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Density and Apparent Location of the Sodium Pump in Frog Sartorius Muscle

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Summary. The binding of the cardiosteroid ^{3}H-ouabain to frog skeletal muscle was determined by studying the kinetics of its uptake and release.

The amount of ouabain bound as a function of drug concentration in the external medium follows a hyperbolic relationship with a maximum binding (B_{max}) of the order of 2500 molecules per square micrometer of surface membrane and an affinity constant (K) of 2.2×10^{-7} M. The data do not suggest a drug-receptor (Na pump site) relation other than one-to-one.

Ouabain molecules are released from whole muscle into ouabain-free media very slowly. The release is a single exponential function of time ($\tau \simeq 25$ hr). When re-binding is prevented by the presence of unlabeled ouabain in the external medium, the loss of labeled ouabain is increased ($\tau \simeq 15$ hr). Increasing $[K^+]_o$ from 2.5 to 10 mM slows the time course of binding without any significant change in binding capacity of the muscle fibers.

Experiments on detubulated muscles indicate that the density of pump sites is considerably higher in the surface than in the T-tubular membrane. These findings agree with the report by Narahara et al. [Narahara, H.T., Vogrin, V.G., Green, J.D., Kent, R.A., Gould, M.K. (1979) *Biochim. Biophys. Acta* **552**:247] on the distribution of $(Na^+ + K^+)$-ATPase among different cell membrane fractions from frog skeletal muscle.

Schatzman (1953) showed that cardiosteriods like ouabain and strophanthidin inhibit active transport of sodium in erythrocytes. Through the years his finding has been extended to a variety of tissues, including frog skeletal muscle. Ouabain is now known to be a specific inhibitor of the membrane $(Na^+ + K^+)$-ATPase (Skou, 1957, 1960), presently thought to be the key element of the Na^+ pump. In freshly isolated frog sartorius muscles, the ouabain or strophanthidin-sensitive fraction of the Na^+ efflux is generally about 50% of the total Na^+ efflux (Horowicz, 1965; Horowicz et al., 1970; Erlij & Leblanc, 1971; Venosa & Horowicz, 1973). The reduction in Na^+ efflux produced by ouabain is maximal at concentrations of the order of 2×10^{-5} M or higher (Horowicz, Taylor & Waggoner, 1970). At these concentrations the onset of inhibition is relatively rapid, having a half time of 5 to 10 min. By comparison, recovery is extremely slow and for most practical purposes inhibition is virtually irreversible. Hence, ouabain appears to bind very tightly to its membrane receptor in skeletal muscle. These characteristics and the availability of tritiated glycosides makes ^{3}H-ouabain a useful marker of Na^+ pumping sites in this preparation. Consequently, ^{3}H-ouabain has been used extensively in binding studies. In frog skeletal muscle Grinstein and Erlij (1974) and Erlij and Grinstein (1976) have estimated a density of the order of 1600 sites per μm^2 of surface membrane.

This value is less than the value of 2500 sites/μm^2 which was found in the experiments reported on here. While there is a discrepancy between the data of Grinstein and Erlij (1974) and our own with regard to the ouabain binding capacity of frog sartorius muscle, there is agreement in the half saturation value of 2×10^{-7} M ouabain. The aim of the experiments reported here was to study, in addition to the ouabain binding capacity, the kinetics of the binding and desorption of the drug, as well as to obtain some information on the location of the Na^+ pumping sites in the sarcolemma of frog skeletal muscle. An early summary of our findings has appeared elsewhere (Venosa & Horowicz, 1975).

Materials and Methods

Sartorius muscles from healthy frogs *Rana pipiens* were used in all the experiments.

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Solutions

The normal Ringer's solution had the following composition (mM): NaCl, 115; KCl, 2.5; $CaCl_2$, 1.8; Na_2HPO_4, 2.15; NaH_2PO_4, 0.85 (pH 7.2). Glycerol Ringer's solution had the same ionic contents as the normal solution with 400 mM glycerol added. High Ca^{++} and Mg^{++} solutions contained 5 mM $CaCl_2$ and 5 mM $MgCl_2$ with NaCl, KCl, and the phosphate buffer at their normal concentrations. In some experiments the concentration of KCl was increased to 10 mM without altering the concentration of the other components of the normal Ringer's solution.

Labeled solutions were prepared by adding ouabain and ^{3}H-ouabain (New England Nuclear) to the Ringer's solution. The total ouabain concentrations used ranged from 1.1×10^{-7} to 7.07×10^{-5} M.

