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Depression of miniature endplate potential frequency by acetylcholine and its analogues in frog

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1 Acetylcholine (ACh), 7.5×10^{-5} M, and carbachol, 5×10^{-6} M (CCh) depressed the frequency of miniature endplate potentials (m.e.p.ps) in the frog (*Rana temporaria*) sartorious neuromuscular junction with active acetylcholinesterase to about 50–55% of the controls.

2 A similar depression was produced by the nicotinic agonists, nicotine, suberyldicholine and tetramethylammonium.

3 The muscarinic agonists, oxotremorine, methylfurmethide and methacholine were without effect on m.e.p.p. frequency. The muscarinic antagonist, atropine and the nicotinic antagonist, (+)-tubocurarine, had no effect on the depression of m.e.p.p. frequency evoked by CCh.

4 The ganglionic blockers, benzhexonium and IEM-1119, were also without effect on the CCh-evoked depression of m.e.p.p. frequency.

5 Pretreatment of muscles with anticholinesterases did not prevent the CCh-induced drop in m.e.p.p. frequency.

6 The effect of CCh was proportionally the same as in the controls in preparations where the m.e.p.p. frequency was changed by elevation of K^+ and in the presence of theophylline, noradrenaline, dibutyryl adenosine 3′:5′-cyclic monophosphate (db cyclic AMP) and db cyclic GMP.

7 An inhibitor of Na^+,K^+-ATPase, ouabain, 5×10^{-5} mol l^{-1}, prevented or reversed the depression of m.e.p.p. frequency by CCh. However, the depression was present in a nominally K^+-free medium. Insulin and adrenaline, which are considered to be Na^+,K^+-ATPase activators, were without effect on depression of m.e.p.p. frequency.

8 The depression of m.e.p.p. frequency by 5×10^{-6} M CCh was the same at temperatures between 5 and 30°C with a Q_{10} near to 1.0. When threshold amounts of CCh were used (6×10^{-7} and 3×10^{-7} M), the depression was less at higher temperatures.

9 The receptive structures responsible for the CCh (or ACh)-evoked depression of m.e.p.p. frequency differ pharmacologically from muscarinic, nicotinic ganglionic and neuromuscular junction ACh-receptors as well as from the synaptic cholinesterase, in contrast to previous reports (Duncan & Publicover, 1979). The low temperature-dependence points to the possibility that physical rather than biochemical processes are limiting in this presynaptic effect of cholinomimetics.

Keywords: Acetylcholine; carbachol; miniature endplate potentials; neuromuscular junction; presynaptic receptors

Introduction

Acetylcholine (ACh) and its analogues increase the frequency of miniature endplate potentials (m.e.p.p.) recorded at the neuromuscular junctions of rats due to activation of presynaptic receptors of the nicotinic type (Miyamoto & Volle, 1974; Nikolsky, 1982; 1984; Bierkamper & Aizenman, 1984). In contrast, the m.e.p.p. frequency in frog skeletal muscle is depressed significantly by ACh and its non-hydrolysable analogue, carbachol (CCh) (Duncan & Publicover, 1979; Bukharaeva *et al.*, 1986). Duncan & Publicover (1979) proposed that the cholinesterase in frog nerve terminals might mediate the inhibitory effect of cholinomimetics. In some of our experiments we observed that not only ACh and CCh but also specific nicotinic cholinomimetics such as nicotine itself lowered the m.e.p.p. frequency at the amphibian endplate (Magazanik & Nikolsky, 1979; Bukharaeva *et al.*, 1986); with respect to the effect of anticholinesterase some discrepancy was found between our preliminary observations (Bukharaeva *et al.*, 1986) and those of Duncan & Publicover (1979).

In the present paper, the presynaptic action of cholinomimetics was therefore re-examined and the m.e.p.p. frequency was followed in the presence of several cholinolytic substances, during Na^+, Cl^-, Ca^{2+} and K^+ changes and under conditions when the activity of membrane Na^+,K^+-ATPase was altered. No simple pharmacological or ionic basis for the CCh depression was found and the negligible temperature-dependence of the presynaptic action of cholinomimetics may indicate that physical rather than biochemical processes determine this phenomenon.

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Methods

The experiments were performed on the sartorius muscle of the winter frog *Rana temporaria* at temperatures of 5–30°C. After rapid decapitation of animals anaesthetized with ether, muscles were dissected and placed in Ringer solution (in mM: NaCl 113.0, KCl 2.5, $CaCl_2$ 1.8 and $NaHCO_3$ 3.0; pH = 7.3) in a 1.7 ml translucid perfusion chamber with Peltier cooling elements. When Br^-, NO_3^- and SO_4^{2-} were substituted for Cl ions, osmotically identical amounts of these salts were used. Drugs were applied to the muscle bath in the perfusion (5 ml min^{-1}). When the ionic composition of the standard medium was changed, isosmolarity was maintained by changes in NaCl concentration or by adding the corresponding amount of sucrose. Acetylcholinesterase (AChE) was inhibited by treating muscles outside the perfusion chamber with the 5×10^{-6} M anti-AChE drug, armin (diethoxyparanitrophenylphosphate), dissolved in Ringer solution, for 30 min and then washing extensively three times with Ringer for 10 min to remove free anti-AChE. In other experiments, the reversible anti-AChE drug, proserin, was present in the bath continually at a concentration of 1×10^{-6} M.

Synaptic m.e.p.ps and resting membrane potentials (RMP) were measured by standard microelectrode techniques. Occasionally a standard two microelectrode voltage-clamp technique was used to measure endplate currents. The microelectrodes were filled with 2.5 M KCl and had resistances ranging from 3–7 MΩ.

M.e.p.ps were processed on a digital analyser-computer system. Spontaneous quantal release was estimated as the mean frequency of at least 100 m.e.p.ps. Postsynaptic effects of cholinergic agents were measured from the change in m.e.p.p. amplitude and resting membrane potential in the synaptic zone of each muscle fibre.

Attention was paid to the m.e.p.p./noise ratio to avoid a possible loss of small amplitude m.e.p.ps during sampling or, at depolarized RMP, due to the action of cholinomimetics or the presence of cholinolytics, as well as to avoid inclusion of some random deflections of the RMP. Only those experiments were evaluated in which the m.e.p.p. amplitude histogram shape was not changed markedly after application of cholinergic agents (see Figure 1).

