Br. J. Pharmac. (1980), **69**, 453–460

COMPARATIVE STUDY OF THE EFFECTS OF 4-AMINOPYRIDINE AND TETRAETHYLAMMONIUM ON NEURO-EFFECTOR TRANSMISSION IN THE GUINEA-PIG VAS DEFERENS

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1 Effects of 4-aminopyridine (4-AP) and tetraethylammonium (TEA) on the neuro-effector junction of the guinea-pig vas deferens were investigated by microelectrode and double sucrose gap techniques.

2 4-AP (0.05 to 0.5 mM) or TEA (0.5 to 1 mM) did not alter the membrane potential, or the membrane input resistance of the smooth muscle cell.

3 The amplitude and frequency of the miniature junction potentials (m.e.j.ps) were not modified by treatment with 4-AP (0.05 to 0.5 mM) or TEA (1 mM).

4 4-AP (1 mM) increased the membrane input resistance, enhanced the spike amplitude of the smooth muscle cells and thereby augmented the amplitude of twitch contraction.

5 4-AP (0.05 to 0.5 mM) or TEA (1 mM) markedly increased the amplitude of excitatory junction potentials (e.j.ps), but the facilitation phenomena produced by repetitive stimulation were not affected.

6 The duration of the extracellularly recorded action potential from the small nerve bundle was prolonged by 4-AP (0.5 mM).

7 The amplitude of the e.j.p. was dependent on the external concentration of calcium. A straight line was produced when the amplitude of the e.j.p. and $[Ca]_o$ was plotted on a double log scale. Application of 4-AP resulted in a parallel shift of this line to the left.

8 These results indicate that 4-AP (0.05 to 0.5 mM) and TEA (0.5 to 1.0 mM) prolonged the action potential generated from the sympathetic nerve terminal thus enhancing the amplitude of the e.j.p. due to an increase in the Ca-influx. However, in the concentrations used, these compounds did not modify the Ca-mobilization in the nerve terminal or the postsynaptic membrane during the resting state.

Introduction

4-Aminopyridine (4-AP) has recently been shown to block the delayed potassium current in giant axons of the cockroach and squid (Pelhate & Pichon, 1974; Meves & Pichon, 1975; Yeh, Oxford, Wu & Narahashi, 1976). Lundh & Thesleff (1977) and Lundh, Leander & Thesleff (1977) studied the effects of 4-AP on the motor endplate and concluded that 4-AP increases the level of free calcium ion inside the nerve terminal and thereby enhances the transmitter release.

4-AP also augments mechanical responses of the rabbit vas deferens to transmural stimulation mainly by increasing transmitter release (Johns, Golks, Lauzon & Paton, 1976), and has been shown to increase noradrenaline output from the perfused cat spleen (Kirpekar, Kirpekar & Prat, 1977). TEA, which is well known for its ability to block K-current, has also been previously shown to enhance transmitter release at the neuromuscular junction (Koketsu, 1958; Katz & Miledi, 1969) and from sympathetic nerve terminals (Thoenen, Haefely & Staehelin, 1967; Kirpekar, Wakade & Prat, 1976).

Thus, it was of interest to investigate and compare the effects of TEA and 4-AP on the neuro-effector junction in smooth muscle. The effects of these agents were observed in guinea-pig vas deferens preparations, since the electrical properties of the neuro-effector junction of this preparation have been studied previously in detail (Kuriyama, 1964; Ito & Tajima, 1979).

Methods

The isolated vas deferens of the guinea-pig was used in all experiments. The animals were stunned, the vas deferens dissected free and pinned out immediately in a Lucite organ bath for microelectrode studies.

For the double sucrose gap method a strip of vas

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deferens was cut to a width of 2.0 to 2.5 mm and a length of about 20 mm. The preparation was bathed in a modified Krebs solution of the following ionic concentrations (mM): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 134.0, $H_2PO_4^-$ 1.2, HCO_3^- 15.5 and glucose 11.5. The solution was aerated with 97% O_2 and 3% CO_2, and the pH adjusted to 7.2 to 7.3.

