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THE BINDING OF TRITIATED OUABAIN TO MAMMALIAN NON-MYELINATED NERVE FIBRES

BY D. LANDOWNE AND J. M. RITCHIE

From the Department of Pharmacology, Yale University School of Medicine, 333 Cedar St, New Haven, Connecticut, 06510, U.S.A.

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SUMMARY

1. A study has been made of the binding of radioactively labelled ouabain by desheathed rabbit vagus nerves, which consist mainly of non-myelinated fibres. The corresponding inhibition of the electrogenic sodium pump was also measured.

2. By varying the ouabain concentration and the external potassium concentration two kinds of binding sites could be distinguished: a first site specifically associated with pumping and whose ability to bind ouabain is dependent on the external presence of potassium; and a second site not associated with pumping and unaffected by external potassium.

3. Just complete inhibition of the sodium pumping mechanism is associated with a specific binding of ouabain of about 4·3 p-mole/mg dry nerve.

4. This gives an upper limit for the density of sodium pumping sites of about 750 per square micron.

5. The turnover rate (i.e. (cation pumped)/(number of sites)) at 20° C is about 22 sec^{-1}.

INTRODUCTION

The bulk of the metabolism of nervous tissue is concerned with maintaining the ionic imbalance between the axoplasm and the external environment. The evidence is now strong that the mechanism involved, the sodium pump, is closely related to the sodium- and potassium-dependent ATPase that is found in membranes, one important piece of evidence being that cardiac glycosides specifically inhibit both the ATPase and the pump. The recent availability of radioactively labelled glycoside, by allowing the kinetics of uptake and binding of ouabain by tissues to be readily determined, has enabled us to characterize further the transport site. Hoffman & Ingram (1969), Hoffman (1969), and Ellory & Keynes (1969) have recently done this for human red cell ghosts, and Baker & Willis (1969) have done

this for a variety of other tissues. The present experiments examine the binding of labelled ouabain to mammalian non-myelinated nerve fibres. The results have revealed that the individual pumping sites in nerve are similar to those of red blood cells, but there are more of them per square centimetre of membrane.

METHODS

Cervical vagus nerves, obtained from rabbits killed by the injection of air into an ear vein, were desheathed under a dissecting microscope.

The Locke solution contained (mM): sodium isethionate, 160; calcium sulphate, 5·0; Tris(hydroxymethyl)aminomethane (as the sulphate, pH 7·2), 2·5; dextrose, 5. Potassium replaced equimolar amounts of part of the sodium where indicated in the text. The temperature of the experiments was about 20° C.

Ouabain binding. Several desheathed nerves were cut in half, each half weighing about 10 mg. The preparations were then allowed to equilibrate for an hour in Locke solutions containing different amounts of potassium. At the end of the hour each preparation was transferred to similar solution that also contained ouabain. These solutions contained enough tritiated ouabain (obtained from New England Nuclear; specific activity, 1–4 mc/m-mole) to bring the radioactivity of 1 ml of solution to about 10^6 counts/min. After being mechanically agitated in this solution, the nerves were removed, washed several times in the corresponding ouabain-free equilibrating solutions (for a total period of 30 min) and dried in an oven for 1 hr at 60° C. They were then weighed dry, dissolved in a Hyamine (Packard) solution (to which some water had been added to facilitate hydrolysis of the nerve protein), added to Bray's solution (Bray, 1960), and the radioactivity in each nerve counted in a liquid scintillation counter; each sample was counted for 4000–10,000 counts. The radioactivity was expressed as p-mole ouabain per mg dry weight of nerve.

The tritiated ouabain was used only as a marker, the bulk of the ouabain in the solution in which the nerves were equilibrated being standard, non-radioactive, drug (Ouabain, U.S.P., supplied by Mann Research Laboratories). A tenfold variation in the specific activity of the radioactive equilibrating solution produced no detectable change in the calculated ouabain binding per gram of nerve. It seems unlikely, therefore, that any impurities in the ouabain may have vitiated the results.

Sodium pumping. The amplitude of the post-tetanic hyperpolarization in chloride-free Locke solution was taken as the index of the activity of the sodium pump (Rang & Ritchie, 1968*a*). The desheathed nerve was mounted in a sucrose-gap and the potentials measured as described previously (Rang & Ritchie, 1968*a*).

