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ACTIVE Na–K TRANSPORT AND THE RATE OF OUABAIN BINDING. THE EFFECT OF INSULIN AND OTHER STIMULI ON SKELETAL MUSCLE AND ADIPOCYTES

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SUMMARY

1. The effect of stimulation or inhibition of active Na–K transport on [^{3}H]ouabain binding has been investigated in isolated soleus muscles and adipocytes.

2. In rat soleus muscle, the ouabain-sensitive component of ^{42}K influx was stimulated by insulin (100 m-u/ml.), adrenaline (6×10^{-6} M), and by pre-incubation with veratrine (10^{-5} M) or in a K-free buffer. In all of these instances, the rate of ouabain binding was increased by 41–113%. Conversely, pre-treatment with tetracaine (0·2 mM) decreased the ^{42}K-influx and diminished the rate of [^{3}H]ouabain binding by 36%.

3. Neither insulin, adrenaline or tetracaine produced any detectable change in the total number of ouabain-binding sites (as measured under equilibrium conditions) in rat soleus muscle.

4. In mouse and guinea-pig soleus muscle and in fat cells isolated from rats, insulin also increased the rate of [^{3}H]ouabain binding without producing any significant change in the total number of ouabain-binding sites.

5. Both in soleus muscle and the epididymal fat pad of the rat, there was a linear correlation between ^{42}K influx and the initial rate of [^{3}H]-ouabain binding.

6. It is concluded that the rate of ouabain binding is determined significantly by the rate of active Na–K transport, but within the time intervals studied (4–6 hr) stimulation or inhibition of the Na pump does not lead to any appreciable change in the total number of Na pumps. It seems unlikely that the stimulation of active Na–K transport by insulin or adrenaline is due to unmasking or *de novo* synthesis of Na pumps.

INTRODUCTION

Cardiac glycosides specifically inhibit and are bound to the Na+K-activated ATPase with high affinity (see review by Schwartz, Lindenmayer & Allen, 1975). A simple stoicheiometric relationship between ouabain binding and the number of Na pumps seems to exist (Barnett, 1970; Hansen, 1971; Jørgensen, 1974). The rate of ouabain binding and the apparent affinity are markedly influenced by the ionic milieu and the temperature. The binding of ouabain is a remarkably slow process, and even in a K-free medium at 37° C, a steady-state binding level may only be achieved after hours of incubation (Tobin & Sen, 1970; Schön, Schönfeld & Repke, 1970; Hansen, 1971; Hansen & Skou, 1973). Determination of the number of Na pumps in fragmented membrane preparations thus requires optimum conditions for ouabain binding and evidence of saturation. Also in intact cells, the binding of ouabain to the outer surface of the plasma membrane may proceed for several hours before equilibrium is reached (Baker & Willis, 1972; Clausen & Hansen, 1974; Joiner & Lauf, 1975; Bodemann &Hoffmann, 1976).

Little is known about how the binding of ouabain to cells is affected by acute or long-term changes in the rate of active Na–K transport. Stimulation of active Na–K transport may either be the result of increased rate of pumping in existing Na pumps (Joiner & Lauf, 1975), *de novo* synthesis of pumps (Jørgensen, 1972), or unmasking of latent pumps (Erlij & Grinstein, 1976). In order to identify the mechanism of action of a given stimulus, it is important to distinguish between these possibilities. In view of the fact that the rate of ouabain binding may show wide fluctuations without any variation in the total number of binding sites, it is essential that measurements are performed under conditions where the entire population of sites has been exposed to labelled ouabain for sufficient time to ensure saturation.

The purpose of the present investigation has been to determine the effect of stimulation or inhibition of active Na–K transport on the rate of ouabain-binding as well as the total number of binding sites. Most of the experiments have been performed with soleus muscles and adipocytes of the rat, but for comparison, a few series were done with muscles obtained from other species which are known to be more susceptible to the action of cardiac glycosides (Lendle, 1935).

METHODS

All experiments were performed with tissues or cells obtained immediately after decapitation from fed animals. Soleus muscles were prepared from Wistar rats (60–70 g), mice of the NMRI strain (25–35 g) or guinea-pigs (70–120 g) using a technique described elsewhere (Kohn & Clausen, 1971). As in earlier experiments (Clausen & Hansen, 1974), the Achilles tendon was left attached to the muscles during incubation. However, since the number of ouabain-binding sites per gram wet weight in this tissue amounted to a few per cent of that found in the muscle cells, the tendon was in most instances resected before weighing and homogenization. The amount of tissue thus removed corresponds to around 12 % of the total mass of tissue incubated. Consequently, the figures given for the total number of ouabain-binding sites are around 14 % higher than those previously published for rat soleus muscles.

Whole epididymal fat pads and free fat cells were prepared from fed Wistar rats (100–120 g) as in earlier studies (Clausen, Gliemann, Vinten & Kohn, 1970). Krebs–Ringer bicarbonate buffer gassed with a mixture of 95 % O_2 and 5 % CO_2 was used as the standard incubation medium. Apart from certain wash-out procedures, all experiments with soleus muscles were performed at 30° C. The experiments with fat cells and fat pads took place at 37° C.

