

Ouabain Binding and Coupled Sodium, Potassium, and Chloride Transport in Isolated Transverse Tubules of Skeletal Muscle

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ABSTRACT The affinity and number of binding sites of [³H]ouabain to isolated transverse (T) tubules were determined in the absence and presence of deoxycholate. In both conditions the K_D was ~ 53 nM while deoxycholate increased the number of binding sites from 3.5 to 37 pmol/mg protein. We concluded that the ouabain binding sites were located primarily on the inside of the isolated vesicle and that the vesicles were impermeable to ouabain. ATP induced a highly active Na^+ accumulation by the T tubules which increased Na^+ in the T tubular lumen by almost 200 nmol/mg protein. The accumulation had an initial fast phase lasting 2–3 min and a subsequent slow phase which continued for at least 40 min. The rate of the initial fast phase indicated a turnover number of 20 Na^+ /s. The Na^+ accumulation was prevented by monensin but was unaffected by valinomycin. Ouabain did not influence Na^+ uptake, but digitoxin inhibited it. At low K^+ the accumulation of Na^+ was reduced 3.7-fold below the value at 50 mM K^+ . ⁸⁶Rb, employed as a tracer to detect K^+ , showed a first phase of K^+ release while Na^+ was accumulated. After 2–3 min, K^+ was reaccumulated while Na^+ continued to increase in the lumen. T tubules accumulated Cl^- on addition of ATP. This suggested that ATP initiated an exchange of Na^+ for K^+ followed by uptake of Na^+ and K^+ accompanied by Cl^- .

INTRODUCTION

In the initiation of muscle contraction, an action potential is conducted from the membrane surface into the muscle fiber through the transverse tubules (T tubules) (Costantin, 1970; Bastian and Nakajima, 1974). The depolarization of the T tubules "triggers" Ca^{++} release from the sarcoplasmic reticulum (SR) (Huxley and Taylor, 1958; Peachey, 1965) which subsequently leads to the interaction of myosin and actin (Ebashi and Endo, 1968; Ebashi et al., 1969).

The depolarization of the T tubules involves the movement of Na^+ and possibly K^+ down their chemical gradients. It is considered that the uphill transport of these ions in relaxed muscle is carried out by the ouabain-sensitive Na^+ pump. The active and passive fluxes of ions across the T tubule membrane have been investigated in physiological experiments largely through indirect observations employing muscles which have been treated to separate the T tubule from the plasma membrane.

Recently, our laboratory has established a method for isolating T tubular vesicles from skeletal muscle (Lau et al., 1977). When [^3H]ouabain, used as an external membrane marker, was injected directly into the muscle or intravenously infused, [^3H]ouabain was trapped and retained within the T tubular vesicles throughout the procedure of isolation. Although we have also demonstrated a ouabain-sensitive ATPase in the T tubular fraction (Lau et al., 1977), the nature of ouabain trapping, the number of ouabain binding sites, and the integrity and direction of the Na^+ pump were not studied. The isolated T tubular system offers the opportunity to investigate directly some of the ion fluxes which occur in T tubules. In this paper, we describe our further characterization of ouabain binding properties of isolated T tubules and extend our investigations to the study of active Na^+ , K^+ , and Cl^- transport.

METHODS

Preparation of T Tubular Vesicles and SR Subfractions

The procedure for fractionation of rabbit sacrospinalis muscle microsomes into longitudinal SR, terminal cisternae (TC) (light band), TC (heavy band), and T tubular vesicles employed the methods described previously (Caswell et al., 1976; Lau et al., 1977) without KCl treatment of the TC/triad vesicles during isolation of T tubules.

Ouabain Binding

Specific ouabain binding of the microsomal fractions was determined in the medium described by Clausen and Hansen (1974) with some modifications. The medium contained 120 mM NaCl, 10 mM MgCl_2 , 1 mM EGTA, 40 mM Tris Cl, (pH 7.4) in the presence or absence of 10 mM Tris-ATP. The [^3H]ouabain concentration in the medium ranged from $2.5 \times 10^{-9}\text{M}$ to 10^{-6}M . The microsomal fraction containing 60–200 μg protein was incubated in 0.3 ml medium for 40–90 min at 37°C . After incubation, the sample was passed through a $0.22 \mu\text{m}$ Millipore filter (Millipore Corp., Bedford, Mass.). Each filter was then washed three times with 1 ml of ice cold medium that contained no ouabain. The filters were digested in a scintillation fluid which contained Triton X-100 (Rohm & Haas Co., Philadelphia), and the retention of [^3H]ouabain was determined from the scintillation counting and by counting standards. Specific binding is defined as the [^3H]ouabain retained per milligram protein in the presence of ATP subtracted from that in its absence. In addition, ouabain-binding assays were repeated in a medium with sodium deoxycholate at final concentrations of 0.015, 0.03, 0.045, and 0.05%. In calculating the Scatchard plots free ouabain was estimated from the ouabain added to the medium by subtraction of that amount which was bound to the vesicles.