For recording the membrane potential of the single cells, a high resistance (50 to 80 MΩ) microelectrode filled with 3 M KCl was inserted from the outer surface of the preparation. Field stimulation (stimulus duration of 0.05 to 0.1 ms) was used to stimulate the sympathetic nerve terminals (Kuriyama, 1964). A chamber for the microelectrode method had a volume of 2 ml and was perfused at a rate of 3 ml/min at a temperature of 35 to 36°C. The double sucrose gap method was also used for recording the membrane potential and tension development in the tissue. The input membrane resistance of the smooth muscle was measured from the electrotonic potentials induced by square pulses (stimulus duration of 1 s) using the double sucrose gap technique. The chamber in which the preparation was mounted was a modification of that used originally by Rougier, Vassort, Garnier, Gargouil & Coraboeuf (1969), and has been described elsewhere (Ito, Suzuki & Kuriyama, 1977). In order to produce the excitatory junction potential, field stimulation was applied through a pair of electrodes placed in the centre pool of the apparatus. The stimulating electrodes consisted of two silver wires (0.2 mm in diameter) separated by 2 mm, and these wires were placed in such a way that the current pulse would pass transversely across the tissue. Single shock and repetitive stimulation (0.1 to 3 Hz) were applied, with pulses of 0.05 ms duration and about 10 V strength.

For the extracellular recordings of action potentials from the small sympathetic nerve bundles (5 to 10 μm), a low resistance (10 MΩ) microelectrode filled with 3 M KCl was used. The time constant of the recording system was 20 to 30 μs.

The drugs used were 4-aminopyridine (Tokyo-Kasei) and tetraethylammonium (Ishizu).

Results

Effects of 4-aminopyridine on resting membrane potential and m.e.j.p.

4-AP in concentrations ranging between 0.05 and 0.5 mM, had no effects on the resting membrane potential and spontaneous excitatory junction potentials recorded by microelectrodes. The resting membrane potential of the smooth muscle cells of vas deferens in normal Krebs solution was -59.3 ± 2.5 mV ($n = 33$), and during exposure to 4-AP (0.5 mM), it was -59.3 ± 2.0 mV ($n = 32$). M.e.j.ps were recorded before and during treatment with 4-AP from the same cell throughout each experiment. The mean amplitudes of m.e.j.ps recorded from a single cell before and during application of 4-AP (0.05 mM) were 0.50 ± 0.29 mV (±s.d., $n = 201$) and 0.51 ± 0.26 mV (±s.d., $n = 220$) respectively, and the corresponding m.e.j.p. frequencies were 0.53 ± 0.16 Hz (±s.d., $n = 201$) and 0.65 ± 0.22 Hz (±s.d., $n = 220$). No changes in the amplitude or the frequency of m.e.j.ps were observed with 4-AP in concentrations of 0.05 to 0.1 mM.

Effects of 4-aminopyridine on e.j.p.

4-AP (0.03 mM) increased the amplitude of the e.j.p. measured either by the double sucrose gap or microelectrode method. As shown in Figure 1, application of repetitive stimulation to the nerve fibre at a frequency of 0.1 Hz facilitated e.j.ps. After the amplitude of the e.j.p. reached a steady level, application of 0.03 mM 4-AP caused a further increase to $170 \pm 67\%$ (±s.d., $n = 4$) of the steady level. However, these concentrations of 4-AP did not modify the membrane input resistance of the muscle cell measured from the electrotonic potentials induced by square wave pulses (1 s duration) and the threshold for the generation of an action potential. However at a concentration of 1 mM, 4-AP slightly depolarized the membrane and increased the membrane input resistance, as measured by the double sucrose gap method. Figure 2 shows an example of the effect of 4-AP on the membrane and mechanical properties of the smooth muscle cells. The mean increase in the input membrane resistance after the application of 4-AP was $135 \pm 8\%$ (±s.d., $n = 4$) of the control value. Furthermore, 4-AP also enhanced the spike amplitude of the muscle cell and thus potentiated the twitch tension evoked by a single pulse (1 s duration).