The procedures for the measurement of Na–K contents, ^{42}K-influx and [^{3}H]-ouabain binding were essentially the same as those described in earlier reports (Clausen & Hansen, 1974; Clausen & Kohn, 1977). Since it was a major purpose of the present study to assess the rate of [^{3}H]ouabain binding, the incubation in [^{3}H]ouabain-containing media was often combined with a subsequent 60–120 min wash in ice-cold unlabelled medium. This procedure was based on the experience that [^{3}H]ouabain already bound to isolated Na + K-ATPase or to soleus muscles remains attached even during long periods of wash-out at 0° C (Tobin & Sen, 1970; Akera & Brody, 1971; Clausen & Hansen, 1974). Control experiments showed that during wash-out at 0° C, the maximum loss of the [^{3}H]ouabain already bound to the cells amounted to 4 % per hour. The disappearance of the [^{3}H]ouabain contained in the extracellular space was assessed by loading the muscles for 15 min at 30° C in buffer containing [^{3}H]ouabain at a concentration (10^{-3} M) far above the saturation level and then following the wash-out at 0° C. After 120 min of wash-out, only $3{\cdot}2 \pm 0{\cdot}3$ % of the amount of ^{3}H-activity taken up during the loading period was left in the tissue. Parallel experiments with [U-^{14}C]sucrose showed that this extracellular marker was retained to the same extent ($2{\cdot}9 \pm 0{\cdot}1$ %). After loading for 240 min with 10^{-3} M [^{3}H]ouabain somewhat more was left after 120 min of wash-out ($12{\cdot}1 \pm 0{\cdot}8$ %). In experiments performed with [^{3}H]ouabain at concentrations around 10^{-6} M or below, this retention of extracellular [^{3}H]ouabain only corresponded to a minute fraction of the amount bound to the cells. However, at 5×10^{-6} M, the retention of [^{3}H]ouabain in the extracellular compartment corresponded to 12–20 % of the total, and in these cases, it was found appropriate to correct the data accordingly (see Fig. 1). Experimental details are given in the legends to Figures and Tables.

Chemicals, hormones and isotopes. Except for the Triton-X-114 and xylene used for the scintillation mixture, all chemicals were of analytical grade. Crystalline ouabain was purchased from the Merck Co., Darmstadt, West Germany, and veratrine and bovine serum albumin (fraction V) from the Sigma Co., St Louis, U.S.A. Insulin (mono-component pork, Lot No MC-S 823081, 25 i.u./mg) were gifts of the Novo Research Laboratories, Copenhagen. Adrenaline (as the bitartrate salt) was a product of Rhone-Poulenc, Paris, France. [^{3}H]Ouabain (12 c/m-mole) was obtained from New England Nuclear, Frankfurt, West Germany; [U-^{14}C]sucrose

(0·4 c/m-mole) from the Radiochemical Centre, Amersham, and ^{42}K (0·1 c/m-mole) from the Danish Atomic Energy Commission, Isotope Laboratory, Risø, Denmark. The purity of the [^{3}H]ouabain used was found to vary from 83 to 93%, and this was taken into account in the calculations of specific binding of the glycoside (Hansen & Skou, 1973).

RESULTS

Even in a K-free buffer, the binding of [^{3}H]ouabain to rat soleus muscles proceeds very slowly, and at concentrations around 10^{-7} M, equilibrium is only achieved after 4 hr incubation (Clausen & Hansen, 1974). Potassium decelerates the binding, and in the standard medium, it is only possible

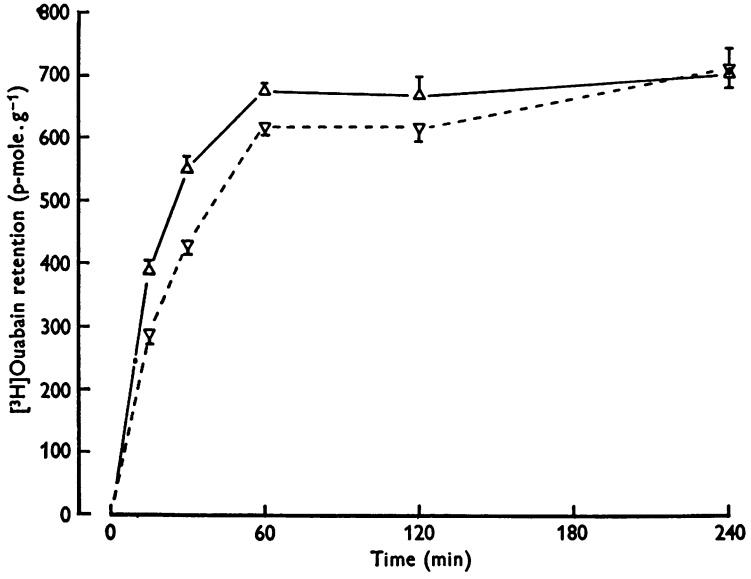

Fig. 1. Effect of insulin on the time course of [^{3}H]ouabain binding. Groups of six rat soleus muscles were incubated at 30° C in 5 ml. standard medium containing 5×10^{-6} M [^{3}H]ouabain (2 μc/ml.) with or without insulin (100 m-u./ml.). After incubation, the muscles were washed 4 × 30 min in four tubes containing 5 ml. ice-cold medium without additions. They were then blotted, the tendons resected, and after weighing and homogenization in 2 ml. 5% trichloroacetic acid, 0·5 ml. clear supernatant obtained by centrifugation was taken for counting. On the basis of the specific activity of the incubation medium, the [^{3}H]ouabain retained was calculated as relative uptake (ml./g wet wt.). This value was corrected for incomplete wash-out of the extracellular space by deducting the corresponding value obtained in experiments where the loading had taken place at a concentration of 10^{-3} M [^{3}H]ouabain (see Methods). From the differences between these two relative values, the amount of ouabain bound was expressed as p-mole/g tissue wet weight. Each point represents the mean of six to twelve observations with bars denoting S.E. of the mean. Controls, ▽; insulin, △.

