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THE EFFECT OF OUABAIN ON NORADRENALINE OUTPUT FROM PERIPHERAL ADRENERGIC NEURONES OF ISOLATED GUINEA-PIG VAS DEFERENS

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SUMMARY

1. The effect of ouabain on the noradrenaline output from peripheral adrenergic neurones has been studied using isolated guinea-pig vasa deferentia.

2. Exposure to ouabain (10^{-4} M) causes a gradual increase in the noradrenaline output. The effect occurs after a delay of 20 min and reaches a maximum during the period from 40–60 min.

3. In the absence of external Ca, exposure to ouabain fails to produce an increase in the noradrenaline output. However, the reintroduction of Ca (2·5 mM) after a 1 hr exposure to ouabain in Ca-free media causes a rapid rise in noradrenaline output which reaches a maximum within the first 20 min.

4. After a 1 hr exposure to a low concentration of ouabain (10^{-5} M) the reintroduction of Ca is almost ineffective in increasing the noradrenaline output. When the concentration of ouabain is increased, the reintroduction of Ca becomes effective and causes a maximum effect with 10^{-4} M ouabain. In the presence of a constant amount of ouabain (10^{-4} M) the noradrenaline output induced by the reintroduction of Ca increases over the range 0·2–2·5 mM.

5. In the presence of ouabain (10^{-4} M) the Ca-induced noradrenaline output increases in a linear fashion with increasing Na concentrations from 25 to 143 mM, as long as NaCl is replaced with equimolar choline chloride or isotonic sucrose.

6. In the presence of the lowest effective concentration of sodium (25 mM) the noradrenaline output induced by the reintroduction of Ca after a 1 hr exposure to ouabain is potentiated by LiCl. However, in the complete absence of Na^+ ions, there is no Li-dependent increase in the Ca-induced noradrenaline output.

7. It is suggested that ouabain may cause an increase in noradrenaline output by an effect on the Na-dependent Ca influx system.

INTRODUCTION

Cardiac glycosides cause a slow but progressive increase in the output of secretory product from a variety of tissues. This has been shown for ACh release from rat and frog motor nerve terminals (Elmqvist & Feldman, 1965*a*, *b*; Birks & Cohen, 1968*a*, *b*; Baker & Crawford, 1975), from parasympathetic nerves of guinea-pig ileum (Paton,

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Vizi & Zar, 1971) and from rat brain slices (Vizi, 1972), for catecholamines from perfused bovine adrenal medulla (Banks, 1967) and for vasopressin from rat posterior pituitary glands (Douglas, 1974). This effect of cardiac glycosides has been explained primarily in terms of inhibition of a sodium pump. Such an inhibition is thought to result in either (1) an increase in Ca influx as a result of the depolarization induced by a loss of intracellular potassium (Banks, 1967), (2) an accumulation of intracellular Na (Birks & Cohen, 1968*b*) which results in the subsequent release of Ca from an internal store or (3) an increase in the effectiveness of internal Ca, induced by changes in the monovalent cation concentration (Baker & Crawford, 1975).

According to Birks & Cohen (1968*b*) the effects of the cardiac glycoside, digoxin, were dependent on the external concentration of Na and Ca. Elmqvist & Feldman (1965*a*, *b*) had also reported a Ca-dependent ouabain action. On the other hand, Baker & Crawford (1975) have shown that the response induced by ouabain was completely independent of external Ca and became sensitive to Na only during the late phase of exposure to the glycoside.

Our concern in the present experiments was to consider the effect of ouabain on noradrenaline output from peripheral adrenergic nerve terminals.

METHODS

Male guinea-pigs weighing 500–700 g were stunned and bled to death. Vasa deferentia, usually bilaterally, were isolated separately, fixed on a length of fine wire inserted into the lumen and immersed in 1 ml. of incubation medium and maintained at 37 °C in a water bath. The media contained either bicarbonate buffer or Tris aminomethane buffer and were bubbled with 5% CO_2 in O_2 or with pure O_2 respectively. The pH was thus maintained at about 7·4. Preceding the test incubation, the preparations were preincubated in Krebs or Ca-free Krebs solution for 40 min during which the solution was changed at 10 min intervals. This allowed the spontaneous release of noradrenaline to reach a low, steady level. The tissue was then transferred to the appropriate test solution and incubated. During this incubation period the solution was replaced at 20 min intervals. Following each incubation period, the medium was collected, acidified with concentrated perchloric acid (final concentration, 0·4 N) and stored in the cold until centrifugation was carried out.

