Chapter 18

Sodium Pump in T-Tubules of Frog Muscle Fibers

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1. INTRODUCTION

It is well known that in the cytosol of most cells the high concentration of K^+ ($[K^+]_i$) and the relatively low concentration of Na^+ ($[Na^+]_i$) are kept constant in spite of their electrochemical gradients, which promotes the loss of K^+ and the gain of Na^+. The steadiness of $[K^+]_i$ and $[Na^+]_i$ is maintained by a metabolic energy-dependent active transport process first proposed by Dean[1] in skeletal muscle and known as the Na^+ pump (Dean coined the name) or more properly as the Na^+/K^+ pump.

Since then, the active transport of Na^+ and K^+ have been extensively studied, particularly in red blood cells, and nerve and muscle fibers (for a recent review, see Ref. 2). Through the years it became clear that the work of translocation of Na^+ from the inside to the outside of the cell and K^+ in the opposite direction was performed by an Na^+/K^+ ATPase located in the cell membrane.[3,4] This enzyme is a protein large enough to be accessible from either side of the membrane.

External ouabain and other cardiosteroids specifically inhibit the enzyme and thereby the active fluxes of Na^+ and K^+. In frog skeletal muscle fibers, about half of the Na^+ efflux is active and maximally blocked by 30 μM ouabain (or strophanthidin) in a practically irreversible manner with an apparent dissociation constant (K_d) of the order of 0.2 μM.[5] Such very poor reversibility is an advantage in binding studies (Figs. 1 and 2).

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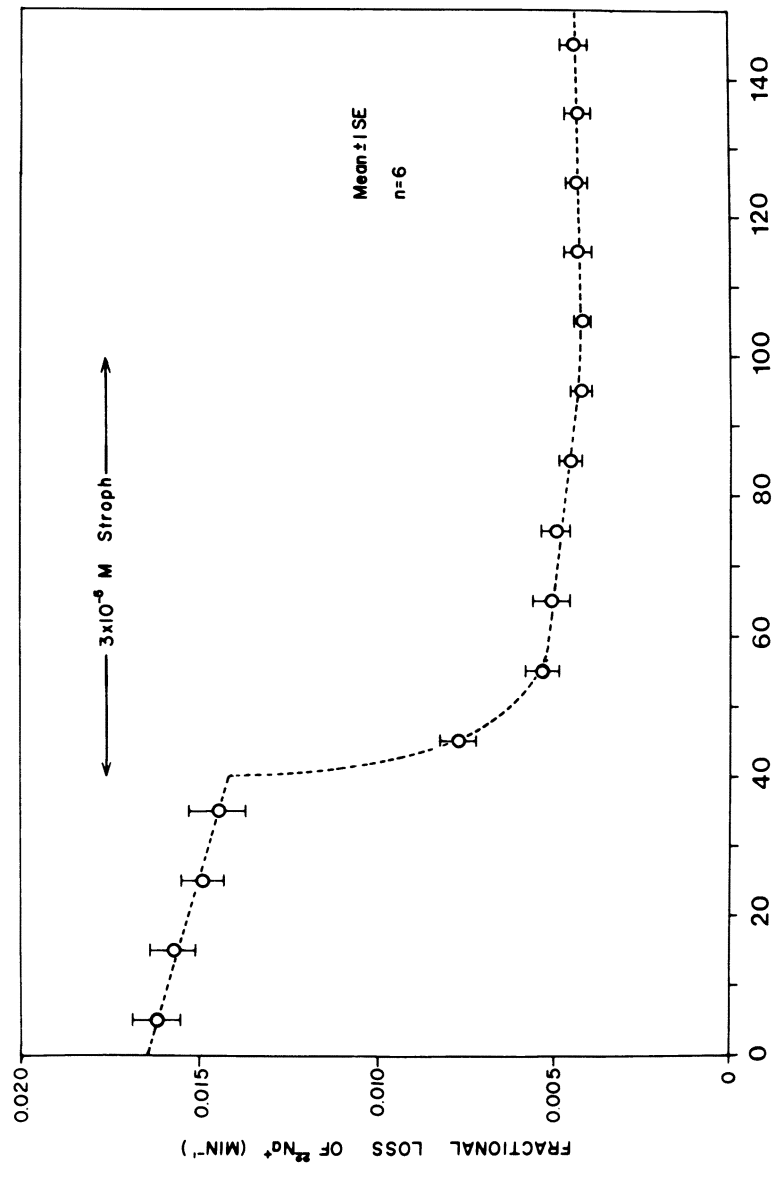

Figure 1. Virtual irreversibility of the inhibition of the active Na^+ transport by 30 μM strophanthidin in frog sartorius muscles (*R. pipiens*). Mean (± 1 SEM) from six experiments.