to obtain saturation of the binding sites either by using inordinately long incubation intervals or by increasing the concentration of the glycoside. However, at 5×10^{-6} M, the amount of [^{3}H]ouabain that may maximally become bound to the cells corresponds to around half the amount contained in the extracellular space, and it is difficult to detect changes in the binding with satisfactory precision. Therefore, the [^{3}H]ouabain present in the extracellular space was washed out in ice-cold buffer, and the amount of ^{3}H-activity retained after the wash taken as [^{3}H]ouabain still attached to the plasma membrane (see methods). Fig. 1 shows the time course of [^{3}H]ouabain binding as assessed with this procedure. With 5×10^{-6} M equilibrium is only reached after at least 2 hr of incubation. The amount of [^{3}H]ouabain retained after 4 hr (717 ± 27 p-mole per gram tissue wet weight) is slightly lower than the value of 820 p-mole/g obtained by measuring the total amount taken up and correcting for the amount present in the space available to [U-^{14}C]sucrose as well as the contribution of the tendons (see Clausen & Hansen, 1974).

When the active Na–K transport is stimulated by insulin (100 m-u/ml.) the uptake of [^{3}H]ouabain proceeds at a faster rate, but the final level is not different from that of the controls. This is also evident from Fig. 2, which shows the effect of insulin on the amount of [^{3}H]ouabain bound after equilibration at various concentrations of ouabain in a K-free incubation medium. In the Scatchard plot the lines nearly coincide, and the ordinate intercepts, which according to the binding model (Hansen, 1971) are equal to the total number of binding sites, do not differ significantly. It should be noted that in these experiments, the tendons were left attached to the muscles after the incubation. Since this part of the tissue binds relatively small amounts of ouabain, the values in Fig. 2 are somewhat smaller than those given in Fig. 1. When the intercept value of Fig. 2 is corrected for the presence of the tendons, a value of 645 ± 22 p-mole/g may be calculated. This is in good agreement with the equilibrium level in Fig. 1.

These data indicated that insulin increases the rate of ouabain binding without producing any detectable change in the total number of binding sites. In order to determine whether this pattern is specific for insulin or simply related to stimulation of the active Na–K transport, several attempts were made to modify the rate of this process and look for possible changes in the binding of ouabain. In the following, changes in ^{42}K influx were compared with the rate of [^{3}H]ouabain binding measured as the amount of labelled glycoside taken up within 15 min of incubation at 10^{-6} M and retained following wash in the cold.

In earlier studies, it was shown that in rat soleus muscle, the ouabain-suppressible component of ^{42}K uptake may be stimulated by insulin,

pre-incubation in K-free buffer (Clausen & Kohn, 1977) or adrenaline (Clausen & Flatman, 1977). From Fig. 3 it can be seen that all of these factors increase the rate of [^{3}H]ouabain uptake by 41–69 %. The rate of active Na-K transport depends on the intracellular Na concentration $[Na]_i$), and this may be modified by altering the rate of passive Na influx. Veratrine, which is known to increase the permeability of the plasma membrane

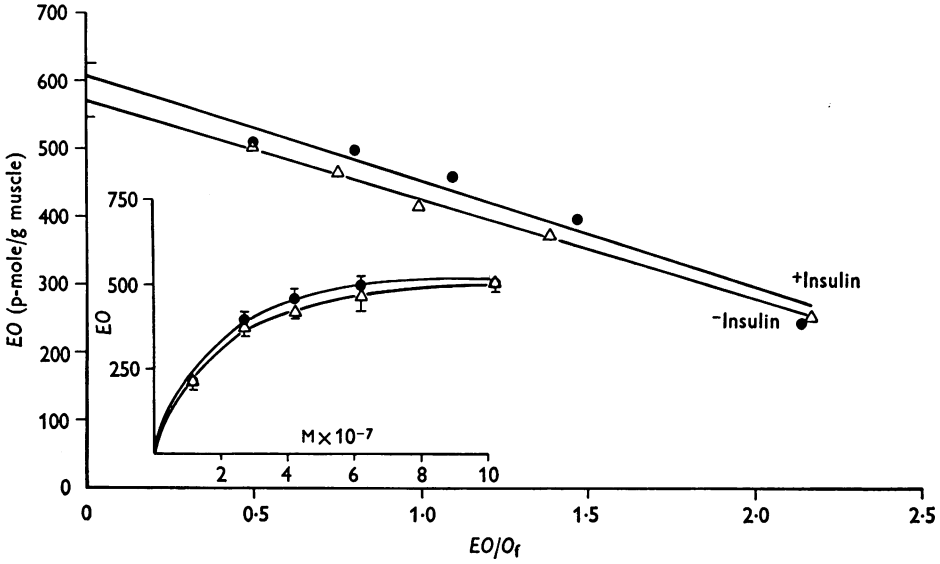

Fig. 2. Plot of bound (*EO*) versus bound/free (EO/O_f) [^{3}H]ouabain in rat soleus muscles at equilibrium of binding. Groups of muscles were incubated for 240 min (except the lowest concentration, for 360 min) in K-free and glucose-free standard medium with or without insulin (100 m-u./ml.). After incubation, the muscles were washed 3 x 20 min in ice-cold medium and the amount of [^{3}H]ouabain retained determined as described in the legend to Fig. 1. The inset shows *EO* as a function of the concentration of free ouabain (O_f), each point representing the mean of five observations with bars denoting S.E. of the mean. The regression lines of the Scatchard plot have been constructed using the method of least squares. Controls, △; insulin, ●.