4-AP (0.05 mM) also increased the amplitude of intracellularly recorded e.j.ps. In the experiment illustrated in Figure 3a, a train of seven e.j.ps was elicited by stimulation at 1 Hz, and after a period of rest for 1 to 2 min the train was repeated several times in the absence or presence of 4-AP. 4-AP increased the amplitude of the first e.j.p. evoked by the first stimulus of the train at 1 Hz to 210% of the control value. When the amplitude of the e.j.p. evoked by the first pulse in the normal solution was defined as 1.0, as shown in Figure 4a and c, the growth of the e.j.p. during short trains of stimulation of 0.5 Hz was in the form of an exponential curve with a time constant of about 5 s. After application of 4-AP 0.05 or 0.1 mM, the amplitude of the first e.j.p. was increased to $196 \pm 12\%$ (±s.d., $n = 4$) or $215 \pm 41\%$ (±s.d., $n = 4$) of the control value respectively, while the facilitation curve was not affected. To analyse further this effect of 4-AP, growth in the amplitude of e.j.p. during

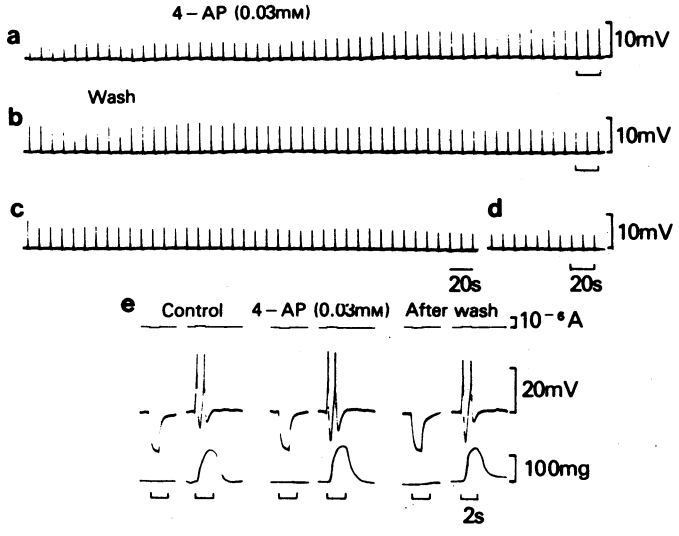

Figure 1 The effects of 4-aminopyridine (4-AP, 0.03 mM) on the e.j.ps elicited by field stimulation using the double sucrose gap method at a stimulus frequency of 0.1 Hz. 4-AP was applied and washed out at the points indicated by dots. (a), (b) and (c) are continuous records; time lag between (c) and (d) was about 7 min; (e) electrotonic potentials (middle record, downward deflections), action potentials (middle record, upward deflections) and twitch tensions (bottom record) evoked by hyperpolarizing and depolarizing pulses of 1 s duration (top record).

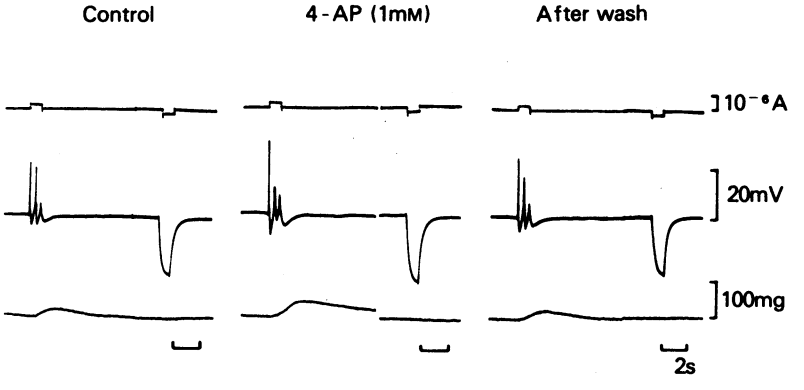

Figure 2 The effect of 4-aminopyridine (4-AP, 1 mM) on the electrical and mechanical properties of smooth muscle cells of guinea-pig vas deferens recorded by the double sucrose gap method. Legend details as in Figure 1e.

trains of impulses was analysed by the method of Mallart & Martin (1967). As shown in Figure 4b, the amplitude of a test e.j.p. evoked at different intervals after application of a conditioning impulse was plotted on a log scale against the time intervals. The changes in the amplitude produced by the conditioning impulse could be classified into two components; the first component occurred with stimulations of short intervals within a few hundred ms, and the second component by intervals of over 1 s. To predict the growth in amplitude of the e.j.p. at a stimulus frequency of 0.5 Hz, the second component was used to calculate the rate constant of decay of the curve. If facilitation (f) is introduced as $(y - y_0)/y_0$ where y_0