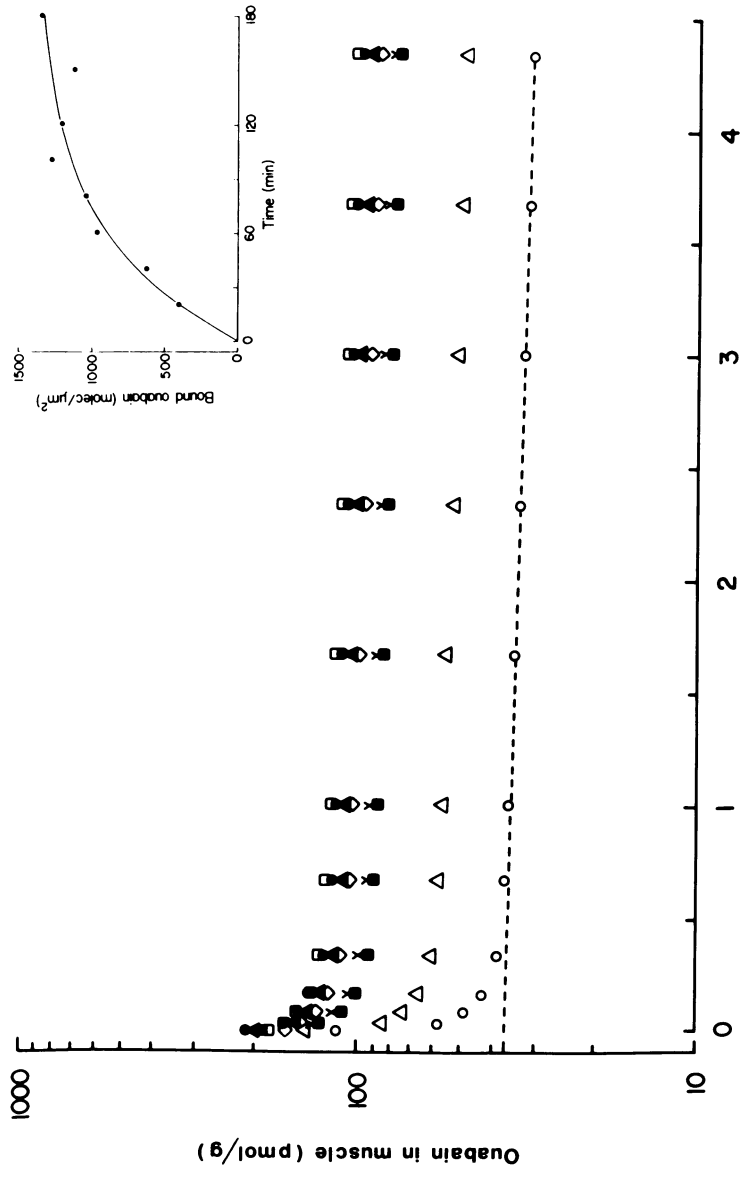

Figure 2. Semilog plot of ouabain washout from eight sartorius muscles (*R. pipiens*) previously exposed to Ringer solution containing 0.36 u*M* labeled (^{3}H) ouabain, each for a different period of time between 20 (○) and 180 (□) min. The dashed line represents the slow single exponential component of the release of the drug. Its extrapolation to time = 0 is a measure of the amount of ouabain bound to specific receptors at the end of the loading period. The inset shows the extrapolated values as a function of the exposure time to ^{3}H-ouabain. (Modified from Ref. 7.)