to Na (Sperelakis & Pappanao, 1969), was found to augment $[Na]_i$ from 18·9 to 25·9 mM (Table 1). From Fig. 3 it appears that this is associated with a rise in ^{42}K influx and the rate of [^{3}H]ouabain binding is more than doubled. Conversely, pre-incubation with tetracaine (0·2 mM) decreased the intracellular Na concentration to 12·2 mM and the rate of [^{3}H]ouabain binding was diminished by 36 %. As in the experiments with insulin, neither adrenaline nor tetracaine produced any significant change in the amount of [^{3}H]ouabain bound after equilibration at a high concentration of the

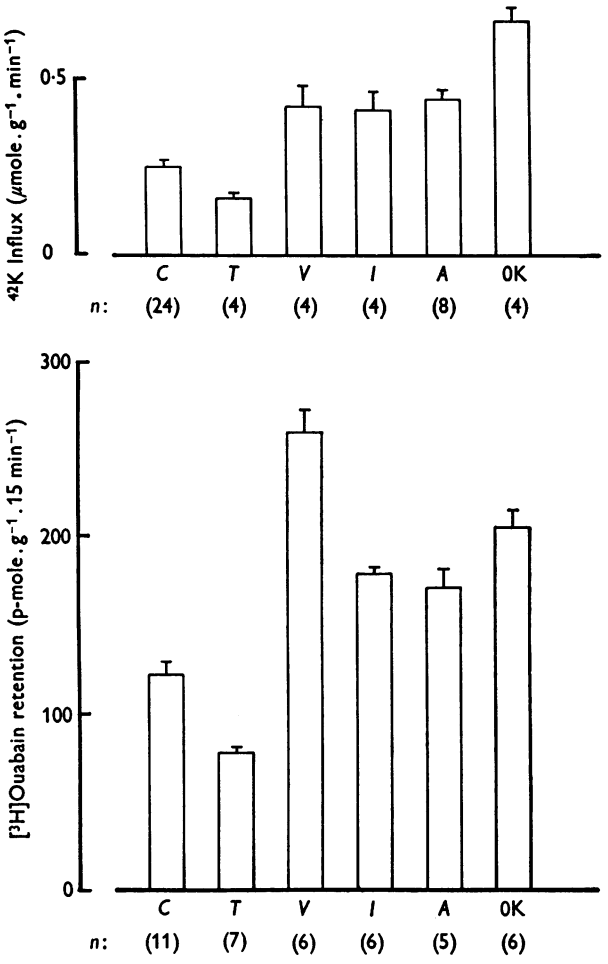

Fig. 3. Comparison between the rates of ^{42}K influx and [^{3}H]ouabain binding in rat soleus muscle. For the determination of the ouabain-suppressible component of ^{42}K influx muscles were pre-incubated for 15 min with or without ouabain (10^{-3} M). They were then transferred into tubes containing 3 ml. buffer with ^{42}K (0·5 μc/ml.) with or without ouabain (10^{-3} M). After 10 min of incubation they were blotted, weighed and the amount of ^{42}K activity taken up determined. On the basis of the specific activity of the incubation medium, this was converted to μmole/g tissue wet weight per minute. The rate of ouabain binding was assessed by incubating muscles for 15 min in standard medium containing 10^{-6} M [^{3}H]ouabain (2 μc/ml.) and determining the amount of ^{3}H-activity retained following 4 × 30 min of wash-out in the cold. The Na content of the muscles was modified by pre-incubating for 75–120 min with either: tetracaine (*T*, 0·2 mM); veratrine (*V*, 10^{-5} M); or K-free buffer (0K). Insulin (*I* 100 m-u/ml.) or adrenaline (*A*, 6×10^{-6} M) when added, were present during the incubation with radio-isotopes only. Each column represents the mean of observations with bars denoting S.E. of the mean. The differences between the controls and each of the experimental values was statistically significant ($P < 0{\cdot}005$).

glycoside. This indicates that in spite of the changes in the rate of binding, there is no change in the total number of ouabain-binding sites (Table 2).

In order to assess whether the increased rate of [^{3}H]ouabain binding induced by adrenaline could possibly be due to a decrease in the $[Na]_i$ *per se*, muscles were pretreated for 75 min with the hormone (6×10^{-6} M) to reduce $[Na]_i$. Under these conditions, $[Na]_i$ was $6{\cdot}7 \pm 3{\cdot}3$ mM ($n = 14$),

TABLE 1. Intracellular Na concentration ($[Na]_i$) and the ratio between the rate of [^{3}H]ouabain binding and ^{42}K influx in rat soleus muscle. Experimental conditions as described in the legend to Fig. 3. For each experimental condition, the ratio between the rate of [^{3}H]ouabain binding (expressed as p-mole.g^{-1}.min^{-1}) and the ouabain suppressible component of ^{42}K influx (expressed in the same units) was calculated. The amount of Na in the space not available to [U-^{14}C]sucrose was determined by flame photometry (see Kohn & Clausen, 1971) and expressed as mM ± S.E. of the mean with the number of observations in parenthesis. In the experiments with insulin or adrenaline it is assumed that $[Na]_i$ at the moment the hormones are added is the same as in the controls.

