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EFFECTS OF SODIUM PUMP INHIBITORS ON SPONTANEOUS ACETYLCHOLINE RELEASE AT THE NEUROMUSCULAR JUNCTION

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The ability of G-strophanthin to influence neuromuscular transmission in voluntary striated muscle has been known since 1955 (Greeff & Westermann, 1955) but the mechanism of this action is not clear. Birks (1963) showed that digitalis glycosides increased the amount of transmitter liberated from the motor-nerve terminals both spontaneously and on nerve stimulation. A similar increase was found in the perfused sympathetic ganglia. Hubbard & Gage (1964) reported that digitalis and sodium azide inhibited post-tetanic potentiation and suggested that they acted upon the mechanism causing post-tetanic hyperpolarization of the motor-nerve terminals (Hubbard & Schmidt, 1963). Gage (1965) only reported post-synaptic effects of the glycosides. In studies of the role of calcium in the process of acetylcholine release at the rat-phrenic motor-nerve terminals (Elmqvist & Feldman, 1965) ouabain was used because of its calcium mobilizing effect (Govier & Holland, 1964). It was then observed that prolonged exposure to high concentrations of ouabain caused a rapid release of acetylcholine recorded as miniature end-plate potentials (m.e.p.p.s) The parameters of this effect were therefore investigated.

METHODS

The experiments were performed on the rat phrenic–diaphragm preparation. The preparations were dissected and mounted as previously described (Elmqvist & Quastel, 1965). The usual techniques for intracellular recording with KCl-filled glass capillary microelectrodes (3–8 MΩ) were employed (Fatt & Katz, 1951, 1952). Potentials were observed on the oscilloscope and recorded on paper with an Elema Mingograf 81. The bathing solution was the same as previously used in this laboratory (Elmqvist & Quastel, 1965). Changes in $CaCl_2$, $MgCl_2$ or KCl concentrations were accompanied by appropriate alteration in NaCl concentration to keep the solution isosmolar. Choline chloride 5×10^{-6} g/ml. was always added to the solution. Experiments were carried out at a temperature of 32° C. Ouabain was prepared in a stock solution of 10^{-2} g/ml. in distilled water or 'normal bathing solution'. Sodium azide was prepared as a stock solution containing 1 m-mole/ml. in distilled water.

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RESULTS

The experiments were concerned with spontaneous activity at the motor-nerve terminals. In this study nerve stimulation was not undertaken, and it was not determined whether the previously reported neuromuscular blockade (Greeff & Westermann, 1955; Birks, 1963) was induced. Post-synaptic effects on m.e.p.p. amplitude, although observed particularly when muscle depolarization was induced by the glycoside, were not systematically investigated.

The effect of ouabain on m.e.p.p. frequency had a long latency, particularly with lower doses. With 80 μg/ml. no change was found in 1 hr; after 5 hr the frequency was 84 ± 20 m.e.p.p.s/sec (mean $\pm$ S.E., 7 fibres). 160 μg/ml. ouabain ($2{\cdot}2 \times 10^{-5}$ M) induced a slow rise for the first 15–30 min and thereafter a rapid rise to 200–400 m.e.p.p.s/sec (Fig. 1). At the same time asynchronous twitching of individual muscle fibres was observed. Within 30–45 min after the application of 160 μg/ml. ouabain high frequencies were consistently observed. This concentration was therefore used for the remainder of the experiments. The time to peak frequency varied from fibre to fibre over a range of about 30 min. After the peak the rate declined with time; 4 hr after application of the drug the rate was 177 ± 42 m.e.p.p.s/sec (mean $\pm$ S.E., 7 fibres).

Liley (1956) has shown that depolarization of the nerve terminals with KCl induces an increase in m.e.p.p. frequency. The rate observed in 20 mM-KCl is comparable to that induced by 160 μg/ml. ouabain. In the rat diaphragm the increase in frequency induced by this concentration of potassium is earlier in onset, and is better maintained than was seen with glycoside induced release (D. Elmqvist and D. Feldman, unpublished observations). There was less variability in the latency of the potassium-induced effect. No systematic studies of resting membrane potential were undertaken, but it was observed that at the time of peak frequency muscle-membrane potentials in the presence of ouabain 160 μg/ml. and of 20 mM-KCl were about 60 mV.