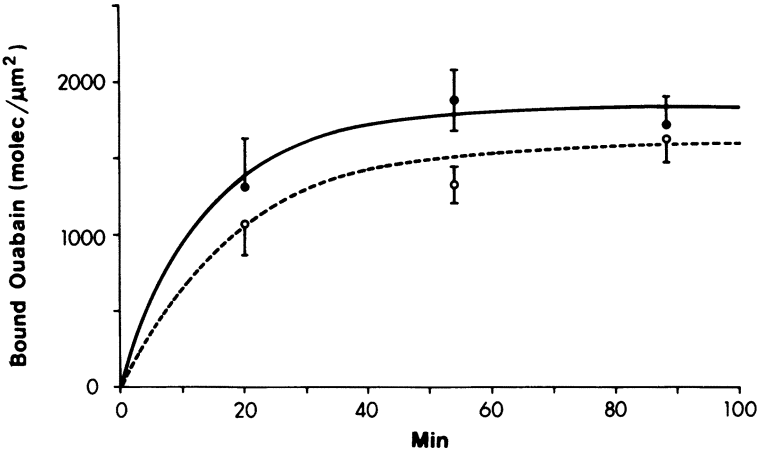

Figure 3. Time course of the ouabain binding to detubulated (○) and control (●) muscles (*R. pipiens*) in the presence of 2 μ*M* ouabain. The paired experimental points are the means (±1 SEM) of at least five pairs of muscles. The curves represent the exponential fit of the experimental points according to $b = B[(1 - \exp(t/\tau)]$ where b and B denote the binding at time = t and time = ∞, and τ the time constant of the uptake. (From Ref. 7.)

Erlij and Grinstein[6] estimated the density of Na^+ pumping sites in frog sartorius muscle (*R. pipiens*) to be of the order of 1600/μm² of surface membrane. Later, Venosa and Horowicz,[7] based on the kinetics of ^3H-ouabain binding and release in the same preparation, found a density of pumps considerably higher (i.e., 2500/μm² of surface membrane). The difference between this value and that of Erlij and Grinstein[6] was apparently due to the fact that the latter authors used a fixed time for the binding period (50 min), which was rather short for equilibration at low ouabain concentrations, and a fixed time for the washout (60 min) of ouabain from nonspecific binding sites that was not long enough, particularly at high concentrations of the drug.[7]

We estimated the density of Na^+ pumping binding sites in the T-tubules by comparing the binding of ^3H-ouabain in pairs of control and detubulated muscles.[8] Previously, measurements of tetrodotoxin (TTX) binding in this preparation, revealed that about half of the voltage-gated Na^+ channels of frog muscle fibers were located in the T-tubules.[9] In the case of the Na^+ pumping sites the ^3H-ouabain-binding measurements indicated that only about 20% of them would be in the T-tubules (Figs. 3 and 4). This meant that the density of pumps in the tubular system would be 15 to 20 times lower than that in the superficial sarcolemma.

In addition, Narahara et al.[10] working with a frog muscle homogenate, had found that the content of Na^+/K^+-ATPase in a membrane fraction rich in superficial sarcolemma was 14-fold higher than that of a T-tubules-rich fraction. Lau et al.,[11] on the other hand, using vesicles formed in a T-tubule rich membrane fraction from rabbit skeletal muscle reported a ouabain binding capacity of 37 pmol/mg of membrane protein, which would correspond to an estimated density of the order of 180 sites/μm²

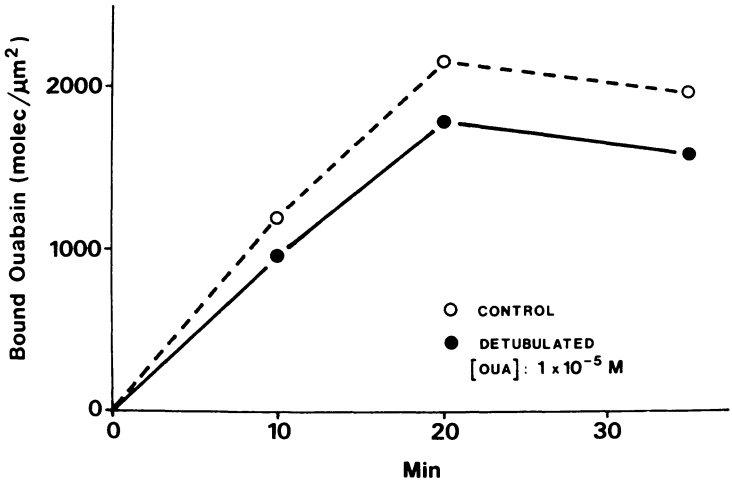

Figure 4. Ouabain binding to paired control (○) and detubulated (●) sartorius muscles from *L. ocellatus*.

