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Ion pathways in transverse tubules. Quantification of receptors in membranes isolated from frog and rabbit skeletal muscle

Enrique Jaimovich, Paulina Donoso *, Jose Luis Liberona and Cecilia Hidalgo **

Departamento de Fisiologia y Biofisica, Facultad de Medicina, Universidad de Chile, Casilla 137-D, Santiago and Centro de Estudios Cientificos de Santiago, Casilla 16443, Santiago 9 (Chile)

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The presence of four cation pathways in membrane vesicles isolated from transverse tubules of frog and rabbit skeletal muscle was studied by measuring binding of specific blockers. Transverse tubules purified from frog muscle have a maximal binding capacity for [^{3}H]nitrendipine (a marker for voltage-dependent calcium channels) of 130 pmol/mg of protein; this binding is strongly dependent on temperature and, at 37°C, on the presence of diltiazem. Receptors for [^{3}H]ethylenediamine tetrodotoxin (a marker for voltage-dependent sodium channels) and for ^{125}I-labeled α-bungarotoxin (a marker for acetylcholine-mediated channels) showed maximal binding values of about 5 pmol/mg. The number of sodium-pumping sites in the isolated tubule vesicles, inferred from [^{3}H]ouabain binding, was 215 pmol/mg. The high purity of this preparation makes feasible the use of these values as a criterion to judge the degree of purity of isolated preparations, and it allows investigation of transverse tubule contamination in other muscle membrane fractions.

Introduction

Muscle physiology, and in particular the coupling between excitation and contraction (E-C coupling), has been the subject of numerous studies in the last 15 years. A major part of this research has been concentrated in frog muscle, which allows easier access to individual fibers than mammalian muscle.

Ion channels and ion transport systems are likely to be of crucial importance in the process of E-C coupling. In the intact muscle fiber, the transverse tubule (T-tubule) membrane is not easily accessible to direct measurements using electrophysiological records. It is not easy either to carry out direct determinations of ion fluxes or to determine binding to T-tubules in intact fibers. In order to study these phenomena in isolated membrane fractions we need to have preparations of known origin and purity. Furthermore, to correlate the information obtained using isolated membrane fractions with physiological parameters, it appears desirable to isolate pure preparations from frog muscle.

Several different methods to isolate skeletal muscle membranes have been described in the

* From the Departamento de Ciencias Preclínicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile.
** On leave from the Department of Muscle Research, Boston Biomedical Research Institute, and the Department of Neurology, Harvard Medical School, Boston, MA, U.S.A.

Abbreviations: E-C coupling, excitation-contraction coupling; T-tubules, transverse tubules.

literature [1]; variable proportions of surface membranes, T-tubule membranes and sarcoplasmic reticulum membranes are present in these isolated membrane fractions. In the last few years, progress has been made towards obtaining membrane fractions of various origins. Thus, methods to isolate membrane fractions containing purified sarcoplasmic reticulum [2,3], T-tubule [4–6], or enriched in plasma membrane [7–9] or triads [10,11] have been reported.

Studies on isolated T-tubules have established that several specific cation pathways are located in this particular structure. Among them are voltage-dependent sodium channels [12], voltage-dependent calcium channels [13], a calmodulin-dependent calcium pump [14] and a sodium pump [15]. The presence of acetylcholine channels [16] remains to be established, since the evidence in the literature is rather indirect.

In the preceding paper [17] we described the isolation of a purified T-tubule membrane fraction from frog skeletal muscle and we characterized its protein and cholesterol contents and various enzyme activities. In this work, we describe and quantify the receptors for blockers of several ion pathways present in this preparation, and we discuss the possible roles that the corresponding channels or transport systems might play in the intact T-tubule.

As a marker for the voltage dependent sodium channel we used tetrodotoxin derivatives [18], that have been shown to have different affinity for surface and tubular receptors [9,12]. As markers for voltage dependent calcium channels, we used the binding of [^{3}H]nitrendipine, a dihydropyridine known to block calcium currents [19] that binds specifically to T-tubule membranes [13,20]. Sodium pump sites and acetylcholine channels were quantitatively determined from [^{3}H]ouabain and ^{125}I-labeled α-bungarotoxin binding studies, respectively.