The results are expressed as the mean of seven or more experiments ($n \pm$ s.e.mean). The statistical significance was evaluated by Wilcoxon's non-parametric test at an indicated probability level, usually 0.05, unless otherwise stated.

The following chemicals were used: acetylcholine chloride, nicotine, oxotremorine and adrenaline (Serva), carbachol chloride (Koch-Light), tetramethylammonium chloride (TMA) (Fluka), (+)-tubocurarine (TC), atropine sulphate (Burroughs-Wellcome), dibutyryl cyclic AMP (db cyclic AMP), dibutyryl cyclic GMP (db cyclic GMP), proserine, metacholine, insulin, ouabain and theophylline (Sigma). Methylfurmethide, benzhexonium, 4-(isopropylammonium butyl) trimethylammonium dibromide (IEM-1119) and diethoxyparanitrophenylphosphate (armin) were synthesized and kindly provided by the Institute of Organic Chemistry, Acad. Sci., U.S.S.R.

Results

Presynaptic effects of cholinomimetics of the nicotinic and muscarinic type

ACh and CCh reduced the frequency of m.e.p.p. which usually ranged from 1.5 to 3 s^{-1} ($2.75 \pm 0.74\,s^{-1}$, Figure 1, Table 2) and depolarized the muscle membrane in a dose-dependent manner (Figure 2a and b). The effects of nicotine, suberyldicholine and TMA were similar, but in concentrations chosen to affect m.e.p.p. frequency similar to CCh, their effects on membrane potential were weaker. Most of experiments in the following sections were therefore done with CCh and no anticholinesterase was used, unless otherwise stated.

The muscarinic agonists oxotremorine, methylfurmethide and metacholine were without effect on m.e.p.p. frequency. Typical muscarinic antagonists, atropine 1×10^{-6} M (Table 1) and pirenzepine 1×10^{-5} M (data of five experiments not given) also did not prevent the depression of m.e.p.p. frequency evoked by CCh (Table 1). In addition the nicotinic antagonist, TC (at 4.5×10^{-7} M), was without effect on CCh-induced m.e.p.p. frequency depression (Figure 2a). Note that this concentration of TC decreased both the postsynaptic action of CCh on the RMP (Figure 2b, see also Pennefather & Quastel, 1981) and the m.e.p.p. amplitude to about 50% (data

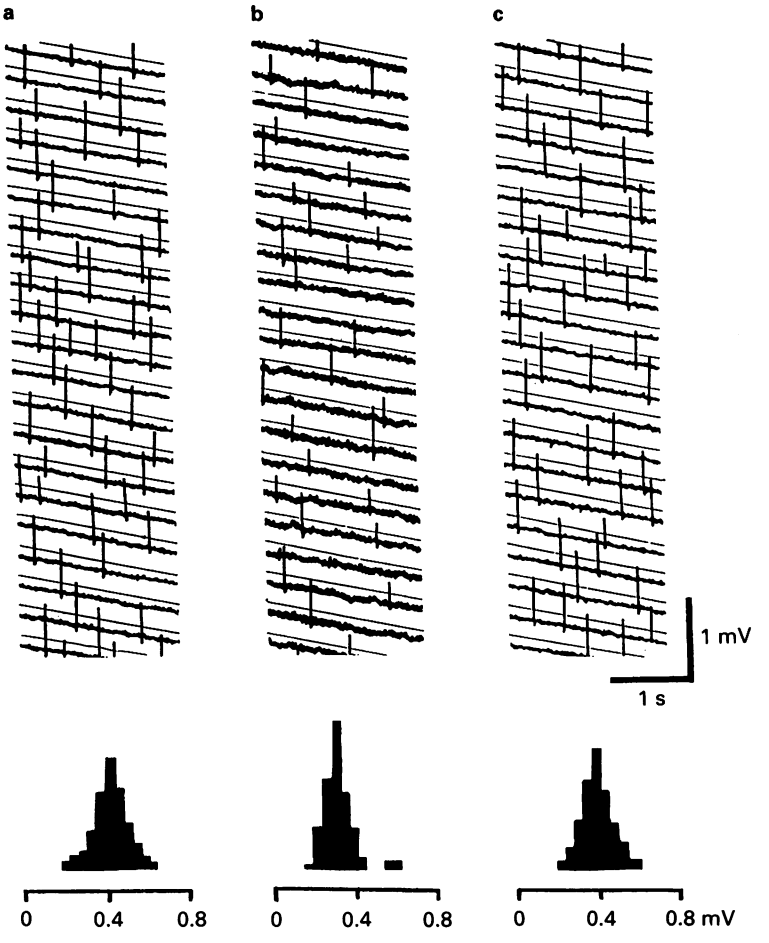

Figure 1 The effect of carbachol (CCh) on the amplitude and frequency of miniature endplate potentials (m.e.p.ps): (a) control; (b) 20 min after bath application of 5×10^{-6} M CCh; (c) 30 min after washing out of the drug. Upper section: E.C. oscilloscopic records of m.e.p.ps on continuously moving film. Upper traces in each record indicate the threshold level for computer counting of m.e.p.ps. Lower section: amplitude histograms constructed from 100 m.e.p.ps.