of tubular membrane. Measurements in rat muscle yield a binding capacity of 310–721 pmol/g wet wt.[2] or a binding site density of 1430–3330/μm^2 of surface membrane (1300 cm^2/g).[12] On the assumption that rabbit muscle has a binding capacity similar to that of rat muscle, it means that 20 to 50% of the sites per unit area of superficial sarcolemma would be located in the T-tubules opening in that portion of the membrane.

Moreover, Seiler and Fleischer[13] found a Na^+/K^+-ATPase activity in plasma membrane vesicles from rabbit muscle about ten times larger than that reported by Lau et al.[14] in a T-tubule rich membrane fraction from the same origin.

Up to this point it would therefore appear that the density of Na^+ pumps in the T-tubules is substantially lower than in the surface membrane, and that the ouabain/TTX binding ratio is higher in the surface than in the tubules. In fact, Moczydlowski and Latorre[15] used this criterion to identify two membrane fractions: the one with the lower ouabain/saxitoxin ratio as rich in T-tubules, and the one with the higher ratio as surface membrane.

More recently, however, Hidalgo et al.[16] reported a Na^+/K^+-ATPase activity in the tubules comparable to that found in the surface membrane and Jaimovich et al.[17] a ouabain-binding capacity of 215 pmol/mg of protein in vesicles of T-tubule membranes and of 163 pmol/mg of protein in a fraction enriched in surface membranes from frog muscles (*Caudiverbera caudiverbera*). The density of pump sites can be estimated from their data by assuming that 1 mg of protein corresponds to about 0.27 m^2 of membrane (thickness = 7.5 nm; 50% protein; density = 1 g/cm^3) and therefore the densities of ouabain molecules (pump sites) per μm^2 would be 480 for the tubular membrane and 364 or 654 for the surface membrane depending on whether or not the folds and caveolae of the sarcolemma are taken into account.[18] If, on the average, there are 4 μm^2 of tubular membrane per μm^2 of surface membrane, then there should be (480 × 4) + 364

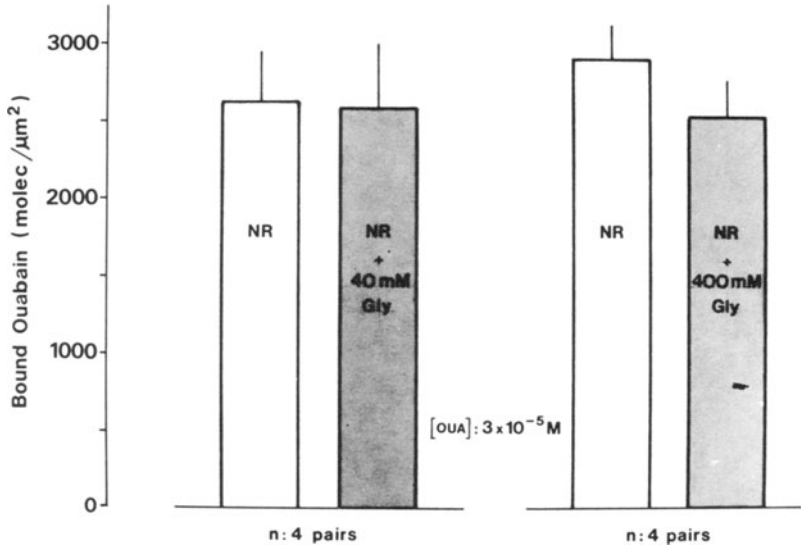

Figure 5. Ouabain binding to paired sartorius muscles (*R. pipiens*) exposed to normal Ringer plus 30 μ*M* ouabain in the absence and in the presence of 40 m*M* and 400 m*M* glycerol. Bars = means + 1 SD. (Venosa and Horowicz, unpublished.)