RESULTS

Binding of ouabain

Fig. 1 shows the time course of binding the ouabain by nerves bathed in a Locke solution containing 2·8 mM potassium and 20 μM glycoside. Binding was rapid in the first 20 min, and subsequently became much slower. Fig. 2 shows how the binding by preparations exposed for 20 min to ouabain depended on the concentration of the glycoside. Clearly, the greater the glycoside concentration the greater was the binding. At higher concentrations the points seem well fitted by a straight line whose slope is

0·085 p-mole.(mg dry weight)$^{-1}$.(μM ouabain)$^{-1}$. This line does not go through the origin, but intercepts the ordinate axis at a value of about 2·0 p-mole/mg dry.

Table 1 (column *a*) shows how the binding of ouabain in preparations exposed to the glycoside in a concentration of 20 μM depends on the external potassium concentration; the greater the external potassium concentration, the less is the binding, as would be expected from the known properties of the ouabain binding site (see Hoffman, 1969).

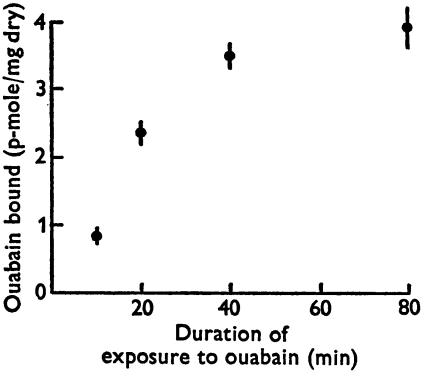

Fig. 1. The binding of ouabain by the rabbit desheathed vagus nerve. The preparations were exposed to 20 μM ouabain (potassium, 2·8 mM) for varying periods of time. The bars represent twice the S.E.

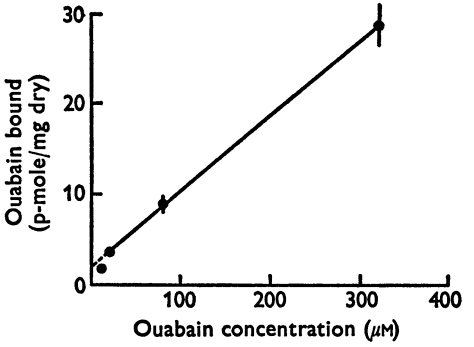

Fig. 2. The binding of ouabain, in different concentrations, by the rabbit desheathed vagus nerve. The time of exposure to ouabain was 20 min. The potassium concentration was 2·8 mM. The bars represent twice the S.E.: sometimes they are smaller than the symbols.

If there were a fixed number of sites that pump sodium and bind ouabain the curve for ouabain binding of Fig. 2 might be expected to flatten at an external concentration of ouabain of about 20 μM because (Table 1) this concentration produces almost complete block of the pumping mechanism. The apparently contradictory finding in the experiments of Fig. 2 could be explained by postulating two different kinds of sites that bind ouabain:

first, specific receptor sites associated with pumping that are saturated by low concentrations of ouabain; and secondly, non-specific ouabain binding sites that are not saturated by concentrations of ouabain much more than sufficient to inhibit pumping. Indeed, the linear portion of Fig. 2 suggests that non-specific binding occurs to the extent of 0·085 p-mole.(mg dry)$^{-1}$.(μM ouabain)$^{-1}$. This means that with an external concentration of ouabain of 20 μM, the non-specific binding would be about 1·7 p-mole/mg dry. The values for the specific binding in the experiments of Table 1, which were obtained by subtracting this amount from the values in column *a*, are given in column *b*.

TABLE 1. The relation between the binding of ouabain and the inhibition of the pumping of sodium in mammalian non-myelinated nerve fibres. The preparations were exposed to the glycoside for 20 min in a concentration of 20 μM. The numbers within brackets indicate the number of experiments

External potassium concentration (mM)	Binding of ouabain (p-mole/mg dry) (*a*)	(p-mole/mg dry) (*b*)	Inhibition of pump (Fractional decrease in amplitude of post-tetanic hyperpolarization) (*c*)	*b*/*c* (p-mole/mg) (*d*)
0	4·91 ± 0·18 (14)	3·21	0·78 ± 0·06 (4)	4·1
2·8	3·93 ± 0·05 (5)	2·23	0·71 ± 0·02 (7)	3·1
5·6	3·54 ± 0·16 (15)	1·84	0·33 ± 0·07 (4)	5·6
11·2	3·70 ± 0·47 (6)	2·00	—	—
22·4	3·11 ± 0·27 (3)	1·41	—	—
80·0	2·46 ± 0·17 (17)	0·76	0·21 ± 0·04 (5)	4·5
			Mean	4·3