Methods

Preparation of isolated membranes. T-tubule membranes were isolated from rabbit skeletal muscle as previously described [21]. T-tubule membranes were obtained from frog skeletal muscle using a similar procedure, with slight modifications as described in detail in the previous article [17]. A fraction rich in surface membranes was obtained from frog skeletal muscle by a procedure that includes a prolonged incubation in high salt solutions [9].

Binding experiments. [^{3}H]Ouabain binding was measured by rapid filtration over Millipore filters as previously described [15,17]; either an excess of unlabeled ouabain or omitting ATP was used to measure the non-specific binding. A protein concentration of 0.01 to 0.02 mg per ml was used.

α-Bungarotoxin binding. Binding of ^{125}I-labeled α-bungarotoxin was measured in a final volume of 0.5 ml of incubation solution containing 115 mM NaCl; 2.5 mM KCl; 1.8 mM $CaCl_2$ and 2.0 mM Tris-HCl (pH 7.2). 0.02 to 0.03 mg of membrane protein per ml were incubated for 30 to 40 min with ^{125}I-labeled α-bungarotoxin (0.2–6 nM with or without unlabeled α-bungarotoxin (2 μM) to measure non specific binding. Saponin (0.2 mg/ml) was present in some experiments. The reaction medium was cooled down and duplicate 0.2-ml fractions were quickly filtered under reduced pressure through HA (0.45 μm) Millipore filters, previously wetted with washing solution. Filters were rinsed twice with 3 ml of a solution containing 120 mM choline chloride and 20 mM Tris-HCl, pH 7.2 (washing solution). Radioactivity remaining in the filters was measured in a liquid scintillation counter.

[^{3}H]Ethylenediamine tetrodotoxin binding. [^{3}H]Ethylenediamine tetrodotoxin derivative II of tetrodotoxin was synthesized and purified as described [12,18]. Binding assays were performed by fast filtering over glass fibers using the incubation conditions described elsewhere [12]. A protein concentration of 0.02 to 0.03 mg per ml was used.

[^{3}H]Nitrendipine binding. [^{3}H]Nitrendipine receptors were quantified as described by Fosset et al. [13]. Special care was taken to filter and rinse the membranes in less than 5 s in the cold. Dihydropyridine containing solutions were always protected from light, and binding experiments were carried out in dim light.

Materials

[^{3}H]Ouabain and [^{3}H]nitrendipine were obtained from New England Nuclear, ^{125}I-labeled

α-bungarotoxin was obtained from Amersham Corp. [^{3}H]Ethylenediamine tetrodotoxin was a kind gift from Professor M. Lazdunski and Dr. A. Lombet, Nice. All reagents used were of analytical grade.

Results

Ouabain receptors

The presence of sodium pump sites in the T-tubule membrane was studied by measuring ouabain receptor density. A saturable component for binding as a function of [^{3}H]ouabain concentration was obtained (Fig. 1), and the non specific (linear) component for binding was almost negligible at low concentrations of ouabain. The Scatchard analysis for the specific binding component (not shown) shows a single family of receptors, with a high maximal binding capacity (237 pmol/mg protein) and a K_d of 10 nM. Ouabain binding in T-tubule membranes isolated from frog muscle is higher than that obtained in rabbit muscle

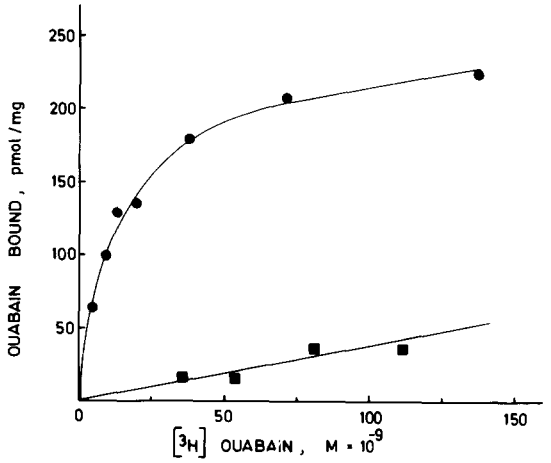

Fig. 1. [^{3}H]Ouabain binding to T-tubule membrane sisolated from frog muscle. Binding was measured as described elsewhere [17]. The points show total (●) and non-specific binding measured in the absence of ATP (■). The continuous curve for the specific (total minus non-specific) binding was fitted to a K_d of 11 nM and a B_{max} of 237 pmol/mg protein.