Table 1 Effects of several cholinomimetics and cholinolytics on the frequency of miniature endplate potentials (m.e.p.ps)

	Concentration (M)	*RMP* (%)	*Amplitude of m.e.p.ps* (%)	*m.e.p.p. frequency* (%)	n
Acetylcholine	7.5×10^{-5}	81.8 ± 3.8*	64.3 ± 5.1*	55.8 ± 5.6*	6
Carbachol (CCh)	5×10^{-6}	91.2 ± 2.5*	78.6 ± 8.7*	52.4 ± 7.6*	16
Nicotine	5×10^{-6}	89.8 ± 2.4*	49.9 ± 4.5*	79.7 ± 7.3*	5
Tetramethylammonium	5×10^{-5}	90.7 ± 2.4*	56.0 ± 9.7*	75.5 ± 3.2*	6
Suberyldicholine	1×10^{-6}	78.6 ± 3.1*	48.2 ± 9.9*	78.3 ± 7.0*	5
Metacholine	5×10^{-5}	99.3 ± 4.3	95.6 ± 4.6	94.3 ± 4.5	5
Oxotremorine	5×10^{-5}	100.1 ± 1.8	97.0 ± 5.4	93.5 ± 6.9	5
Methylfurmethide	5×10^{-5}	100.3 ± 3.1	98.6 ± 3.9	95.4 ± 6.5	5
CCh + atropine 1×10^{-6}	5×10^{-6}	91.0 ± 1.8*	75.6 ± 4.6*	54.6 ± 6.8*	6
CCh + benzhexonium 1×10^{-5}	5×10^{-6}	94.8 ± 2.3*	82.6 ± 4.5*	58.7 ± 5.0*	5
CCh + IEM-1119 4×10^{-5}	5×10^{-6}	92.1 ± 3.0*	81.8 ± 5.0*	54.8 ± 6.9*	5

Resting membrane potential (RMP), amplitude of m.e.p.ps and m.e.p.p. frequency are expressed as percentage of control values (100%) measured at the beginning of each experiment. n = number of experiments.
Asterisks indicate the values which are significantly different from controls ($P < 0.05$).
All values in the Table and in the text are the mean ± s.e.mean, significance tested by t test.

not given). Ganglionic blockers (Brown, 1980) benzhexonium, hexamethonium and IEM-1119 were also without effect on the depression of m.e.p.p. frequency evoked by CCh (Table 1). Thus, the presynaptic structures involved in the CCh-evoked depression of m.e.p.p. frequency differ pharmacologically from nicotinic and muscarinic endplate ACh-receptors and as well as from those of the ganglionic postjunctional membrane.

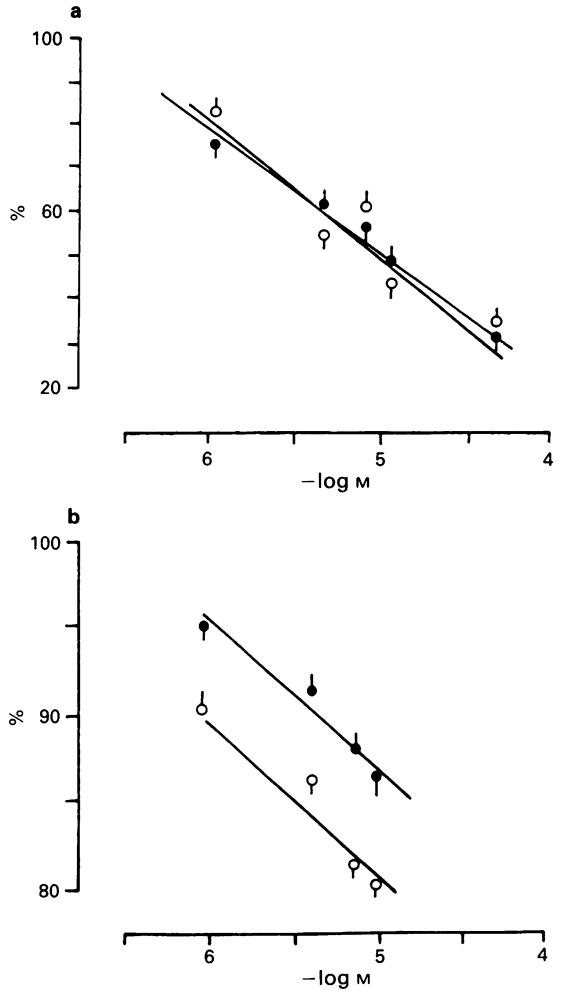

Figure 2 Effects of different concentrations of carbachol (−log M) on the frequency of miniature endplate potentials (a) and on the resting membrane potential (b) expressed as a percentage of the average values obtained 30 min before carbachol application. The average value of frequency was 1.32 ± 0.41 imp s^{-1} and resting membrane potential was 85.3 ± 1.2 mV in control experiments: (○)without (+)-tubocurarine; (●) in presence of (+)-tubocurarine, 4.5×10^{-7} M. Each point represents the mean value (s.e.mean shown by vertical lines) obtained from 5 experiments. Temperature 21°C.

Presynaptic action of cholinomimetics on anticholinesterase-treated preparations

It has been suggested that the presynaptically localized cholinesterase might be the target for the CCh and ACh effects on m.e.p.p. frequency in the frog (Duncan & Publicover, 1979). Therefore the effects of a reversible anticholinesterase, proserin, and an irreversible drug, armin, on CCh effects were examined. Armin, 5×10^{-6} M, and proserin, 1×10^{-5} M, inhibit the specific as well as the non-specific cholinesterase (Aluf, 1955; Prozorovskij & Savatjeev, 1976); both drugs increased the m.e.p.p. amplitude by about 80%, as expected. However, pretreatment of muscles with 5×10^{-6} armin or 1×10^{-6} M proserin had no measurable effect on m.e.p.p. frequency. Carbachol, 5×10^{-6} M, when added 30 min after pretreatment of muscles with armin or 45 min after application of proserine, decreased m.e.p.p. frequency by 46.3 ± 5.3% ($n = 4$, $P < 0.05$) and by 50.3 ± 6.7% ($n = 7$, $P < 0.05$). The decreases in m.e.p.p. frequency were similar to the values obtained without cholinesterase inhibition. Thus, the absence of an effect of inhibition of cholinesterase on the CCh-induced depression of m.e.p.p. frequency suggests that cholinesterase does not directly regulate the cholinolytic-induced modulation of spontaneous quantal release at the frog endplate.

Presynaptic action of carbachol during variation in the ionic composition of the medium

Changes in ion permeabilities, ion gradients and corresponding shifts in membrane potential of the nerve terminal might be involved in the cholinergic modulation of m.e.p.p. frequency. To check this possibility, we measured the effect of ionic composition changes, namely in Na^+, Cl^-, Ca^{2+} and K^+ on m.e.p.p. frequency influenced by CCh.

Na^+ and Cl^- variations First, sucrose was substituted for 2/3 of NaCl in the medium (Table 2A). This led to an increase of initial m.e.p.p. frequency (cf. Duncan & Publicover, 1979). The new level was stable for at least 90 min; the depressive effect of CCh on m.e.p.p. frequency was, however, the same as in a normal Ringer solution.