^{3}H-Ouabain Uptake

In each experiment four to eight small frog sartorius muscles (2 to 4 pairs) were carefully dissected and fastened to light stainless steel holders by means of thin surgical threads attached to both tendons. After one-half to one hour in normal Ringer's solution, they were drained by blotting the tendons and the holders with filter paper and weighed. With this procedure the surface-to-weight ratio in sartorius from *Rana pipiens* is 552 cm^2/g (Venosa, 1974).

The muscles were then exposed to normal Ringer's solution containing a known concentration of ^{3}H-ouabain, each for a different period of time. Subsequently, they were washed by transferring them through a series of tubes containing 3 ml of the same solution without ouabain. The muscles were then solubilized overnight in counting vials containing 1 ml of Protosol (New England Nuclear) at 45 °C. The solubilized preparations were mixed with 10 ml of liquid scintillation cocktail of the following compositions: POPOP, 250 mg; PPO, 5 g; ethanol, 500 ml, and toluene, 2 liters. After assaying their radioactivity, 10 µl of the labeled loading solution was added to each vial and recounted. This procedure allows one to measure the sample and loading solution activities with the same degree of quenching, and no correction due to this factor is needed (internal standard). The total volume of each of the washout samples was transferred to a vial containing 15 ml of liquid scintillation cocktail of composition similar to the one just described, except that in this instance it contained in addition 1.25 liters of Triton X-100. The specific activity of the loading solution was also determined under conditions similar to those of the washout samples (10 µl of loading solution, 3 ml of Ringer, and 15 ml of cocktail). With this technique the ouabain content of muscles at the end of the experiment and at any time during the washout period can be easily calculated and expressed in terms of moles/g of tissue or molecules/μm^2 of surface membrane area.

Results

Figure 1 shows the release of ^{3}H-ouabain into ouabain-free, normal Ringer's solution from eight muscles (four pairs) which had been exposed to 3.6×10^{-7} labeled ouabain for periods from 20 to 180 min.

There is at first a rapid initial loss due to the washout of the extracellular space and release of the drug from unspecific binding sites. This is followed by a second, slow single exponential component having a time constant of about 27 hr. The mean value of this time constant was 25.3 ± 2.5 hr (± 1 SD; $n = 105$) and was independent of the amount of bound glycoside. It seems reasonable to assume that it represents the release of ouabain bound to the cell membrane. This slow release is consistent with the practically irreversible effect of the glycoside on Na^+ efflux found in frog muscle. The amount of ouabain bound to specific sites at the end of the exposure to the drug is determined by extrapolation of the slow component to the start of the washout. These values plotted as a function of the time of exposure to ^{3}H-ouabain give the time course of ouabain binding, as illustrated in Fig. 2. As might be expected, the experimental points are reasonably well fit by an exponential function of time of the form $b = B(1 - e^{-t/\tau})$ where b and B represent the ouabain binding at time $= t$ and time $= \infty$, respectively, and τ is the time

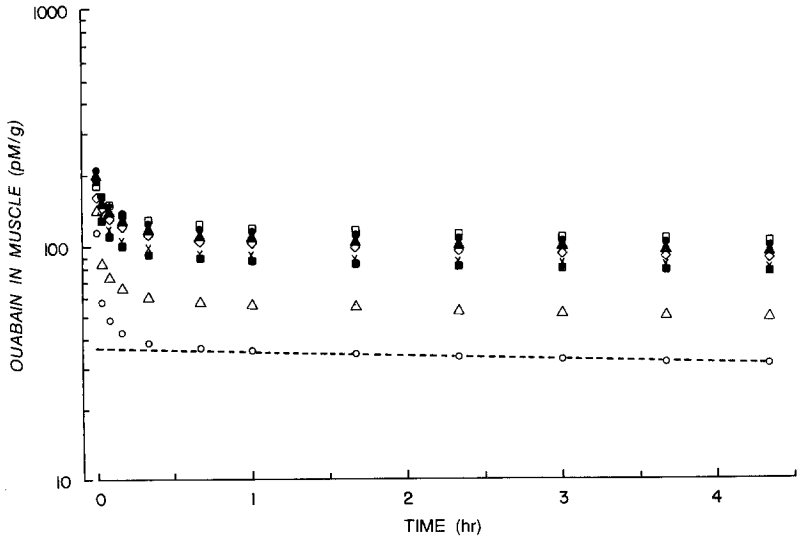

Fig. 1. Semilog arithmic plot of ouabain washout from eight sartorii which had been exposed to normal Ringer's solution containing 3.6×10^{-7} M labeled (^{3}H) ouabain for 20 (○), 40 (△), 60 (■), 80 (×), 100 (◇), 120 (▲), 150 (●), and 180 (□) min. The dashed regression line fitted to the last five points of muscle (○) is the slow single exponential component of the release of glycoside. Its extrapolation to time = zero represents the amount of ouabain per gram of muscle bound to specific receptors. The amounts of ouabain specifically bound to the other muscles were obtained in a similar way

constant of the uptake. In each experiment (4 to 8 muscles) the amount of ouabain bound was taken to be equal to the calculated value of B. In this particular experiment τ was 58 min and $B=1400$ molecules/μm^2. If a drug-receptor reaction of the type $D+R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} DR$ is assumed, in which D, R and DR represent the drug, the receptor, and the drug-receptor complex, respectively, and k_1 and k_2 are the association and dissociation rate constants of the reaction, one should expect the uptake to be faster the higher the concentration of the glycoside to which the muscles are exposed (Hill, 1909). This seems to be the case in the present experiments, at least qualitatively.