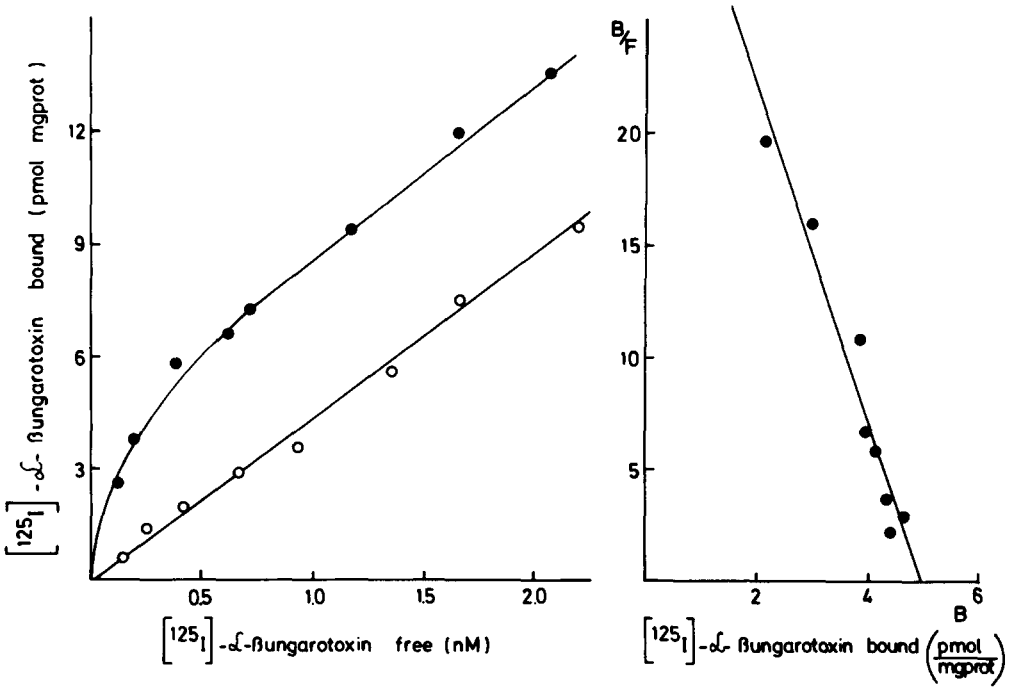

Fig. 2. α-Bungarotoxin binding to T-tubule membranes from frog muscle. Binding was measured as described under Methods. Left: total binding (●) and non-specific binding measured in the presence of an excess of unlabelled α-bungarotoxin (○). Right: Eady-Scatchard plot for the specific (total minus non-specific) binding component. The curve was fitted to a K_d of 0.13 nM and a B_{max} of 5 pmol/mg protein.

membranes (Table I) when both are fully expressed in the presence of detergent (0.2 mg/ml saponin). It is important to compare binding in the presence of detergents, since ATP-dependent ouabain binding involves sites present in both sides of the membrane and T-tubule vesicles are usually more than 80% sealed [17].