Incubation media. The principal incubation medium was Krebs solution of the following composition (mM): NaCl, 118; KCl, 4·8; $CaCl_2$, 2·5; $MgSO_4$, 1·2; $NaHCO_3$, 25; KH_2PO_4, 1·2; glucose 10. Low Na solution was prepared by replacing a part or all of NaCl with equimolar amounts of choline chloride or isotonic sucrose. For the sodium (25 mM)-containing Li solution all of the NaCl was replaced by equimolar amount of LiCl. For the Na-free lithium solution, $NaHCO_3$ and KH_2PO_4 were replaced by 2·5 mM Tris amino methane buffer in addition to the replacement of all of the NaCl with LiCl. The low Li solution was made by reducing the concentration of LiCl and adding the appropriate amount of choline chloride. For Ca-free or low Ca solutions, $CaCl_2$ was omitted or reduced. Atropine (10^{-5} M) was added to the solutions containing choline chloride to prevent cholinergic effects. All the solutions contained phenoxybenzamine (5×10^{-5} M) and ascorbic acid (6×10^{-5} M) to inhibit the uptake and oxidation of noradrenaline, respectively. Ouabain was simply added to the appropriate solution to give the required concentration between 10^{-5} M and $1{\cdot}25 \times 10^{-4}$ M. In a few experiments the exposure to phenoxybenzamine was ended 1 hr before ouabain was applied. Similar results were obtained.

Assay of noradrenaline. Following incubation, the acidified media were centrifuged at 30,000 g at 5 °C for 10 min. The clear supernatants were then transferred to small test-tubes and stored on ice until assay. The assay was performed by the fluorimetric method of Anton & Sayre (1962).

RESULTS

Increase in noradrenaline output induced by ouabain

Exposure of vasa deferentia to ouabain (10^{-4} M) caused an increase in noradrenaline output from the peripheral adrenergic neurones. The response induced by ouabain occurred after a delay of 20 min, reached a maximum at 40–60 min, and then gradually declined to a resting level at 140–160 min (Fig. 1*A*). In the absence of external Ca, exposure to ouabain failed to increase the noradrenaline output (Fig. 1*B*).

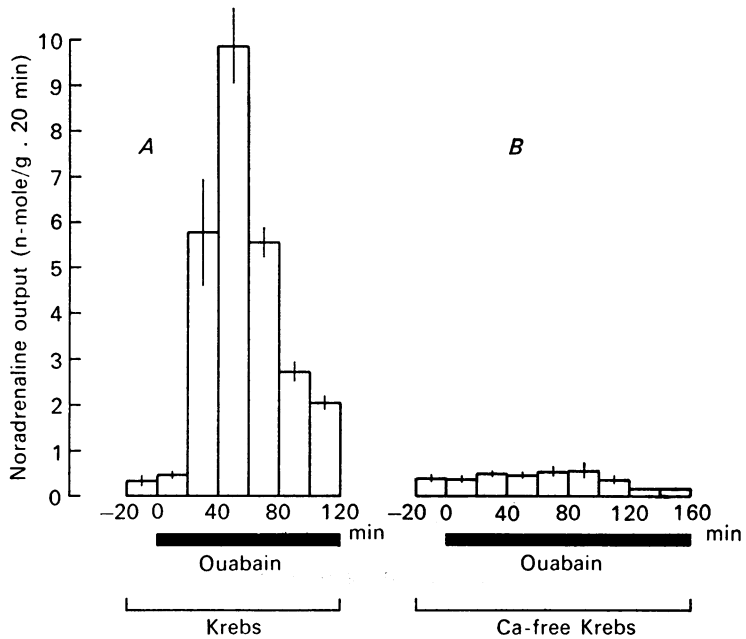

Fig. 1. Effects of ouabain on noradrenaline output from isolated guinea-pig vas deferens in the presence (*A*) or absence (*B*) of Ca. Columns represent the mean (± S.E.) of noradrenaline output obtained from five (*A*) and four (*B*) experiments. Horizontal heavy bars indicate the period of exposure to ouabain (10^{-4} M).