Figure 3 shows the time course of the uptake of ouabain in the presence of 2.8×10^{-5} M of the cardiosteriod. Here B was 2834 molecules/μm^2 and $\tau = 8.3$ min. The dependence of the time course of the uptake on ouabain concentration is illustrated in Fig. 4. It is clear that below 10^{-5} M ouabain the time constant of the uptake is markedly affected by changes in the concentration of ouabain, while above 10^{-5} M, τ changes little. This is not surprising if one considers the diffusion delays in the extracellular space. Figure 5 shows the time constant of the filling of the extracellular space as a function of $[OU]_o$. It can be seen that this parameter is not much affected by $[OU]_o$ and in addition its value at high $[OU]_o$ is similar to that of τ for the binding. It is clear, therefore, that at high $[OU]_o$ binding is rate limited by the diffusion time of the drug in the extracellular space. Nevertheless, at the lowest ouabain concentrations ($<4 \times 10^{-7}$ M), the time constant of the extracellular space increases to about 10 min

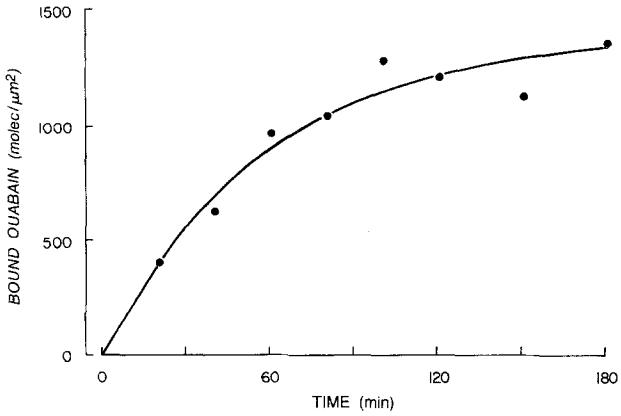

Fig. 2. Time course of the uptake of ouabain. The experimental points are the ordinate values of the slow components extrapolated to time = zero in Fig. 1. The curve gives the exponential function which was fit to the data (*see* text). Time constant of the uptake (τ): 58 min. Binding at time = ∞ (B): 1399 molecules/μm^2

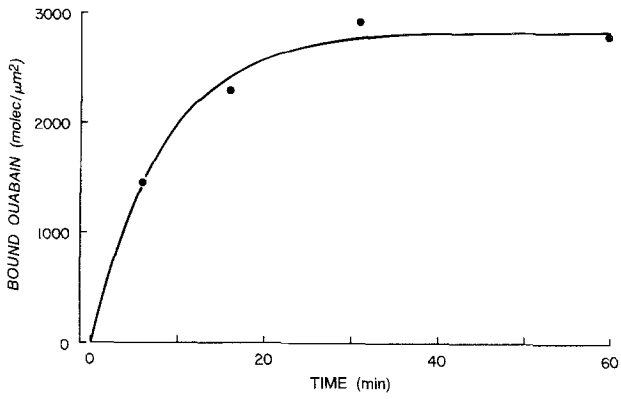

Fig.3. Uptake of ouabain similar to that shown in Fig. 2 except that the muscles were exposed to 2.8×10^{-5} M ouabain. $B=2834$ molecules/μm^2; $\tau = 8$ min

