 1) or

on adenosine-induced depression of the Ca^{2+} current. Although other second messengers cannot be ruled out, these results indicate that two known protein kinase A- or protein kinase C-coupled second messengers may not necessarily play a role.

Cholinergic inhibition

ACh

In addition to its postsynaptic function, ACh also inhibited presynaptic Ca^{2+} currents at the neuromuscular junction (Fig. 5*A*). This effect was reversible and was

TABLE 2. Effects of cholinergic agents on nerve terminal calcium currents

Compound (mM)	Number of experiments	Calcium current (percentage of control) Peak	Total
ACh (0·1)	8	78±4	73±4*
ACh (0·2)	4	77±4	63±6*
ACh (0·1) 2 mM-Ba^{2+} substituted for Ca^{2+}	4	73±9	76±7*
ACh (0·1) Pre-incubated in pertussis toxin (4 h)	3	96±16	107±5
Methane sulphonyl fluoride (MSF) (1·0)	5	67±4	55±9*
ACh (0·1) following 1 mM-MSF	3	134±23	103±11
Muscarine (0·1)	6	79±6	73±4*
Atropine (0·2) following 0·1 mM-muscarine	9	116±8*	156±22†
Pirenzipine (4 μM) following 0·1 mM-muscarine	6	123±14	149±15†

Percentage (±S.E.M.) changes in control levels of peak and total calcium movement in response to cholinergic agents. Averages ± standard error of the mean, values of changes in averaged records (thirty or fifty traces) obtained before and after drug application. Stimulation frequency was 0·5–2 Hz. * Significantly different (0·05 level) from control values in normal saline. † Significantly different (0·05 level) from data obtained in the presence of muscarine.

concentration dependent. At concentrations of 100 and 200 μM, ACh reduced total Ca^{2+} currents by 27 and 37% of control values, respectively (Table 2).

Supporting evidence is provided by the observation that 1 mM-methane sulphonyl fluoride (MSF), an anticholinesterase (Ziskind-Conhaim, Inestrosa & Hall, 1984), caused a 45% reduction in the total inward Ca^{2+} current (Fig. 5*B*, Table 2). When 100 μM-ACh was added following the addition of 1 mM-MSF treatment, no additional reduction was observed. Thus, the 45% reduction may represent an upper limit to the effectiveness of ACh.

Muscarinic receptors

Muscarine (100 μM) inhibited the peak and total inward Ca^{2+} current to levels comparable to ACh (Table 2). Individual traces in Fig. 5*C* demonstrate the dose response of the Ca^{2+} current to micromolar concentrations of muscarine. The muscarinic antagonists atropine (100 μM) and pirenzipine (3–4 μM) both increased the total Ca^{2+} current by about 50% following cholinergic inhibition (Table 2). Thus, the blockage of the muscarinic receptors reversed the inhibitory effect of ACh and muscarine on the terminal Ca^{2+} current.

Atropine (100 μM) had no effect on the adenosine-mediated inhibition of Ca^{2+} currents. Conversely, theophylline (100 μM) had no effect on the muscarine-induced

depression of Ca^{2+} currents. In three control experiments, addition of atropine (100 μM) to normal saline (in the absence of adenosine or ACh) increased total Ca^{2+} currents by 8, 14 and 18% during 2 Hz stimulation.

Nicotinic receptors

Since nicotinic antagonists such as *d*-tubocurarine or α-bungarotoxin were required to block postsynaptic currents, tests for presynaptic nicotinic receptors were inherently ambiguous. However, there were no changes in Ca^{2+} current as *d*-tubocurarine or α-bungarotoxin concentrations were raised progressively above threshold concentrations needed to block endplate currents. Thus, there was no clear evidence for the presence of nicotinic autoreceptors.

Potassium currents not involved

Additionally, as seen with adenosine, ACh (100 μM) was also capable of reducing the terminal Ca^{2+} current when 1 mM-Ba^{2+} was added or when Ca^{2+} was replaced by equimolar Ba^{2+} (Fig. 5*D*; Table 2). Likewise, addition of ACh (100 μM) had no effect on the magnitude of the K^+ current recorded in control saline with inward currents blocked by Co^{2+} and focal application of tetrodotoxin. This argues against activation of a K^+ conductance as an explanation for the cholinergic inhibition of Ca^{2+} inward current.

Sensitivity to pertussis toxin

Furthermore, the muscarinic effect is also probably mediated by a G protein interaction. When the EDL preparation had been incubated in pertussis toxin for 4 h prior to the start of the experiment (Table 2), no significant change in the total Ca^{2+} current was observed in response to 100 μM-ACh.

Cyclic AMP and protein kinase C may not be involved

The depression of terminal Ca^{2+} currents by ACh was not reversed by addition of the cyclic AMP analogues 8-bromoadenosine 3′,5′-cyclic monophosphate (10 μM) and 8(4-chlorphenylthio)-cyclic AMP (50 μM), or stimulation of adenylate cyclase with 10 μM-forskolin (Table 1). As with adenosine-mediated inhibition, compounds associated with activation of the protein kinase C pathway (PMA and OAG) were also without effect on ACh-depressed Ca^{2+} currents at these motor nerve terminals.

Adenosine and ACh effects are non-additive

When 100 μM-ACh was added following 50 μM-adenosine ($n = 3$) no additional reduction in the inward Ca^{2+} current was observed. These data suggest that adenosine and ACh may be functioning through the same pathway to mediate similar inhibitory effects on the motor nerve terminal Ca^{2+} current.