However, the output was almost completely restored by the subsequent reintroduction of Ca (Fig. 2). The reintroduction of Ca (2·5 mM) after a 1 hr exposure to ouabain in the absence of Ca, caused a rapid increase in noradrenaline output, the maximal effect being attained during the first 20 min following Ca reintroduction. The noradrenaline output during the first 1 hr following the reintroduction of Ca accounted for more than 80 % of that obtained throughout the entire period following Ca reintroduction. In the absence of ouabain, there was no increase in the noradrenaline output following the reintroduction of Ca. In the following experiments the noradrenaline output during the first 1 hr after the reintroduction of Ca was used as an indicator of the effect of ouabain.

Concentration response relationships

To determine the effectiveness of ouabain in increasing the noradrenaline output, the response induced by the reintroduction of Ca was observed following a 1 hr exposure to ouabain over the range of concentrations 10^{-5} M to $1{\cdot}25 \times 10^{-4}$ M (Fig. 3). In the presence of low concentration of ouabain (10^{-5} M), the reintroduction of Ca failed to increase the noradrenaline output. However, as the concentration of

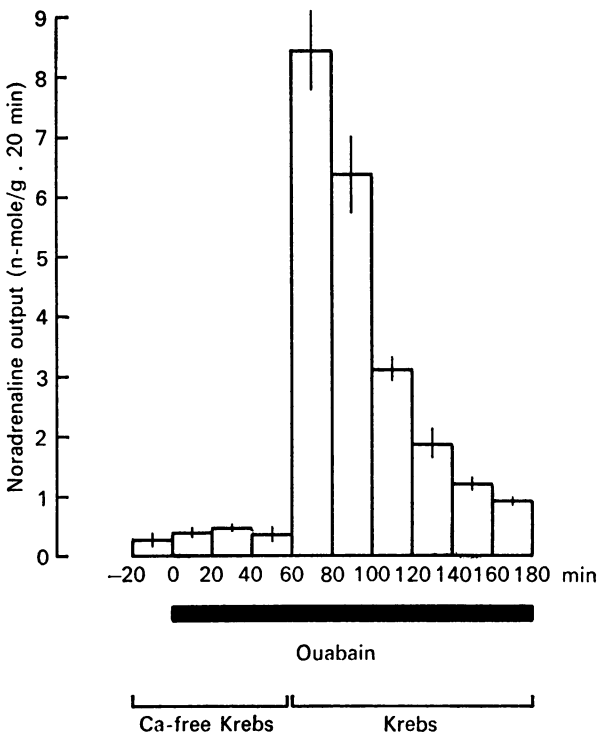

Fig. 2. Noradrenaline output induced by reintroduction of Ca after a 1 hr exposure to ouabain in the absence of Ca. Columns represent the mean (± S.E.) of noradrenaline output obtained from six experiments. Horizontal heavy bar indicates the period of exposure to ouabain (10^{-4} M). After exposure to ouabain for 1 hr in the absence of Ca, the vas deferens was transferred to the standard Krebs solution containing ouabain.

ouabain was increased the output of noradrenaline increased until it reached a maximum at 10^{-4} M ouabain. Further increases in the concentration of ouabain seemed to inhibit noradrenaline output. Thus, 10^{-4} M ouabain was used as an optimal concentration in all of the following experiments.

