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4-AMINOPYRIDINE AND EVOKED TRANSMITTER RELEASE FROM MOTOR NERVE ENDINGS

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1 In the presence of tetrodotoxin, electrotonic depolarization of frog motor nerve terminals causes the appearance of stimulus-graded endplate potentials. When 4-aminopyridine is added, the graded endplate potential is converted into a triggered all-or-none response resulting in giant endplate potentials of about 70 mV amplitude and 50 ms duration. The triggered endplate potentials are abolished in Ca^{2+}-free saline and are blocked by Mn^{2+} ions. Sr^{2+} but not Ba^{2+} can replace Ca^{2+} in supporting transmitter release. Mg^{2+} fails, even in concentrations as high as 32 mM, to affect the amplitude and the shape of the endplate potential but abolishes it when the Ca^{2+} concentration is reduced to 0.2 mM.

2 Despite the large amplitude of the triggered endplate potential in the presence of 4-aminopyridine and tetrodotoxin, repetitive stimulation up to 10 Hz causes only a small decline in amplitude of successive endplate potentials. However, in the presence of (+)-tubocurarine or gallamine, repetitive nerve stimulation produces a marked decline in successive endplate potential amplitude. The fall is counteracted when evoked transmitter release is reduced in the presence of 0.2 mM Ca^{2+}. The results suggest that in the presence of 4-aminopyridine such large amounts of transmitter are released that even during repetitive stimulation (5 to 10 Hz) endplate potentials are of maximal amplitude.

3 4-Aminopyridine causes a parallel shift to the right of the dose-response curve to Mg^{2+} for blockade of nerve *impulse*-evoked transmitter release (in the absence of tetrodotoxin). A similar parallel shift occurs in the presence of tetraethylammonium and guanidine.

4 It is concluded that 4-aminopyridine increases transmitter release by enhancing the transport efficacy for Ca^{2+} across the nerve terminal membrane during nerve terminal depolarization.

Introduction

At the neuromuscular junction 4-aminopyridine (4-AP), guanidine and tetraethylammonium (TEA) are known to potentiate greatly transmitter release evoked by nerve impulses. As demonstrated for TEA by Katz & Miledi (1969) and for the two other drugs by Lundh & Thesleff (1977) their mechanism of action is to enhance the influx of calcium ions during nerve terminal depolarization. Under conditions of complete blockade of inward sodium current by tetrodotoxin (TTX) these drugs, in response to electrotonic depolarization of the nerve terminals, trigger a non-conducted regenerative calcium flux across the nerve terminal membrane and thereby cause the appearance of a giant end-plate potential in all-or-none fashion.

The purpose of the present study was to investigate the effects of divalent cations and postsynaptic neuromuscular blocking agents on conducted and non-conducted transmitter release evoked in the presence of 4-AP. This substance is more than 20 times as potent as guanidine and TEA in eliciting a regenerative calcium current and it lacks the curare-like postsynaptic blocking effect of TEA (Lundh, Leander & Thesleff, 1977). The experiments were carried out *in vitro* on the frog neuromuscular junction.

Methods

Experiments were performed *in vitro* on the sartorius nerve-muscle preparation from *Rana temporaria*. The composition of the Ringer solution was (mM): NaCl 115, KCl 2, $CaCl_2$, 1.8 and NaH_2PO_4 1. The phosphate buffer maintained the pH at 6.8 to 6.9 in the presence of 4-AP. In some of the experiments the muscles were immersed for 30 to 45 min in a nominally Ca^{2+}-free solution after which 1.8 mM $CaCl_2$, $SrCl_2$, $BaCl_2$ or $MgCl_2$ was added to the bath. Neither in these experiments, nor in the experiments with different concentrations of $CaCl_2$ or $MgCl_2$ was any adjustment made for changes in tonicity. The temperature of the bath was kept at 14°C, when trans-

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mitter release in response to electrotonic depolarization of nerve terminals was studied (Katz & Miledi, 1969) and at 21°C when release in response to conducted nerve impulses was investigated.