= 2284, or (480 × 4) + 654 = 2574 pump sites per μm² of surface membrane and its associated T-tubules. These figures are quite close to the value found by Venosa and Horowicz[7] in intact muscles (2500 sites/μm²).

Detubulation produced by glycerol treatment[8] disconnects 90% of the T-tubules from the external medium.[19] On the basis of the data of Jaimovich et al.,[17] after detubulation there should be expected an 80% reduction in ouabain binding instead of the above mentioned 20% observed by Venosa and Horowicz.[7]

Although this discrepancy between the data from isolated membrane fractions and intact muscle fibers could be due to several factors, two likely possibilities are apparent. On the one hand, if the binding in intact muscle fibers were mostly confined to surface membrane it would mean that the agreement between the data of Jaimovich et al.[17] and Venosa and Horowicz[7] on the magnitude of the total binding is fortuitous. On the other hand, if the binding sites were homogeneously distributed, detubulation by glycerol osmotic shock should, somehow, produce a binding increase of its own which roughly cancels out the expected reduction due to the disconnection of the T-tubules from the surface. At a ouabain concentration of 2 μ*M* no sign of nonspecific binding in detubulated muscles was found.[7] In addition, the binding is not significantly affected by the presence of either 40 m*M* or 400 m*M* glycerol Ringer (see Fig. 5). This seems to rule out a chemical effect of glycerol on ouabain binding.

The study of Na^+ movements and ouabain binding under anisotonic conditions have recently provided some clues on this subject. Some of those results and their possible relation with the data from detubulated muscles are reviewed next.

2. THE HYPOTONIC EFFECT

It is generally accepted that the active Na^+ transport in most cells, including skeletal muscle fibers, is a function of $[Na]_i$. This suggests that a decrease in $[Na^+]_i$ should be followed by a fall in the activity of the Na^+ pump. It would be expected, then, that a reduction in the osmolarity of the external medium should produce a decrease of the active Na^+ extrusion. Quite on the contrary, 10 years ago I found that a reduction of the tonicity of the external medium promoted an increase of the Na^+ efflux, which is completely blocked by strophanthidin (or ouabain) (Fig. 6), not due to depolarization and independent of external Na^+.[20] This stimulation of the Na^+ pump by hypotonicity is quickly reversible and the lower the osmolarity, the larger the stimulation. In Fig. 7 it can be seen that a reduction in osmotic pressure of the external medium to one-half (π = 0.5) of its normal value (π = 1) produces an increase close to threefold in the active transport of Na^+. A 2.5-fold increase in active K^+ influx (not shown) was also observed in π = 0.5 (in π = 1, half of the osmolarity was provided by sucrose, replacing NaCl; different π's were obtained by changing the sucrose content).

Na^+ and K^+ permeabilities (P_{Na}, P_K), on the other hand, are reduced by 36 and 20% respectively in π = 0.5 (Venosa, unpublished). The fall in P_K precludes the possibility that the stimulation of the active Na^+/K^+ transport was due to an increased K^+ leak, which would raise $[K^+]_0$ and thereby the activity of the pump.