Na⁺ and Rb⁺ Transport

Active Na⁺ and Rb⁺ transport of the isolated T tubular vesicles were examined by the radioisotope-Millipore filtration technique. The assay medium contained 5 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 30 mM imidazole, pH 7.2, and a concentration of NaCl, KCl, and choline Cl as specified in the legends of figures. The final anion and cation concentration was 60 mM. The exact concentrations of K⁺ and Na⁺ in the final medium were checked using a flame photometer. ²²Na⁺ and ⁸⁶Rb⁺, when added together as tracers, were present at concentrations of about 1 μCi per ml and 30 μCi per ml, respectively. ⁸⁶Rb⁺ was used instead of ⁴²K⁺ as a tracer for K⁺ because of its longer half-life. T tubular vesicles containing 40–130 μg protein were first incubated in 0.5 ml of the above assay medium at 37°C for 35–50 min. Tris-ATP (prepared by treatment of Na⁺ ATP with Dowex X-50 (Dow Corning Corp., Midland, Mich.) followed by neutralization with Tris base) or Tris Cl (5 mM) was then added, and incubation continued for a period described in each experiment. The experiment was terminated by rapid filtration through a 0.22 μm Millipore filter, followed by rapid washing of 1 ml of identical medium except containing no radioisotopes. When Na⁺ alone was assayed two washes were employed, whereas in the assay of Na⁺ and Rb⁺ three washes were carried out.

Radioisotope Assays

When ²²Na was the only isotope present, the activity was estimated in a scintillation fluid using a β counter. When ⁸⁶Rb and ²²Na were employed in double-labelling experiments, the different β- and γ-emitting properties of these two isotopes were employed to estimate the isotope contents individually. ²²Na could be estimated with negligible ⁸⁶Rb interference in a γ counter since only 8% of ⁸⁶Rb disintegrations are associated with emission of γ rays and it was further possible to employ pulse height separation to reduce ⁸⁶Rb interference. ⁸⁶Rb was estimated in a β counter. Since both ⁸⁶Rb and ²²Na emit β particles of high energy, it was necessary to employ a 30-fold higher ⁸⁶Rb activity in order to assay this isotope. ²²Na contribution to the total activity was subtracted using the γ-counting data. Accordingly, the dry filters were first counted in a γ counter and then transferred to a scintillation cocktail for β counting. Each γ tube was washed with 1 ml of water to ensure total transfer of isotope to the cocktail. Controls were always employed in which incubation medium without vesicles was passed through the filtration and assay procedure.

Cl⁻ Transport

ATP-induced Cl⁻ transport was assayed in a medium which contained 2.5 mM KCl, 2.5 mM NaCl, 2.5 mM Na³⁶Cl, 2.5 mM MgCl₂, 10 mM imidazole, 1 mM Tris EGTA, 100 mM sucrose, pH 7.2. 0.5 ml of medium containing approximately 100 μg of membrane protein was incubated for 50 min before addition of 10 mM Tris-ATP. At the end of the incubation period the suspension was filtered on a 0.22 μm Millipore filter and washed rapidly three times with 1.5 ml of ice cold suspension medium which lacked ³⁶Cl. The filters were counted in a β counter.

RESULTS

Ouabain Binding in T Tubules

The specific binding of ouabain and other cardiac glycosides to the Na-K ATPase from a variety of tissues and the requirement of the presence of ATP

and Mg^{++} for binding have been extensively characterized (Matsui and Schwartz, 1968; Tobin and Sen, 1970; Skou et al., 1971; Tobin et al., 1972; Erdmann and Schoner, 1973). The time-course of ouabain binding was first determined. Our data showed that maximal binding was obtained within 40 min. In subsequent experiments the incubation period was 1 h. Fig. 1 is the Scatchard plot of the ATP-dependent [3H]ouabain binding to isolated T tubular vesicles. The experimental conditions were described in Methods. This figure shows that the number of binding sites is 3.5 pmol/mg protein. The ouabain binding shows a single class of binding sites with a dissociation constant (K_D) of 5.41×10^{-8} M. This K_D value is similar to those found in HeLa cells (Baker and Willis, 1970), fat cell (Clausen and Hansen, 1974), an ox brain Na-K ATPase preparation (Hansen and Skou, 1973), and rat soleus muscle (Clausen and Hansen, 1974).