An attempt was made to determine the specific binding more directly by adding 50 mM caesium to the Locke solution. But this procedure, although it has been shown to inhibit non-specific binding in red blood cells (Hoffman, 1969), had no effect on the total binding of ouabain in the present experiments.

That there are indeed two types of binding is also suggested by the finding in an experiment similar to that of Fig. 2 but carried out in Locke solution containing 80 mM potassium. In this experiment in high potassium solution the relation between ouabain uptake and concentration was again found to be linear between 40 and 320 μM ouabain, and its slope was 0·073 p-mole.(mg dry)$^{-1}$.(μM ouabain)$^{-1}$. The near equality of this slope with high potassium concentration and that obtained with a low potassium concentration (Fig. 2) shows that, unlike the binding obtained with low concentrations of ouabain, which is markedly potassium-sensitive (Table 1),

the binding with concentrations much larger than those required to inhibit the pump is hardly, if at all, sensitive.

Activity of the sodium pump

The values obtained for ouabain binding can readily be related to the sodium pumping mechanism in this preparation. For as Rang & Ritchie (1968*a*) have pointed out there is an electrogenic component of the sodium pump in these nerve fibres that provides a convenient and reliable index of the activity of the sodium extrusion mechanism. Desheathed vagus nerves, mounted in a sucrose-gap, were stimulated in a Locke solution for 5 sec at 30 shocks/sec, and the amplitude of the post-tetanic hyperpolarization that ensued was measured and taken to reflect the peak sodium efflux (see Rang & Ritchie, 1968*b*, for full account of experimental details). The preparations were then exposed for 20 min to ouabain under the same conditions as obtained for the ouabain binding experiments. They were then washed in ouabain-free solution for 30 min and the post-tetanic hyperpolarization again elicited; in the experiments with 80 mM potassium the post-tetanic responses were elicited in a Locke solution containing 2·8 mM potassium shortly before, and 30 min after, exposure to the ouabain in the high potassium Locke (in which the preparation was inexcitable). The reduction in size of the response after ouabain was taken as a measure of the inhibition of the sodium pumping mechanism and is shown in column *c*, Table 1. Thus, exposure to 20 μM ouabain in Locke solution containing 2·8 mM potassium reduced the size of the post-tetanic response to 0·29 of its original value, i.e. the pump was 71 % inhibited. As might be expected from the known, apparent, competition between ouabain and potassium, the inhibition was less when the preparation had been exposed to the ouabain in the presence of a high potassium concentration.

Site density

The total specific ouabain binding associated with just complete inhibition of the pump in non-myelinated fibres calculated from the degree of inhibition of the sodium pump given in column *c* and the corresponding specific uptake given in column *b* is given in column *d* of Table 1. Clearly, there is reasonably good agreement between the values calculated from the experiments at different potassium concentrations; and it seems that, on the average, there are about 4·3 p-mole binding sites per mg dry nerve.

That the correction for non-specific binding made in Table 1 is probably valid, is suggested by the results obtained with a lower concentration of glycoside. Eight preparations were exposed for 20 min to 10 μM ouabain (in 2·8 mM potassium) and were found to bind $1{\cdot}78 \pm 0{\cdot}13$ p-mole/mg dry. Application of the same correction as was used above suggests that there

is a non-specific binding of 0·85 p-mole/mg dry wt., the remaining, supposedly specifically bound, glycoside being 0·93 p-mole/mg dry wt. This binding was found to be associated with an inhibition of the post-tetanic hyperpolarization of $0{\cdot}26 \pm 0{\cdot}02$ (five experiments). The binding for total inhibition of the pump is thus 3·6 p-mole/mg dry, which is close to the values in Table 1 obtained with the higher ouabain concentration.

