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EFFECT OF PROSTAGLANDIN E_1 ON NEUROMUSCULAR TRANSMISSION IN THE RAT

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1 The effect of prostaglandin E_1 on neuromuscular transmission in the phrenic nerve-diaphragm muscle preparation of the rat was studied with intra- and extracellular recording techniques.

2 Prostaglandin E_1, in concentrations from 10 nM, induced intermittent failures in the generation of the end-plate potential in response to repeated indirect stimulation.

3 Failures appeared abruptly, the end-plate potential behaving in an all-or-nothing fashion. The effect occurred only at 36–38°C when the nerve was stimulated at 30–80 Hz and was reversible upon washing with drug-free solution.

4 Since miniature end-plate potentials were not affected, such failures must be attributed to a presynaptic action of prostaglandin E_1.

5 Extracellular recording suggested that prostaglandin E_1 prevented the action potential from reaching the nerve terminal.

Introduction

The modulation, by prostaglandins, of the release of noradrenaline from adrenergic nerve endings during stimulation of sympathetic nerves is well documented (Hedqvist, 1970b; Hedqvist, Stjärne & Wennmalm, 1970; Wennmalm, 1971). As regards cholinergic nerves, however, the role of prostaglandins in the release of acetylcholine is still controversial. Stimulation of the rat phrenic nerve-diaphragm muscle preparation (Ramwell, Shaw & Kucharski, 1965; Laity, 1969), of the cholinergic nerves to the rat stomach (Coceani, Pace-Asciak, Volta & Wolfe, 1967) or of the rabbit heart vagal nerves (Junstad & Wennmalm, 1974) liberates substantial amounts of prostaglandins. The results of several studies suggest moreover that prostaglandins may influence cholinergic neurotransmission (see Hedqvist, 1973). Some reports indicate that prostaglandins facilitate, others that they inhibit the release of acetylcholine from cholinergic nerve endings (Wennmalm & Hedqvist, 1971; Hahn & Patil, 1972; Ehrenpreis, Greenberg & Belman, 1973; Heilbronn, 1973; Hahn & Patil, 1974). Still other reports suggest, by showing that prostaglandins do not affect the release of acetylcholine, that prostaglandins do not act as modulators of neurotransmission in the parasympathetic neuroeffector system (Ginsborg & Hirst, 1971; Hadházy, Illés & Knoll, 1973; Marco & Coceani, 1973; Botting & Salzmann, 1974; Illés, Vizi & Knoll, 1974). To tackle this problem at a cellular level, the effects of prostaglandin E_1 on cholinergic neurotransmission were studied *in vitro* by intra- and extracellular recording techniques in the rat phrenic nerve-diaphragm muscle preparation. A preliminary report of this work has already appeared (Jansson, Hyvärinen, Tolppanen & Gripenberg, 1974).

Methods

Animals and preparations

The experiments were carried out *in vitro* with isolated left diaphragm muscles from adult male rats (180–220 g) of the Sprague–Dawley strain. The animals were anaesthetized with ether and killed, and the diaphragm muscle with the attached nerve was removed and immersed in a bathing solution. The muscle was stretched about 10% beyond its resting length and attached with stainless steel needles to a paraffin-lined Perspex plate that had a lens in the centre. The phrenic nerve was kept in mineral oil in a separate chamber. To avoid muscle twitches upon stimulation of the nerve, the ends of the muscle fibres were cut transversely (Barstad, 1962; Hubbard & Wilson, 1973).

Recording details

The experiments were begun 1 to 1.5 h after the muscle fibres had been cut. By that time the resting membrane potential (RMP) of the muscle fibres had decreased to a level at which an action potential could no longer be generated. Only surface fibres were studied. Intracellular recordings were made with glass microelectrodes drawn with internal glass fibres (Tasaki, Tsukakava, Ito, Wayner & Yu, 1968) and filled with 3 M KCl (resistance 5–20 MΩ). Most extracellular recordings were carried out with glass-insulated platinum microelectrodes (Wilska, 1940; Wolbarsht, MacNichol & Wagner, 1960). In some experiments, extracellular recordings were made with 3M NaCl-filled glass microelectrodes that had a resistance of less than 3 MΩ.