The author is not aware of a similar behavior of the ouabain-sensitive Na^+/K^+

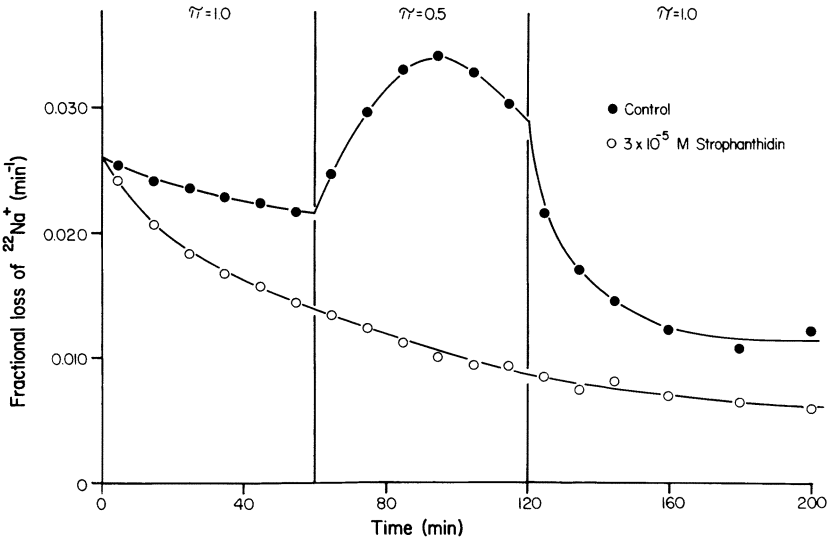

Figure 6. Effect of the reduction of the osmolarity to one-half (π = 0.5) of its normal value (π = 1) on the fractional loss of $^{22}Na^+$ from a pair of sartorii one of them in the presence of 30 μM strophanthidin from time zero to the end of the experiment (○) and the other in glycoside free media (●). The hypotonic response is absent when the Na^+ pump is inhibited. (From Ref. 20.)

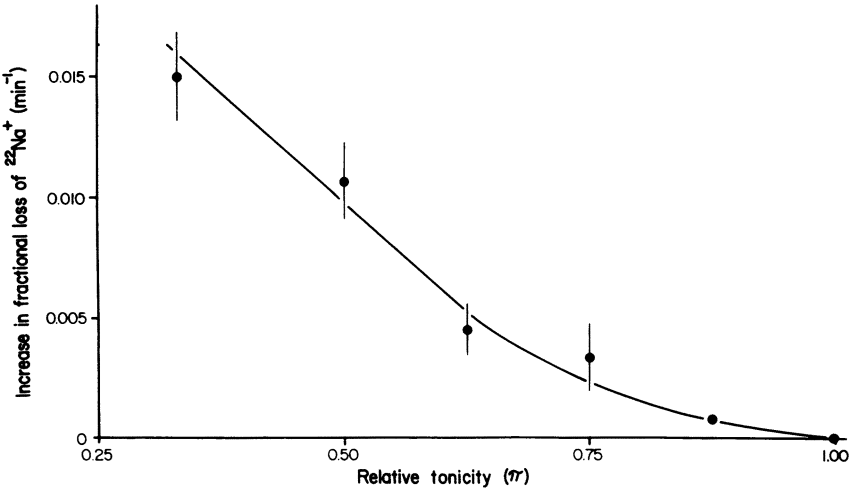

Figure 7. Increase of $^{22}Na^+$ efflux as a function of tonicity. In $\tau = 1$ the strophanthidin (ouabain)-sensitive portion of the efflux (expressed as fractional loss) was about 0.006 min^{-1}, which means that in $\pi = 0.5$ the active Na^+ efflux was roughly three times higher than in $\pi = 1$. Means: + 1 SEM. (From Ref. 20.)

transport in other cell types exposed to hypotonic media. More recently, it was found in frog sartorii from *Leptodactylus ocellatus* (Argentine frog) that the increase in the active fluxes of Na^+ and K^+ that occurs under hypotonic conditions is accompanied by an increase in the apparent density of pump sites. Preliminary results indicate that a reduction of 25% in osmotic pressure ($\pi = 0.75$) produces an increase in ouabain-binding capacity of the order of 30%, and in $\pi = 0.5$ the increase was on the average 40% (Fig. 10). In what follows it is shown that this response to hypotonicity might bear on the ouabain-binding measurements in detubulated muscles.

Muscles are detubulated by exposing them to a Ringer's solution made hypertonic by addition of 400 m*M* glycerol for 60–80 min, then to a glycerol-free Ringer containing 5 m*M* Ca^{2+} and 5 m*M* Mg^{2+} for 60–80 min, to reduce depolarization,[21] and thereafter to normal Ringer. Since glycerol permeates the plasma membrane, the fibers swell upon return to isotonic media. This change should be transient as glycerol (and water) flows out and the cell volume returns toward its normal value. Control experiments showed that the shrinking of the fibers back to their normal volume is rather slow. Thus, 2 to 2.5 hr after glycerol withdrawal, the time at which ouabain binding was previously determined,[7] the mean fiber volume is about 1.3 times larger than the initial one in normal Ringer (Fig. 8). Frog muscle fibers behave virtually as perfect osmometers. A relative fiber volume of 1.3 is produced by $\pi = 0.66$. It can be estimated that this osmotic pressure increases the ouabain-binding capacity by about 37%. This means that in detubulated muscles the observed modest reduction in the number of pump sites to about 80% of the controls in muscles from *R. pipiens* and *L. ocellatus* (Figs. 3 and 4) would probably represent the sum of two opposite effects: (i) an increase of about 35%

promoted by the swelling of the fibers; (ii) a decrease due to the disconnection of the T-tubules from the surface.