DISCUSSION

According to Keynes & Ritchie (1965) the amount of nerve membrane in mammalian C fibres is 6 cm^2/mg wet. A value for the (dry weight)/(wet weight) ratio of 0·178 (Keynes & Ritchie, 1965) gives a membrane area of 34 cm^2/mg dry. The concentration of specific binding sites (and hence presumably of sodium pumping sites) of 4·3 p-mole/mg dry obtained above (Table 1) thus gives an upper limit to the number of sites per square micron of just over 750. The density of sites on mammalian non-myelinated fibres is thus quite different from that in red blood cells where there is only about 1 site per square micron. This difference may well be related to the difference in the requirements for cation pumping in the two tissues. It will clearly be of interest to compare the value for the number of pumping sites in mammalian non-myelinated fibres with those obtained in other non-myelinated nerves, particularly the squid giant axon where Moore, Narahashi & Shaw (1967) have suggested, on the basis of experiments with tetrodotoxin, that the number of sodium permeability channels is fewer than thirteen per square micron.

The mammalian fibres, although differing from red blood cells in respect of the density of ouabain binding sites, do, however, resemble many of the tissues studied by Baker & Willis (1969), who found in cultured HeLa cells, for example, that there are about 500 ouabain binding sites per square micron. Furthermore, the density of sites in mammalian non-myelinated fibres is the same order of size as that in guinea-pig brain, which contains 14×10^{11} binding sites per mg wet (Baker & Willis, 1969); for the value obtained in the present experiments of 4·3 p-mole/mg dry corresponds with a value of $4{\cdot}6 \times 10^{11}$ molecules/mg wet (the (dry weight)/(wet weight) ratio being taken to be 0·178).

Turnover rate

How fast can each site work, in other words, what is the greatest number of sodium ions that can be extruded by an individual site in unit time? The answer to this question depends on knowing the sodium efflux, which unfortunately has not yet been measured directly in this nerve using radioactively labelled sodium (see Keynes & Ritchie, 1965). Because the

direct approach has failed, two indirect approaches have been used. In the first the total sodium movement associated with activity has been determined and the maximum efflux calculated; in the second approach, the site has been characterized in terms not of the sodium efflux but rather of the active potassium uptake, which can readily be estimated.

One simple assumption made by Rang & Ritchie (1968*a*) is that the amount of sodium extruded depends on the internal sodium concentration and equals $k[\mathrm{Na}]_i$ where $[\mathrm{Na}]_i$ is the internal sodium concentration; a more general assumption would have taken it to be $k[\mathrm{Na}]_i^n$. In this general case if a constant inward passive leak of sodium (L) is assumed, the net *amount* of sodium gained by the fibres is given by

$$\frac{\mathrm{dNa_i}}{dt} = -k[\mathrm{Na}]_i^n + L. \tag{1}$$

Dividing both sides by the fibre volume (V, in l.) this gives

$$\frac{\mathrm{d}[\mathrm{Na}]_i}{dt} = \frac{-k}{V}[\mathrm{Na}]_i^n + \frac{L}{V}.$$

For small changes $\delta[\mathrm{Na}]_i$ in the internal sodium concentration this equation becomes

$$\frac{\mathrm{d}}{dt}(\delta[\mathrm{Na}]_i) = \frac{kn}{V}[\mathrm{Na}]_i^{n-1}.\delta[\mathrm{Na}]_i$$

or, solving for $\delta[\mathrm{Na}]_i$,

$$(\delta[\mathrm{Na}]_i)_t = (\delta[\mathrm{Na}]_i)_0 \exp\left(\frac{-kn[\mathrm{Na}]_i^{n-1}.t}{V}\right), \tag{2}$$

where $(\delta[\mathrm{Na}]_i)_t$ represents the increment of the internal sodium concentration above its steady-state value at rest at any time t following a sudden small increment $(\delta[\mathrm{Na}]_i)_0$ as a result of electrical stimulation. Equation (2) means that recovery from a period of repetitive activity would be exponential (with a rate constant which is equal to $kn[\mathrm{Na}]_i^{n-1}/V$) as indeed Rang & Ritchie (1968*a*) have shown experimentally.