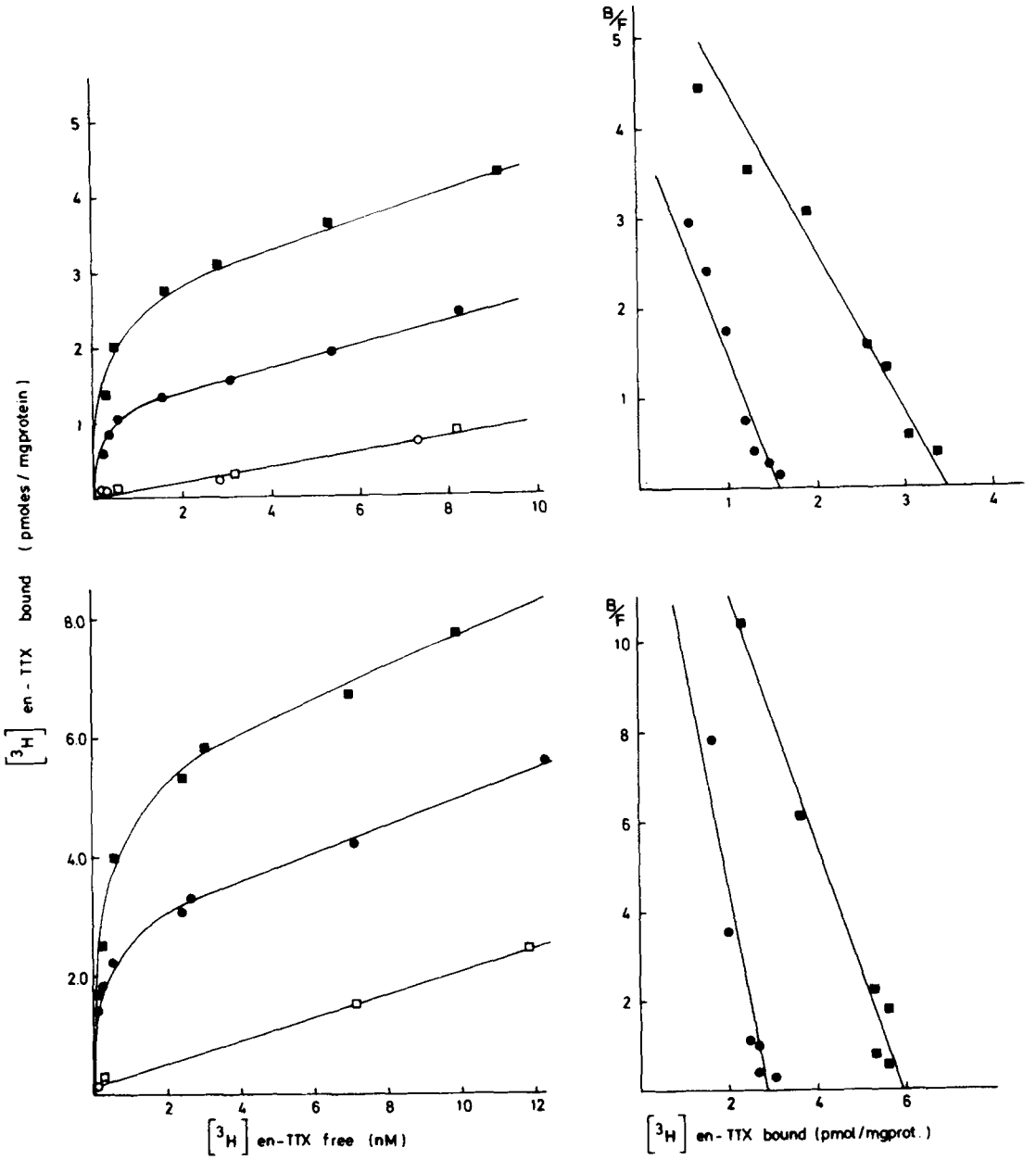

Fig. 3. [^{3}H]Ethylenediamine tetrodotoxin binding to T-tubule membrane isolated from frog and rabbit skeletal muscle. Binding to membranes from rabbit muscle (upper panel) and from frog muscle (lower panel) was measured as described [12] both in the presence (■) and in the absence (●) of 0.2 mg/ml saponin. Non-specific binding, measured in the presence of 10 μM tetrodotoxin (□, ○), was subtracted for all points. Curves in the Eady-Scatchard plots (right) were fitted to equations that give values of K_d and B_{max} described in the text.

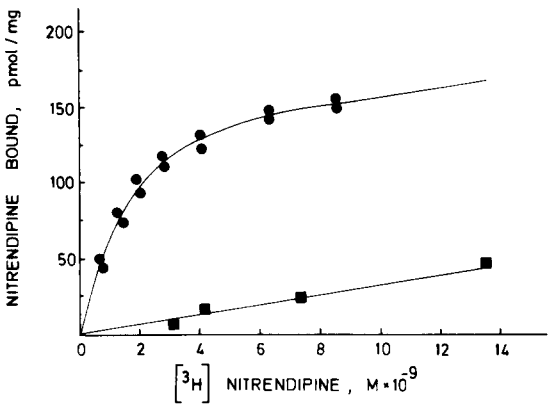

Fig. 4. [^{3}H]Nitrendipine binding to T-tubule membranes from frog muscle. Total binding was measured as described [13] both in the presence (non-specific binding) (■) and in the absence (●) of 1 μM unlabeled nifedipine. The continuous curve for the specific (total minus non-specific) binding component was fitted to a K_d of 1.1 nM and a B_{max} of 154 pmol/mg protein.