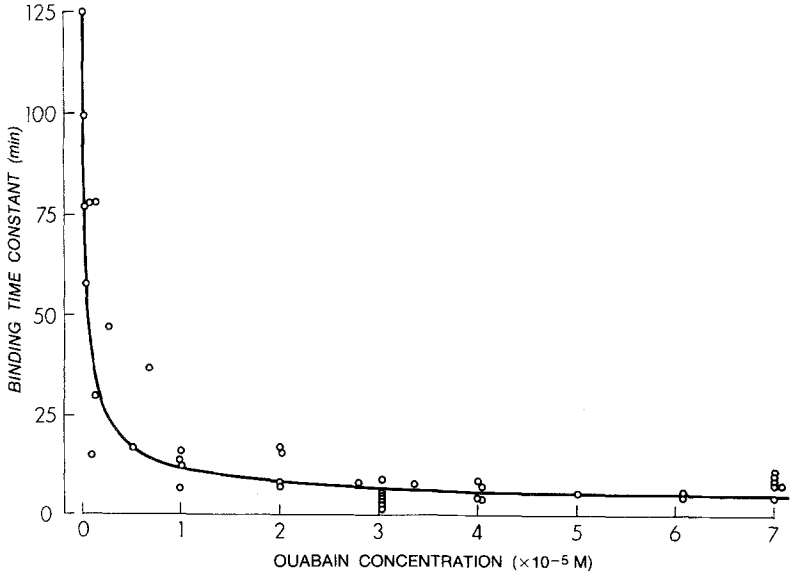

Fig. 4. Binding time constant as a function of ouabain concentration. Each point represents a single experiment where 4 to 8 muscles were used. The values of τ were obtained from curves like those shown in Figs. 2 and 3. Curve was drawn by eye

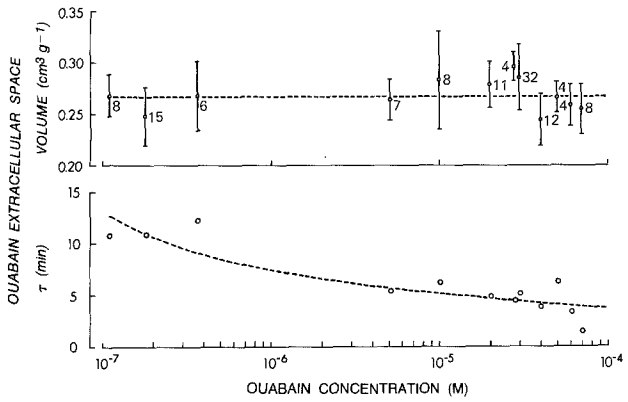

Fig. 5. *Upper panel:* Ouabain extracellular space as a function of $[OU]_o$. The figures next to the points indicate number of muscles and the bars are ± 1 SD. The dashed line represents the mean of all values: 0.267 ± 0.016 cm$^3 \times$ g^{-1}. *Lower panel:* Time constant for the equilibration of the extracellular space as a function of $[OU]_o$. The amount of ouabain present in the extracellular space at the end of a given loading period (zero time of the washout) was taken as the difference between the total amount of ouabain present in the muscle and that specifically bound. In Fig. 1, for instance, the amount of ouabain present in the extracellular space of muscle (○) at the end of the loading period is the difference between the extrapolated value of the slow component (dashed line) and the total amount of ouabain at time zero of the washout. The values of the time constants (τ) were calculated from fitting an exponential function to the filling of the extracellular space. Logarithmic abscissa

or somewhat longer, suggesting that under such conditions the ratio of free ouabain molecules to binding sites is sufficiently low as to prolong the apparent diffusion time of the cardiosteriod in the extracellular compartment.

For example, with a density of 2496 sites/μm^2, an extracellular space of 0.27 cm^3/g, and a surface to weight ratio of 552 cm^2/g muscle (Venosa, 1974), it can be estimated that in the presence of 4×10^{-7} M ouabain at equilibrium, 1 g of muscle contains 1.1×10^{-10} molecules of free ouabain and 2.3×10^{-10} sites. This is a free ouabain molecule to site ratio of 0.5. This represents a maximum ratio since there may be nonspecific binding sites present as well.

Figure 5 also shows that the size of the ouabain space is of the order of 0.27 cm^3/g, independent of $[OU]_o$ and close to the Na^+ space (0.23 cm^3/g) measured under similar experimental conditions (Venosa, 1974). The fact that this space does not increase at low external ouabain concentrations implies that there are few, if any, rapidly equilibrating, nonspecific binding sites in the extracellular space.

Release of ^{3}H-Ouabain in the Presence of Unlabeled Ouabain

As illustrated in Fig. 1 the washout of ouabain from specific sites is a very slow process. It is possible that the release of ^{3}H-ouabain molecules from the muscle might be prolonged by multiple detachment from and re-binding to receptor sites as ouabain diffuses out. To test this possibility, four pairs of sartorii were exposed to 5×10^{-5} M ouabain, each pair for different periods of time varying between 9 and 34 min. One member of each pair was then washed as usual in an ouabain-free medium, while the other was washed in solutions to which 5×10^{-5} M of unlabeled ouabain was added. In this situation the likelihood of any ouabain molecule being re-bound after dissociating from a receptor becomes negligible. The mean time constant of release from the muscles washed in ouabain-free medium was 28.8 ± 2.6 hr (SEM) and that from muscles washed in ouabain containing medium was 17.1 ± 0.5 hr (SEM). This reduction of about 40% in τ, which is statistically significant ($P < 0.02$) suggests that re-binding does, indeed, play a role in the slow release of ^{3}H-ouabain from frog sartorius. Nevertheless, the binding capacities of these two groups of muscles were similar: 2411 molecules/μm^2 for muscles washed in the presence of ouabain and 2329 molecules/μm^2 for those washed in ouabain-free medium. These results agree well with those obtained in rat muscle by Clausen and Hansen (1974).