Intracellular recording of endplate potentials (e.p.ps) from superficial muscle fibres was performed by conventional techniques with glass capillary electrodes filled with 3 M KCl. Conducted nerve impulses were initiated by stimulation at supramaximal voltage and 0.2 ms duration through a glass capillary suction electrode. In experiments with electrotonic depolarization of nerve terminals, TTX 10^{-6} M was added to the bathing solution, and current pulses of 4 ms duration were applied to the nerve via the glass capillary suction electrode. An endplate was localized as close to the stimulating electrode as possible. In this way the pre-terminal part of the nerve fibre could be depolarized, and the potential change allowed to spread electrotonically into the terminals. The current through the suction electrode was measured by monitoring the potential over a 1 kΩ resistance placed in series with the pipette.

Contact time was 15 to 20 min for all the ions and substances tested.

Drugs used were: 4-aminopyridine (Sigma), guanidinium chloride (BDH), tetraethylammonium chloride (BDH), tubocurarine chloride (Burroughs Wellcome), gallamine triethiodide (May & Baker), decamethonium bromide (Burroughs Wellcome) and tetrodotoxin (Sankyo).

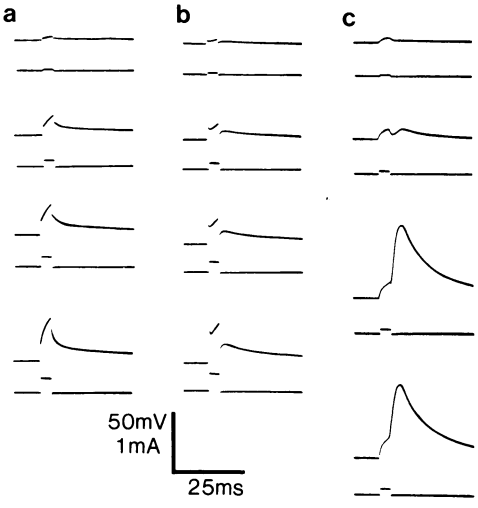

Figure 1 Electrotonic depolarization of nerve terminerals in the presence of tetrodotoxin (TTX) 10^{-6} M and in the absence of extracellular Ca^{2+} failed to release transmitter as shown in (a). When Ca^{2+} 1.8 mM was readmitted to the bath a stimulus-graded endplate potential appeared (b). The addition of 4-aminopyridine (4-AP) 1.3×10^{-4} M converted the graded endplate potential into an all-or-none response as shown in (c). Upper trace shows intracellular potential and lower trace monitors the stimulus current.

Results

Effect of 4-aminopyridine on transmitter release in the presence of calcium and/or other divalent cations

Endplate potentials triggered electrotonically in tetrodotoxin. In Ca^{2+}-free saline the endplate region was localized by looking for miniature endplate potentials (m.e.p.ps) with a fast rising phase. In the presence of TTX (10^{-6} M), i.e. in the absence of nerve impulses, depolarization applied electrotonically to the nerve terminal failed to release transmitter. However, when Ca^{2+} 1.8 mM was readmitted to the bath a small and stimulus-graded e.p.p. appeared, showing that the release of the transmitter is dependent on Ca^{2+} (Figure 1). On the administration of 4-AP (1.3×10^{-4} M) an almost explosive increase of transmitter release occurred, resulting in giant e.p.ps of about 70 mV amplitude. The mean duration of the e.p.p. at 50% of its amplitude was 9.9 ± 1.2 ms in 4 experiments and its total duration about 50 ms. A very small increment of current intensity triggered the e.p.p. virtually in an all-or-none fashion (Figure 1). The giant e.p.ps were abolished in Ca^{2+}-free saline but otherwise they were independent of the ambient Ca^{2+} concentration, e.p.ps of about equal amplitude being triggered in the presence of 0.2 and 20.0 mM Ca^{2+} as shown in Table 1.

The addition of 1.8 mM Sr^{2+} to Ca^{2+}-free solution allowed the release of transmitter in response to depolarization of nerve terminals (Table 1). The addition of 1.8 mM Ba^{2+} or Mg^{2+}, instead of Sr^{2+}, failed to replace Ca^{2+} in supporting electrotonically evoked transmitter release. Manganous ions (10 mM), which block the membrane permeability to Ca^{2+} (Meiri & Rahamimoff, 1972), completely abolished transmitter release in the presence of Ca^{2+} or Sr^{2+} (Table 1).