In 'effective concentration' ouabain induces an increase in m.e.p.p. frequency in calcium-free solution, and a decline in the rate when EDTA is present (Elmqvist & Feldman, 1965). Twenty millimolar KCl does not influence the m.e.p.p. rate in calcium-free solutions whether or not EDTA is present. Although high rates were induced by ouabain in calcium-free solution these declined more rapidly than when 2 mM-Ca^{2+} was present. At the peak the frequency was 335 ± 17 m.e.p.p./sec (mean $\pm$ S.E., 4 fibres) and 3 hr later it was 81 ± 10 (11 fibres). When Ca^{2+} was reintroduced the rate returned to the peak level within about 1 min.

An increase in m.e.p.p. frequency which can be induced by depolariza-

tion with electric current or increased K^+ can be prevented or reduced by increasing the Mg^{2+} concentration (Liley, 1956; Hubbard, 1961). In Liley's study 12·5 mM-Mg^{2+} reduced the rate by a factor of five at KCl concentrations from 20 to 30 mM. The addition of 10 mM-Mg^{2+} either before or after the effect of ouabain had occurred had only a slight and transient, if any, effect on the m.e.p.p. rates observed.

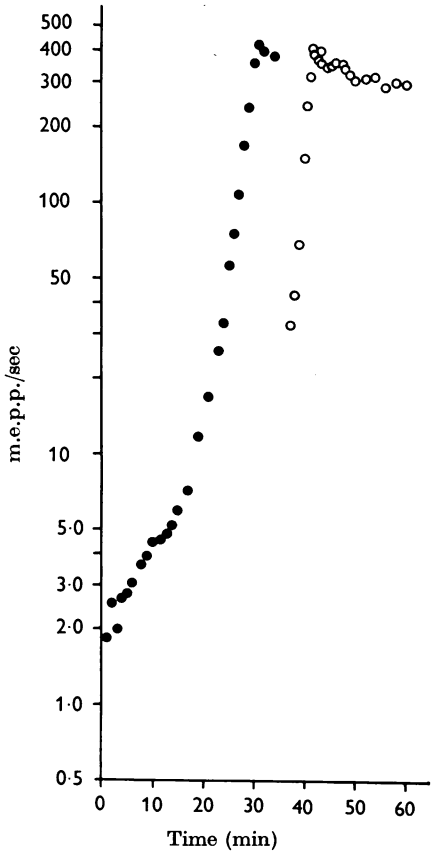

Fig. 1. M.e.p.p. frequency at single rat phrenic-neuromuscular junctions. At zero time ouabain (160 μg/ml.) was added to normal bathing solution. Continuous records were obtained. Frequencies counted at 1–2 min intervals are shown in two fibres.

Synergism between cardiac glycosides and calcium ions on myocardial contraction is well known (Glynn, 1964). It was therefore surprising to find that specimens pre-incubated with 8 mM-Ca^{2+} in otherwise normal solution did not respond with an increased m.e.p.p. rate upon the addition of ouabain. Increasing the calcium ion concentration from 2 to 8 mM when a high discharge rate was induced by ouabain reduced the frequency

to about the level which was observed in this calcium concentration alone. These effects were reversible (Figs. 2 and 3). The full effect on m.e.p.p. frequency occurred sooner when calcium was added than the increase observed when the excess was removed. When the frequency in ouabain