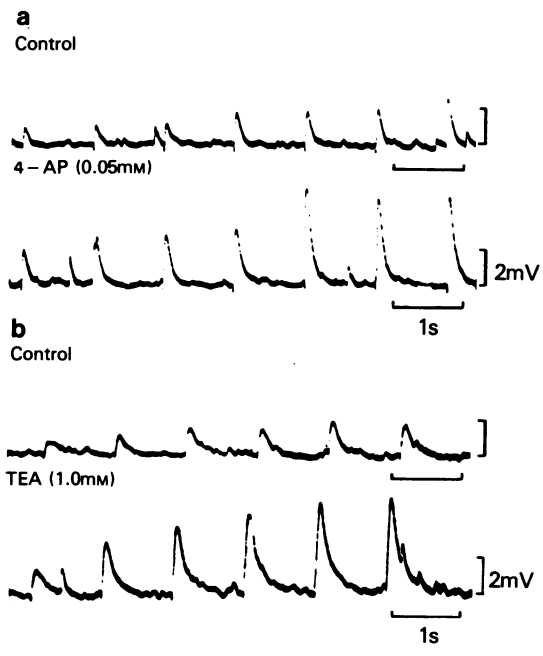

Figure 3 The effects of (a) 4-aminopyridine (4-AP, 0.05 mM) or (b) tetraethylammonium (TEA, 1 mM) on the amplitude of e.j.p. recorded with microelectrodes during a short train of impulses.

is the amplitude of a conditioning e.j.p., and y is the amplitude of a test e.j.p., then the second component in Figure 4b can be expressed by the equation $f = f_1 \exp(-bt)$, where f_1 is the facilitation at zero time (equal to 0.46) and b is the rate constant of decay of the curve (equal to 0.16 s^{-1}). As shown in Figure 4d, 4-AP (0.05 mM) did not affect the facilitation parameters of f_1 and b observed in Krebs solution. When the above parameters were inserted into the equation $f = f_1(e^{b\Delta t} - 1)^{-1}(1 - e^{-bt})$ (Mallart & Martin, 1967), the predicted facilitation of e.j.p. with the rate constant measured in the normal Krebs solution (all the curves in Figure 4a and c) fit well with experimental data obtained within the time course of 10 s. Although this theoretical analysis seems to fit the results, the time scale of the facilitation is very different from that in the experiments of Mallart & Martin (1967) done on the amphibian motor endplate, i.e. the time constant of the decay of the second component of facilitation was in the order of 5 s, and that of the e.p.p. was in the order of 250 ms. These results indicate that the facilitation process of e.j.ps is not affected by 4-AP (0.05 to 0.1 mM), while the amplitude of e.j.p. is enhanced.

Effects of $[Ca]_o$ *on the e.j.p. and 4-aminopyridine*

The amplitude of the e.j.p. was recorded by the double sucrose gap method in various concentrations

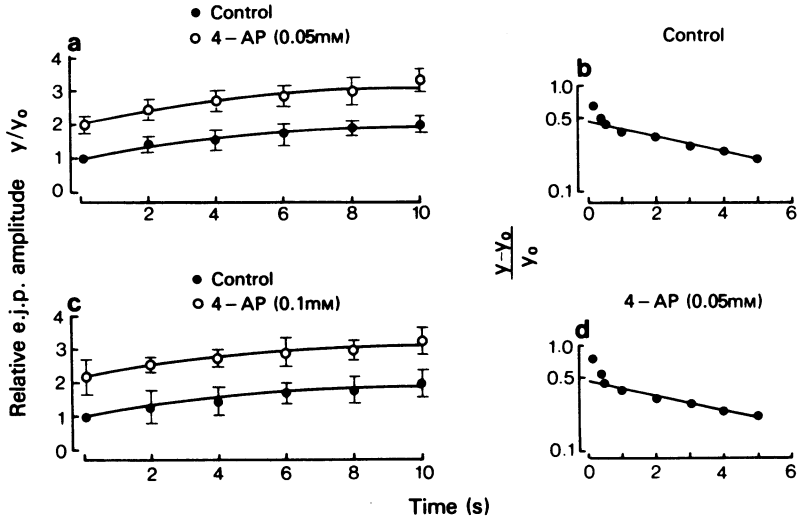

Figure 4 (a and c) Changes in amplitude of e.j.ps during short trains of impulses at 0.5 Hz. (●) Control; (○) during treatment with 4-aminopyridine (4-AP, 0.05 and 0.1 mM). (b and d) Effect of a conditioning impulse on the amplitude of the e.j.p. evoked by a subsequent test impulse, where y_0 is the amplitude of conditioning e.j.p. (mV) and y is the amplitude of a test e.j.p. Each point gives the mean amplitudes of several trials. Vertical bars represent s.d.