α-Bungarotoxin binding

^{125}I-labeled α-bungarotoxin receptor density was measured using either cold bungarotoxin or d-tubocurarin to quantify the non-specific binding component; both compounds were equally effective in blocking specific binding. A saturable (specific) component was easily seen in these membranes (Fig. 2) although the non-specific binding sites were always present in a significant amount (10–15% in the K_d region). A dissociation constant in the order of 0.1 nM was found both for frog (Fig. 2) and rabbit membranes (Table I), the maximal binding capacity being between 4 and 5 pmol/mg. The unmasking effect of saponin on this binding was less effective than that obtained when measuring ouabain [17] or ethylenediamine-tetrodotoxin binding (Fig. 3). Only 30% stimulation of α-bungarotoxin binding was observed after saponin addition (data not shown); this may reflect limited access of the polypeptide toxin to the receptor even in the presence of saponin, so that the maximal number of binding sites might be underestimated.

Tetrodotoxin receptors. As previously seen [9,12], a single family of tetrodotoxin receptors was found in T-tubule membranes isolated from rabbit or frog muscle (Fig. 3). Binding increased when vesicles were treated with saponin, indicating that an important fraction of the vesicles have cryptic

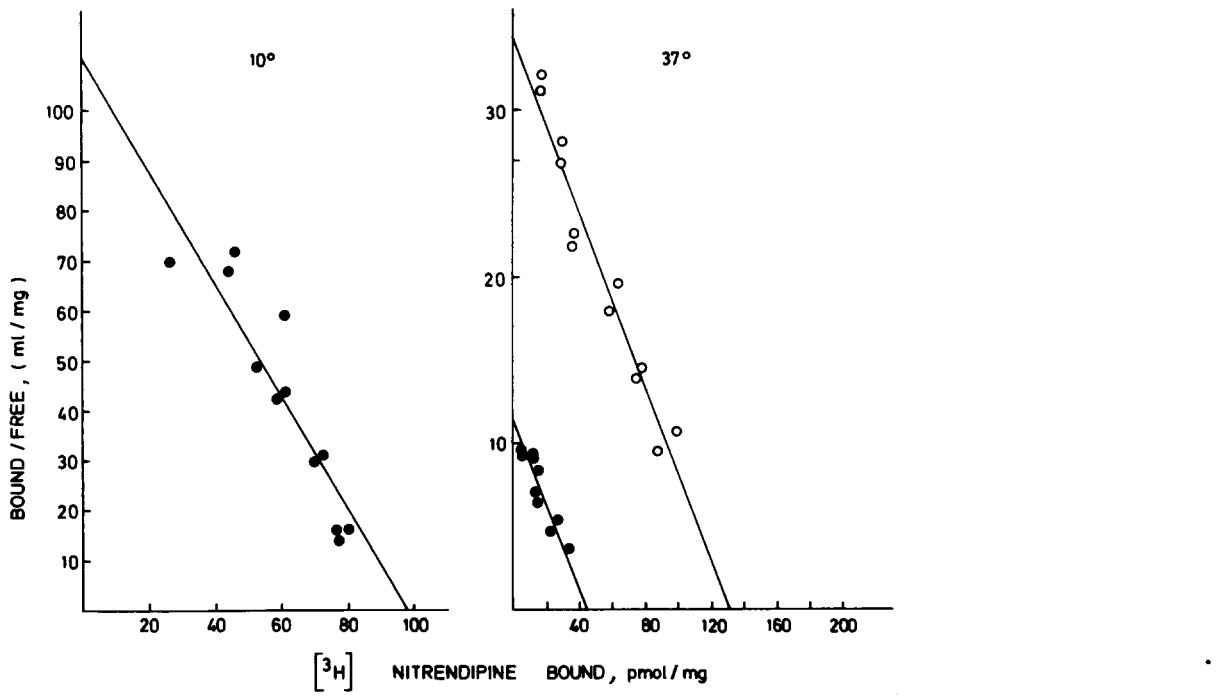

Fig. 5. Effect of temperature and diltiazem on [^{3}H]nitrendipine binding to T-tubules from frog muscle. Right panel: [^{3}H]nitrendipine binding at 37°C with (○) or without (●) 10 μM diltiazem.