The relationship between ouabain binding and $[OU]_o$ is illustrated in Fig. 6. Each experimental point (B, as defined above) was obtained by following the time course of the uptake of the cardiosteroid in three to eight muscles as already described. Thus, this graph represents the data from more than 200 muscles. The points are fitted by Eq. (1), a hyperbolic function of the Michaelis-Menten type,

$$B = \frac{B_{max}[OU]_o}{K + [OU]_o} \tag{1}$$

where B_{max} represents the maximum binding or binding capacity and K the dissociation constant or the ouabain concentration at which $B/B_{max} = 0.5$. The values of B_{max} and K were calculated from regression analysis of Eq. (2), which is a rearrangement of Eq. (1),

$$B = B_{max} - K\left(\frac{B}{[OU]_o}\right). \tag{2}$$

The values obtained were $B_{max} = 2496$ sites/μm^2 of surface membrane (transverse tubules excluded) and $K = 2.2 \times 10^{-7}$ M. The latter value is in excellent agreement with the concentration (2×10^{-7}) that inhibits about 50% of the ouabain-sensitive Na^+ efflux (Horowicz et al., 1970) and very close to the value of K previously reported by Erlij and Grinstein (1976) (2.6×10^{-7} M) for the same preparation.

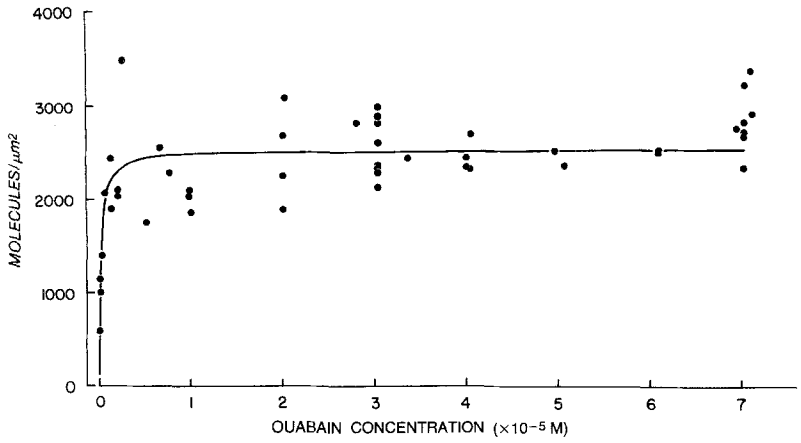

Fig. 6. Density of ouabain binding sites (in molecules/μm^2 of surface membrane) as a function of $[OU]_o$. The curve represents the fit of the experimental points to Eq. (1) with $B_{max}=2496$ molecules/μm^{-2} and $K=2.2\times10^{-7}$ M (*see* text)

Other muscle types exhibit K's for ouabain binding similar to those found in frog sartorius. Thus, Clausen and Hansen (1974) reported a value of 2.1×10^{-7} M in rat soleus and Brading and Widdicombe (1974) obtained a value of 1.2×10^{-7} M in smooth muscle. It should be pointed out that when the abscissa in Fig. 6 is expanded, the experimental points in the rising part of the curve do not show any sign of being sigmoidal in shape. Furthermore, fitting the data corresponding to ouabain concentrations below 1.3 μM yields a binding capacity (B_{max}) of 2300 molecules/μm^2 and a K of 1.9×10^{-7} M. These values are close to those obtained when all of the data are considered. ($B_{max}=2496$ molecules/μm^2; $K=2.2\times10^{-7}$ M.) It seems reasonable, therefore, to assume a 1:1 interaction between ouabain molecules and pump sites.

The expression relating τ and $[OU]_o$ derived from a simple drug-receptor reaction ($OU+R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} OU\text{-}R$) is:

$$\tau=(k_1[OU]_o+k_2)^{-1} \tag{3}$$

(Hill, 1909) where the dissociation rate constant, k_2, represents the release of ouabain molecules from their receptors. As discussed above, the time constant of ouabain washout in ouabain-free medium is of the order of 25 hr.