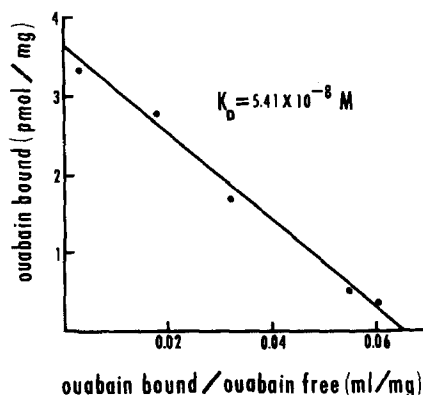


FIGURE 1. Scatchard plot of ATP-dependent [3H]ouabain binding to T tubular vesicles. The assay procedure was described in Methods. Each determination was derived from the binding assay of 60 μ g protein of T tubular vesicles in 0.3 ml medium with specific [3H]ouabain concentration ranges from 5×10^{-9} to 10^{-6} M.

When the T tubular vesicles were perturbed by the presence of deoxycholate, the specific ouabain binding sites increased sharply. We have assayed for the number of ouabain binding sites under a range of concentration of deoxycholate from 0.015 to 0.065% with a constant suspension of T tubular vesicles of 0.2 mg/ml. The highest number of binding sites is obtained when the deoxycholate present reaches 0.045%. At this deoxycholate concentration, the vesicle suspension retains its cloudy appearance and appears not to be dissolved. We detected that less than 1% of T tubular protein had passed through the 0.22 μ m Millipore filter at this detergent concentration. The Scatchard plot of [3H]ouabain binding under such a condition is shown in Fig. 2. The ATP-dependent ouabain binding reaches a maximum of 37 pmol/mg protein, which is about 10-fold that of the ouabain binding sites determined in the absence of detergent treatment. The Scatchard plot shows that the binding again exhibits a single class of binding sites with a K_D of $5.23 \times$

10^{-8} M. This K_D value is almost identical to the K_D value determined in the absence of deoxycholate (Fig. 1), indicating that the binding affinity of ouabain to its receptor is not affected by the presence of deoxycholate at this concentration. These data are consistent with our previous conclusion that the ouabain binding sites are on the inner surface of the lumen of the resealed T tubular vesicles (Caswell et al., 1976; Lau et al., 1977). The inaccessibility of external ouabain to the binding sites of the vesicles, except after detergent treatment, gives further support to the conclusion that the isolated vesicles are mainly intact and resealed to provide an effective barrier to nonspecific permeation of solutes.

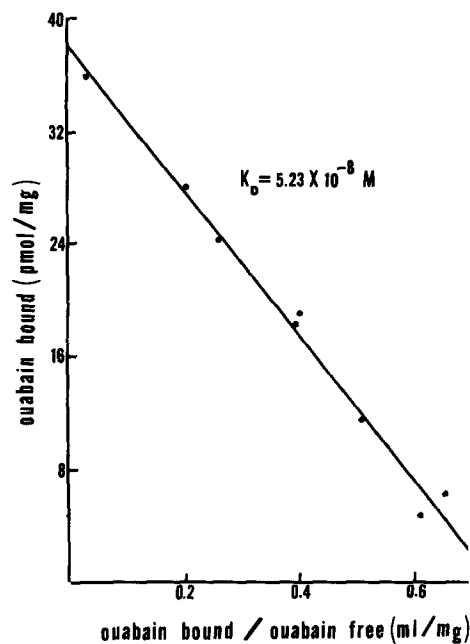


FIGURE 2. Scatchard plot of ATP-dependent [3 H]ouabain binding to T tubular vesicles in the presence of deoxycholate. The assay condition is the same as in Fig. 1 except that the medium contains 0.04% deoxycholate.

Active Na⁺ Transport in T Tubules

The existence of a sodium pump in the T tubules, the integrity of these resealed vesicles, and their apparent orientation with the cytoplasmic side on the outside of the vesicles led us to examine sodium transport in these vesicles. Isolated T tubular vesicles were incubated in the sodium uptake medium for 45 min to 1 h as described in Methods, so that equilibrium distribution of ions inside and outside the vesicles could be approached. Then ATP was added and the vesicle suspension was incubated for a specific time interval before filtering through the Millipore filter. The active Na⁺ uptake process is illustrated in Fig. 3. Upon the addition of ATP at time 40 min, there is a

rapid phase of Na^+ transport into the T tubular vesicles in the first 2–3 min, followed by a much slower phase of Na^+ accumulation that does not level off 50 min after the initiation of uptake. During the first 2 min, the T tubular vesicles accumulate 90 nmol Na^+ per mg protein. Since we have determined that the T tubular vesicles have ouabain binding density of 37 pmol/mg, we estimated from the initial slope of this figure that the initial turnover rate of the Na^+ pump is 20 Na^+ per ouabain binding site per second at 37°C. Clausen and Hansen (1974) estimated that the Na^+ transport rate per ouabain binding site in intact rat muscle fibres was eight per second. They attributed the slow rate of transport to their partially K^+ -depleted cell and assay at a lower temperature (30°C).