Sodium efflux. The total increase in internal sodium content after 900 impulses in nerves poisoned with ouabain is about 21 μ-mole/g dry(Rang & Ritchie, 1968*b*); the increase after stimulation for 5 sec at 30/sec (i.e. 150 impulses), which causes a nearly maximal post-tetanic hyperpolarization, and presumably a nearly maximal stimulation of the sodium pump, would thus be about 3·5 μ-mole/g dry. Since the rate constant of recovery (in Locke solution containing 2·8 mM potassium) is 0·01 sec (den Hertog & Ritchie, 1969) the maximal rate at which sodium is extruded at the beginning of the recovery period is 35 p-mole/mg dry.sec^{-1}. This, taken in conjunction with a site density of 4·3 p-mole/mg, means that the turnover

rate of the site (in terms of sodium ions) is 8 sec^{-1}. There is, however, a considerable degree of uncertainty about the value of 21 μ-mole/g dry for the sodium influx associated with 900 impulses (the S.E. of eight estimates was 10 μ-mole/g dry, Rang & Ritchie, 1968*b*) so it is probably better to ignore this calculation and rely on the potassium calculation below.

Potassium uptake. Stimulation of mammalian non-myelinated fibres for 5 sec at 30 shocks/sec causes a net loss of potassium into potassium-free, isethionate Locke solution of $150 \times 0{\cdot}175 \times 0{\cdot}000211$ μ-mole/mg dry (Rang & Ritchie, 1968*b*). An additional amount is also released, but is quickly reabsorbed; for in four of five experiments ouabain was found to increase the potassium loss by 46 ± 4 %; in the fifth experiment the potassium loss was also increased but by an abnormally large amount, 171 % (Rang & Ritchie, 1968*b*). The total deficit of potassium in the axoplasm immediately after stimulation in potassium-free solution is thus 8·1 n-mole/mg dry. The same amount will be lost into Locke solution containing up to 5·6 mM potassium; for although the rate constant for potassium loss on stimulation increases by 36 % when the external potassium is reduced from 5·6 mM to zero (Keynes & Ritchie, 1965), the internal steady-state concentration of potassium falls by a corresponding amount (Rang & Ritchie, 1968*b*), so that the product remains almost constant. This potassium must, of course, be recaptured during recovery, presumably with the same rate constant as for sodium extrusion. This means that in 2·8 mM potassium, where the rate constant is 0·01 sec^{-1}, the maximum uptake of potassium immediately after stimulation is 81 p-mole/mg dry.sec. In addition there is an ouabain-sensitive active resting influx of 14 p-mole/mg dry.sec (152 p-mole.mg wet.min^{-1}, Table 10, Keynes & Ritchie, 1965; dry weight/wet weight = 0·178, p. 356, Keynes & Ritchie, 1965). The total peak influx of potassium thus seems to be 95 p-mole/mg dry.sec. This means that with a site density of 4·3 p-mole/mg dry the turnover rate (in terms of potassium ions) is 22 sec^{-1} at 20° C. The value of about 30 sec^{-1} reported earlier (Landowne & Ritchie, 1969) resulted from taking only the values in Table 1 obtained in 2·8 mM potassium solution.

Comparison with other tissues. Ellory & Keynes (1969) obtained a value of 175 sec^{-1} for the ATPase catalytic centre activity in human red blood cells, i.e. the ratio (ATPase activity)/(ouabain binding). Direct measurement of the ratio (sodium efflux)/(ouabain binding) in red blood cells at 37° C gives a value of 100 sec^{-1} (Hoffman & Ingram, 1969) to 150 sec^{-1} (Hoffman, 1969). The value of this latter ratio obtained in the experiments in mammalian non-myelinated nerve fibres is 22 sec^{-1} at 20° C. Part of the reason why this is smaller than the corresponding values for red blood cells may be that the present experimental conditions (intact nerve bathed in Locke solution containing 2·8 mM potassium) may not have been optimal

for pumping activity. Furthermore, if the value in nerve is to be compared with those for red blood cells, it ought probably to be at least doubled because of the difference in temperature (20° C for nerve; 37° for red blood cells); for den Hertog & Ritchie (1969) have shown that the rate constant of decay of the post-tetanic response (and hence of the extrusion of sodium from the fibres) at 37° C is twice that at 20° C. When this is done it is clear that the ratio (efflux of sodium)/(binding sites) in mammalian C fibres is somewhat smaller than, but is nevertheless the same order of size as, that obtained for red blood cells. Thus it seems that the sodium pumping sites in the different tissues do not differ dramatically from each other in turnover rate.

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