

# Biochemical characterization of plasma membrane isolated from human skeletal muscle

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Specific components of ion translocation systems were studied in excitable plasma membranes isolated from normal human muscle. Na<sup>+</sup> – K<sup>+</sup> ATPase and ouabain-sensitive K<sup>+</sup> phosphatase activities were  $8.9 \pm 1$   $\mu\text{mol P}_i/\text{h}$  per mg protein and  $96 \pm 9$  nmol/min per mg protein, respectively. Scatchard analysis of equilibrium binding assays with [<sup>3</sup>H]ouabain showed non-linear curves consistent with high- and low-affinity sites (estimated  $K_d$  3 nM and 0.22  $\mu\text{M}$ ). Two families of receptors with different affinities for a tritiated TTX derivative (estimated  $K_d$  0.4 and 4 nM) were also identified suggesting the existence in human muscle of at least two classes of voltage-dependent Na<sup>+</sup> channels. In addition (+)-[methyl-<sup>3</sup>H]PN200-110, a potent Ca<sup>2+</sup> antagonist used for labeling voltage-dependent Ca<sup>2+</sup> channels, was observed to bind to a homogeneous population of receptors in the plasma membrane ( $K_d$  = 0.2 nM).

<i>Human muscle</i>	<i>Plasma membrane</i>	<i>Na<sup>+</sup> – K<sup>+</sup> ATPase</i>	<i>Ouabain receptor</i>	<i>Tetrodotoxin receptor</i>
			<i>Dihydropyridine receptor</i>	

## 1. INTRODUCTION

Human neuromuscular diseases such as muscular dystrophies with or without myotonia are often assumed to be associated with disorders of the skeletal muscle plasma membrane (surface and transverse tubular (T-tubule) membranes) [1–3]. Therefore it may be of pathogenic value to compare the molecular composition of the membrane in normal and pathological subjects. No reports have been published concerning their characterization and purification. Membrane preparations obtained from a variety of animal species [4–7] are of little significance in pathology since no animal models are available for human neuromuscular diseases. The present procedure derives from one

we reported for rabbit [7]. It allows for the first time analysis of highly purified muscle plasma membrane from human. Membrane fractions were identified by the presence of specific ion translocation components. The Na<sup>+</sup> pump was assessed by determination of Na<sup>+</sup>–K<sup>+</sup> ATPase, ouabain-sensitive K<sup>+</sup> phosphatase and ouabain binding. Special attention was paid to voltage-dependent Na<sup>+</sup> and Ca<sup>2+</sup> channels which were studied through their interaction with TTX and dihydropyridine derivatives, respectively.

## 2. MATERIALS AND METHODS

### 2.1. Human muscle samples

Human muscle samples (4–8 g) were removed from serratus magnus after informed consent from patients undergoing thoracic surgery with no specific clinical features of muscular diseases, and frozen at –80°C. About 20 g (2–4 samples) were used for each assay.

*Abbreviations:* [<sup>3</sup>H]en-TTX<sub>II</sub>, tritiated ethylenediamine tetrodotoxin derivative;  $B_{\text{max}}$ , maximal binding capacity

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## 2.2. Purification of plasma membranes

Purification of plasma membranes was achieved using our procedure described for rabbit [7] with some modification. (i) The contractile protein-free homogenate Ho was obtained by homogenization of the pellet  $P_1$  in a 50 mM triethanolamine-HCl buffer, pH 7.4 (standard buffer) supplemented with 0.15 M sucrose. (ii) The fraction  $S_2$  was centrifuged at  $5000 \times g$ . (iii) 7 ml of the French press-treated microsomal fraction  $P_4$  (1–2 mg protein/ml) were layered on the top of a discontinuous sucrose gradient composed of 10 ml of 40% and 18 ml of 20% (w/w) sucrose. After centrifugation at  $113\,000 \times g$  for 14–16 h in a Beckman SW 28 swinging bucket rotor, the particles banding between 7–20% and 20–39% sucrose were separately withdrawn yielding fractions G1 and G2, respectively.

## 2.3. Enzymatic activities

The ouabain-sensitive  $K^+$  phosphatase and  $Ca^{2+}$ -stimulated phosphatase activities were determined as in [7], cytochrome oxidase as in [8]. The  $Na^+$ - $K^+$  ATPase activity was evaluated in triethanolamine-Cl buffer, pH 7.4, containing 20 mM KCl, 3 mM  $MgCl_2$ , 130 mM NaCl and the membrane (15  $\mu$ g proteins/ml) with or without 0.4 mM ouabain (Sigma). The reaction was started by addition of 0.3 mM ATP (disodium salt, Sigma) and stopped after 10 min at 37°C by 100  $\mu$ l of 30% trichloroacetic acid. The liberated  $P_i$  was determined as in [9].

## 2.4. Equilibrium binding assays

Dihydropyridine binding sites were assayed as described in [10] using (+)-[methyl- $^3H$ ]PN200-110 (Amersham France, 84 Ci/mmol) already used in skeletal muscle studies [11,12]. TTX-binding sites were evaluated using [ $^3H$ ]en-TTX<sub>II</sub> (25 Ci/mmol; radiochemical purity 90%) obtained as described in [13]. TTX derivatives have been shown to titrate the same receptors as STX in human muscle [2]. Moreover [ $^3H$ ]en-TTX and [ $^3H$ ]STX bind to frog muscle with similar  $B_{max}$  and  $K_d$  values [13]. Equilibrium assays and calculation of specific binding were performed as described [2,13]. Equilibrium binding assay with [ $^3H$ ]ouabain (NEN France, 20.9 Ci/mmol) were carried out as in our previous experiments [2,7]. All data were analysed as in [2].