Table I summarizes further experiments in the characterization of the Na^+ transport in the T-tubular vesicles. Experiment I shows that these vesicles are

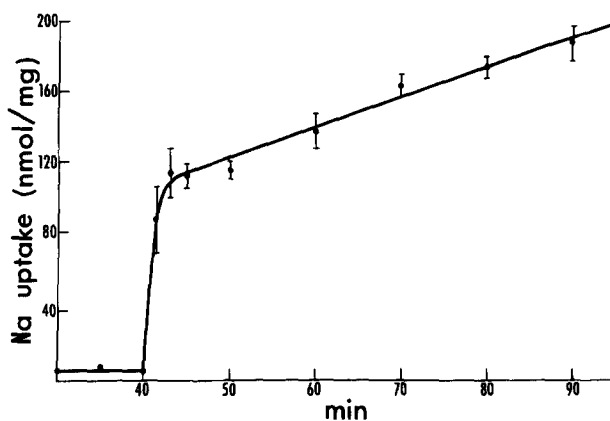


FIGURE 3. Active Na uptake of T tubular vesicles. 30–40 μg protein of T tubular vesicles was incubated in 0.5 ml of 5 mM NaCl, 50 mM KCl, 5 mM MgCl_2 , 1 mM EGTA, 30 mM imidazole, and NaCl (0.2 $\mu\text{Ci/ml}$) at 37°C. After 40 min, 5 mM Tris-ATP was then added and incubated for a further time interval as shown in the graph before Millipore filtration. Each point represents the average of three determinations. Each bar represents 1 SD.

able to accumulate 198 nmol Na^+ per mg protein in 40 min after the addition of ATP, whereas, under identical conditions, the TC (light band) and TC (heavy band) accumulate only 1.7 nmol/mg. This insignificant amount of Na^+ uptake may be accounted for by the contamination of a few T tubular vesicles in these microsomal subfractions.

Experiment II in Table I shows the effects of the ionophores, monensin and valinomycin, on the Na^+ transport process (Pressman, 1976). Monensin is an exchange diffusion carrier which is able to exchange Na^+ for K^+ or H^+ while valinomycin transports K^+ selectively in electrophoretic transport. When 1×10^{-6} M monensin is present in the incubation medium, the active Na^+ accumulation has been almost completely abolished. The data indicate that active Na^+ accumulation in the vesicles results in the formation of a Na^+ activity gradient across the membrane which is abolished when the membrane

becomes highly permeable to Na^+ due to the presence of monensin. On the other hand, the presence of 9×10^{-7} M valinomycin in the uptake medium has no effect on Na^+ accumulation of the T tubular vesicles. Since the initial stock solutions of monensin and valinomycin were made up in ethanol, a control run which showed that an equivalent concentration of ethanol (0.005–0.02%) had no significant effect on Na^+ accumulation (data not shown).

We have also examined the effects of ouabain and digitoxin on the Na^+ transport of T tubular vesicles. Ouabain at 1×10^{-5} M has no significant effect on this transport process. On the other hand, 2×10^{-6} M digitoxin inhibits more than 80% of the active Na^+ accumulation in comparison with its control. Since ouabain and digitoxin have similar potencies as inhibitors of

TABLE I
ACTIVE Na^+ TRANSPORT

Experiment	Samples	Additions	Na^+ uptake (mean \pm SD) <i>nmol/mg</i>
I	T tubules		198.0 \pm 26.0
	TC (light band)		1.7 \pm 0.8
	TC (heavy band)		1.7 \pm 0.7
II	T tubules		168.0 \pm 38.7
	T tubules	1×10^{-6} M monensin	6.5 \pm 1.4
	T tubules	9×10^{-7} M valinomycin	155.0 \pm 11.3
III	T tubules		168.0 \pm 38.7
	T tubules	1×10^{-5} M ouabain	158.5 \pm 22.0
	T tubules	0.05% ethanol	97.5 \pm 3.5
	T tubules	2×10^{-6} M digitoxin + 0.05% ethanol	19.0 \pm 8.5

All the assay media contain 5 mM NaCl, 50 mM KCl, 5 mM MgCl_2 , 1 mM EGTA, 30 mM imidazole, and ^{22}Na as tracer except for the medium used for determining the effect of ouabain or digitoxin, which in addition contains 1 mM NaH_2PO_4 . Other reagents added were as indicated in the Table. After 40 min incubation, 5 mM Tris-ATP or 5 mM Tris Cl was added and the sample was incubated for another 40 min before filtration. Each value represents the average of three to four determinations \pm SD.