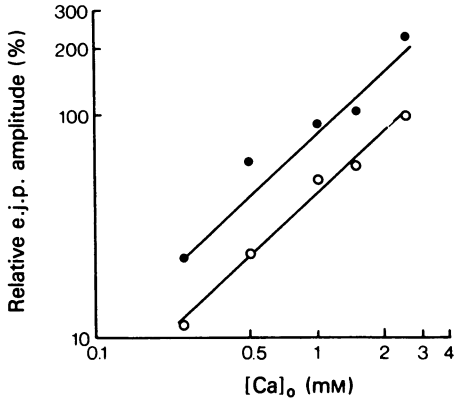

Figure 5 The effects of 4-aminopyridine (4-AP, 0.05 mM) on the relationship between Ca concentration and relative amplitude of e.j.p. plotted on a double logarithmic scale (Control e.j.p. amplitude at Ca 2.5 mM = 100). Each point was determined from at least 3 series of experiments, changing $[Ca]_o$ in a step-wise manner. (○) Control; (●) 4-AP.

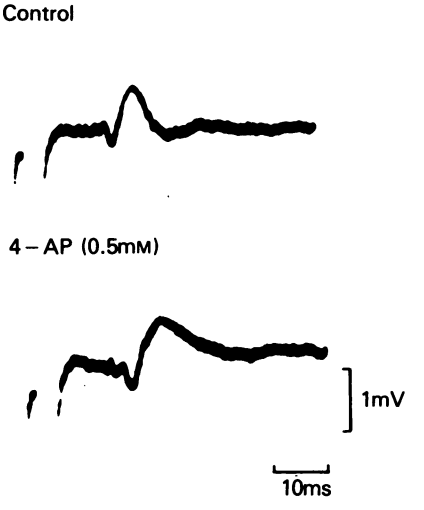

Figure 6 Action potentials recorded extracellularly by a microelectrode from small sympathetic nerve bundles which innervate the vas deferens, before and after application of 4-aminopyridine (4-AP, 0.5 mM).

of $[Ca]_o$ (0.25 to 2.5 mM). As shown in Figure 5, when the log of relative amplitude of e.j.p. was plotted against log $[Ca]_o$, a straight line with a slope of approximately 1 was obtained as previously found by Ito & Tajima (1979). In the presence of 4-AP (0.05 mM), increasing the $[Ca]_o$ produced an increase in the amplitude of the e.j.p., which was linear, although it showed a parallel shift to the left as compared to that in the absence of 4-AP.

Effects of 4-aminopyridine on sympathetic nerve action potential

To investigate the mechanisms of the augmenting action of 4-AP on the e.j.p. of the guinea-pig vas deferens, the extracellular action potentials were recorded by a microelectrode from the small sympathetic nerve trunk which innervates the vas deferens (Figure 6). The mean duration of the extracellular action potential was 9.3 ± 1.1 ms (±s.d., $n = 5$) in Krebs solution, and it increased to 16.3 ± 1.3 ms (±s.d., $n = 5$) by treatment with 4-AP (0.5 mM).

Effects of tetraethylammonium on the e.j.p.

To compare the effects of 4-AP and TEA, e.j.ps were recorded before and during application of TEA (0.5 to 1.0 mM). Figure 3b shows the effects of 1 mM TEA on the amplitude of e.j.p. recorded by the microelectrode method. The amplitude of the first e.j.p. during repetitive stimulation was increased to 130 ± 37% (±s.d., $n = 3$) and to 193 ± 28% (±s.d., $n = 5$) by the application of 0.5 and 1.0 mM TEA, respectively. The membrane resistance was not affected by treatment with 1.0 or 0.5 mM TEA. The increase in amplitude of the e.j.p. after 0.05 mM 4-AP was 196 ± 12% (±s.d., $n = 3$), and 193 ± 28 after 1 mM TEA. Thus 4-AP seems to be about 20 times more potent in enhancing the amplitude of e.j.p.