The phrenic nerve was stimulated with a bipolar platinum electrode. Pulses were of 0.04 ms duration and 2 to 3 times threshold strength. Microelectrodes were considered to be located in the end-plate region when end-plate potentials (e.p.ps) or miniature end-plate potentials (m.e.p.ps) were recorded that had a rise time of 0.8 ms or less. A criterion for the appropriate extracellular position of the microelectrode was the simultaneous recording of the arrival of the action potential at the nerve ending and the resultant end-plate current (e.p.c.). In the present paper, the abbreviation e.p.c. thus refers to an extracellular current and not to a true transmembrane current. Potentials were preamplified (using in intracellular recordings a d.c.-preamplifier with capacitance neutralization and an input resistance of 10^{12} Ω or, in extracellular recordings, a Tektronix 122 a.c.-preamplifier), displayed on an oscilloscope and photographed or recorded on FM-tape. M.e.p.p. amplitudes were measured from the screen of a storage oscilloscope after FM-recorded material had been replayed.

Solutions

The composition of the bathing solution was as follows (mM): NaCl 136.0, KCl 5.6, $MgCl_2$ 1.3, $CaCl_2$ 2.2, PO_4^{3-} 1.3, $NaHCO_3$ 16.0 and glucose 11.0. Because 5 mM KCl has been shown to induce nerve block in cut preparations (Randić & Straughan, 1964), the potassium concentration was reduced to 2.8 mM when e.p.ps or e.p.cs were studied. The solution was continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2 which resulted in a pH of 7.3 at room temperature. A peristaltic pump infused the solution into the bathing chamber at a constant rate of 3–6 ml min^{-1}. The temperature of the bathing medium was recorded with a thermistor. Prostaglandin E_1 was added to the bathing medium from a 10 mM stock solution in 95% ethanol.

Results

Effect of prostaglandin E_1 on resting membrane potential of muscle fibres and on spontaneous m.e.p.ps

The effects of prostaglandin E_1 on the RMP and spontaneous m.e.p.ps were studied in uncut preparations that had been bathed in solutions containing 5.6 mM KCl. Prostaglandin E_1 at 0.1 μM had no definite effects on either of these parameters. The RMP of muscle fibres in control preparations maintained at 37°C was 76.7 ± 5.8 mV, whereas during exposure to prostaglandin E_1, the RMP was 76.7 ± 7.6 mV (means ± s.d. of 82 and 87 muscle fibres, respectively, in 12 preparations). The mean amplitude of m.e.p.ps after at least 10 min of treatment with 0.1 μM prostaglandin E_1 was 0.54 ± 0.26 mV, as compared with 0.52 ± 0.23 mV under control conditions (mean ± s.d.). Corresponding m.e.p.p. frequencies were 9.60 ± 5.24 and 7.37 ± 5.20 m.e.p.ps s^{-1}, respectively. Prostaglandin E_1 did not affect the distribution of m.e.p.p. amplitudes in a way indicative of a clear-cut biological effect (Figure 1).

Neuromuscular transmission in the cut diaphragm muscle preparation

One hour after the muscle fibres had been cut, with the preparation having been maintained at room temperature, the RMP of the muscle fibres had decreased to about −35 mV. During the following 3 to 4 h, there was a further gradual depolarization. The RMP of muscle fibres during this period was -31.6 ± 10.9 mV (mean ± s.d. of 116 fibres in 31 preparations). At such a low RMP, no action potential was generated, but an e.p.p. 1–10 mV in amplitude was elicited in response to nerve stimulation. When a tetanic stimulation was applied to the nerve, the amplitude of the first e.p.ps decreased. In cut preparations, unlike those treated with tubocurarine, the amplitude of the e.p.p. remained at a conveniently recordable level after this initial run-down (see also Lilleheil, 1965; Hubbard & Wilson, 1973).

Failures in the e.p.p. response

As the frequency of the indirect stimulation was progressively increased, the e.p.p. sooner or later failed intermittently. The stimulation frequency at which such failures occurred varied considerably among different junctions. In 12 preparations, 56% of all end-plates tested (86 fibres) at 37°C showed no failures when stimulated for a total of 100 s, the stimulation frequency being increased from 10 to 100 Hz in 10 Hz steps every tenth second. The remaining 38 cells failed somewhere between 20 and 90 Hz. When failures started to appear, the e.p.p. did not decrease in amplitude but rather responses to some stimuli in the