Nevertheless, the washouts carried out in ouabain-containing medium suggest that the actual time constant of the release from the receptors would be about 40% shorter, i.e., 15 hr. Therefore if $k_2=(15\times60\text{ min})^{-1}=1.11\times10^{-3}\text{ min}^{-1}$; solving for k_1 we have

$$k_1=\frac{\tau^{-1}-1.11\times10^{-3}}{[OU]_o}. \tag{4}$$

The mean k_1 and $K(=k_2/k_1)$ calculated with this equation from 38 experiments in which $[OU]_o$ ranged between 1.1×10^{-7} and 7.07×10^{-5} M were $1.4\times10^4\pm3\times10^3\ \text{M}^{-1}\times\text{min}^{-1}$ and $2.4\times10^{-7}\pm4\times10^{-8}$ M (±1 SEM), respectively. This value of K is not significantly different from that obtained at equilibrium using Eq. (2). However, as mentioned before, at high $[OU]_o$'s the rate of binding is probably limited by the diffusion time in the extracellular space. When only the experiments done with ouabain concentrations of 10^{-5} M or lower are considered, the mean k_1 is $2.4\times10^4\pm8\times10^3\ \text{M}^{-1}\times\text{min}^{-1}$ while the mean K is $1.0\times10^{-7}\pm2\times10^{-8}$ M ($n=13$). This value of the equilibrium dissociation constant, although lower, is reasonably close to that obtained using Eq. (2), considering the experimental variability involved.

Although our results agree well with those of Erlij and Grinstein (1976) with regard to K, they differ with regard to their estimates of B_{max}. Thus, Erlij and Grinstein (1976) obtained a value of 1600 sites/μm^2 as compared to 2496 sites/μm^2 obtained in these experiments. Possible causes of this discrepancy are discussed below.

Two binding components have previously been described in frog muscle (Erlij&Grinstein, 1976) as well as in several other cells (Baker&Willis, 1972*a*). One is saturable and presumably represents the binding of ouabain to specific receptors, and the other is a linear function of $[OU]_o$. With an analysis similar to that used by Grinstein and Erlij (1974) and by Erlij and Grinstein (1976), i.e., taking the binding at relatively short washout times (1 hr), we also obtain a linear component of the ouabain binding. However, when the binding is measured after the muscles have been washed for long periods of time (4.5 to 12 hr), as in the present experiments, the linear component is absent, as can be seen in Fig. 6.

Effect of Raising $[K^+]_o$

In mammalian cells (Baker&Willis, 1972*a*) and in squid axon (Baker & Willis, 1972*b*) an increase in

$[K^+]_o$ decreases the rate of ouabain binding. We performed an experiment using eight pairs of sartorii to compare the rate of ouabain uptake and the binding capacity of muscles exposed to 10 mM $[K^+]_o$ with the parameters measured in control muscles exposed to normal Ringer's fluid ($[K^+]_o = 2.5$ mM). Both sets of muscles were exposed to 6.1×10^{-5} M ouabain for periods varying from 5 to 20 min. In $[K^+]_o = 10$ mM the binding capacity was 2612 molecules/μm^2 and the uptake τ was 10.4 min, while the corresponding values for the control muscles were 2662 molecules/μm^2 and 6.1 min. These data indicate that under our experimental conditions an increase in $[K^+]_o$ which produces a pronounced stimulation of the Na^+ pump does not affect the binding capacity of muscle fibers but tends to reduce the rate of ouabain uptake, as it does in other preparations (Baker & Willis, 1972*a*). No attempt was made in the present series of experiments, however, to study in detail the effect of different $[K^+]_o$'s on the ouabain binding parameters of muscles in the presence of various $[OU]_o$.

Binding to Detubulated Muscles

An obvious question with regard to the density of Na^+ pumping sites in frog muscle fibers is to what extent ouabain binds to the transverse tubular membrane. We have attempted to answer this question by measuring the binding of ouabain in paired muscles in which one member of the pair had been detubulated by glycerol tratment (Howell & Jenden, 1967), a procedure that disconnects, in isolated frog sartorius muscle, 90% of the transverse tubules from the external medium (Franzini-Armstrong, Venosa & Horowicz, 1973). Due to the fact that the area of the tubular membrane is 4 to 5 times larger than that of the surface membrane, it was hoped that even a moderate density of tubular pumping sites would be detected by this technique. The experiments with detubulated muscles at high $[OU]_o$ (2 to 7×10^{-5} M) were complicated by the appearance in this preparation of a nonsaturable binding component particularly for long exposure times.