receptors. This observation agrees with the previous findings that most vesicles are sealed with the inside-out configuration [17]. It is interesting to note that saponin caused a slight variation in K_d for both frog and rabbit membranes; the values in this case are 0.19 nM without saponin and 0.35 nM with saponin for frog membranes, and 0.34 nM and 0.60 nM without and with saponin for rabbit membranes, respectively. The significance of such differences remains to be studied.

[^{3}H]Nitrendipine receptors. In agreement with results previously described [13], T-tubules displayed a large number of dihydropyridines binding sites (Fig. 4). Although the K_d values were identical to those found previously [13], the maximal binding capacity was somewhat higher for rabbit, and 3–4-times higher for frog membranes, with respect to those previously reported [13]. This higher density probably reflects the fact that we have isolated a more purified preparation. It is important to note that nitrendipine binding was also measured in the presence of saponin (data not shown) and no difference was seen either in K_d or in B_{max} when compared to the binding in the absence of detergents.

An interesting observation regarding dihydropyridine binding to muscle membranes is the effect of temperature and the possible allosteric interactions of the nitrendipine receptor with the calcium channel modulator diltiazem [22]. The Scatchard plots shown in Fig. 5 evidence a marked effect of temperature on B_{max} for [^{3}H]nitrendipine binding. At 37°C, B_{max} is 44 pmol/mg (right panel), about one third of the number of receptors measured at 10°C (125 pmol/mg, left panel). Whereas the presence of diltiazem has little or no effect on binding at 10°C [23], at 37°C it increases the maximal binding capacity from 44 to 131 pmol/mg, reaching the membranes a B_{max} similar to that obtained at 10°C (Fig. 5).

Table I shows a summary of the binding results obtained for the different cation pathway blockers in isolated T-tubule membranes from frog and rabbit muscle. The mean values of B_{max} and K_d for T-tubules isolated from rabbit muscle are compared with those obtained for two different preparations isolated from frog muscle: T-tubules and a membrane fraction that contains both T-tubule and surface membrane markers [9]. The density of receptors for the four markers used is roughly similar in both T-tubule preparations, with [^{3}H]ouabain binding being slightly higher in frog membranes. Ouabain receptor density does not differ significantly between T-tubules and the surface membranes enriched fraction. This finding suggests that ouabain receptors are homogeneously distributed between surface and T-tubules, whereas nitrendipine receptor density is clearly higher in T-tubule membranes, and both tetrodotoxin and α-bungarotoxin receptor density seem to be higher in the fraction containing surface membranes.

TABLE I

BINDING RESULTS FOR CATION PATHWAY BLOCKERS

Binding of [^{3}H]ouabain, of [^{3}H]ethylenediamine tetrodotoxin and of ^{125}I-α-bungarotoxin was measured in the presence of saponin, as described in the text.

	Rabbit T-tubules		Frog			
			T-tubules		surface [a]	
	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)
[^{3}H]Ouabain	169	32	215 ±38 (4)	10.4	163	9
[^{3}H]Nitrendipine	173	1.7	124 ±18 (4)	1.0	63	1.15
[^{3}H]Ethylenediamine tetrodotoxin	3.5	0.54	4.8 ± 0.6 (4)	0.52	9.0	0.3; 6.0
^{125}I-α-Bungarotoxin	4.0	0.43	5.0	0.13	15.2	0.39

[a] This preparation, referred to as F_o in the text, was isolated by high salt extraction [9] and, although enriched in surface membranes, is not homogeneous.

Discussion

The T-tubule membranes isolated from frog skeletal muscle described in this and in the preceding paper [17], can be quantified in terms of several markers. Given the purity of the preparation, estimates for the density of ion pathways in the T-tubule system can be obtained, and they can be compared to values described in the literature for other preparations.