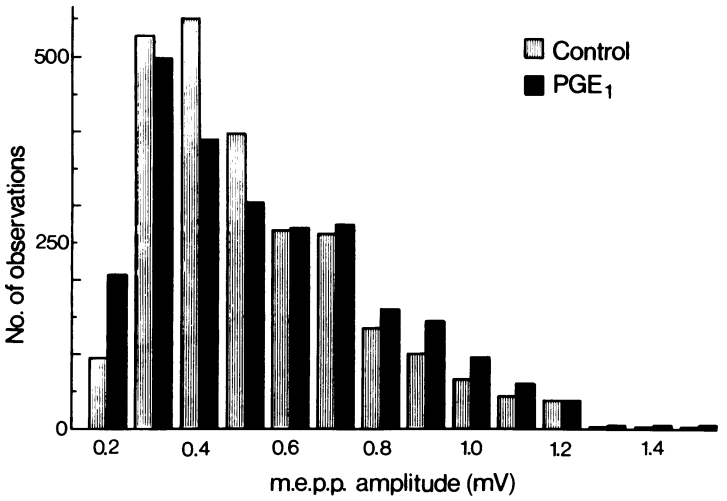

Figure 1 Lack of effect of prostaglandin E_1 (PGE_1) 0.1 μM on m.e.p.p. amplitude in the rat diaphragm muscle preparation *in vitro*. A total of 12 preparations were used. In eight of these, recordings were first made from seven control cells after which prostaglandin E_1 was infused into the bathing chamber. Recordings were begun 10 min after the addition of prostaglandin E_1. In the other four preparations, recordings were first made in prostaglandin E_1 and control recordings were begun after 15–30 min of washing with drug-free solution. All experiments were carried out at 37°C. A total of 2496 and 2453 m.e.p.ps were analyzed during control (lined columns) and prostaglandin E_1-conditions (solid columns) respectively.

train disappeared altogether, the e.p.p. behaving like an all-or-nothing phenomenon. When the stimulation frequency was decreased from, for example, 50 to 5 Hz, failures disappeared. If the stimulation frequency was resumed or increased, a total block of the e.p.p. occurred. This kind of failure has previously been described by Krnjević & Miledi (1958; 1959) as presynaptic failure of neuromuscular propagation and results from an impaired conduction of the action potential in the terminal branch of motor nerve fibres.

Prostaglandin E_1-induced failures in the e.p.p. response

When the effects of prostaglandin E_1 were being studied, only such cells were examined which did not exhibit spontaneous failures in the e.p.p. response to tetanic stimulation. Prostaglandin E_1, concentrations from 10 nM, induced intermittent failures in the generation of the e.p.p. within a few minutes when the nerve was stimulated at 30–80 Hz at 37°C. As a rule, continuing high-frequency stimulation after prostaglandin E_1-induced failures had appeared, resulted in a complete block of the e.p.p. As was also found to be true for spontaneous failures, prostaglandin E_1-induced failures occurred abruptly. Only occasionally did the e.p.p. (or e.p.c.) amplitude definitely decrease before failures occurred. The amplitude of single e.p.ps appearing after failures was as a rule greater than average. Further, intermittent prostaglandin E_1-induced failures, like spontaneous failures, were abolished by decreasing the stimulation frequency. Blockade of the e.p.p. was often preceded by a prolongation of the latency between the stimulation artefact and the beginning of the e.p.p.

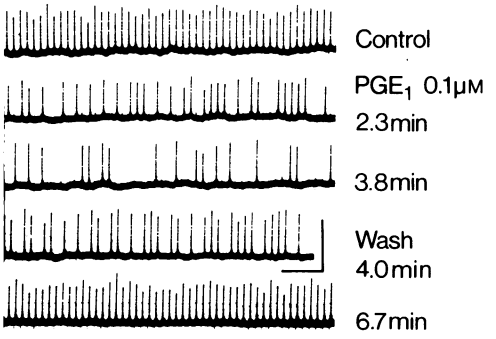

Figure 2 Effect of prostaglandin E_1(PGE_1) 0.1 μM on the generation of e.p.ps in the cut phrenic nerve-diaphragm muscle preparation of the rat in response to continuous indirect stimulation at 80 Hz *in vitro*. All records are from the same cell. The temperature was maintained at 36–37°C and the RMP varied between −30 and −28 mV throughout the experiment. Calibrations: vertical, 3 mV; horizontal, 0.1 s.