Magnesium ions, which compete with Ca^{2+} and inhibit the liberation of acetylcholine by nerve impulses failed, even in concentrations as high as 32 mM, to affect the amplitude or the shape of the e.p.p. in response to electrotonic depolarization (Table 1). However, in the presence of 0.2 mM Ca^{2+}, 32 mM Mg^{2+} blocked the all-or-none response (Table 1) and converted it to a stimulus-graded e.p.p.

Endplate potentials in response to conducted nerve impulses. Figure 2 shows the effect of a range of Mg^{2+} concentrations on the amplitude of the e.p.p. in curarized ((+)-tubocurarine, Tc, 5×10^{-6} M) muscle. A semi-logarithmic plot of Mg^{2+} concentrations against

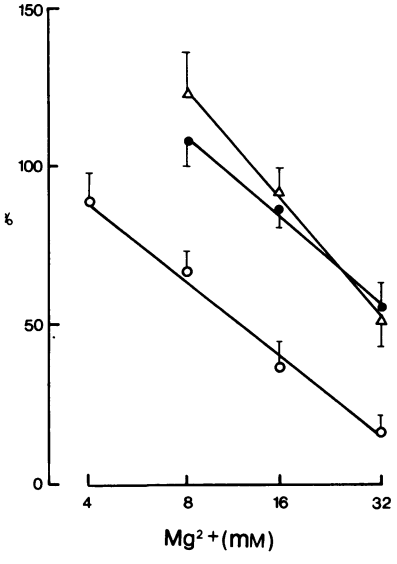

Figure 2 Dose-response curves to Mg^{2+} for blockade of endplate potentials in response to conducted nerve impulses in curarized muscles; (○): before and (●): after the addition of 1.3×10^{-4} M 4-aminopyridine (4-AP); (△): effect of 3×10^{-3} M tetraethylammonium (TEA). To block neuromuscular transmission tubocurarine (Tc) 5×10^{-6} M was used in the absence of 4-AP or TEA, and 2×10^{-5} M in the presence of these drugs. The values are means ± s.e. mean of 4 to 5 experiments and are expressed as % of endplate potential amplitude in the absence of Mg^{2+}.

a percentual decrease of e.p.p. amplitude yielded a straight line.

Since 4-AP (1.3×10^{-4} M) drastically increased the amplitude of the e.p.ps, high concentrations of Tc (2×10^{-5} M) were required to block muscle twitches. 4-AP caused a parallel shift to the right of the dose-response curve to Mg^{2+} for blockade of transmitter release, 35 mM Mg^{2+} reducing transmitter release to about one half which is a 3.5 times higher concentration than that required in the absence of 4-AP. A similar parallel shift occurred in the presence of both TEA (3×10^{-3} M) and guanidine (3×10^{-3} M, not shown).

Effect of 4-aminopyridine on transmitter release in the presence of cholinoceptor blocking drugs

Endplate potentials triggered electrotonically in tetrodotoxin. Figure 3a shows triggered e.p.ps in the presence of 4-AP (1.3×10^{-4} M) when the nerve terminal was depolarized at the frequency of 5 Hz. Despite the large amplitude of the e.p.p. (≃70 mV) and its prolonged duration, which indicate an enormous release of transmitter, only a slight decline occurred in successive e.p.p. amplitudes. Doubling stimulation frequency to 10 Hz failed to cause a further reduction in e.p.p. amplitude during the period of 1 s of stimulation. Judging from these results, no obvious depletion occurred of the pool of transmitter available for release despite the large number of quanta released by each pulse.

When a receptor blocking agent such as Tc (10^{-6} to 10^{-5} M) or gallamine (10^{-5} to 10^{-4} M) was added to the solution the picture changed as shown in Figure 3b and Table 2. Repetitive nerve depolarization at 5 Hz now caused a rapid decline in amplitude and a marked shortening of the time course of successive e.p.ps (Fig. 3b), the fall being graded with drug concentration (Table 2). Decamethonium (10^{-4} M) on the other hand failed to cause the e.p.p. amplitude to decline during repetitive stimulation although this drug had a blocking effect similar to that of Tc or gallamine, i.e., it reduced the size of the initial e.p.p. by about one half (Table 2).