Experimental conditions	$[Na]_i$	Rate of [^{3}H]-ouabain binding / Ouabain-suppressible ^{42}K influx
Control	$18{\cdot}9 \pm 0{\cdot}8$ (6)	$3{\cdot}3 \times 10^{-5}$
75 min pre-incubation with tetracaine (0·2 mM)	$12{\cdot}2 \pm 1{\cdot}0$ (6)	$3{\cdot}3 \times 10^{-5}$
75 min pre-incubation with veratrine (10^{-5} M)	$25{\cdot}9 \pm 0{\cdot}7$ (6)	$4{\cdot}2 \times 10^{-5}$
120 min pre-incubation with K-free medium	$32{\cdot}0 \pm 0{\cdot}8$ (6)	$2{\cdot}1 \times 10^{-5}$
Insulin (100 m-u./ml.)	$18{\cdot}9 \pm 0{\cdot}8$ (6)	$2{\cdot}9 \times 10^{-5}$
Adrenaline (6×10^{-6} M)	$18{\cdot}9 \pm 0{\cdot}8$ (6)	$2{\cdot}6 \times 10^{-5}$
75 min pre-incubation with adrenaline (6×10^{-6} M)	$6{\cdot}7 \pm 3{\cdot}3$ (6)	$3{\cdot}8 \times 10^{-5}$

TABLE 2. Effect of insulin, adrenaline and tetracaine on steady-state binding of [^{3}H]ouabain to rat soleus muscles. Experimental conditions as described in the legend to Fig. 1. All muscles were incubated with [^{3}H]ouabain (5×10^{-6} M) for 4 hr and the results are given as p-mole/g tissue wet weight ± S.E. of the mean. The number of observations are given in parenthesis.

	[^{3}H]Ouabain retained after wash-out in the cold (p-mole/g tissue wet weight)	
Control	717 ± 27 (18)	
Insulin (100 m-u./ml.)	704 ± 24 (12)	$P > 0{\cdot}70$
Adrenaline (6×10^{-6} M)	732 ± 47 (6)	$P > 0{\cdot}90$
Tetracaine (0·2 mM)	707 ± 35 (6)	$P > 0{\cdot}60$

the ouabain-suppressible component of ^{42}K influx 0·276 μmole/min per gram tissue wet weight, and the rate of [^{3}H]ouabain uptake was 156 ± 5 p-mole/g wet weight per 15 minutes (as compared to a control value of

141 ± 9 p-mole/g wet weight per 15 minutes; six versus six observations, $P > 0{\cdot}10$). From this it seems reasonable to conclude that the effect of adrenaline on [^{3}H]ouabain binding is not caused by a decrease in $[Na]_i$. The rate of [H^3]ouabain binding seems to be related to the magnitude of the ouabain-suppressible component of ^{42}K influx rather than the cytoplasmic Na–K content. When the concentration of Na in the space not available to [U-^{14}C]sucrose was varied over a fivefold range, the ratio between the rate of [^{3}H]ouabain binding and ^{42}K influx showed considerably smaller variation (Table 1).

In the experiments performed with 10^{-6} M ouabain, up to 32 % of the total number of binding sites became occupied within the 15 min incubation periods used. Thus, the active Na–K transport is inhibited to some extent, and in order to determine whether this might be of significance for the acceleration of binding seen under stimulation of the Na pump, other experiments were performed with ouabain at a concentration of 10^{-8} M. Also under these conditions, where a maximum of 2 % of the total population of ouabain-binding sites were occupied, both insulin, adrenaline and pre-incubation with veratrine or in a K-free buffer produced a significant stimulation of the rate of [^{3}H]ouabain binding.

These observations indicated that the rate of ouabain binding is to a significant extent determined by the rate of active Na–K transport, but may also be further influenced by the particular conditions used to stimulate the Na pump. Therefore, it was of interest to determine the influence of a single condition applied so as to produce a graded stimulation of the Na pump. This was achieved by pre-incubating muscles for 60–180 min in K-free buffer, whereby $[Na]_i$ could be varied over a wide range. Since it was possible to measure the initial rate of [^{3}H]ouabain binding without occupying more than a few per cent of the sites, the ^{42}K influx was not inhibited to any significant extent and could be measured simultaneously. Hence, following the Na loading, both of these parameters were determined using a 15 min incubation period. It is clear that there is a rather good correlation between the rate of [^{3}H]ouabain uptake and the ouabain-suppressible component of ^{42}K influx (Fig. 4).

In all of the above experiments, the binding of ouabain was determined by measuring the amount of ^{3}H-activity retained by the tissue following wash-out in the cold. Other experiments showed that provided the concentration of ouabain was low enough to ensure a high relative uptake, it was possible to estimate the rate of [^{3}H]ouabain binding simply by measuring the total amount of isotopic ouabain taken up and correcting for the fraction contained in the extracellular space. (The latter is measured as the relative uptake at a concentration of ouabain (10^{-3} M) where only a negligible fraction of the ouabain taken up is bound to the

cells. The relative uptake of ouabain at this concentration was found to be almost identical to that of [U-^{14}C]sucrose (Clausen & Hansen, 1974). Using this procedure, it was found that in a K-free medium containing 10^{-7} M ouabain, insulin (100 m-u./ml.) increased the rate of ouabain uptake from 60 ± 4 to 90 ± 4 p-mole/g tissue wet weight per 30 minutes ($P < 0{\cdot}005$, four versus four observations).