Similar results were also obtained in muscles bathed in a solution where NaCl (Statham & Duncan, 1977) was exchanged for NaBr and $NaNO_3$ in a molar ratio 1:1 or Na_2SO_4 in a molar ratio 3:2 (Table 2B). The substituted anions differ in their membrane permeabilities but they are less permeable than chlorides in all cases (Saint *et al.*, 1987). In these solutions, the m.e.p.p. frequency was almost identical

Table 2 Effects of decreases in Cl^- and in NaCl concentrations on presynaptic action of 5×10^{-6} M carbachol (CCh)

	Original ion or solution	*Exchanged for*	*% of NaCl exchange*	*Concentration of exchanged ion* (mM)	*m.e.p.p. frequency without CCh* (control)	*m.e.p.p. frequency with CCh*	*Decrease of m.e.p.p. frequency* (% of control)	*Number of experiments* n
A	Control solution	—	0	118.6	2.75 ± 0.74	1.28 ± 0.42	48.8 ± 6.5	6
	Na^+	Sucrose	66.7	43.2	4.62 ± 1.22	2.10 ± 0.52	49.1 ± 7.4	6
B	Control solution	—	0	119.1	0.97 ± 0.23	0.41 ± 0.10	51.1 ± 6.6	5
	Cl^-	$NaNO_3$	100	6.1	0.50 ± 0.13	0.50 ± 0.13	46.2 ± 7.6	5
	Cl^-	Na_2SO_4	100	6.1	0.39 ± 0.09	0.39 ± 0.09	58.7 ± 7.2	6
	Cl^-	NaBr	100	6.1	0.43 ± 0.11	0.43 ± 0.11	51.2 ± 6.4	5

Miniature endplate potential (m.e.p.p.) frequency expressed as number of events per s. Chlorides of KCl and $CaCl_2$ in Ringer solution were not exchanged. A, B – experiments with lowered Na^+ or Cl^- respectively. Mean ± s.e.mean are given. Temperature 20°C.

and no statistically significant difference was observed as far as the action of CCh on m.e.p.p. frequency was concerned. These experiments indicate that the CCh-induced drop in frequency is not directly connected with ionic channels for Na^+ and Cl^- in the nerve terminal membrane.

Effect of Ca^{2+} and K^+-depolarization Duncan & Publicover (1979) proposed that Ca^{2+} permeability of the nerve terminal membrane is decreased during the action of cholinomimetics. We therefore followed the CCh effect on m.e.p.p. frequency during changes in extra- and intracellular Ca^{2+} concentration and also in the presence of agents which are known to modify Ca^{2+} action within the terminal.

First of all, the extracellular Ca^{2+} ($[Ca^{2+}]_o$) was removed from the Ringer solution. This decreased m.e.p.p. frequency from 1.4 ± 0.4 ($n = 4$) in 1.8 mM $[Ca^{2+}]_o$ to 0.5 ± 0.2 imp s^{-1} ($n = 7$, $P < 0.05$) after 30 min in a nominally Ca^{2+}-free medium. Subsequent addition of 3.5×10^{-6} M CCh further decreased the m.e.p.p. frequency. This decrease was, however, smaller in nominally Ca^{2+}-free solution (by 29.3 ± 3.5%, $n = 7$, $P < 0.05$) than in the controls with 1.8 mM $[Ca^{2+}]_o$ (by 46.0 ± 3.5). An increase of $[Ca^{2+}]_o$ above 1.8 mM (Figure 3) increased m.e.p.p. frequency which may well be explained by a larger influx of Ca^{2+} into the terminals. In parallel, a greater presynaptic effect of CCh was observed (Figure 3, open symbols), namely when $[Ca^{2+}]_o$ exceeded 6 mM. At a concentration of 7.2 mM $[Ca^{2+}]_o$, for example, CCh depressed the m.e.p.p. frequency by 62.4 ± 3.4% ($n = 6$, $P < 0.05$), i.e. by about 16% more than in the controls with 1.8 mM $[Ca^{2+}]_o$.

Further experiments were done during potassium depolarization which is known to increase the Ca^{2+} entry into the terminals due to activation of the voltage-dependent Ca^{2+}-channels (Liley, 1956; Gardos, 1958; Elmquist & Feldman, 1965) and to stimulate the spontaneous quantal release of the transmitter (for review, see Cohen & Van der Kloot, 1985).

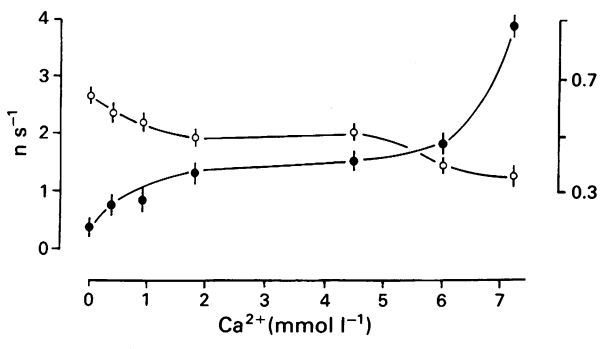

Figure 3 Dependence of miniature endplate potential (m.e.p.p.) frequency (●) and ratio of frequencies before and after 5×10^{-6} M carbachol (right ordinate scale, ○) on calcium concentration in the bath. Each point is mean from 7 experiments; s.e.mean shown by vertical lines.

An increase of extracellular K^+ above 0.5 mM produced a concentration-dependent increase in m.e.p.p. frequency (Figure 4) but there was no change in the percentage decrease of the frequency with CCh at any K^+ concentration (Figure 4, open symbols). Moreover, if the m.e.p.p. frequency was already decreased by CCh in normal potassium, the percentage increase of frequency produced by elevation of external K^+ was similar to that in the absence of CCh (data not given). Interestingly, the percentage reduction of m.e.p.p. frequency by CCh was independent of the original frequency in individual muscle fibres (Table 3). The effect of CCh or ACh on m.e.p.p. frequency was therefore not connected directly with the K^+-induced depolarization and seemed not to depend on the intraterminal calcium level.