Ionic dependence of the effect of ouabain

Calcium. To determine the effect of the concentration of Ca on noradrenaline output in response to ouabain, various concentrations of Ca (0·2–5 mM) were reintroduced after a 1 hr exposure to ouabain (10^{-4} M) in the absence of the external Ca. As shown in Fig. 4, in which the amount of noradrenaline output during the 1 hr period

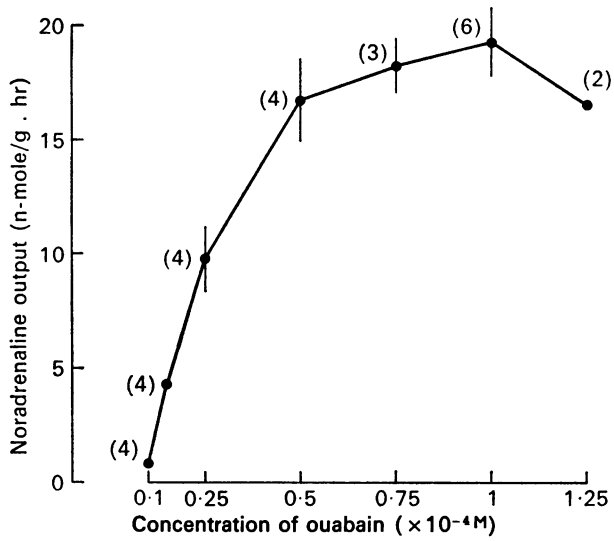

Fig. 3. Dose–response curve for the Ca-induced noradrenaline output as a function of ouabain concentration. The ordinate is the amount of noradrenaline released in n-mole/g.hr. The abscissa is the concentration of ouabain. Symbols indicate the mean (± S.E.) of noradrenaline output induced by the reintroduction of Ca (2·5 mM) after 1 hr exposure to ouabain in concentrations ranging from 10^{-5} M to $1{\cdot}25 \times 10^{-4}$ M. The numbers in brackets are the number of experiments.

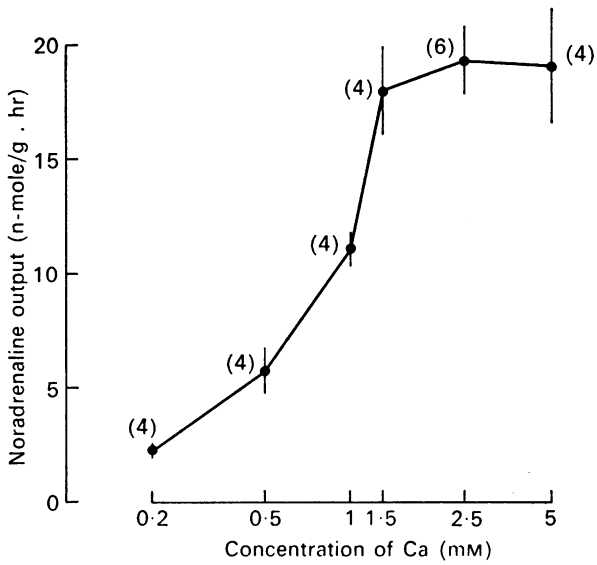

Fig. 4. Dose–response curve for the increase in the noradrenaline output induced by reintroduction of Ca after exposure to ouabain. The ordinate is the amount of noradrenaline released in n-mole/g.hr. The abscissa is the concentration of Ca on a logarithmic scale. Symbols indicate the mean (± S.E.) of noradrenaline output induced by reintroduction of Ca in concentrations from 0·2 to 5 mM after 1 hr exposure to ouabain (10^{-4} M) in the absence of Ca. The numbers in brackets are the number of experiments.

immediately following the reintroduction of Ca was plotted against the concentration of Ca, the lowest effective concentration of Ca was 0·2 mM, and the maximum output was reached at 2·5 mM Ca.