In view of the effect of swelling on ouabain binding it seems appropriate to reevaluate the data from detubulated muscles. Because hidden in the reduction of the binding capacity of detubulated muscles to about 80% of the controls there would be a 35% increase due to the volume increase, the percentage of sites left after detubulation would be 80/1.35 = 59. In detubulated fibers up to 10% of the tubules might remain connected to the surface.[19] Therefore, assuming as we did above that there are 4 μm^2 of tubular membrane per μm^2 of superficial sarcolemma, it can be estimated that the actual binding would approximately be 42% for the surface and 58% for the tubules, and, accordingly, the density of pump sites in the surface membrane of intact muscle fibers would be two to three times higher than in the tubular membrane. This distribution of Na^+ pump sites resembles that of the voltage-gated Na^+ channels determined in the same preparation as well as in isolated membrane fractions.[9,17]

The opposite effect of glycerol and detubulation may explain in part early results of Venosa and Horowicz.[22] They found that 1 to 1.5 hr after glycerol withdrawal (when the fibers were maximally swollen) the active Na^+ transport was increased, while later on (6–8 hr) when the fiber volume had presumably diminished or returned to its normal value, the active Na^+ efflux was reduced by about 50%.

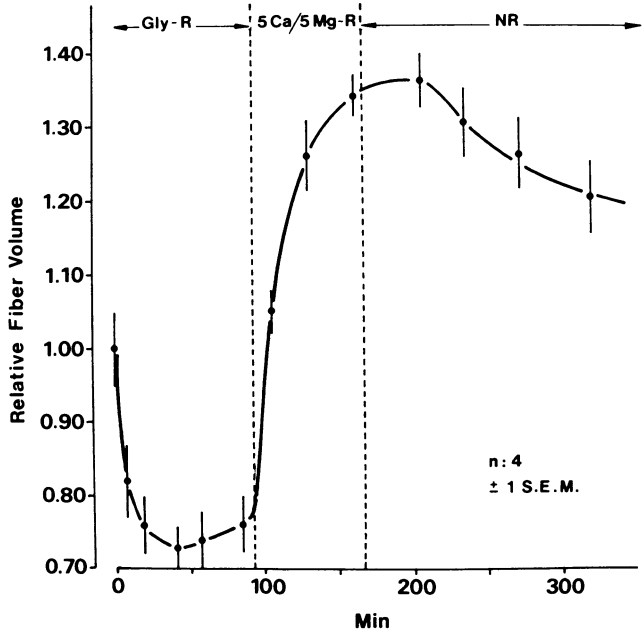

Figure 8. Time course of fiber volume changes in four sartorii from *L. ocellatus* during glycerol treatment. The fiber volumes were estimated by subtracting the extracellular space from the muscle weight. The values from each muscle were normalized with respect to that in normal Ringer and averaged. Means: ± 1 SEM.

It is clear that although the differences between the data from isolated membrane fractions and intact muscle are narrowed by considering the swelling component, and possibly the presence of remnants of tubular membrane accessible to ouabain in glycerol-treated muscles, they are still far from being insignificant.