This conclusion is supported by experiments with magnesium and manganese which inhibit Ca^{2+} entry (for review see Silinsky, 1985). The addition of 6 mM Mg^{2+} or 3 mM Mn^{2+} to the muscle led to a small increase (by about 8%) in m.e.p.p. frequency. The decrease produced by subsequent application of 5×10^{-6} M CCh was not significantly different from the control (61.1 ± 7.8, $n = 6$ for Mg^{2+}, 62.1 ± 3.2, $n = 7$ for Mn^{2+}).

Effects of db cyclic AMP, db cyclic GMP and theophylline

Regulatory mechanisms of transmitter release might be connected with shifts in cyclic nucleotides (Goldberg & Singer,

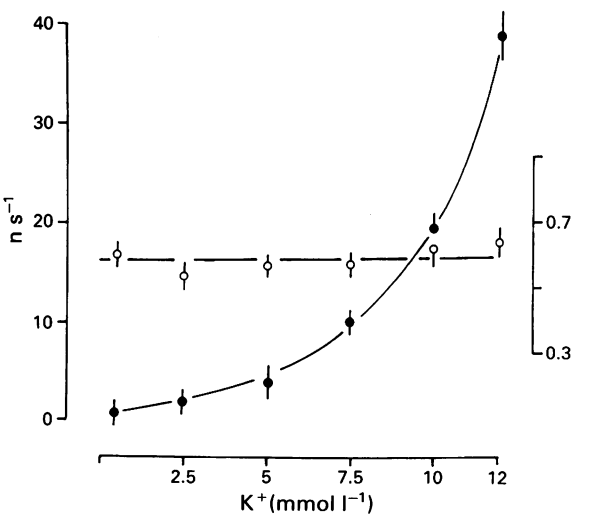

Figure 4 Dependence of miniature endplate potential (m.e.p.p.) frequency (the number, n, of m.e.p.ps per 1 s, ●) on the concentration of potassium ions (K^+) in the bath; (○) the ratio of the frequency of m.e.p.ps after 20 min of 5×10^{-6} M carbachol (right ordinate scale) to that before carbachol. Average values from 5–7 experiments.

Table 3 Effects of changes in miniature endplate potential (m.e.p.p.) frequency on the effect of 5×10^{-6} M carbachol (CCh) on m.e.p.p. frequency

Range of m.e.p.p. frequency without CCh (control)	*% of m.e.p.p. frequency in CCh*	*Number of experiments* (n)
0.3–0.5	54.6 ± 2.6	11
0.6–0.8	56.2 ± 4.5	11
0.8–1.1	57.9 ± 5.5	9
1.1–1.4	49.4 ± 3.8	11
1.4–2.0	52.5 ± 6.3	9
2.0–2.5	49.6 ± 4.8	7
2.5–3.0	47.9 ± 6.8	5
3.0–4.5	53.9 ± 7.9	8

m.e.p.p. frequency = number of events per s. Mean ± s.e.mean. Temperature 20°C.

1969; for review, see Greengard, 1979). Agents known to decrease the hydrolysis of cyclic AMP by phosphodiesterase, e.g. theophylline, increase the m.e.p.p. frequency and the quantum content of evoked ACh released during stimulation (Goldberg & Singer, 1969). Catecholamines which stimulate the production of cyclic AMP (Sutherland & Robison, 1966) also increase the m.e.p.p. frequency (Kuba, 1970) as well as the permeable analogue of cyclic AMP, db cyclic AMP (Goldberg & Singer, 1969).

In our experiments the application of theophylline or noradrenaline, db cyclic AMP or db cyclic GMP did not influence significantly the effect of CCh on m.e.p.p. frequency (Table 4).

Presynaptic action of carbachol during inhibition and activation of Na^+-K^+-ATPase

The Mg^{2+}-dependent, Na^+,K^+-activated ATPase (E.C. 3613) has been shown to participate in the regulation of transmitter release (Baker & Crawford, 1975; Vizi, 1978; 1979; Vyskočil, 1979; Vizi & Vyskočil, 1979) and can be influenced by cholinomimetic and cholinolytic drugs (Kometiani *et al.*, 1975; Dlouhá *et al.*, 1979). Moreover, there is considerable homology between the nicotinic ACh receptor and a catalytic subunit of Na^+,K^+-ATPase (Elman *et al.*, 1982).

Ouabain (5×10^{-5} M) blocked the inhibitory action of CCh on m.e.p.p. frequency (Figure 5); it even reversed the depression when added to the bath after CCh. The presence of ouabain in the bath was restricted to only 15–20 min to avoid the increase in m.e.p.p. frequency usually found after 30 min, when ionic gradients are already distorted and RMP of terminals decreased (Baker & Crawford, 1975). The effect of ouabain on the dose-response curves for CCh showed no signs