Dihydropyridine receptors

Independent studies have suggested that dihydropyridine receptors are located preferentially, if not solely, in the T-tubules [13,20]. Thus, nitrendipine binding seems to be a suitable marker to establish the purity of T-tubule membrane preparations. The maximal values of nitrendipine binding sites obtained in this work, 120–130 pmol/mg (Fig. 4), is the highest reported up to now in the literature when measured under similar experimental conditions (Table II). Hence, we propose that this value can be considered, at least for T-tubule membranes from frog muscle where enough data are available, as a criteria for a preparation close to 100% purity. It has been reported that binding of nitrendipine at 37°C is increased by diltiazem in a subcellular fraction isolated from guinea pig muscle [31] (see Table II). In T-tubules isolated from frog muscle, diltiazem caused a 3-fold stimulation in maximal binding capacity at 37°C (Fig. 5). However, this effect is much less marked at 10°C [22], where all nitrendipine receptors seem to be expressed. Thus, our maximal binding values at 10°C without diltiazem are equal to those obtained at 37°C in the presence of diltiazem (Table II). It is conceivable that increasing temperature from 10°C to 37°C masks a fraction of the receptors and increases the K_d more than 3-fold. Diltiazem, presumably by an allosteric interaction [31] unmasks the cryptic receptors but does not change the K_d value. In addition to diltiazem, depolarization of the T-tubule membrane seems to increase the number of binding sites in frog muscle [32]. It is likely that the isolated T-tubule vesicles are depolarized. If this is the case, our binding data at 10°C would reflect the maximal number of receptors present in the T-tubule membrane. Given the average T-tubule vesicular diameter of 0.12 μm (data not shown), and assuming a vesicular volume of 4–5 μl per mg of protein – in analogy to that of sarcoplasmic reticulum vesicles of comparable diameters – the maximal binding of 120–130 pmol per mg of protein would correspond to 250–300 binding sites per μm^2 of T-tubule membrane surface. This number of sites is similar to the 230 receptor sites per μm^2 of T-tubule surface calculated by Schwartz et al. [32] in depolarized frog muscle.

Tetrodotoxin receptors

In addition to receptors for nitrendipine, receptors for tetrodotoxin derivatives have proved to be a useful tool to distinguish between surface and T-tubular membranes [9,12]. Thus, T-tubule preparations display a single type of receptors (Fig. 3), whereas membrane fractions that contain both surface and T-tubular elements (Fo) exhibit two types of receptors [9]. Furthermore, in frog muscle the total density of receptors for ethylenediaminetetrodotoxin is higher in a preparation enriched in plasma membranes (F_o) than in T-tubules (Table I). If we consider the nitrendipine binding capacity as a criterium for T-tubule content, the F_o preparation would have about 50% T-tubules, since it has about one half the binding sites for nitrendipine – 63 pmol/mg – as compared to 120–130 for T-tubules. We have found that highly purified T-tubule membranes isolated from frog muscle bind less than 5 pmol tetrodotoxin per mg of protein in the presence of unmasking agents. We can them conclude from the data on Table I that purified surface membranes should contain at least 13 pmol of tetrodotoxin binding sites per mg of protein. These values, showing 3-fold higher density in surface membranes, are in agreement with the higher density of tetrodotoxin receptors postulated for the surface membrane as compared to T-tubules in whole frog muscle [34]. Furthermore, other studies in T-tubules isolated from rabbit muscle [28] have described saxitoxin binding of 3.3 pmol per mg of protein, in the same range as the values found in this work, but considerably lower than those reported elsewhere [35]. Thus, it seems likely that highly purified T-tubules have about 5 pmol per mg of protein for tetrodotoxin or saxitoxin receptors.