The effects of TEA on the facilitation of the e.j.p. during repetitive stimulation were also observed. The facilitation curve of the e.j.p. obtained by short trains of stimuli was not affected. All the curves in Figure 7 show the predicted relationship using the rate constant measured in Krebs solution. The curves fit well with the experimental data, thus indicating that the amplitude of the e.j.p. was enhanced while the facilitation of the e.j.p. was not affected by TEA.

Effects of the combined use of 4-aminopyridine and tetraethylammonium on the amplitude of e.j.p.

The combined use of 4-AP and TEA on the amplitude of the e.j.p. was studied. Figure 8 shows the effects of 0.05 or 0.1 mM 4-AP on the e.j.ps in the presence and absence of 0.5 mM TEA. When 0.1 mM 4-AP and 0.5 mM TEA were applied simultaneously the amplitude of the e.j.p. was enhanced to 337 ± 39% of the first e.j.p. (±s.d., $n = 3$), an additive action of the separate effects of the two drugs on the amplitude of e.j.ps. The facilitation curves of e.j.p. during short train impulses

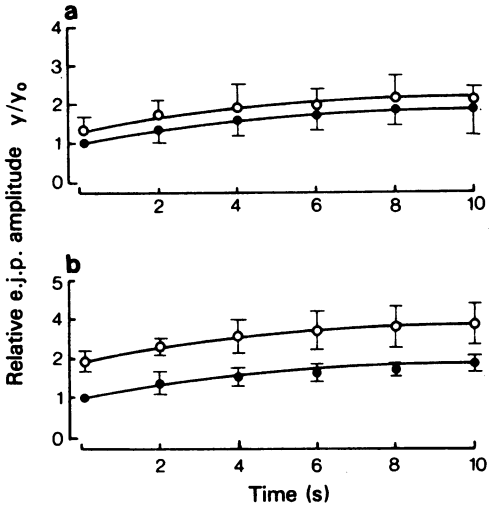

Figure 7 The effect of tetraethylammonium (TEA) 0.5 mM in (a) and 1.0 mM in (b) on the amplitude and facilitation process of the e.j.ps recorded with microelectrodes. All the continuous curves are the predicted relationship. Vertical bars represent s.d. (●) Control; (○) TEA.

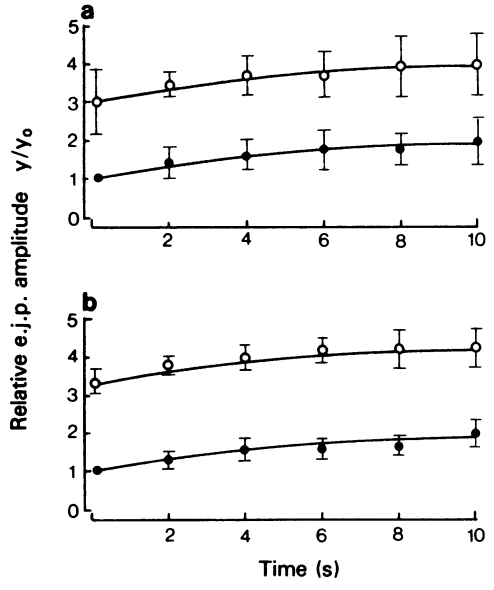

Figure 8 The effects of combined use of 4-aminopyridine (4-AP) and tetraethylammonium (TEA) on the amplitude and facilitation process of the e.j.ps during a short train of impulses recorded with microelectrodes. Continuous curves are the predicted relationship. In (○), control (●); 4-AP 0.05 mM plus TEA 0.5 mM (○). In (b), control (●); 4-AP 0.1 mM plus TEA 0.5 mM (○). Vertical bars represent s.d.

were not affected by the combined application of 4-AP and TEA, and the curves were compatible with the rate constant obtained in Krebs solution.

Discussion

The present results showed that 0.05 mM 4-AP approximately doubled the amplitude of e.j.p. of guinea-pig vas deferens, although the amplitude and frequency of m.e.j.p. remained unaffected. During enhancement in the amplitude of e.j.p., the membrane potential and the input membrane resistance of the smooth muscle cells determined by the microelectrode or double sucrose gap method were not affected.