Sodium. Complete removal of Na^+ icns from the incubation medium has been shown to cause a moderate increase in the noradrenaline output from adrenergic neurones (Nakazato, Onoda & Ohga, 1977). It was suggested that Na deprivation caused an accumulation of intracellular Ca through the reversal of Na gradient across the plasma membrane (Baker, 1972) and the increase in intracellular Ca produced an increase in noradrenaline output. It is well known that ouabain is a potent inhibitor of Na-K ATP-ase and, because of this, causes an accumulation of intracellular Na

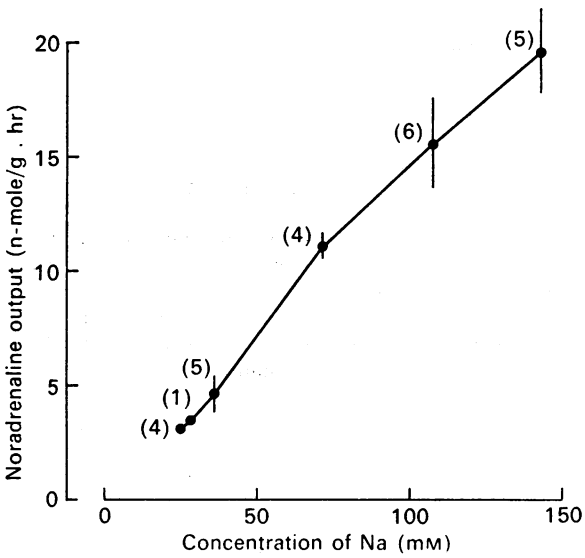

Fig. 5. Effects of varying the concentration of external Na on the noradrenaline output induced by reintroduction of Ca after exposure to ouabain. The ordinate is the amount of noradrenaline released in n-mole/g.hr. The abscissa is the concentration of Na. The NaCl was replaced by equimolar amounts of choline chloride. Symbols indicate the mean (± S.E.) of noradrenaline output induced by reintroduction of Ca (2·5 mM) after 1 hr exposure to ouabain (10^{-4} M) in the absence of Ca. The numbers in brackets are the number of experiments.

and a loss of intracellular potassium (Glynn, 1964). According to Birks & Cohen (1968*b*), the effects of digoxin on transmitter release from motor nerve terminals are likewise produced by the accumulation of intracellular Na and modified by the loss of intracellular K. If this is the case in the present experiments, it could be expected that the noradrenaline output induced by the reintroduction of Ca after exposure to ouabain depends on the concentration of extracellular Na. To test this, the concentration of Na in the bathing medium was varied between 25 and 143 mM by replacing it with the corresponding concentration of choline chloride, sucrose or LiCl. In the presence of choline chloride, it was found that the noradrenaline output induced by the reintroduction of Ca increased in proportion to the extracellular concentration of Na (Fig. 5). A similar result was obtained when NaCl was replaced by isotonic sucrose. However, if LiCl was used to replace NaCl the results became

more complicated. The addition of LiCl (46·5 mM), whether added to the incubation medium simultaneously with calcium or prior to the reintroduction of Ca, potentiated the output of noradrenaline obtained with the lowest effective concentration of Na (25 mM) (Fig. 6). This suggests that Li can substitute for Na in the ouabain action (Gorman & Marmor, 1970; Baker & Willis, 1972). Accordingly, the effect of Li on the noradrenaline output induced by the reintroduction of Ca in the presence of ouabain was studied.