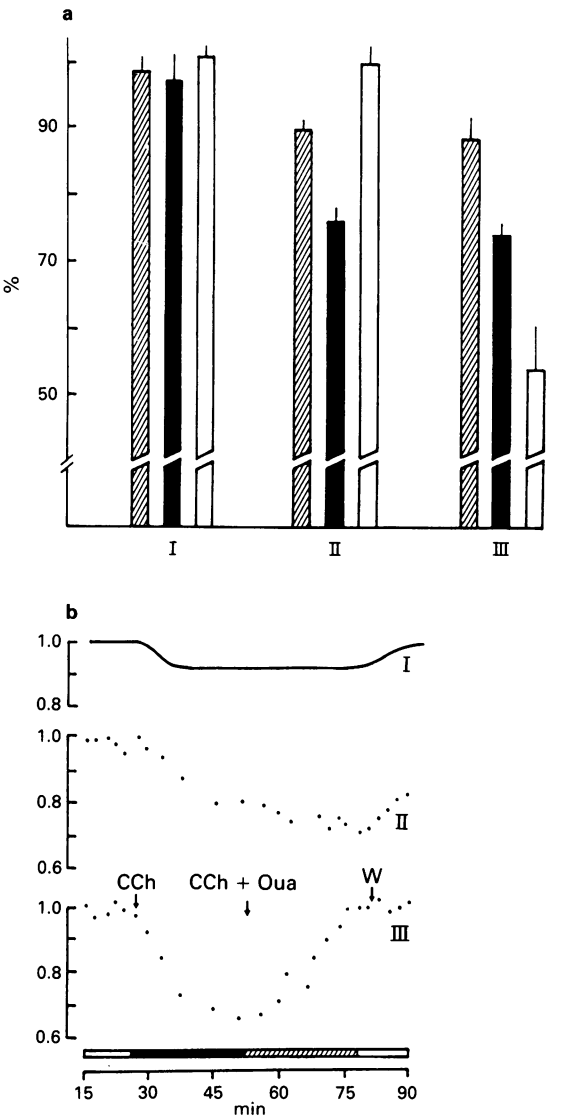

Figure 5 Pre- and postsynaptic effects of 5×10^{-6} M carbachol (CCh) in the absence and presence of 5×10^{-5} M ouabain (Oua). (a) Percentage changes of membrane potential (hatched columns), miniature endplate potential (m.e.p.p.) amplitude (closed columns) and frequency (open columns) in muscles treated for 20 min with (I) ouabain, (II) ouabain plus carbachol and (III) carbachol only. Seven fibres were measured in each group between 15 and 20 min of the treatment. (b) Single fibre membrane potential (I), m.e.p.p. amplitude (II) and frequency (III) before CCh, after CCh, CCh + Oua and washing (W) respectively. Ordinates: ratio of experimental and control values.

Table 4 The effects of theophylline, dibutyryl cyclic AMP (db cyclic AMP) and dibutyryl cyclic GMP (db cyclic GMP) on the frequency of miniature endplate potentials (m.e.p.ps) in the absence and presence of carbachol (CCh)

	A	B	C	D
Drug	*Concentration* (M)	*Increase without CCh* (%)	*Drug + CCh* (%)	n
Theophylline	1×10^{-3}	198.3 ± 4.6	58.8 ± 3.4	8
Noradrenaline	1×10^{-5}	189.2 ± 3.6	61.3 ± 2.9	7
db cyclic AMP	1×10^{-6}	217.1 ± 6.1	63.3 ± 3.7	10
db cyclic GMP	1×10^{-6}	262.4 ± 5.3	57.5 ± 5.0	10

Muscles (*n*) were bathed first without drugs for 20 min; then the fibre was impaled with a microelectrode and m.e.p.p. frequency was measured. Then drug was added (column A) and a new series of m.e.p.ps was collected during 20–30th min of drug action (column B). Carbachol was then added and during the next 20 min the last series of m.e.p.ps was measured.
Values in column B were taken as 100% for column C.
Temperature 20°C.

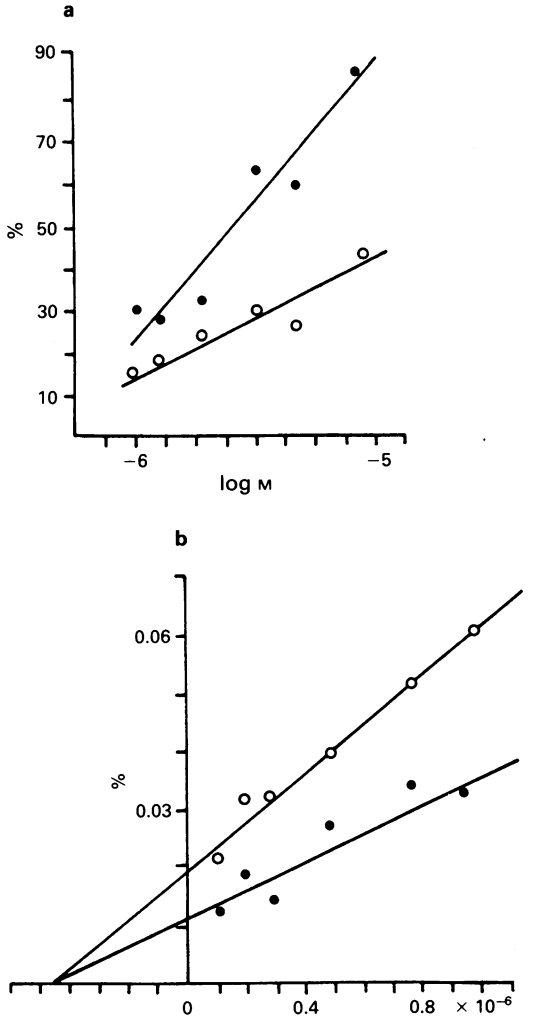

Figure 6 Logarithmic (a) and double-reciprocal (b) plots of the depressive effect of carbachol at several concentrations on the frequency of miniature endplate potentials (m.e.p.ps) without (●) and with 3.5×10^{-5} M (○) ouabain (20 min) in the perfusion bath. (a) Ordinate scale percentage inhibition

$$\left[\left(1 - \frac{\text{m.e.p.p. frequency in carbachol}}{\text{m.e.p.p. frequency in control}}\right) \times 100\right]$$

Abscissae: log of carbachol concentration in mol l^{-1} (M). (b) Ordinate scale: reciprocal values of m.e.p.p. frequency depression by carbachol; abscissae: reciprocal values of carbachol concentration. Each point represents a mean from 3 independent experiments.

of competitive interaction between ouabain and CCh which suggests, that the drugs interact with different sites on the enzyme molecule (Figure 6).

Na^+,K^+-ATPase can also be inhibited by removing potassium from the bath solution (Kernan, 1962; Vyskočil & Illés, 1977; Vizi & Vyskočil, 1979; Marunaka, 1986). Application of 5×10^{-6} M CCh to muscles bathed for 30 min in K^+-free solution, decreased the m.e.p.p. frequency by 41.6 ± 5.2% ($n = 6$, $P < 0.05$). To reduce the likelihood of K^+ release from the muscle fibres, which could activate the Na^+,K^+-ATPase, several experiments were done on fibres voltage-clamped at a holding potential of −110 mV to prevent, or at least drastically reduce K^+ efflux. The results were similar. After addition of 5×10^{-6} M CCh, the clamp current increased as expected, but the frequency of miniature endplate currents was decreased by 36.5 ± 0.5% ($n = 5$, $P < 0.05$). Thus, under conditions where the Na^+,K^+-ATPase was blocked in a K^+-free solution, no significant inhibition of the CCh-induced effect on m.e.p.p. frequency was observed, in contrast to ouabain. Apparently, the blocking effect of ouabain on CCh-induced frequency inhibition is not connected with Na^+,K^+-ATPase function.