Increase in either the 4-AP concentration from 1.3×10^{-4} M to 5.6×10^{-4} M or the strength of the

Table 1 Effects of divalent cations on electrotonically-induced endplate potentials in the presence of 4-aminopyridine

	Divalent cations	*Number of experiments*	*All-or-none response*	*Amplitude of e.p.p. (mV)*
Ca^{2+}	1.8	13	+	71.7 ± 2.5
	0.2	6	+	63.7 ± 4.9
	20.0	3	+	76.0 ± 9.5
	1.8 + Mg^{2+} 32.0	4	+	69.0 ± 3.1
	0.2 + Mg^{2+} 32.0	3	−	
	1.8 + Mn^{2+} 10.0	3	−	
Sr^{2+}	1.8	3	+	76.0 ± 3.5
	1.8 + Mn^{2+} 10.0	2	−	
Ba^{2+}	1.8	4	−	
Mg^{2+}	1.8	3	−	

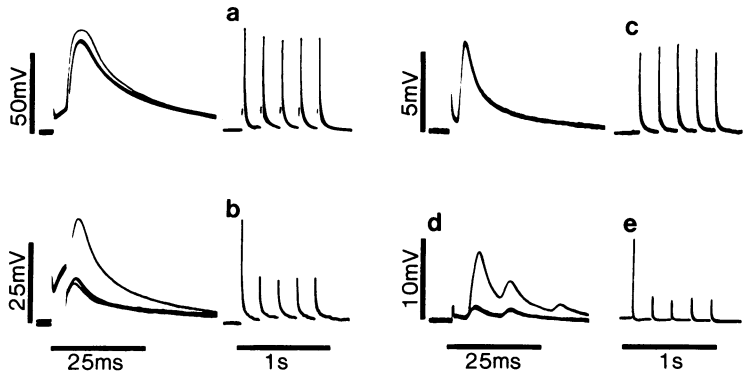

Figure 3 Records (a) show endplate potentials in tetrodotoxin (TTX) obtained by electrotonic depolarization of the nerve terminals at the frequency of 5 Hz. In the record on the left 5 endplate potentials are superimposed; they are shown consecutively on the right. When tubocurarine (Tc) 10^{-5} M was added to the solution a marked decline occurred in the amplitude of successive endplate potentials as shown in (b); (c) shows the effect of Tc 5×10^{-6} M on 5 successive endplate potentials triggered by conducted nerve impulses at 5 Hz. The addition of 4-aminopyridine (4-AP) 1.3×10^{-4} M caused repetitive endplate potentials in response to a single nerve stimulus as shown in (d) and a fall in amplitude of successive endplate potentials as shown in (e). In (e) the extracellular Ca^{2+} concentration was 10 mM in order to abolish repetitive nerve firing.

depolarizing current 3 times failed to affect the tetanic decline of e.p.ps observed in the presence of Tc or gallamine. Substitution of 4-AP by guanidine (6×10^{-3} M) which similarly converts, in the presence of TTX, motor nerve terminal depolarization into a triggered release of transmitter also failed to alter the observed effects of Tc on e.p.p. amplitude during tetanic stimulation (Table 2): TEA has also been reported to increase, in an all-or-none fashion, transmitter release evoked by depolarizing pulses in the presence of TTX (see Introduction). However, in contrast to results obtained in a solution containing 4-AP (1.3×10^{-4} M) or guanidine (6×10^{-3} M), when TEA (6×10^{-3} M) was present in the bath the amplitude of triggered e.p.ps declined during tetanic stimulation (Table 2). This may be due to the fact, that TEA in the above concentration, apart from increasing the release of acetylcholine, also has a curare-like postsynaptic action (Ing & Wright, 1931). Therefore this substance seems to mimic the combined effects of 4-AP and Tc on the neuromuscular junction of the frog.