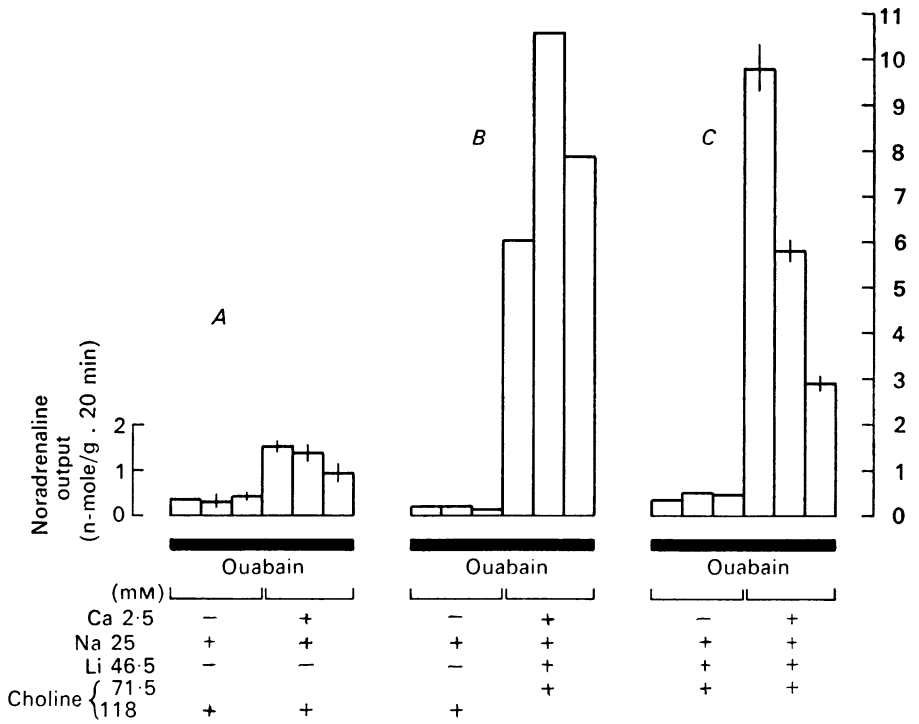

Fig. 6. Effects of partial replacement of Na with choline and/or Li on the noradrenaline output induced by reintroduction of Ca after 1 hr exposure to ouabain. Horizontal heavy bars indicate the presence of ouabain (10^{-4} M). The signs below the light bars show the presence (+) or absence (−) of Na (25 mM), Li (46·5 mM), choline (71·5 or 118 mM) and Ca (2·5 mM) in the incubation media. Columns represent the mean (± S.E.) of noradrenaline output obtained from four experiments in *A* and *C* and the mean of two experiments in *B*.

Lithium. In the absence of external Na, LiCl at various concentrations (from 0 to 143 mM) was added to the incubation medium. The tonicity was maintained by the addition of an appropriate amount of choline chloride. As shown in the previous paper (Nakazato, Onoda & Ohga, 1977), during exposure to the medium in which *all* the Na was replaced by choline chloride and external Ca was omitted, the reintroduction of Ca caused a moderate increase in the noradrenaline output. This response was not significantly affected by exposure to ouabain. The addition of LiCl, at any concentration, did not significantly change the response in the presence of ouabain (Fig. 7, open circles). On the other hand, when the concentration of Li was varied in the presence of Na (25 mM), the noradrenaline output induced by the reintroduction of Ca increased with an increase in the concentration of Li until it attained a

maximum at 46·5 mM (Fig. 7, filled circles). The maximum response was almost the same magnitude as that obtained in the presence of normal Na (143 mM). Further increases in the concentration of Li caused no additional release of noradrenaline.

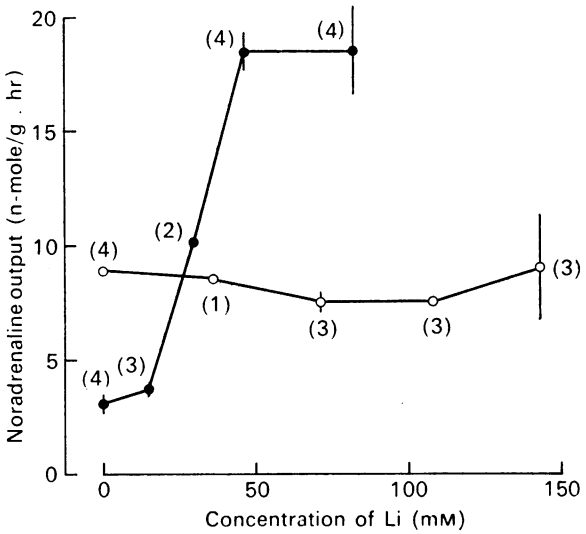

Fig. 7. Effects of varying the concentration of Li on the noradrenaline output induced by reintroduction of Ca after 1 hr exposure to ouabain. The ordinate is the amount of noradrenaline released in n-mole/g.hr. The abscissa is the concentration of Li. All the NaCl was replaced by equimolar amounts of LiCl. The concentration of LiCl was changed by a corresponding addition of choline chloride. Symbols indicate the mean (± S.E.) of noradrenaline output induced by reintroduction of Ca (2·5 mM) after 1 hr exposure to ouabain (10^{-4} M) in the presence (●, bicarbonate and phosphate buffers) and absence (○, Tris aminomethane buffer) of sodium (25 mM).