DISCUSSION

The results of this study demonstrate that purinergic and cholinergic compounds inhibit the motor nerve terminal Ca^{2+} currents. Since ATP and ACh are co-ordinately released from these terminals, this inhibition most probably involves autoreceptors. Moreover, these autoreceptors appear to be coupled to a G protein.

Pharmacologically, the adenosine autoreceptor responds like an A_1 receptor. The adenosine response is inhibitory, mediated by the analogue L-phenylisopropyl adenosine, and blocked by theophylline; each of these actions is characteristic of the A_1 receptor type (Phillis & Wu, 1981). Adenosine A_1 receptors have been shown to exist on the nerve terminals of the rat perforant path (Dragunow, Murphy, Leslie & Robertson, 1988), and adenosine decreases synaptic transmission via an A_1 receptor-mediated reduction in presynaptic Ca^{2+} currents in rat hippocampal slices (Reddington, Lee & Schubert, 1982; Schubert *et al.* 1986) and rat (Dolphin *et al.* 1986) and mouse (Gross *et al.* 1989) dorsal root ganglion cells. Although A_1 receptors may inactivate adenylate cyclase in some preparations (reviewed in Phillis & Wu, 1981), our results indicate that their inhibition of rat motor nerve terminal Ca^{2+} currents may be independent of adenylate cyclase (and cyclic AMP) activity. In cultured dorsal root ganglion neurons, adenosine-mediated inhibition of N-type Ca^{2+} currents is also independent of adenylate cyclase activity (Gross *et al.* 1989).

The ACh autoreceptor appears to be muscarinic. In other tissues, such as hippocampal neurons, muscarinic receptors also mediate presynaptic inhibitory effects (Gähwiler & Brown, 1987; Toselli & Lux, 1989). Moreover, blockage of the ACh-mediated Ca^{2+} current depression by low levels of pirenzipine are also indicative of an M_1-type ACh receptor-mediated pathway (Hammer & Giachetti, 1984). This, too, is consistent with results from other systems; activation of M_1-muscarinic receptors also inhibits Ca^{2+} currents in rat sympathetic neurons (Wanke *et al.* 1987).

Whether nicotinic ACh receptors are also located presynaptically at the neuromuscular junction is unclear. Biochemical assays of ACh release indicate enhancement by nicotine and suppression by α-bungarotoxin (Miledi *et al.* 1978; Bierkamper *et al.* 1986). However, autoradiographic studies using ^{125}I-labelled α-bungarotoxin failed to detect nicotinic ACh receptors located on vertebrate motor nerve terminals (Jones & Salpeter, 1983). Although nicotinic ACh receptors cannot be rule out, we have seen no indication that would suggest their presence.

Since purinergic and cholinergic inhibitory effects were blocked by pertussis toxin, the adenosine and ACh autoreceptors are presumably coupled to G proteins. Although this conclusion must be considered preliminary, it is consistent with mounting data implicating a number of ion channels, including Ca^{2+} channels, as effectors in receptor-coupled G protein pathways (Dunlap *et al.* 1987). In cultured sympathetic and sensory neurons, muscarinic and adenosine receptors, respectively, have been found to depress Ca^{2+} currents through apparent G protein effects directly on the ion channels (Wanke *et al.* 1987; Gross *et al.* 1989). Moreover, adenosine-mediated inhibition of transmitter release from frog motor nerve terminals appears to involve G proteins (Hirsh & Silinsky, 1989). Analogous muscarinic control mechanisms have been found in cardiac tissue, where K^+ channels appear to be regulated directly by either the α- (Yatani, Hamm, Codina, Mazzoni, Birnbaumer & Brown, 1988) or the β, γ-subunit (Kim, Lewis, Graziadei, Neer, Bar-Sagi & Clapham, 1989) of the G protein.

Since adenosine and ACh appear to act in a similar, non-additive fashion to reduce the motor nerve terminal Ca^{2+} current, the question arises as to whether they might share a common G protein. The results of this study, though, do not permit an answer to this intriguing question.

Calcium-dependent transmitter release from mammalian motor nerve terminals is thus under modulatory control that may be a direct consequence of the vesicular release of ATP and ACh. This feedback represents a mechanism for a graded limitation of transmitter release during stimulation. Cleft concentrations of adenosine and ACh following an action potential have been calculated to be in the 100–300 μM range in this preparation (Smith, 1990, 1991). At these concentrations, adenosine and ACh reduce the inward Ca^{2+} current by up to 45 and 37%, respectively. In some preparations, a tetrodotoxin-sensitive Na^+ component of the inward current may comprise up to 22% of the inward current (authors' observation). This Na^+ component is not affected by either adenosine or ACh, however. Thus, the inhibitory effects of adenosine and ACh on the Ca^{2+} components of the inward current may be underestimated. At normal concentrations of extracellular Ca^{2+} (2 mM), the relationship between quantal release and bath Ca^{2+} levels at the rat neuromuscular junction deviates only slightly from linearity (Hubbard, Jones & Landau, 1968). Therefore, it is likely that a 45% reduction in the inward Ca^{2+} current should have a comparable influence on quantal release from those terminals. The muscle response may not be affected greatly by a reduction of this magnitude. However, Ca^{2+} entry into the terminal during repetitive activity may be limited in this way. Therefore this modulatory system may prevent detrimental increases in presynaptic Ca^{2+} levels.

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