Increase in the ambient Ca^{2+} concentration from 1.8 to 20 mM failed to affect the Tc-induced fall in

Table 2 Effects of postsynaptic neuromuscular blocking agents on repetitive electrotonically-induced endplate potentials in the presence of 4-aminopyridine (4-AP) or guanidine

Treatment	*Number of experiments*	*Amplitude of 1st e.p.p. (mV)*	*Amplitude of 5th e.p.p. as % of the 1st e.p.p.*
4-AP 1.3×10^{-4} M	18	76.6 ± 3.1	89.8 ± 3.2
plus Tc 10^{-6} M	5	78.6 ± 4.2	79.5 ± 4.1*
10^{-5} M	5	51.2 ± 4.4***	38.3 ± 3.1***
plus Gallamine 10^{-5} M	7	61.0 ± 5.9*	75.7 ± 4.1**
10^{-4} M	7	50.7 ± 5.7***	40.2 ± 8.9***
plus Decamethonium 10^{-4} M	3	42.0 ± 6.1***	89.1 ± 6.4
plus TEA 6×10^{-3} M	3	61.3 ± 4.4**	34.1 ± 11.3***
Guanidine 6×10^{-3} M	5	60.0 ± 6.3	88.0 ± 1.9
plus Tc 10^{-5} M	5	33.0 ± 5.2**	43.3 ± 4.7***
TEA 6×10^{-3} M	3	72.0 ± 7.0	40.6 ± 4.9***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ (Student's t test).
Tc = (+)-tubocurarine; TEA = tetraethylammonium.

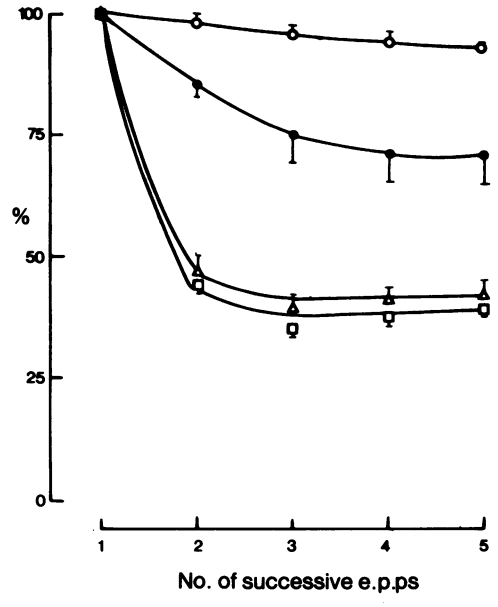

Figure 4 Effect of various extracellular Ca^{2+} concentrations on the successive fall in amplitude of 5 endplate potentials triggered electrotonically at 5 Hz in the presence of 4-aminopyridine (4-AP) 1.3×10^{-4} M and tubocurarine (Tc) 10^{-5} M. The values are expressed as % of the amplitude of the first endplate potential in the train and are means ± s.e. mean of 3 to 4 experiments. (○): In the presence of 0.2 mM extracellular Ca^{2+} but without Tc present; (●): in the presence of 0.2 mM Ca^{2+} and Tc; (△): in the presence of 1.8 mM Ca^{2+} and Tc; (□): in the presence of 20 mM Ca^{2+} and Tc.

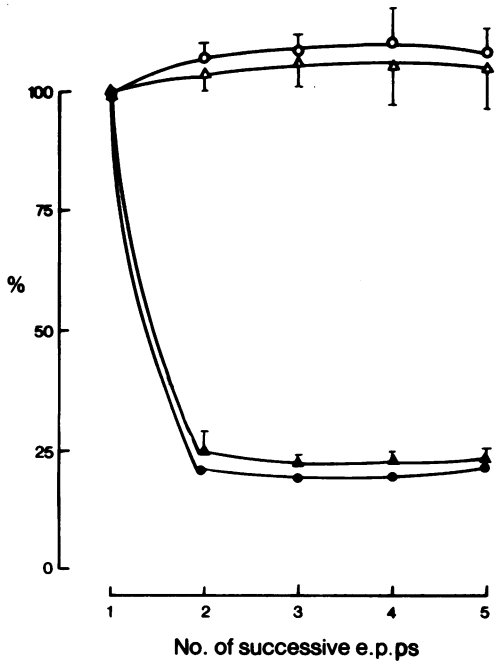

Figure 5 Effects of tubocurarine (Tc) on the amplitude of 5 successive endplate potentials in response to conducted nerve impulses at 5 Hz. The values are expressed as % of the amplitude of the first endplate potential and are means ± s.e. mean of 5 to 9 experiments. (○): In the presence of 1.8 mM Ca^{2+} and 5×10^{-6} M Tc; (●): same as above but with 1.3×10^{-4} M 4-aminopyridine and 2×10^{-5} M Tc; (△) and (▲): results of similar experiments but at an extracellular Ca^{2+} concentration of 10 mM.