TABLE II

BINDING OF DIHYDROPYRIDINES TO ISOLATED T-TUBULES MEMBRANES

Preparation	Ligand	Temperature (°C)	B_{max} (pmol/mg)	K_d (nM)
Rabbit				
Fosset et al. [13]	[^{3}H]nitrendipine	10	56.0	1.7
Kirley and Schwartz [22]	[^{3}H]nitrendipine		12	1.7
	[^{3}H]nitrendipine + diltiazem	10	17.5	1.44
Curtis and Catterall [23]	[^{3}H]nitrendipine	4	2.1–5.9	
Galizzi et al. [24]	[^{3}H]nitrendipine	10	55	1.8
Borsotto et al. [25]	[^{3}H]PN 200-110	10	90	0.4
Galizzi et al. [26]	[^{3}H]nitrendipine	10	60–80	
Barhanin et al. [27]	[^{3}H]nitrendipine	10	77	
Brandt et al. [19]	[^{3}H]nitrendipine	23	27.2	3.5
Kraner et al. [28]	[^{3}H]nitrendipine		41	
This work	[^{3}H]nitrendipine	10	173	1.7
Guinea pig				
Glossmann et al. [29]	[^{3}H]nimopidine	37	11.7	1.30
	[^{3}H]nimopidine + diltiazem	37	61.6	1.29
Rat				
Chin and Beeler [30]	[^{3}H]nitrendipine		45	
Frog				
This work	[^{3}H]nitrendipine	10	125	1.0
	[^{3}H]nitrendipine	37	44.5	3.9
	[^{3}H]nitrendipine + diltiazem	37	131	3.8

α-Bungarotoxin receptors

The distribution of α-bungarotoxin receptors between surface and T-tubule membranes in frog muscle seems to follow a pattern similar to that of tetrodotoxin receptors; in the presence of saponin the Fo fraction (enriched in surface membranes) has a density of receptors 2–3-times higher than T-tubules (Table I). Acetylcholine channels concentrate near the neuromuscular junction and only following denervation they distribute over the whole muscle surface [36]. Assuming that α-bungarotoxin binding reflects functional acetylcholine receptors this would be the first evidence of their presence in the T-tubular system. The physiological role that acetylcholine channels may play in T-tubules is obscure. The fact that both sodium channels [37,38] and acetylcholine channels [39] are concentrated in particular spots of the sarcolemma makes it tempting to speculate [40] that a gradient of channel density exists in muscle. This gradient might be established as the channel proteins migrate in the plane of the membrane after synthesis, to finally reach the T-tubules, where their mean density would be lower. However, surface and T-tubules receptors for tetrodotoxin derivatives differ not only in their density but also in their affinity for the ligand. Thus, differences in tetrodotoxin receptor properties cannot be the attributed solely to channel diffusion in this case [38].

Ouabain receptors

The presence and density of Na^+ pump sites in T-tubule membranes has been a matter of some controversy. Venosa and Horowicz [41] measured [^{3}H]ouabain binding to isolated normal frog sartorius muscles and to paired muscles 'detubulated' by glycerol-induced osmotic shock. The number of binding sites was rather similar in both experiments, so that they concluded that not more than 20% of the receptors are located in the T-tubules. Considering the membrane areas for surface

and T-tubules, this finding implies that the density of receptors in the tubule would be only 4–5% of that in the surface membrane. Membranes isolated from skeletal muscle, on the other hand, show a relatively high ($Na^+ + K^+$)-ATPase activity in preparations where the T-tubular origin of the membranes has been established [1,11]. Our results on [^{3}H]ouabain binding agree with the ($Na^+ + K^+$)-ATPase data [17], in that they both show high values. Furthermore, in frog muscle we see no difference in ouabain receptor density between T-tubules and the preparations enriched in surface membranes (Table I). It remains to be established why ouabain binding to the T-tubules is not expressed in intact muscles [41]. The apparent lack of binding in the intact muscle may reflect differences in the accessibility of the receptors, different requirements for binding (ATP, ions) between surface and tubules, or an artificially high binding following detubulation, which increases nonspecific binding [41]. In any case, our results indicate that ouabain binding cannot be used to distinguish between surface and T-tubule membranes, as proposed elsewhere [35], since both systems have comparable receptor densities.

In conclusion, the results presented in this and in the preceding paper [17] reveal that T-tubules isolated from frog muscle share many properties with the T-tubules isolated from rabbit muscle. They are not identical, though, since their Mg^{2+}-ATPases behave differently [17]. Furthermore, preliminary studies indicate that the T-tubules membranes from frog muscle differ in their permeability to ions from T-tubules of mammalian origin, and exhibit different ionic channels when fused to lipid bilayers. Thus, the studies described in these two papers constitute a foundation for future development.

Acknowledgements

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