the Na^+ pump, the ineffectiveness of ouabain in inhibiting the Na^+ transport is likely to be associated with its much poorer membrane permeability compared to digitoxin (Greenberger et al., 1969). This finding is in accord with our [^3H]ouabain binding data that the binding sites are on the inner surface of the membrane of these resealed vesicles. Hence, only digitoxin which is able to penetrate the membrane is able to interact with the Na^+ pump and subsequently leads to the inhibition of Na^+ transport. In this case the ethanol (0.05%) control for digitoxin shows some inhibition of Na^+ accumulation.

Fig. 4 shows the K^+ dependency of active Na^+ accumulation in T tubular vesicles. Over the range from 0.02 to 50 mM K^+ , there is a continuous increase of Na^+ accumulation with increase of K^+ concentration in the medium.

Further analysis of the K^+ dependency is complicated by the dual influence of K^+ on the generation of the Na^+ gradient. A coupled translocation of K^+ and Na^+ is required for the operation of the Na^+ pump; also, the steady-state gradient of Na^+ will depend on the K^+ gradient generated. Thus, the K^+ dependency of Na^+ accumulation may have a kinetic component and an equilibrium component since K^+ may be required to activate the Na^+ pump, and also the K^+ gradient will influence the final Na^+ gradient. In our experiments, active Na^+ accumulation occurs slowly over a long time period without reaching equilibrium. The incubation time in the presence of ATP for the experiment was 40 min. Skou (1964) examined the effect of increasing concentrations of K^+ on Na-K ATPase of crab nerve at a fixed concentration

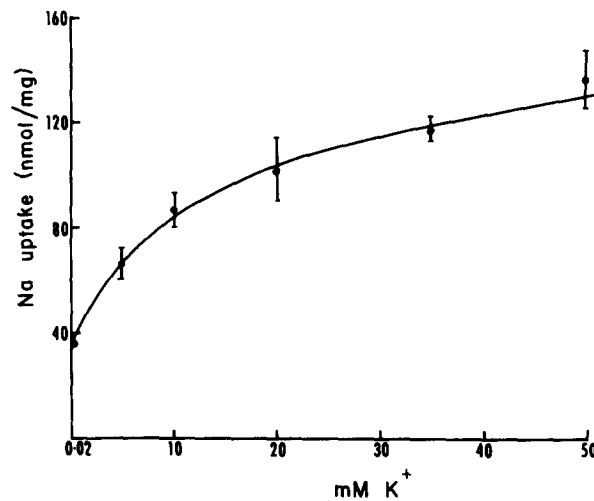


FIGURE 4. K dependency of active Na uptake in T tubular vesicles. The assay conditions are the same as described in the legend to Fig. 3 except that the incubation medium contains variable concentrations of KCl and choline Cl with their sum concentration equal to 50 mM. Each point represents the average of three determinations. Each bar represents 1 SD.

of Na^+ in the medium (100 mM). He found that the rate of ATP hydrolysis increases with increased concentration of K^+ until about 50 mM. Fig. 4 shows that Na^+ accumulation still occurs to a considerable level even at the low concentration of K^+ of 20 μM . This suggests that an obligate K^+ flux may not be necessary for operation of the Na^+ pump. It is possible either that Cl^- is able to replace K^+ and serve as the co-ion for Na^+ in a low K^+ environment or that there is a high-affinity binding site for K^+ on the enzyme. Also an ATP-stimulated exchange of $^{22}Na^+$ for $^{23}Na^+$ may occur although our long preincubation in the medium containing $^{22}Na^+$ would suggest that the T tubules have already approximated equilibrium distribution of Na^+ . A similar finding has been reported for purified renal Na^+ pump by Goldin and Tong (1974).

Coupled Na, K and Cl Transport in T Tubules

We have further studied the ion translocation effected by the Na^+ pump in isolated T tubular vesicles using ^{22}Na and ^{86}Rb as tracers in double labelling experiments. $^{86}\text{Rb}^+$ was used instead of $^{42}\text{K}^+$ as a tracer for K^+ because of its longer half-life and lower expense. It has been shown to substitute for K^+ both in stimulation of Na^+ - K^+ hydrolytic activity and in the ouabain-sensitive active transport of K^+ across the membrane of several types of cells (Bonting, 1970). Sweadner and Goldin (1975) reported that Rb^+ and K^+ have a similar K_m and induce comparable V_{\max} for Na-K ATPase activity in brain microsomes. Sandow and Mandel (1951) and Sjodin (1961) have shown that the muscle membrane permeability to Rb^+ lies between one-third and one-half of that of K^+ . Therefore Rb^+ has been established and employed by many workers as an adequate tracer for K^+ flux in biological membranes with similar kinetic properties.