The results of a typical experiment are illustrated in Figure 2. The nerve was continuously stimulated at

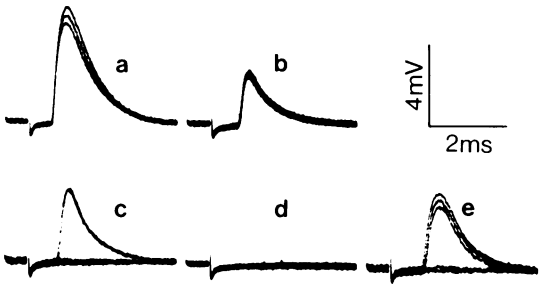

Figure 3 Effect of the stimulation frequency on the generation of e.p.ps in the cut phrenic nerve-diaphragm preparation of the rat treated with prostaglandin E_1 0.1 μM *in vitro*. The temperature was maintained at 37–38°C and the RMP varied between −40 and −34 mV except in (e), in which the RMP was −24 mV. The nerve was initially stimulated at 30 Hz (a) after which the stimulation frequency was gradually increased in 5–10 Hz steps. After 1 h of continuous stimulation at increasing frequencies, the stimulation frequency reached 80 Hz (b) whereupon the frequency was increased to 100 Hz (c) and a few minutes later to 105 Hz (d). Record (e) was made 16 min after washing with prostaglandin E_1-free solution was begun, the stimulation frequency being maintained unchanged at 105 Hz. Each frame is composed of 3–10 superimposed traces.

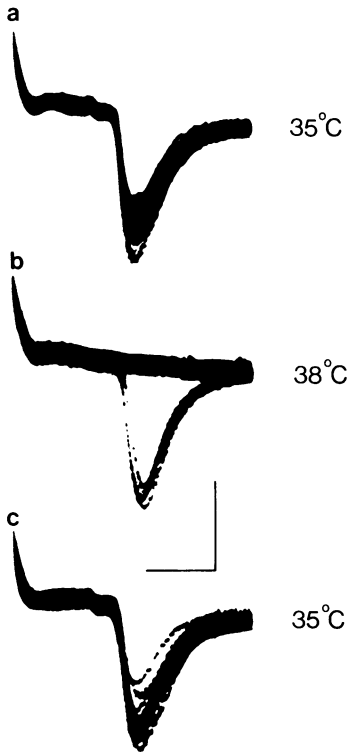

Figure 4 Temperature-dependence of the prostaglandin E_1-induced block of the terminal nerve spike and subsequent e.p.c. failure. Records were obtained extracellularly from a cut phrenic nerve-diaphragm muscle preparation of the rat *in vitro*. Prostaglandin E_1 was used in a concentration of 1 μM throughout the experiment. The stimulation frequency was 70 Hz. In (a) the temperature was 35°C and the preparation had been exposed to prostaglandin E_1 for 19.5 min; (b) was made 1 min after the temperature had been raised to 38°C. Note that although the e.p.c. fails in the majority of traces, a few stimulations have resulted in the generation of an e.p.c. while the corresponding nerve spikes are obscured by the baseline; (c) was obtained less than 1 min after the temperature had been readjusted to 35°C. Each frame is composed of about 35 superimposed traces. Calibrations vertical, 0.3 mV; horizontal, 1 ms.

80 Hz, and during control conditions every stimulation resulted in the generation of an e.p.p. While stimulation of the nerve was continued, prostaglandin E_1 was infused into the bathing chamber at 0.1 μM and within 2 to 3 min the e.p.p. suddenly and intermittently failed. Upon washing with drug-free solution, the number of failures gradually decreased until the e.p.p. response was completely normal. In cells where the e.p.p. was totally blocked, washing with drug-free solution resulted in a sudden appearance of the full-size e.p.p. In one single experiment, the reappearing e.p.p. gradually increased in size until normal amplitude was achieved.

Two experiments were carried out with uncut, curarized preparations and altogether 7 experiments were conducted with preparations treated with high concentrations of Mg^{2+} to block muscle contractions. Extensive experiments with curarized preparations were hampered by the pronounced tetanic run-down of the e.p.p. during these conditions. In Mg^{2+}-treated preparations, positive effects with prostaglandin E_1 were recorded. It seemed, however, that high concentrations of Mg^{2+} opposed the prostaglandin E_1-effect. Instead of appearing within 2 to 3 min as in a cut preparation, prostaglandin E_1-induced failures often did not appear until after about 10 minutes. It is not clear if this is a specific Mg^{2+}-effect or whether the cut preparation is in some way more susceptible to the effects of prostaglandin E_1.

Effect of the concentration of prostaglandin E_1

The effect of prostaglandin E_1 remained the same when used in a wide range of concentrations. The same abrupt intermittent failure in the tetanic e.p.p.

response and subsequent block of the e.p.p. occurred within a few minutes of treatment with prostaglandin E_1 in a concentration ranging from 10 nM to 10 μM. Concentrations lower than 10 nM were not tested. The possibility of an inverse correlation between the concentration of prostaglandin E_1 and the stimulation frequency required to obtain a positive prostaglandin E_1-effect, was not examined.