Adrenaline and insulin activate Na^+,K^+-ATPase and the electrogenic Na^+,K^+ pump (cf. Clausen, 1986). The m.e.p.p. frequency was increased slightly 30 min after adding 1×10^{-5} M adrenaline to 111.3 ± 2.3% ($n = 6$, $P > 0.05$) of the control and muscle fibre RMP was hyperpolarized by 3.3 ± 1.1 mV ($n = 6$, $P < 0.05$). CCh application reduced the m.e.p.p. frequency by 34.3 ± 3.5% ($n = 5$, $P < 0.05$). Insulin (0.5 iu ml^{-1}) also hyperpolarized the resting membrane by 3.3 ± 1.1 mV ($n = 12$, $P < 0.05$), and reduced m.e.p.p. frequency by 18.9 ± 5.0% to 81% ($n = 12$, $P < 0.05$). A higher dose (2 iu ml^{-1}) hyperpolarized the resting membrane by 6.3 ± 2.8 mV ($n = 3$, $P < 0.05$) and decreased the m.e.p.p. frequency by 20.5 ± 5.2% ($n = 3$, $P < 0.05$). Three iu ml^{-1} of insulin were without further effect. In the presence of insulin, the percentage depression of frequency by 5×10^{-6} M CCh was similar to that in the controls (to 43.1 ± 22%).

Temperature and presynaptic action of carbachol

If an enzyme or receptor were the target for the CCh presynaptic action one would expect a significant effect of temperature on the response. A pronounced temperature-dependence is characteristic for many enzymatic and ion channel functions as well as for processes of transmitter release. Temperature coefficients (Q_{10}) range between 1.5–3.0 (e.g. Frankenheuser & Moore, 1963, for Na^+ and K^+ permeability; Anderson & Stevens, 1973, for mean open time of ACh-operated channel) and 10–12 (e.g. Thesleff *et al.*, 1983, for

Table 5 The temperature (t) dependence of different concentrations of carbachol (CCh) action on frequency of miniature endplate potentials (f.m.e.p.ps)

	Carbachol (M)	t (°C)	*Control f.m.e.p.ps*	*f.m.e.p.ps with CCh*	*% of control*	*Number of experiments* (n)
A	1×10^{-5}	10	0.35 ± 0.11	0.18 ± 0.04	51.4 ± 7.8	5
		20	2.25 ± 0.86	0.97 ± 0.25	43.1 ± 5.8	6
		25	11.20 ± 2.76	5.10 ± 2.16	45.5 ± 9.8	5
		30	19.17 ± 4.33	8.71 ± 1.70	45.4 ± 6.6	6
B	5×10^{-6}	5	0.36 ± 0.11	0.18 ± 0.03	50.0 ± 5.4	6
		10	0.14 ± 0.024	0.08 ± 0.01	57.1 ± 7.8	8
		15	0.39 ± 0.10	0.20 ± 0.05	51.3 ± 8.0	6
		20	2.63 ± 0.71	1.31 ± 0.40	49.8 ± 6.5	6
		25	4.72 ± 0.95	2.04 ± 0.29	43.2 ± 6.9	6
		30	23.50 ± 5.53	11.54 ± 4.02	49.1 ± 4.0	6
C	6×10^{-7}	8.5	0.12 ± 0.01	0.072 ± 0.01	60.0 ± 4.7	6
		30	21.26 ± 4.24	17.71 ± 2.11	83.3 ± 3.2	6
D	3×10^{-7}	6	0.103 ± 0.010	0.073 ± 0.010	56.2 ± 2.1	6
		26	9.72 ± 2.16	9.45 ± 1.91	97.2 ± 4.3	6

f.m.e.p.ps = number of events per s. Mean ± s.e.mean.

calcium insensitive and slow m.e.p.p.; Hartzel *et al.*, 1977, for muscarinic ACh receptors).

Therefore, the effects of CCh were examined at temperatures between 5° and 30°C (Table 5). The basal m.e.p.p. frequency increased from about $0.14\,s^{-1}$ at 10°C to about $23\,s^{-1}$ at 30°C as shown by Fatt & Katz (1952). Surprisingly, the inhibition of m.e.p.p. frequency by 5×10^{-6} and 1×10^{-5} M CCh was the same throughout the temperature-range with a Q_{10} close to 1.0. These concentrations of CCh were maximal in that a further increase of the CCh in the bath did not produce a greater depression of m.e.p.p. frequency. On the other hand, with lower (threshold) concentrations (6×10^{-7} and 3×10^{-7} M) of CCh, the depression of frequency was much less at 26° and 30°C (97% and 84% respectively) than at 6° and 8.5°C (71 and 62% respectively, Table 5A,B).

Discussion

Several possibilities should be taken into account when discussing the observed effect of acetylcholine analogues on the frequency of m.e.p.p. in the frog neuromuscular junction.