amplitude of successive e.p.ps. When the Ca^{2+} concentration was decreased from 1.8 to 0.2 mM no significant fall occurred in the e.p.p. amplitude, however, the Tc-induced fall in amplitude of successive e.p.ps was partially counteracted (Figure 4). Addition of 3 mM Mn^{2+} to the Ringer solution had a similar effect.

The effect of Tc or gallamine occurred under specialized conditions, i.e. when nerve impulse conduction was blocked by TTX and 4-AP or guanidine were present to induce triggered e.p.ps in response to nerve terminal depolarization. It was therefore of interest to see if Tc had a similar effect under more physiological conditions i.e. when the e.p.ps were elicited by ordinary conducted nerve impulses.

Endplate potentials in response to conducted nerve impulses. Figures 3c and 5 show the effect of Tc (5×10^{-6} M) on the amplitude of successive e.p.ps elicited by conducted nerve impulses (in the absence of TTX) at a stimulation frequency of 5 Hz. Note that under these conditions the initial e.p.p. amplitude is less than one tenth of that ($\simeq 5$ mV) triggered by a regenerative calcium current induced in the presence of TTX and 4-AP. Tc, under these conditions failed to induce a fall in successive e.p.p. amplitude. If, however, 4-AP (1.3×10^{-4} M) was added a prompt decline of e.p.ps occurred (Figure 5). At the same time a single nerve stimulus elicited repetitive firing as shown by the recording of multiple e.p.ps in response to a single nerve impulse in Figure 3d. Increasing the extracellular Ca^{2+} concentration to 10 mM abolished repetitive firing but had no influence on the fall of e.p.p. amplitude (Figures 3e and 5).

The possibility that this effect of Tc on e.p.ps caused by conducted nerve impulses in the presence of 4-AP was different in nature from that on e.p.ps triggered electrotonically was investigated by determinations of the time course of recovery of e.p.p. amplitude under the two conditions. Figure 6 shows the effect of Tc 1 to 2×10^{-5} M on recovery of e.p.p.

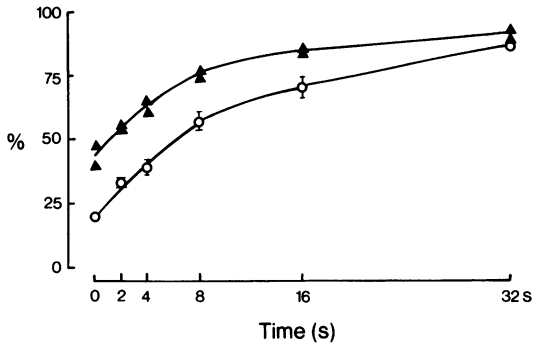

Figure 6 Effect of tubocurarine (Tc, 1.2×10^{-5} M) on the recovery of endplate potential amplitude following a period of 1 s stimulation at 5 Hz in the presence of 1.3×10^{-4} M of 4-aminopyridine (4-AP). The values are expressed as % of the amplitude of the first endplate potential in the train. (○): Values (means ± s.e. mean of 4 experiments) obtained with endplate potentials induced by conducted nerve impulses; (▲): results from two experiments with endplate potentials triggered electrotonically in the presence of tetrodotoxin (TTX).

amplitudes after a period of 1 s stimulation at 5 Hz in the presence and absence of TTX. The time course of recovery was rather similar under both conditions suggesting a common mode of Tc action on e.p.ps in response to non-conducted and conducted impulses.

Discussion

In the presence of 4-AP and TTX nerve terminal depolarization triggered giant e.p.ps of about 70 mV amplitude, i.e. up to the reversal potential level for acetylcholine (Takeuchi & Takeuchi, 1960; Magleby & Stevens, 1972) and with a duration approaching 50 ms. These e.p.ps must represent an enormous release of transmitter, possibly several thousands of acetylcholine quanta.