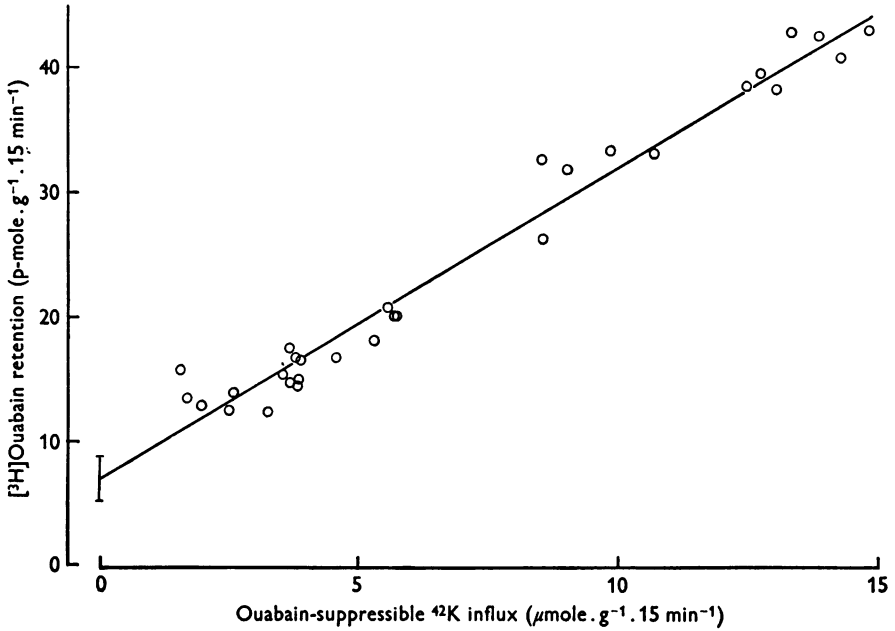

Fig. 4. The relationship between the rates of ^{42}K influx and [^{3}H]ouabain binding following graded Na loading. Groups of rat soleus muscles were loaded with Na by incubation for 60–180 min in K-free standard medium. They were then transferred into tubes containing ^{42}K (0·5 μc ml.) and 10^{-7} M [^{3}H]ouabain (0·3 μc ml.). After 15 min of incubation, they were washed 3×20 min in ice-cold medium, and the amount of ^{3}H-activity retained determined as described in the legend to Fig. 1. The amount of ^{42}K taken up was calculated by adding the amount retained to the amount released during the wash-out in the cold and correcting for the amount taken up into the extracellular space during the incubation with ^{42}K. The ouabain-suppressible component of ^{42}K-influx was calculated by deducting the ^{42}K-uptake measured in the presence of ouabain (10^{-3} M).

In order to assess the general significance of the effect of insulin on ouabain binding, some of the experiments described above were repeated using other tissues which are known to respond to the hormone. Adipose tissue and isolated fat cells are very sensitive to insulin, and due to their relatively high affinity to ouabain, equilibrium of binding can be achieved within a rather short incubation time in normal medium. Fig. 5 shows the

time course of [^{3}H]ouabain binding to isolated fat cells at two different concentrations of ouabain. At 10^{-8} M even after 2 hr of incubation equilibrium is not reached, but the rate of binding is clearly accelerated by insulin.

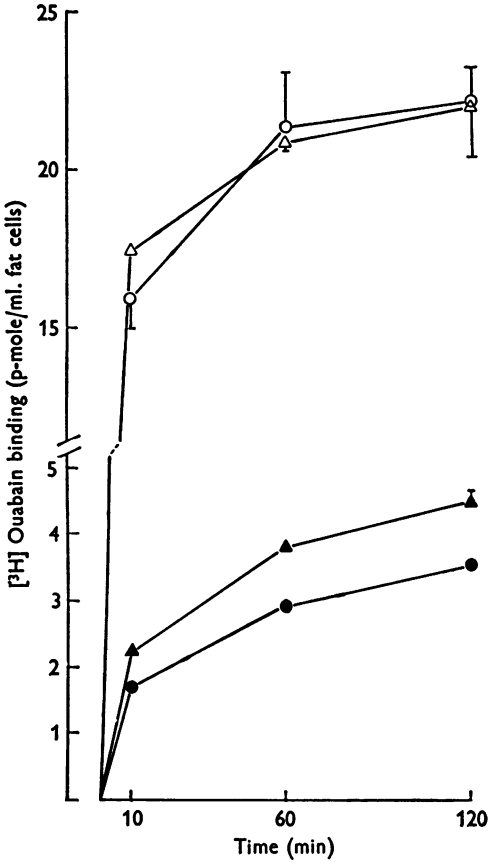

Fig. 5. Effect of insulin on the time course of [^{3}H]ouabain binding to adipocytes. Isolated fat cells were incubated in standard medium containing 1% dialysed bovine serum albumin, 5 mM D-glucose and 10^{-8} (●, ▲) or 10^{-7} M (○, △) [^{3}H]ouabain. At the time indicated, triplicate samples of the cells were separated from the incubation medium by centrifugation through oil (Clausen & Hansen, 1974). The [^{3}H]ouabain taken up was expressed as p-mole/ml. fat cells with bars denoting S.E. of the mean where this exceeds the size of the symbols. Controls, ○, ●; insulin (1 m-u/ml.), △, ▲.

At 10^{-7} M, the equilibrium binding level, which is nearly identical with the total number of binding sites previously determined (Clausen & Hansen, 1974), seems unaffected by insulin.