Another difference between the data from intact muscle and those from isolated membrane fractions refers to the dissociation constant (K_d) of the ouabain binding to the receptor. In intact sartorius muscle from *R. pipiens* it was found to be 0.22 μM,[7] a value practically identical to that required for 50% inhibition ($K_{0.5}$) of the ouabain-sensitive Na^+ efflux in the same preparation.[5] In muscles from *L. ocellatus* the K_d for the ouabain-receptor interaction is somewhat higher (1–5 μM). In isolated membrane fractions from *C. caudiverbera* on the other hand, Jaimovich et al.[17] reported K_ds of 10.4 and 9 nM for T-tubule and surface membranes, respectively. A ouabain concentration of 10 nM does not have any appreciable effect on the ouabain-sensitive Na^+ efflux in frog muscle from at least three species (*R. pipiens, R. temporaria,* and *L. ocellatus*). It would be interesting, therefore, to determine the $K_{0.5}$ of the ouabain-sensitive fraction of the Na^+ efflux in muscle fibers from *C. caudiverbera.* If the $K_{0.5}$ for the inhibition of active Na^+ transport were similar to those found in other frog species it would suggest that the high-affinity receptors to which ouabain binds under their experimental conditions may be other than those related to the pumping sites which maintain Na^+ far from equilibrium across the sarcolemma under physiological conditions. If, on the contrary, the active Na^+ transport were inhibited by ouabain with a $K_{0.5}$ of the order of 10 nM, it would strongly support the notion that the magnitude of the ouabain binding and the distribution of sites correspond to the Na^+ pump under physiological conditions in the intact muscle fibers from *C. caudiverbera.*

3. CONCLUSION

Whether or not the Na^+ pumps are evenly distributed between T-tubules and surface membrane in frog muscle might partly depend on the species used. What seems likely, however, is that the density of Na^+ pumps in the T-tubules found by comparing the ouabain binding in detubulated and control-paired muscles is low because the volume increase (30%) in detubulated fibers, which in turn increases the ouabain binding and partially offsets the actual reduction of binding sites due to disconection of T-tubules from the surface.

The mechanism of the rise in the apparent number of Na^+ pump sites produced by volume increase (hypotonic effect) is not clear. The possibility that it might be due to a reduction of the intracellular K^+ concentration or of ionic strength does not seem likely because, as shown in Fig. 9, in muscles where the continuity of the sarcolemma was disrupted by cutting them in 15 to 20 places along their length the hypotonic effect was not detected. It has been shown that under isotonic conditions the disruption of the sarcolemma per se does not alter ouabain binding.

It would not be unreasonable to assume that under hypotonic conditions and after glycerol treatment the swelling of the fibers could activate latent pumping sites. It is tempting to speculate that the tension in the sarcolemma might modulate the number of active pumps.

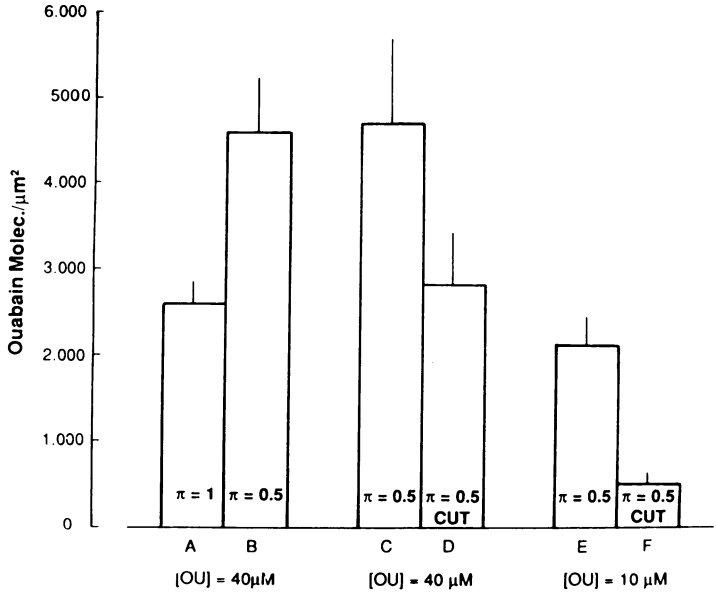

Figure 9. Effect of hypotonicity ($\pi = 0.5$) on ouabain binding (molecules/μm^2) in intact and membrane-disrupted (cut fibers) sartorii from *L. ocellatus*. A–B: eight intact muscles equilibrated in $\pi = 1$ (A) and their paired companions in $\pi = 0.5$ (B) all exposed to 40 μM ouabain. C–D: three intact muscles equilibrated in $\pi = 0.5$ (C) and their cut paired companions (D) all exposed to 40 μM ouabain. E–F: four paired muscles, same conditions as in C–D except that they were exposed to 10 μM ouabain. The increase in ouabain binding promoted by $\pi = 0.5$ in intact muscles is not apparent in membrane-disrupted muscles. Exposure time: 30 min. Bars: means + 1 SEM.

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