Dependence on stimulation frequency

At no concentration tested did prostaglandin E_1 induce failures or transmission blockade when the nerve was stimulated below 10 Hz. There appeared to be a threshold frequency below which, at a particular junction, no prostaglandin E_1-effects could be obtained. In one-fifth of all trials in prostaglandin E_1, e.p.p. failures were recorded at a stimulation frequency of 15 Hz. Upon stimulation at 30 or 50 Hz, 45% and 60%, respectively, of all end-plates showed a constant failure or block of the e.p.p. which was reversed upon washing with drug-free solution. At higher stimulation frequencies, the incidence of conduction failures in control solution was high (cf. page 388), making the study of the prostaglandin E_1-effect difficult. In the experiment illustrated in Figure 3, there was no failure upon stimulation at 30 Hz despite exposure to prostaglandin E_1 at 1 μM. When the stimulation frequency was progressively increased, failures and, ultimately, a block of the e.p.p. occurred at 100 and 105 Hz, respectively.

Temperature dependence

The prostaglandin E_1 effect proved extremely temperature-dependent. The effect was readily obtained at 37–38°C but was not seen at temperatures below 35°C. As may be seen in Figure 4, there was no failure in the generation of the extracellularly recorded e.p.c., despite exposure to 1 μM prostaglandin E_1, as long as the temperature was maintained at 35°C. When the temperature was raised, the e.p.c. started to fail intermittently within 1 min after the temperature had reached 38°C. Readjusting the temperature to 35°C immediately restored the response. In this particular experiment, prostaglandin E_1 was washed out at this stage of treatment. The preparation could now be maintained at 38°C for 15 min while a normal e.p.c. was recorded from the same cell that had previously failed at this temperature when exposed to prostaglandin E_1.

Effect of prostaglandin E_1 on the nerve terminal spike and end-plate current

Currents generated by action potentials in the presynaptic nerve terminal and by the action of concomitantly-released transmitter on the postsynaptic receptor can be recorded simultaneously with extracellular techniques (Katz & Miledi, 1965a, b). The results of the present m.e.p.p. experiments and the mode of failure of the e.p.p. favour a presynaptic site of action for prostaglandin E_1 in inhibiting the stimulus-evoked release of acetylcholine. Extracellular recordings were made from the end-plate to see if the e.p.p. failures were due to a failure of invasion of the nerve terminal by the nerve spike or whether the impulse invaded the terminal but failed to release transmitter.

With extracellular techniques, positive effects with prostaglandin E_1 were obtained at seven junctions in seven preparations. The generation of the extra-

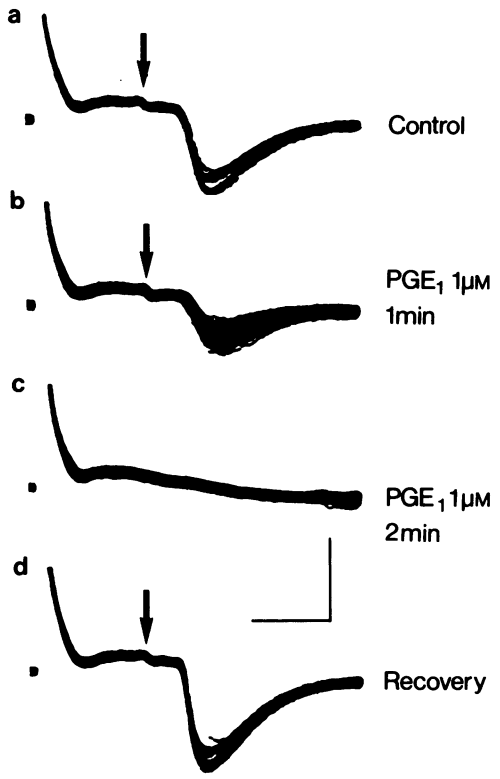

Figure 5 Effect of prostaglandin E_1 (PGE$_1$) 1 μM on the extracellularly-recorded terminal nerve spike and the e.p.c. in the cut phrenic nerve-diaphragm muscle preparation of the rat *in vitro*. The stimulation frequency was 60 Hz and the preparation was maintained at 38°C. The terminal nerve spike is recorded as a small deflection in the baseline (arrow) preceding the e.p.c. In (c) the blockade of the e.p.c. is preceded by a block of the terminal nerve spike. Each frame is composed of about 30 superimposed traces. Calibrations: vertical, 0.2 mV; horizontal, 1 ms.