Fig. 5 shows the Na^+ and K^+ transport process of T tubular vesicles. ATP was added to the uptake medium (indicated by arrow) after an incubation interval of 50 min at 37°C . Upon the addition of ATP, there is a rapid phase of Na^+ accumulation and K^+ release in the first 1.5 mins. Within this interval, the Na^+ content of the T tubular vesicles rises from about 4.4 to 47 nmol/mg, whereas the K^+ content drops from about 65 to 28 nmol/mg. The rapid Na^+ uptake phase is then followed by a gradual slowing of Na^+ uptake that does not level off 20 min after the addition of ATP. On the other hand, the rapid phase of K^+ release that accompanied Na^+ uptake in the first 2 min is immediately reversed to give a distinct K^+ reuptake. The Na^+ and K^+ contents of the T tubular vesicles at 10 min after ATP addition are about 70 and 36 nmol/mg, respectively. In the second phase of Na^+ accumulation both Na^+ and K^+ move into the vesicle. This requires the simultaneous movement of a counterion or co-ion in order to maintain a balanced movement of ions. Technical difficulties in assaying the very low levels of ^{86}Rb retained in the T tubules when the levels of ^{86}Rb in the medium are high have caused significant random variation in the assays. However, Fig. 5 represents the average of those experiments in which the basic character of the K^+ and Na^+ flux have been the same including the initial fast exchange of Na^+ for K^+ and the subsequent accumulation of K^+ when Na^+ is also accumulated.

The data of Fig. 5 suggested the presence of ion movements other than those of Na^+ and K^+ . Since Cl^- represented the other significant ion in the medium, we tested to determine whether ATP stimulated an influx of Cl^- . We were not able to employ the same medium as that of Fig. 5 since the high external Cl^- caused high residual counts on the filter compared with the Cl^- retained by the vesicle. Hence $[\text{Cl}^-]$ was lowered to 12.5 mM. Fig. 6 shows the average of two experiments in which ATP stimulates Cl^- uptake into isolated T tubules. Upon the addition of ATP after 50 min of prior incubation in ^{36}Cl there is an increase of T tubular Cl^- . The control curve in the absence of ATP (dashed line) shows no accumulation and also suggests that Cl has distributed passively to equilibrium before ATP administration. The high degree of variation in the data is intrinsic to these Cl^- measurements and will be

discussed later. However, the increase in T-tubular Cl^- after administration of ATP is clear and unambiguous. An unpaired *U* test evaluation of the aggregate data obtained after ATP addition compared with those in the absence of the ATP indicates a *P* value of <0.001 which is highly significant. Several other experiments were performed which confirmed the observation of an increase in Cl^- in the T tubule after ATP addition. The triangles (dotted line) represent an identical experiment in which TC (light band) were employed in place of T tubules. The experiment shows that ATP causes no stimulated Cl^- movement in this medium (presence of EGTA) and hence that the Cl^- flux in the isolated T tubules is not a consequence of the presence of contaminating terminal cisternae.

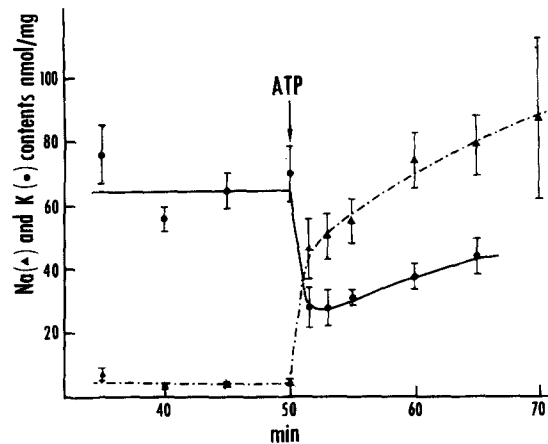


FIGURE 5. Na and K transport in T tubular vesicles in the presence of ATP. 120 μg protein of T tubular vesicles was incubated in 0.5 ml medium of 5 mM NaCl, 50 mM KCl, 5 mM MgCl_2 , 1 mM EGTA, 30 mM imidazole, pH 7.4, and ^{22}Na (1 $\mu\text{Ci/ml}$) and $^{86}\text{Rb}^+$ (30 $\mu\text{Ci/ml}$). After 50 min incubation 5 mM Tris-ATP was added and incubated for further time intervals before Millipore filtration. Each value represents the average of seven determinations in three separate preparations. Each bar represents the standard error of the mean.