4-AP reduces the potassium current in the giant axon of squid (Meves & Pichon, 1975; Yeh, *et al.*, 1976), cockroach (Pelhate & Pichon, 1974), and frog node of Ranvier (Ulbricht & Wagner, 1976). In the present experiments the duration of the extracellular action potential recorded from the small sympathetic nerve bundle was prolonged by 4-AP. Therefore, it is reasonable to assume that 4-AP suppresses the K-conductance, prolonging the duration of the action potential which is associated with a large increase in transmitter release from the nerve terminals as demonstrated at the skeletal neuromuscular junction for TEA and 4-AP (Katz & Miledi, 1969, Illes & Thesleff, 1978).

At the skeletal neuromuscular junction (Lundh *et al.*, 1977) the frequency of m.e.j.ps was not affected by 4-AP. The frequency of the m.e.p.p. is determined, at least in part, by the resting concentration of Ca^{2+} inside the presynaptic terminal (Miledi & Thies, 1971). The relationship between log $[Ca]_o$ and the log amplitude of e.j.p. was linear and the parallel shift to the left after treatment with 4-AP, indicates that 4-AP enhanced the effect of $[Ca]_o$ on the presynaptic nerve terminals with $[Ca]_o$ between 0.25 and 2.5 mM. These observations also indicate that 4-AP enhances Ca entry during the prolonged nerve action potential, but not during the resting state of the nerve terminals. TEA is also known to prolong the spike duration and to enhance the spike amplitude of excitable tissues without membrane depolarization (Beaulieu & Frank, 1967; Kao & Stanfield, 1970; Ito, Kuriyama & Sakamoto, 1970), and enhances acetylcholine release from the neuromuscular junction (Koketsu, 1958; Lundh, *et al.*, 1977). Furthermore it is suggested that this agent enables the Ca-inward current to become regen-

erative in the motor nerve terminal (Katz & Miledi, 1969), and also to increase release of catecholamines from the sympathetic nerve (Thoenen, *et al.*, 1967; Kirpekar *et al.*, 1976; 1977).

In the present studies, 1 mM TEA also approximately doubled the amplitude of the e.j.p., without affecting the membrane potential and input membrane resistance of smooth muscle cells of the guinea-pig vas deferens. Thus it appears that 4-AP is about 20 times more potent than TEA in potentiating the amplitude of e.j.p. of guinea-pig vas deferens. A similar relative potency was found for the restoration of neuromuscular transmission in botulinum-treated rats (Lundh *et al.*, 1977).

Lundh *et al.* (1977) also suggested that 4-AP allows a larger amount of calcium to enter the terminal during the spike and at the same time allows the accumulation of intracellular Ca by slowing its binding to intracellular substructures. However, in the present experiments, the facilitation curves of e.j.ps during short trains of impulses were not affected by treatment with 4-AP (0.05 mM) or TEA (1 mM), although the amplitude of the e.j.ps was almost doubled. That is, the facilitation during the repetitive stimulation at a low frequency (0.5 Hz) could be predicted from the rate constant (b) and facilitation at zero time (f_1) measured in normal Krebs solution. Thus it seems that 4-AP and TEA do not affect the facilitation process in sympathetic nerve terminals. On the assumption that the facilitation process is closely related to the intracellular free Ca ions, it appears that 4-AP or TEA do not increase the 'spare' calcium ion per impulse during short trains.

The combined use of 4-AP and TEA had no effect on the facilitation process, thus providing no clue as to the mode of action of the two drugs.

Hara, Kitamura & Kuriyama (1980) found that in the smooth muscle cells of the pulmonary artery, 4-AP (1 mM) markedly depolarized the membrane, increased the membrane resistance and suppressed the rectifying property of the membrane. However, 4-AP 1 mM was less potent in the smooth muscle cells of the guinea-pig portal vein. In the present study, this concentration of 4-AP slightly depolarized the membrane, increased the membrane resistance and enhanced the spike amplitude. Possibly the membrane of the sympathetic nerve terminal is more sensitive to 4-AP than are smooth muscle cell membranes.

This work was supported in part by the Matsunaga Science Foundation and Ministry of Education, Science and Culture of Japan. The authors are grateful to Prof. H. Kuriyama for continuous encouragement, and to M. Ohara, Kyoto University, for assistance with the manuscript.

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(Received June 4, 1979.
Revised October 15, 1979.)