Receptor hypothesis

The depression of m.e.p.p. frequency has already been studied by Duncan & Publicover (1979). This depression is not caused by a loss of small-amplitude m.e.p.ps in the presence of the agonists because the histograms of m.e.p.p. amplitude retained a normal Gaussian character (Figure 1). The fact that current and voltage clamp experiments were similar suggests that this phenomenon was not caused by ionic or metabolic influences from depolarized muscle fibres (Magazanik & Nikolsky, 1979; Hohlfeld *et al.*, 1981) and point to a direct action on the nerve terminal. It was not mediated by muscarinic autoreceptors which ensure negative feed-back in other nerve tissue, including poikilothermic vertebrates (Michaelson *et al.*, 1979; Kilbinger & Kruel, 1981), since different specific muscarinomimetics such as methylfurmethide, oxotremorine and metacholine did not affect m.e.p.p. frequency. Moreover, atropine, which blocks muscarinic ACh receptors, did not prevent the depression of m.e.p.p. frequency evoked by cholinomimetics. Our data are thus not in accordance with the results of Duncan & Publicover (1979) who reported that muscarinic antagonists have a depressant effect on m.e.p.p. frequency. The discrepancy might be ascribed to differences in the experimental condition. In the above mentioned paper, the preparation was first cooled to 10°C and then warmed to 22.5°C. During this temperature change, a decrease of m.e.p.p. frequency was reported in the controls as well as in the presence of muscarine, atropine and anticholinesterase. Lowering of temperature to or below 10°C may well switch off a temperature-dependent system, e.g. the Na^+,K^+-ATPase, which is then activated after warming the bath (Vizi, 1978; Vizi & Vyskočil, 1979). Our finding that other nicotinomimetics depress m.e.p.p. frequency in a similar way to ACh and CCh supports the 'autoreceptor' hypothesis. Furthermore, the reduction of the quantal content of the evoked e.p.p. after application of cholinomimetics in the frog (Ciani & Edwards, 1963; Nikolsky & Giniatullin, 1979) supports the possibility that quantal release is modulated by receptors. However, in contrast to evoked e.p.ps, which curare blocks (Nikolsky & Giniatullin, 1979), the depression of spontaneous m.e.p.p. frequency was not affected by this drug (Figure 2). Other pharmacological tests (Table 1) of the depression in frequency of m.e.p.p. showed that the responsible structures were different from the pharmacologically well-characterized ACh receptors of the muscle fibre and autonomic ganglia.

Ca^{2+} hypothesis

Because of the dependence of transmitter release on membrane potential, it is possible that hyperpolarization mediates the cholinomimetic action. However, the hyperpolarization induced by potassium removal and potassium depolarization did not change the effect of cholinomimetics and a simple connection between nerve polarization and m.e.p.p. frequency depression probably does not exist.

Quantal release depends on the external level of Ca^{2+} ions (Elmquist & Feldman, 1965). It is also possible that the Ca^{2+} regulating systems in the nerve terminal are connected with m.e.p.p. frequency depression. If cholinomimetics simply blocked Ca^{2+} entry, their effect should be dependent on external Ca^{2+}. This was true, however, only at very low or relatively high (6–7 mM) concentrations. The CCh inhibition of m.e.p.p. frequency was well developed in a Ca^{2+}-free solution. Furthermore, the fact that the calcium antagonists, Mg^{2+} and Mn^{2+}, had no effect on the CCh-induced depression shows that Ca^{2+} entry was not a limiting step. This was most clearly supported by the experiments with potassium depolarization where the depression of m.e.p.p. frequency was always the same.

The entry of Ca^{2+} from the outside is not the only mechanism by which the intracellular Ca^{2+} concentration might be altered. Changing the intracellular Ca-buffering processes e.g. via the cyclic nucleotide system would also alter the free intracellular Ca^{2+}. However, we did not find any alternation in the CCh effect even when m.e.p.p. frequency was increased by db cyclic AMP or db cyclic GMP or when cyclic AMP splitting was inhibited by theophylline. Thus, the effect of CCh is similar to the Ca^{2+}-independent stimulation of m.e.p.p. frequency in frog muscle by trinitrobenzene sulphonic acid (Kijima & Tanabe, 1988) which acts by an as yet unknown mechanism even in a Ca^{2+}-free medium, when Ca^{2+} channels were blocked by ω-conotoxin and internal calcium was buffered with 1,2-*bis*(2-aminophenoxy)ethane tetraacetic acid (BAPTA). It also resembles the effect of ethanol and dimethyl sulphoxide described by Quastel *et al.* (1971) and McLarnon *et al.* (1986).

Na^+-K^+-ATPase

Because ouabain, a well-known inhibitor of membrane Na^+,K^+-ATPase, decreased the effect of cholinomimetics on m.e.p.p. frequency, this enzyme was thought to be a candidate for this cholinergic modulation. However, the absence of an effect of K^+-free solution on the CCh-induced depression, together with the ambiguous effects of adrenaline and insulin suggested that this enzyme is not directly involved in the CCh presynaptic effect. The protective effect of ouabain might be connected with its rather nonspecific mode of action. It can, for example, increase Na permeability (Robbins, 1977) or modulate the vesicle recycling (Haimann *et al.*, 1985) and non-quantal ACh release (Zemková *et al.*, 1990).

Acetylcholinesterase

Our observation that anticholinesterase drugs had little or no effect on the depression of m.e.p.p. frequency by carbachol indicates that cholinesterases do not mediate the carbachol effect, in contrast to the conclusions of Duncan & Publicover (1979).

Temperature

The absence of temperature-dependence at saturating concentrations of CCh was surprising. Either two parallel processes exist, both temperature-dependent, acting under CCh in an opposite direction on m.e.p.p. frequency, or the limiting step in presynaptic CCh action is purely of a physical nature. According to the classical physical concept of adsorption (Bikerman, 1948; Cassidy, 1951), not only would the temperature-dependence in the case of saturating concentrations be negligible, but the temperature-dependence, when threshold concentrations are used, would be opposite. Free presynaptic hypothetical sites for CCh might be occupied to a greater extent at lower temperatures. Experiments with

6×10^{-7} and 3×10^{-7} M CCh are in agreement with this, showing that the CCh effect is much smaller at higher temperatures than at lower ones. Because the effective doses of ACh as well as of CCh are in the micromolar range, it is probably not the whole surface of the nerve terminal membrane but only some specific parts (e.g. active zones of ACh release; Heuser *et al.*, 1974) which are affected by CCh. Despite the fact that the percentage drop in m.e.p.p. frequency was not temperature-dependent, the presence of CCh or ACh did not prevent the increase or decrease of m.e.p.p. frequency as such e.g. by external K^+ changes, application of cyclic nucleotides or by temperature itself.

It should also be mentioned that the same degree of inhibition by CCh was observed in the case of stimulation-evoked ACh release, where a drop of endplate potentials to about one half was found with 5×10^{-6} M CCh (Nikolsky & Giniatullin, 1979) irrespective of quantum content size.

Whatever the most probable explanation, the temperature independence of this process in the frog might reflect the fact that for amphibians living in a broad range of temperatures, the cholinergic depression is of physiological significance. For example, one can speculate that the post-tetanic increase in m.e.p.p. frequency (Magleby & Zengel, 1976) is compensated for, to some extent, by the feedback action of the released acetylcholine.

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