In whole epididymal fat pads, the K content may be diminished by

incubation in a K-free medium (Clausen, 1969). This was found to lead to a stimulation of ^{42}K uptake as measured during a subsequent incubation in standard medium with normal K-content. As in soleus muscles, this stimulation of the active Na–K transport was associated with an increase in the rate of [^{3}H]ouabain binding, and the correlation between these two parameters is statistically significant (Fig. 6).

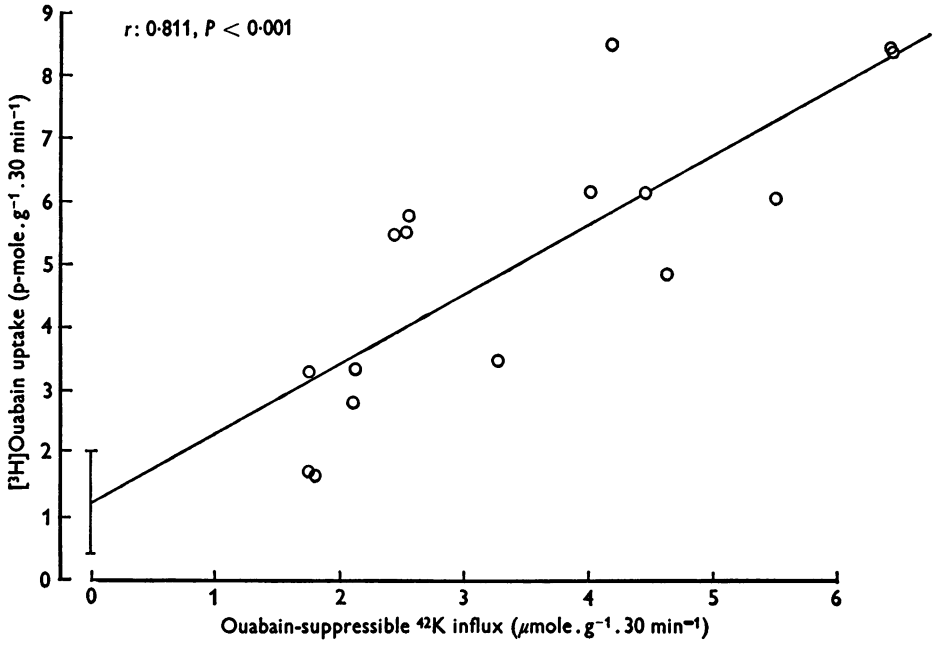

Fig. 6. The relationship between the rates of ^{42}K influx and [^{3}H]ouabain binding following graded Na loading in adipose tissue. Whole epididymal fat pads were loaded with Na by pre-incubation for 120 min in K-free standard medium. They were then transferred into plastic counting vials containing ^{42}K (0·5 μc ml.) and 10^{-8} M [^{3}H]ouabain (0·12 μc/ml.). After 30 min incubation at 37° C, the fat pads were blotted, weighed and homogenized in 2 ml. 5% trichloroacetic acid. The extract was centrifuged and 0·5 ml. clear interphase taken for determination of ^{3}H and ^{42}K activity. From parallel experiments performed with buffer containing 10^{-3} M ouabain, it was possible to correct for the amount of [^{3}H]ouabain taken up into the extracellular space as well as the ouabain-resistant component of ^{42}K influx. Each point represents one fat pad, and the regression line was constructed using the method of least squares.

Since it is often pointed out that the rat is less sensitive to ouabain than other animals, it seemed appropriate to repeat some of the experiments using tissues from other species. It is evident that in soleus muscles from both the mouse and the guinea-pig, insulin produces almost the same

increase in the rate of [^{3}H]ouabain binding (Table 3). Again, following longer incubation (120–180 min), the relative increase in binding is considerably diminished. It is interesting that the level reached after this interval of time is close to the equilibrium level measured in the soleus muscle of the rat (cf. Fig. 1 and Table 2).

TABLE 3. Effect of insulin on [^{3}H]binding to soleus muscles from mice and guinea-pigs. The muscles were prepared as described elsewhere (Kohn & Clausen, 1971) and incubated in standard medium containing [^{3}H]ouabain (10^{-6} to 5×10^{-6} M) for the intervals indicated. The amount of [^{3}H]ouabain retained following wash-out in the cold was determined as described in the legend to Fig. 1 and expressed as picomoles per gram tissue wet weight ± S.E. of the mean. The number of observations is given in parenthesis, and the significance of difference between controls and insulin-treated muscles given by P values.

	[^{3}H]Ouabain (p-mole/g wet wt.)			
Experimental conditions	Controls	Insulin (100 m-u./ml.)	% change	*P*
	Mice			
10^{-6} M ouabain, 15 min	224 ± 18 (4)	291 ± 20 (4)	+30	< 0·05
10^{-6} M ouabain, 30 min	348 ± 7 (4)	443 ± 18 (4)	+27	< 0·005
10^{-6} M ouabain, 180 min	583 ± 22 (4)	625 ± 34 (4)	+7	> 0·30
	Guinea-pigs			
5×10^{-6} M ouabain, 15 min	329 ± 19 (4)	418 ± 21 (4)	+27	< 0·025
5×10^{-6} M ouabain, 120 min	554 ± 7 (4)	593 ± 9 (4)	+7	< 0·02