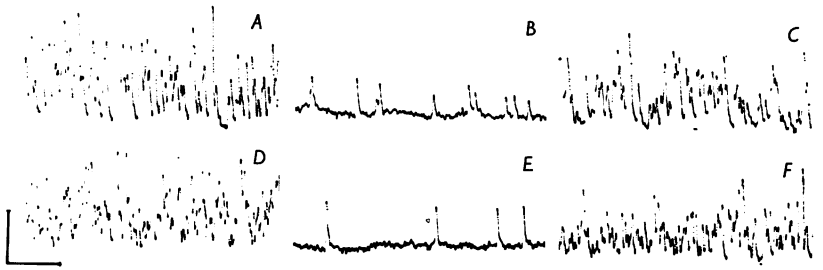

Fig. 2. Intracellular recording of m.e.p.p.s. fibre 1: *A*. After 3 hr in ouabain and 2 mM-Ca^{2+} (normal bathing solution); *B*. 5 min after increasing Ca^{2+} to 8 mM; *C*. 15 min after return to 2 mM-Ca^{2+}. Fibre 2: *D*. After 4 hr in ouabain and 2 mM-Ca^{2+} (normal bathing solution); *E*. 30 min after increasing Ca^{2+} to 8 mM; *F*. 5 min after K^+ concentration was increased to 20 mM (8 mM-Ca^{2+} still present). Ouabain (160 μg/ml.) was present throughout these experiments. Calibrations: 0·5 mV and 50 msec.

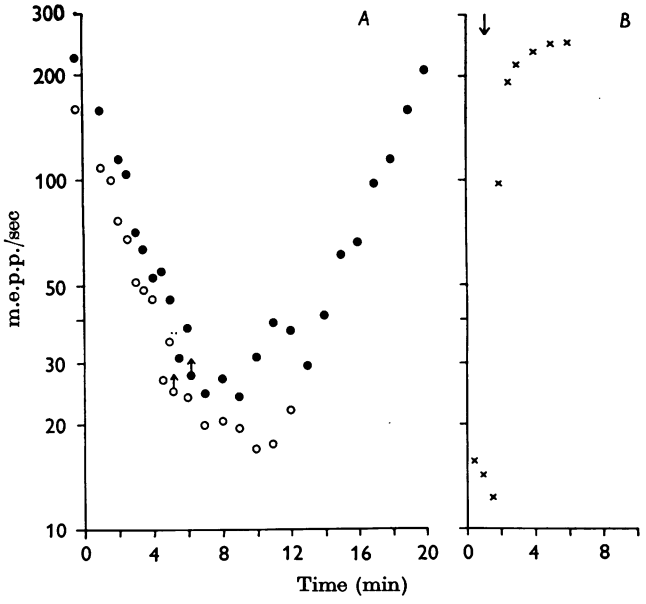

Fig. 3. M.e.p.p. frequency at single rat phrenic-neuromuscular junctions. *A*. Effect of changing Ca^{2+} concentration after 3 hr in ouabain (two fibres are illustrated). At zero time Ca^{2+} concentration was increased from 2 to 8 mM. At ↑ Ca^{2+} concentration was reduced to 2 mM. *B*. Effect of increasing K^+ concentration (↓) to 20 mM when m.e.p.p. frequency in the presence of ouabain had been reduced by 8 mM-Ca^{2+}. Ouabain (160 μg/ml.) was present throughout the experiments.

was reduced by the addition of Ca^{2+} addition of 20 mM-K^+ promptly induced a high frequency discharge (Fig. 3*B*). The inhibiting effect of the excess on the action of ouabain was not antagonized by the addition of 1–10 mM-Mg^{2+}.

A parallel action at the neuromuscular junction of sodium azide and ouabain in abolishing post-tetanic potentiation was observed by Hubbard & Gage (1964). As azide, in addition to its other effects, blocks the sodium pump (Hodgkin & Keynes, 1955), as do the glycosides (Glynn, 1964), its effects on m.e.p.p. frequency were compared with ouabain. In normal calcium-ion concentration azide induced a rapid discharge of acetylcholine packages. The latency of onset was similar to that observed with ouabain. High frequencies obtained were not sustained and rates declined to zero in about half an hour. The increase in frequency was not inhibited with 8 mM-Ca^{2+}.

DISCUSSION

The results show that the cardiac glycosides can induce a rapid spontaneous release of ACh packages from the mammalian motor-nerve terminals similar to that observed in the frog. The concentration required for this effect is several orders of magnitude higher. The concentrations of ouabain used by Gage (1965) were half those employed here for most of the experiments. Half the 'effective concentration' had an effect but required several hours before it was seen.