cellularly recorded e.p.c. was blocked by prostaglandin E_1 (0.1 μM and 1 μM) in the same way as was the generation of the e.p.p. The e.p.c. failed abruptly although in one cell the e.p.c. amplitude decreased gradually before failures occurred. In six out of seven successful experiments, a failure in the e.p.c. response was consistently preceded by a block of the action current. In the seventh experiment, a summation of potentials originating from different junctions was apparently recorded which resulted in a terminal spike persisting even when the e.p.c. was blocked by prostaglandin E_1. A typical experiment is illustrated in Figure 5. Under control conditions, the action potential arriving at the nerve terminal is recorded as a small deflection preceding the e.p.c. Within 1.5 min after the introduction of prostaglandin E_1, the amplitude of the e.p.c. was decreased but the nerve spike continued to invade the nerve terminal. After an additional 1.5 min the terminal spike as well as the e.p.c. were blocked. One minute of washing with prostaglandin E_1-free solution restored both the spike and the e.p.c.

Discussion

The present results confirm and extend preliminary observations (Jansson *et al.*, 1974) showing that prostaglandin E_1 depresses release of acetylcholine evoked by nerve impulses. Under these conditions, the e.p.p. or e.p.c. abruptly begins to fail intermittently in an all-or-nothing manner. As judged from present extracellular recordings, this probably results from a partial or complete failure of the nerve impulse to invade the nerve terminal. The presence of m.e.p.ps of unchanged amplitude excludes the possibility of a direct effect on the postsynaptic membrane.

The simplest explanation for our observations is that prostaglandin E_1 depolarizes the presynaptic nerve fibre membrane (see Sjöstrand, 1972) thereby causing a presynaptic inhibition of acetylcholine release (Eccles, Schmidt & Willis, 1962). However, the unchanged m.e.p.p. frequency as well as the all-or-nothing character of the e.p.p. block after treatment with prostaglandin E_1 indicate that the unmyelinated nerve terminal was not affected by the hypothetical membrane depolarization (see Miledi & Slater, 1966). Instead the block is apparently located proximally to the nerve terminal, perhaps at one of the last nodes of Ranvier (Katz & Miledi, 1968) or at the vulnerable branching of peripheral nerve ramifications (Krnjević & Miledi, 1958; 1959). As judged from extracellular recordings (Hedqvist, 1970a; Jansson *et al.*, 1974), conduction in the nerve trunk is not affected by prostaglandins.

The absolute refractory period of fast mammalian nerve fibres is about 0.5 ms. This is compatible with a stimulation frequency of 2000 Hz, which is possible for only short periods (Gasser & Grundfest, 1936; Lowitzsch & Hopf, 1972). Spikes elicited even at millisecond intervals do progressively level off. But as pointed out by Krnjević & Miledi (1959), it is surprising that conduction failure appears at a stimulation frequency well below 100 Hz. The mechanisms involved in spontaneous failure of the e.p.p. have been discussed at some length by Krnjević & Miledi (1959) who suggested that the deleterious effect of prolonged nerve stimulation may be focused on the terminal nerve branches which, while running within the diaphragm muscle, '... are fully exposed to the effects of substances released by active muscles'. Our results raise the possibility that spontaneous failure of the e.p.p. might be mediated by prostaglandins released from either the muscle or the nerve. This possibility is supported by the demonstrated release of prostaglandins upon stimulation of a rat phrenic nerve-diaphragm muscle preparation (Ramwell *et al.*, 1965; Laity, 1969). On the other hand, prostaglandin E_1 was effective only during certain critical conditions (temperature 36–38°C, tetanic stimulation rate). In other words, e.p.p.-failures induced by exogenous prostaglandin E_1 occurred only when the safety factor for impulse conduction was brought close to one (see Krnjević & Miledi, 1958; 1959). The marginal nature of the prostaglandin E_1-effect could be taken as an indication against a profound physiological role of prostaglandins in neuromuscular transmission.

In conclusion, exogenous prostaglandin E_1 induces, upon repetitive indirect stimulation, intermittent failures and ultimate block of the e.p.p. in the cut phrenic nerve-diaphragm muscle preparation of the rat. This effect is reversible and it is achieved by preventing the invasion of the nerve terminal by the action potential. A protective role of prostaglandins in neuromuscular transmission might be considered. Only from the results of further studies will a fuller understanding of these processes become available.

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