DISCUSSION

The major purpose of the present study has been to establish possible relationships between the rate of active Na–K transport and the rate of ouabain binding to intact cells. We have tried to make a clear distinction between the experimental conditions giving the rate of ouabain binding and those allowing a determination of the total number of ouabain-binding sites. As in earlier studies (Clausen & Hansen, 1974) it was found that provided the concentration of ouabain is sufficiently high and the time of exposure long enough, it is possible to reach a steady state where no further increase in the binding of ouabain can be detected. From the data obtained under these equilibrium conditions, it was possible to draw a straight line in the Scatchard plot, giving the total number of sites at the ordinate intercept. The slope of the line gave an apparent ouabain affinity ($1{\cdot}5 \times 10^{-7}$ M) similar to that found in isolated plasma membranes from rat soleus muscles (Erdmann, Philipp & Tanner, 1976), and one order of magnitude lower than reported for various tissues from other mammals. The good agreement between the number of sites obtained from the Scatchard plot and that found by measuring the amount of [^{3}H]ouabain retained by the muscles following long incubation at a

high concentration made it reasonable to assume that we have measured the total number of [^{3}H]ouabain-binding sites accessible from the outside of the cell. It should be noted that at variance with what has been suggested by the work of other investigators (Baker & Willis, 1972; Erlij & Grinstein, 1976), we have not been able to detect any appreciable penetration of [^{3}H]ouabain into the intracellular compartment (defined as the space not available to [U-^{14}C]sucrose).

Due to the complex structure of muscular tissue, not all Na pumps are equally accessible from the extracellular phase (Caswell, Lau & Brunschwig, 1976) and therefore, some of them may not contribute to any major extent to the net extrusion of Na from the cytoplasm. Furthermore, partial blocking of the active Na–K transport leads to an increase in $[Na]_i$. This will stimulate the remaining unoccupied pumps, and since the activation of the Na+K-ATPase is not a simple linear function of $[Na]_i$, the contribution of these pumps to the net extrusion of Na is even more difficult to quantify. For these reasons, even though the rate coefficient of ^{22}Na wash-out has reached its minimum level, this does not imply that all ouabain-binding sites are occupied. Consequently, we preferred to establish full occupancy by saturation or extrapolation from equilibrium data with respect to [^{3}H]ouabain binding rather than from measurements of ^{22}Na efflux. When this criterion was used, we were unable to detect any significant change in the total number of binding sites with insulin, adrenaline or tetracaine. Thus, within the time intervals used for the present *in vitro* experiments, we have not obtained any evidence for a change in the *de novo* synthesis of Na pumps. Neither do the data support the idea that Na pumps may be unmasked by insulin (Erlij & Grinstein, 1976). This phenomenon may be a reflexion of the fact that insulin increases the rate of ouabain binding.

Our results show that insulin increase the rate of [^{3}H]ouabain binding not only in rat soleus, but also in rat adipocytes and soleus muscles obtained from mice and guinea-pigs, which are considerably more sensitive to cardiac glycosides than the rat. The stimulation of [^{3}H]ouabain binding was not only induced by insulin, but equally well when the active Na–K transport was accelerated by adrenaline or Na loading. In fact, there seemed to be a rather close correlation between the rate of Na–K-pumping and the rate of ouabain binding. Such a relationship has earlier been demonstrated for red cells (Joiner & Lauf, 1975). The present data indicate that it may be of a rather general nature, ranging from subnormal rates to ones considerably above the basal level.

Whereas insulin and adrenaline induce a decrease in the intracellular Na/K ratio, pre-incubation with veratrine or in a K-free buffer leads to an increase. Since all of these conditions increased the rate of [^{3}H]ouabain

binding, it seems unlikely that this is due to an increase in $[Na]_i$ *per se* In a recent study with red cell ghosts (Bodemann & Hoffman, 1976) it was concluded that the rate of ouabain binding was inversely related to the rate of Na/K translocation. On one hand it was shown that an increase in $[Na]_i$ at a very low K concentration leads to a decrease in the rate of [^{3}H]ouabain binding. On the other hand, it was also demonstrated that when $[Na]_i$ was kept constant and the intracellular Na/K ratio increased, both the ouabain-suppressible component of ^{22}Na efflux and the rate of [^{3}H]ouabain binding were considerably increased. Furthermore, when the intracellular K concentration was kept at 109 mM, a ninefold increase in $[Na]_i$ (from 4 to 36 mM) did not lead to any inhibition of the ouabain binding. Since the present experiments were carried out with muscles in which $[Na]_i$ varied between 6·7 and 32 mM, these data give no reason to expect that the rate of [^{3}H]ouabain binding should be influenced by $[Na]_i$ *per se*. It cannot be excluded, however, that at $[Na]_i$ further above the physiological range, inhibitory effects may also be observable in soleus muscles.

It is reasonable to assume that the binding of cardiac glycosides requires a certain configuration of the binding site (see review by Schwartz *et al.* 1975). This particular structure of the region involved in the binding may occur once during each Na–K pump cycle. Provided all other conditions remain the same, an isolated increase in the rate of active Na–K transport (as brought about by insulin, adrenaline or an increase in $[Na]_i$), will allow the Na–K pump to assume the optimum configuration for the binding of ouabain more frequently. In rat soleus muscles, the ratio between the rate of [^{3}H]ouabain binding and the ouabain-suppressible component of ^{42}K influx was around $3{\cdot}3 \times 10^{-5}$ in the basal state. If it is assumed that two K ions are transported for each cyclical operation of the Na–K pump, this result indicates that at the concentration of ouabain used (5×10^{-6} M), [^{3}H]ouabain may become bound in one out of every 15,000 cycles.

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