At high $[OU]_o$ there was no significant difference between control and detubulated muscles in the uptake of ouabain during the first 20 to 30 min of the exposure to the drug. For longer times, however, the binding to detubulated muscles continued to increase monotonically while binding to control muscles was stationary. The nature of such an increase in nonspecific binding to detubulated muscles is not clear at present. However, a leak of ouabain into the fibers and its subsequent intracellular binding can be ruled out. This possibility was tested by an experiment similar to that performed by Clausen and Hansen (1974) in rat muscle. Four pairs of muscles were used. One muscle of each pair was cut so that all the fibers were sectioned in at least fifteen places along their length. These muscles were kept in a large volume of normal Ringer's solution for about 2 hr before exposing them to 20 μM ouabain for various periods of time. The result is shown in Fig. 7. It is apparent that the time course and the amount of bound glycoside are quite similar in both sets of muscles. This suggests that the pronounced increase of the binding in detubulated muscles is not due to a leak of ouabain into the fibers. Moreover, cut muscles exhibited a large increase in ouabain space, indicating that the intra- and extracellular spaces were equally accessible to the cardiosteroid. From these results one can safely conclude that, first, ouabain does not bind intracellularly to any significant extent and, second, the binding does not depend on parameters which require the integrity of the cell membrane such as a normal resting potential. These

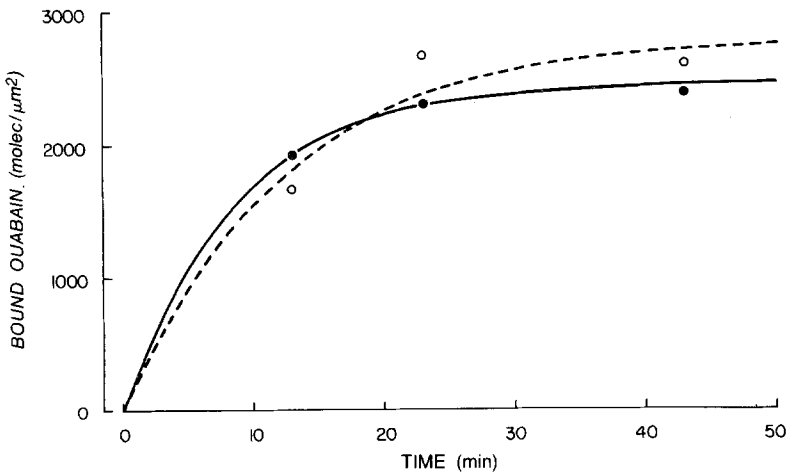

Fig. 7. Time course of the uptake of ouabain by paired cut (--o--) and control (-●-) muscles in the presence of 2×10^{-5} M ouabain. Cut muscles: $B = 2830$ molecules/μm^{-2}, $\tau = 16$ min. Control muscles: $B = 2474$ molecules/μm^{-2}, $\tau = 13$ min

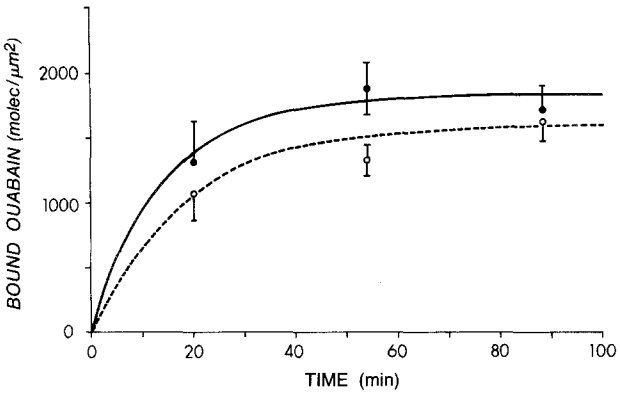

Fig. 8. Time course of the ouabain binding to paired detubulated (○) and control (●) muscles in the presence of 2.0×10^{-6} M ouabain. Each point is the mean ±1 SEM of at least five muscles. The curves represent the fit of an exponential function to the two groups of points. Detubulated muscles: $B = 1593$ molecules/μm^{-2}, $\tau = 18$ min. Control muscles: $B = 1830$ molecules/μm^{-2}, $\tau = 14$ min

results are in excellent agreement with those of Clausen and Hansen (1974). It is also worth mentioning here the fact that HeLa cells bind the same amount of ouabain whether they are intact or lysed (Baker & Willis, 1972*a*).

When the binding was measured in detubulated muscles in the presence of 2×10^{-6} M ouabain, the nonspecific component observed at concentrations of the drug one order of magnitude higher was not detectable. The advantage of using this lower concentration of cardiosteroid is that the binding time constant is considerably longer than that of the filling of the extracellular space and yet the B values are not too far from the saturation level (*see* Fig. 6). The results of these experiments are illustrated in Fig. 8. Although the binding is somewhat lower in the detubulated than in control muscles, the difference is not statistically significant.