Transmitter release was Ca^{2+}-dependent since it was abolished in Ca^{2+}-free solution and in the presence of manganous ions, a blocker of membrane permeability to calcium (Meiri & Rahamimoff, 1972). On the other hand the dependence on calcium was surprisingly low, e.p.ps of approximately similar size being generated in extracellular Ca^{2+} concentrations of 0.2 to 20.0 mM. Similarly, giant e.p.ps were triggered in the presence of 1.8 mM Sr^{2+} despite this ion being only one seventh as effective as Ca^{2+} in supporting nerve impulse evoked transmitter release (Rahamimoff, 1976). However, Ba^{2+} which has only 1/20 of the effectiveness of Ca^{2+} (Rahamimoff, 1976) failed to substitute for Ca^{2+} at 1.8 mM concentration. Magnesium ions, which inhibit transmitter release (del Castillo & Katz, 1954) failed at 32 mM to affect triggered all-or-none e.p.ps but when the Ca^{2+} concentration was reduced to 0.2 mM this amount of Mg^{2+} abolished triggered e.p.ps.

These results indicate that 4-AP must enhance enormously the influx of calcium ions during depolarization of the nerve terminal. Such an enhancement could result from an increase in the number of active Ca^{2+} channels or alternatively from a greater transport capacity of each channel. To investigate these possibilities we used solutions with high concentrations of Mg^{2+} since this ion competes with Ca^{2+} and acts as an inhibitor of the transmitter release mechanism (del Castillo & Katz, 1954). The study was carried out on conducted nerve impulses, i.e. in the absence of TTX, and Tc was used to block neuromuscular transmission. 4-AP produced a parallel shift to the right in the dose-response curve to Mg^{2+} for blockade of transmitter release. An increase in the number of active calcium channels would not have altered the Mg^{2+}-sensitivity of the preparation. Therefore, the results imply that 4-AP enhanced the transport efficacy of Ca^{2+} movement across the terminal membrane. TEA and guanidine also caused a parallel shift in the dose-response curve for Mg^{2+} which might indicate that they act on Ca^{2+} influx by a similar mechanism. 4-AP and TEA have been shown to prolong the duration of inward cationic currents by blocking the potassium conductance increase of excitable membranes (Hille, 1967; Yeh, Oxford, Wu & Narahashi, 1976). This could account for a greater influx of divalent cations. On the other hand, it is also possible that the drugs have a direct facilitatory effect on the movement of Ca^{2+} as discussed by Lundh & Thesleff (1977) and Jankowska, Lundberg, Rudomin & Sykova (1977). The present experimental results do not allow a decision between these possibilities.

Despite the enormous increase of evoked transmitter release in the presence of 4-AP, repetitive stimulation at 5 or 10 Hz caused only a slight decline in amplitude of successive e.p.ps. However, when a receptor blocking drug such as Tc or gallamine was present, a dose-dependent decline in the amplitude and a marked shortening of the time course of successive e.p.ps occurred. Such a decline could result from a block of postsynaptic receptors. A competitive block of postsynaptic cholinoceptors would result in a decline in the amplitude of successive e.p.ps provided that each nerve stimulus released successively smaller amounts of acetylcholine and that, in the absence of the blocking drug, the amount of transmitter released by each stimulus was so great as to produce a maximal postsynaptic response and therefore e.p.ps of unchanged amplitude. To test this possibility,

in the presence of 4-AP, we reduced evoked transmitter release by reducing the extracellular Ca^{2+} concentration to 0.2 mM. In these conditions the Tc-induced fall in amplitude of successive e.p.ps was counteracted. This finding indicates that in the presence of 4-AP evoked transmitter release by each stimulus is so great that e.p.ps of maximal amplitude are produced, even during repetitive stimulation up to 10 Hz. Thereby a successive reduction in transmitter release is masked and the reduction becomes evident only when a competitive cholinoceptor blocking agent such as Tc or gallamine is present, but not when depolarizing and desensitizing compounds like decamethonium are used.

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