As has been reported digitalis appears to mobilize calcium, not only in cardiac muscle (Govier & Holland, 1964; Lüllman & Holland, 1962) but also at the motor nerve terminal (Elmqvist & Feldman, 1965). Ouabain increases the m.e.p.p. frequency in the absence of calcium but decreases it when EDTA also is present. Depolarization of the nerve terminals by electric current or by raised external K^+ concentration (Liley, 1956; Hubbard, 1961) did not cause an increased m.e.p.p. frequency when Ca^{2+} was not present.

The high rates induced by ouabain are not explained only by an increased activity or availability of calcium. Elevation of the external calcium concentration to 10 mM (Hubbard, 1961) about doubled and an increase to 30 mM (D. Elmqvist and D. Feldman unpublished observation) gave a fourfold change in the resting frequency. Caffeine induced an about fourfold increase in m.e.p.p. rate and this could not be further increased by prior or further addition of calcium (Elmqvist & Feldman, 1965). The ability of calcium *per se* to affect the m.e.p.p. rate appears to be limited to about this amount.

It is well established that depolarization of the nerve terminals causes a great increase in the rate of release of acetylcholine packages (del

Castillo & Katz, 1954; Liley, 1956; Katz, 1962). The limited ability of calcium and caffeine to increase m.e.p.p. rate suggests that ouabain has an action on acetylcholine release in addition to its calcium mobilizing capability. In other tissues the glycosides have been demonstrated to block the active sodium extrusion from cells (Glynn, 1964). A block of the sodium pump results in accumulation of sodium in the cells and lowering of the membrane potential. At the time when the peak m.e.p.p. frequencies were achieved the muscle potential was lowered to about 60 mV. It is reasonable to relate the observed increase in m.e.p.p. frequency to inhibition of the sodium pump. Our data do not determine whether release was initiated by depolarization or by intracellular accumulation of sodium ions. More generally acting metabolic poisons such as sodium azide and 2,4-dinitrophenol (Kraatz & Trautwein, 1957) also induce a rapid release of acetylcholine packages as well as muscle membrane depolarization. This action, too, appears after a considerable latency. It is at present not possible to measure directly the membrane potential or the sodium content of the motor-nerve terminals.

Membrane depolarization alone does not suffice to explain the observation that magnesium ions had virtually no inhibiting effect on the rapid release induced by ouabain. It was shown by Hubbard (1961) that there is a competitive antagonism between the external calcium and magnesium concentration on m.e.p.p. frequency. Ouabain has significant effects on the availability of active calcium in cardiac muscle (Govier & Holland, 1964). It has been shown that this is also the case in the motor nerve terminals (Elmqvist & Feldman, 1965). The failure of magnesium to reduce the m.e.p.p. frequency elevated by ouabain is best explained by increased calcium availability. It may tentatively be suggested that ouabain has two actions in causing release of ACh packages. This may well be an oversimplification of a more complex interaction between sodium and calcium (cf. Kelley, 1965; Orkand & Niedergerke, 1964).

The effect of 8 mM calcium in antagonizing the ouabain-induced augmented release of ACh packages may be related to the ability of calcium to limit the movement of sodium ions across biological membranes (Frankenhaeuser & Hodgkin, 1957; Adelman & Moore, 1961; Curran, Herrera & Flanigan, 1963). An incomplete inhibition of the sodium pump by the concentration of ouabain utilized might then be insufficient to cause a major accumulation of intracellular sodium. If this is so the resulting depolarization would be less and the m.e.p.p. frequency correspondingly reduced. The failure of high calcium concentrations to inhibit the release induced by sodium azide suggests that this metabolic inhibitor causes a more complete inhibition of the energy-dependent sodium pump. The failure of magnesium to antagonize this effect of calcium is explained

by the synergistic effects of these divalent ions on sodium permeability. Magnesium is less effective than calcium in decreasing sodium permeability (Frankenhaeuser & Hodgkin, 1957) and this fact may explain the inability of magnesium (10 mM) to inhibit ouabain induced release.

SUMMARY

1. Ouabain and sodium azide induce after a latency of about 30 min a rapid release of acetylcholine packages from the motor-nerve terminals.

2. Increased concentrations of calcium but not of magnesium block the ouabain-induced augmented release of ACh packages.

3. The effects of ouabain on transmitter release are explained by its calcium mobilizing and sodium pump inhibiting effect.

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