Judging from the results obtained with detubulated muscles, it is apparent that if there are binding sites in the tubular membrane their density should be much lower than that of the surface membrane. Conversely, if the density of the Na^+ pumping sites were nearly the same in both structures, it would almost certainly have been revealed by detubulation. The tentative conclusion, therefore, is that the Na^+ pumping activity in frog skeletal muscle is mostly confined to the surface membrane. It is interesting that TTX binding studies have shown that the density of voltage-gated Na^+ channels in the surface is also higher (~4:1) than in the T-tubules (Jaimovich, Venosa, Shrager & Horowicz, 1976).

Discussion

The results indicate that for measurements of ouabain binding to be reliable, at least in frog muscle, the methods should incorporate: (i) long washouts of muscles after exposure to drug, and (ii) determination of the time course of uptake at each concentration used. Long washouts reveal more adequately the presence of the slow exponential component of ouabain binding after the withdrawal of the drug from the external medium. Extrapolation of the slow component to the start of the washout provides an estimate of the amount of ouabain bound to specific receptors at the end of the uptake period. The extrapolated values obtained from several muscles exposed to the same concentration of drug for different periods represent the kinetics of ouabain binding to specific receptors. The relation between binding and ouabain concentration in the medium (Fig. 6) can be reasonably well fitted by Eq. (1).

About one-half of the Na^+ efflux (~2.5 pmol/cm^2 sec^1) is ouabain sensitive. If the density of pump sites is 2500/μm^2 the extrusion rate is, therefore, of the order of 360 Na^+ ions/site min. If one assumes 3 Na^+ are expelled per cycle of the pump, this gives a pump turnover rate of 120 cycles/site min.

Detubulated sartorii have proven to be a useful preparation to estimate the density of voltage-gated Na^+ channels in the T-tubules through tetrodotoxin binding measurements (Jaimovich et al., 1976). In the present experiments the use of detubulated muscles was complicated by the fact that glycerol appears to have an effect of its own on the binding of ouabain which was particularly noticeable in the presence of high $[OU]_o$ (3×10^{-5} M) and for relative long periods of exposure. This problem was circumvented by lowering $[OU]_o$ (2×10^{-6} M). At this ouabain concentration, the data indicate that the density of Na pump sites is substantially greater in the surface than in the tubular membranes. From these experiments at most 20% of the ouabain sites might be located in T-tubule membranes. Since the tubular membranes in sartorius muscles have an area 4 to 5 times greater than the surface membranes, this means that the average density of pump sites is about 4 to 5% of that bound on the surface. This implies that the gradient of Na-pump sites is steep in the radial direction of the T-tubular lattice.

This relative paucity of pump sites in the tubular membranes is supported by the recent findings of Narahara et al. (1979) in frog muscle. Their data show that the $(Na^+ + K^+)$-ATPase activity in a membrane fraction containing the sarcolemma is as much as 14 times higher than that in the fraction containing the T-tubules. Recently Lau, Caswell, Garcia and Letellies (1979), using T-tubular vesicles from rabbit, found a ouabain binding density of 37

pmol per mg of protein. An approximate conversion of this value in terms of tubular membrane area can be obtained by assuming that: (i) half of the membrane is made of protein (density ~ 1.4 g/cm^3) with the other half being lipids, and (ii) the tubular membrane is about 75 Å thick. The density so calculated is of the order of 180 site/μm^2. In rat muscle Clausen and Hansen (1974) obtained a density of 3350 binding sites per μm^2 of surface membrane. If, in rabbit muscle, the binding is similar to that in rat muscle, and the tubular membrane area is four times larger than the surface membrane, the ouabain bound to the T-tubules would therefore represent about 20% of the total binding. This apparently lower density of Na^+ pump sites in the T-tubules as compared with that of the surface is consistent with the findings of Narahara et al. (1979) and the results presented here in frog muscle.

The discrepancy between our data and those of Grinstein and Erlij (1974) and Erlij and Grinstein (1976) with regard to binding capacity is probably the result of several factors. As shown in Figs. 2, 3 and 4, the time course of the ouabain uptake is very much dependent on $[OU]_o$, particularly at concentrations below 10^{-5} M. Furthermore, long washouts are needed after the exposure to ouabain to adequately reduce the extracellular ouabain concentration, particularly at higher concentrations. Erlij and Grinstein (1976) used fixed periods of 50 min for uptake and 60 min for washouts for all concentrations of ouabain. Our data indicate that at ouabain concentrations lower than 10^{-6} M a 50 min uptake period would be insufficient to reach equilibrium (*see* Figs. 2 and 4). In addition, washouts of 60 min after exposure to ouabain concentrations higher than 10^{-6} M would be too short and hence yield apparently high binding values which increase in proportion to the ouabain concentration during the exposure period. Conceivably, the combination of these factors could produce both the presence of a nonspecific binding component and a saturable binding capacity lower than that found in our results.

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