DISCUSSION

The present experiments show that a relatively high concentration of ouabain causes a gradually developing increase in the noradrenaline output from the peripheral adrenergic nerurones of guinea-pig vas deferens. Such an effect of cardiac glycosides has also been observed with the release of transmitter from rat (Elmqvist & Feldman, 1965*a*, *b*) and frog (Birks & Cohen, 1968*a*, *b*; Baker & Crawford, 1975) motor nerve endings.

Elmqvist & Feldman (1965*a*, *b*) have found that ouabain caused a Ca-dependent increase in the m.e.p.p. frequency. Birks & Cohen (1968*b*) have also reported that digoxin caused a sodium-dependent increase in e.p.p. size and in m.e.p.p. frequency, and suggested that these effects arose from an acceleration of Ca influx caused by the accumulation of intracellular Na being modified by the loss of intracellular K. In contrast to these reports, Baker & Crawford (1975) have shown that ouabain did not require external Ca to induce the increase in the m.e.p.p. frequency. They suggested that the changes in monovalent cation concentration resulting from inhibition of the Na pump could release Ca from internal stores or make internal Ca more effective in releasing transmitter, perhaps by increasing the affinity for Ca of

the sites that bind Ca in the release process. The reason for the discrepancy of the dependence of Ca is not known, but it might be attributed to the difference of the species or the cardiac glycosides used.

In the present experiments the increase in the noradrenaline output induced by ouabain was clearly dependent on the presence of Ca. Furthermore, the response evoked by the reintroduction of Ca after a 1 hr exposure to ouabain (10^{-4} M) increased with the concentrations of Ca (0·2–2·5 mM) reintroduced and external Na (25–143 mM) used, as long as Na was replaced by either choline or sucrose. In squid giant axon, it is known that anything reducing the electrochemical gradient for Na – for instance, a rise in internal Na or fall in external Na – will tend to reduce the Na-dependent Ca efflux and increase Ca uptake in exchange for Na loss (Baker, 1972). This sodium-dependent mechanism may explain the effect of ouabain on the noradrenaline output obtained in the present experiments as was proposed by Birks & Cohen (1968*b*); ouabain may cause the accumulation of Na in the adrenergic nerve terminals as the result of the inhibition of Na pump and an increase in the intracellular level of Ca and thus trigger the output of noradrenaline. In the absence of Ca the accumulation of Na seems to be facilitated by an amount dependent on the external concentration of Na, because of the lack of the stabilizing effect of Ca (Frankenhaeuser & Hodgkin, 1957). Once Ca is added to the medium, Ca could be taken up into the nerve terminals by exchange with the internal Na.

In the present experiments it is evident that Na^+ ions are indispensable for the effect of ouabain. Even Li, which is known not to interfere with the inhibition of Na pump by ouabain (Gorman & Marmor, 1970; Baker & Willis, 1972), and can substitute for sodium as a carrier of inward current (Hille, 1970) and thus produce action potentials (Hodgkin & Katz, 1949; Huxley & Stämpfli, 1951), failed to completely substitute for Na. A certain amount of Na was always required. Recently we found that both tetrodotoxin (5×10^{-7} g/ml.) and excess Mg (20 mM) were ineffective in inhibiting the noradrenaline output induced by the reintroduction of Ca after exposure to ouabain, unless they were added to the medium before ouabain (Nakazato, Ito & Ohga, 1977). Taken together, the present results suggest that depolarization-induced Ca influx may not be the primary mechanism of the Ca-induced noradrenaline output, although it could contribute to the response.

An interesting result was that in the presence of a low concentration of Na, the response induced by reintroduction of Ca after exposure to ouabain was potentiated by the addition of Li. The mechanism is unknown; increased depolarization of the nerve endings could play a part, but it is also possible that Na could become more effective in producing the action in the presence of Li or that Li could be converted into an effective substitute for Na in the presence of Na.

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