DISCUSSION

Previous electrophysiological and morphological investigations (Porter and Palade, 1957; Huxley and Taylor, 1958; Falk and Fatt, 1964) have demonstrated the functional role of the T tubule in excitation-contraction coupling. However, up to the present, the nature of the signal transmitted through the triad junctions and the mechanism of rapid release of Ca^{2+} from the SR both remain unclear (Endo, 1977). The difficulties arise in part from the inaccessibility of the T tubule to biochemical and electrophysiological studies as a single system without the interference of other membrane components of the muscle fiber. The isolated T tubule provides a unique opportunity to study directly its functional properties and its interaction with the SR.

This study demonstrated the existence of an Na pump in the T tubule.

[³H]ouabain binding studies show that it has specific ouabain binding sites of 37 pmol/mg with a K_D of 5.2×10^{-8} M. These ouabain binding sites are manifested only in the presence of detergent. We interpret the data to indicate that the ouabain binding sites are on the inner surface of these vesicles and ouabain is accessible to these sites only when the membrane becomes leaky in the presence of deoxycholate. The argument may be proposed that the enhancement of [³H]ouabain binding on addition of detergent is due to enhancement of membrane leakiness to ATP and that binding of ATP to specific sites on the inner surface of the vesicles is required for the binding of

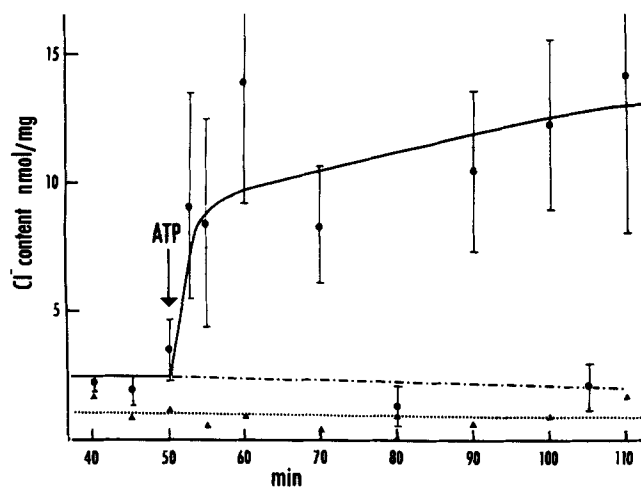


FIGURE 6. Active Cl^- uptake of T tubule vesicles. The medium contains 2.5 mM Na^{36}Cl (84 $\mu\text{Ci}/\text{ml}$), 2.5 mM KCl , 2.5 mM NaCl , 2.5 mM MgCl_2 , 10 mM imidazole, 1 mM Tris EGTA, 100 mM sucrose pH 7.2. After 50 min, 10 mM Tris-ATP was added as indicated by the arrow and incubated for different times before Millipore filtration. Each point represents the average of six determinations from two separate preparations. Each bar represents the standard error of the mean. The dashed line represents the base line of Cl^- content in T tubules in the absence of ATP. The dotted line (\blacktriangle) is the Cl^- content in terminal cisternae (light band) from the same preparation as one of those employed for the T tubule uptake studies. ATP was added to this preparation at the arrow. The experimental conditions are identical to those of the T tubule vesicles.

[³H]ouabain on the external surface. However, we have reported previously (Caswell et al., 1976; Lau et al., 1977) that when [³H]ouabain is injected into the muscle before homogenization, [³H]ouabain is trapped inside the lumen of resealed T tubules of triads in the microsomal fraction. All the [³H]ouabain remains trapped inside these T tubular vesicles after they are detached from the TC vesicles by a French press procedure. Hence, throughout the isolation process, the T tubular vesicles remain as resealed vesicles with the extracellular spaces enclosed within. The T tubules are clearly oriented with the extracellular space on the inside when they form part of the isolated triad junction.

Also, we have found that the active Na transport was inhibited by externally added digitoxin but not by ouabain. In view of the fact that digitoxin has a very much higher membrane permeability than ouabain (Greenberger et al., 1969), this finding is in accord with our interpretation that the ouabain binding sites are on the inner surface of the T tubular vesicles. Thus the integrity of the vesicle membrane is high and nonspecific leakage appears to be negligible.

The high degree of impermeability of T tubules to ouabain gives evidence of the structural integrity of the isolated preparation. This is particularly remarkable in view of the period of 3 d which separates the initial stages of the preparation from the final solution and assay of vesicle integrity. An effective barrier to permeation of lipid insoluble solutes is a prerequisite for study of physiological solute fluxes. Our data showing the impermeability of T tubules establishes the potential for obtaining further information on permeability and active transport of ions. This has constituted the second phase of this investigation.

We have employed radioactive tracers and ultrafiltration as our means of following ion fluxes. This represents the most direct and unequivocal method of following the fluxes of ions. However, some experimental difficulties are manifest when very low levels of ions are trapped within very small spaces. The ultrafiltration effectively separates external ions from trapped ions only if the filter is washed. Although we could make effective measurements of Na⁺ content after two washes, we found it necessary to wash three times in assays of K⁺ or Cl⁻ movements owing to the low accumulation of the ions within the vesicles and the high concentration of ions present in the incubation medium. Thus, some ion efflux or isotope exchange may occur during the wash. However, our findings indicate that the T tubule is permeable to both K⁺ and Cl⁻. The variability in the data particularly with Cl⁻ may indicate that efflux of ³⁶Cl has occurred during washing and suggests that our estimate of T tubular ³⁶Cl may be low.

We observe that the active Na⁺ uptake of the T tubular vesicles is a two phase process: a rapid phase in the first 2 min followed by a much slower phase that does not level off 20–40 min after the addition of ATP (Figs. 3 and 5). The slow phase of Na uptake may be associated with a slower turnover rate of Na pump after the Na⁺- and K⁺ gradients across the membrane have been generated and is possibly associated with a swelling of the T tubular vesicles as the internal osmotic pressure increased following ion accumulation. The possibility that some of the vesicles accumulate Na⁺ at a very slow rate has not been precluded.

Our studies using ²²Na and ⁸⁶Rb as tracers enable us to monitor the translocation of both Na⁺ and K⁺ by the Na pump in resealed T tubular vesicles. Upon the addition of ATP, there is a rapid Na accumulation of 43 nmol/mg coupled to rapid K⁺ release of 37 nmol/mg within the first 1.5 min. Hence, during this rapid phase, there is a coupled Na and K translocation. Our data do not permit us to establish the stoichiometry accurately especially since this phase of exchange lasts only for 1 or 2 min. It is possible that initially Na⁺ and K⁺ exchange account for the full ion movement of the pump in a 1:

l exchange. After this rapid phase of counter transport of Na^+ and K^+ , the uptake of Na^+ becomes slower and this is accompanied by reuptake of K^+ . Hence, during the 3–10 min interval after the ATP addition, there are net uptakes of 20 nmol/mg Na^+ and 10 nmol/mg K^+ . In order to maintain the electrical neutrality, the influx of both K^+ and Na^+ must be accompanied by the influx of 30 nmol/mg of an anion during this time interval. Although we have not been able to assay Cl^- under identical conditions to those of Fig. 5, we have demonstrated that ATP induces influx of Cl^- which is therefore consistent with the supposition that Na^+ and K^+ reuptake is accompanied by Cl^- accumulation. A Cl^- permeability in T tubules of rat diaphragm has been described (Palade and Barchi, 1977).

As with most methods for assaying ion fluxes in isolated organelles, the use of radioactive tracers gives information about the net amount of ion accumulated but does not give direct information on the internal concentration. Technical difficulties may well limit the accuracy or time resolution of assays of internal volume in these small vesicles. An increase in internal volume may occur during Na^+ accumulation in order to maintain osmotic equilibrium across the membrane especially in the slow second phase in which Cl^- is being accumulated.

We observed that the active Na uptake of T tubular vesicles is completely abolished by the presence of monensin, a neutral exchange ionophore which is capable of exchanging Na^+ for K^+ or H^+ (Pressman, 1976), whereas the presence of valinomycin, a K^+ selective ionophore, has no effect on the Na^+ uptake. We interpret this to indicate that an Na^+ gradient across the T-tubular membrane is generated by the Na pump and this gradient is then abolished by the increased membrane permeability to Na by the added monensin. On the other hand, the increase of membrane permeability to K^+ has no effect on the Na^+ gradient generated in the steady state. These data and the K^+ influx studies discussed above suggested that during the resting state of a muscle fibre, the Na^+ gradient across the T tubular membrane is maintained by an active Na pump and the intrinsic low membrane permeability to Na^+ .

This communication establishes for the first time the physiological integrity of our isolated T tubular preparation and indicates the potential for studying in depth the ion movements and alterations of electrical potential which T tubules undergo under physiological stimuli. Furthermore, the high stability of the preparation suggests its value as a model for general biochemical studies on excitable membrane.

This work was supported by research grants from the U.S. Public Health Service (1 R01 AM-21601) and the American Heart Association. Dr. Lau is a Muscular Dystrophy Association Postdoctoral Fellow; Dr. Letellier is a North Atlantic Treaty Organization Postdoctoral Fellow.

Received for publication 12 June 1978.

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