## 2.5. Chemical analysis

Membrane lipids corresponding to 0.5–1 mg proteins were extracted as in [14]. Free and esterified cholesterol was determined as in [15] using the Merckotest kit. Total lipid phosphorus was evaluated as in [16]. Proteins were determined as in [17] using Bio-Rad reagents. SDS gel electrophoresis were run using 7.5–12% polyacrylamide slab gels according to [18].

## 3. RESULTS

Centrifugation through a sucrose gradient of human muscle microsomes pretreated in a French press separated a lighter fraction, G1, banding around 13% sucrose (limits: 7%–20%) and a heavier fraction, G2, centered around 29% sucrose (limits: 20%–39%). G1 contained 0.23% and G2 0.76% of the proteins present in the contractile protein-free homogenate Ho (table 1).

### 3.1. Enzymatic activities

Table 1 also shows that the bulk of ouabain-sensitive  $K^+$  phosphatase and  $Na^+$ - $K^+$  ATPase is concentrated in G1 (specific activities 94 nmol/min per mg protein and 8.9  $\mu$ mol  $P_i$ /h per mg protein, respectively). Mitochondrial cytochrome oxidase is very low in the fraction and  $Ca^{2+}$ -stimulated phosphatase characteristic of muscle sarcoplasmic reticulum is not detectable. As expected [7] G2 is rich in  $Ca^{2+}$ -stimulated phosphatase and mitochondrial debris. Specific enzyme activities in Ho and  $P_4$  are also given in table 1 to control the purification.

### 3.2. Equilibrium binding assay data

Equilibrium binding assay data are presented in table 2 and fig.1. Typical saturation curves of [ $^3H$ ]ouabain and [ $^3H$ ]en-TTX<sub>II</sub> binding to their specific receptors are given in fig. 1A and B (insets). The corresponding Scatchard plots (main panels of the figures) are consistent with high- and low-affinity binding sites for ouabain and en-TTX<sub>II</sub> (estimated  $K_d$  = 3 nM and 0.22  $\mu$ M for ouabain, 0.36 nM and 4.1 nM for TTX), the majority of both types of sites being in G1. The relative concentrations of high- and low-affinity ouabain- and TTX-binding sites in G1 and G2 are similar to those indicated above for enzymatic markers of the  $Na^+$  pump. A typical example of total and non-

Table 1  
Enzymatic activities in membrane fractions

Fractions	Proteins (%)	Specific activities			
		Ouabain-sensitive K <sup>+</sup> -stimulated phosphatase	Na <sup>+</sup> -K <sup>+</sup> ATPase	Ca <sup>2+</sup> -stimulated phosphatase	Cytochrome oxidase
H <sub>0</sub>	100	4.5 ± 1	/	4.3 ± 0.5	22.6 ± 2
P <sub>4</sub>	2.7	32 ± 4	/	25.5 ± 1.5	136.4 ± 30
G1	0.23	96 ± 9	8.9 ± 1	n.d.	2 ± 0.4
G2	0.76	28.5 ± 7	3.8 ± 0.2	79.2 ± 15	205 ± 38

Average of 5 determinations. Specific activities are expressed in nmol/5 min per mg protein for all enzyme determinations except Na<sup>+</sup>-K<sup>+</sup>ATPase which is expressed in μmol P<sub>i</sub>/h per mg protein. n.d., not detected; /, not determined

specific (+)-[methyl-<sup>3</sup>H]PN200-110 is given in fig.1C (inset). The specific binding was found to be a saturable process in the investigated radioligand range. The corresponding Scatchard plot (fig.1C) is linear in this case, suggesting a single class of receptors ( $K_d = 2$  nM).

### 3.3. Membrane lipids and proteins

Total cholesterol (free + esterified) and lipid phosphorus were 0.32 and 0.8 μmol/mg protein in G1 (average of 3 determinations) leading to a cholesterol/phospholipid molar ratio of 0.4. The ratio is only 0.16 in G2. SDS gel electrophoresis patterns of the proteins in G1 and G2 are reproduced in fig.2. The strong band at about 100 kDa in the G2 pattern probably corresponds to Ca<sup>2+</sup>ATPase. The number of major protein bands in the G1 pattern is low as compared to G2.

## 4. DISCUSSION

This paper describes the first purification of excitable plasma membranes from normal human

skeletal muscle and gives preliminary information about their molecular composition. Fraction G1 is strongly enriched in plasma membrane since it contains the majority of Na<sup>+</sup> pump enzyme markers. Its contamination by components of the fraction G2 is weak since it is nearly devoid of Ca<sup>2+</sup>-stimulated phosphatase and Ca<sup>2+</sup>ATPase, and its cholesterol/phospholipid ratio is stronger than in G2. About 75% of the excitable membrane markers present in the G1 + G2 mixture are found in G1. The human plasma membranes are markedly lighter (peak at 13% sucrose) than those from several animal species (19–25% sucrose [5]) thus probably indicating a lesser protein content compared to lipids. Nevertheless, the specific activities of the enzymes are of the same order in human and other mammals [4–7].

We have characterized in human skeletal muscle at least 2 classes of ouabain binding sites with different affinities for the ligand. This finding was recently reviewed by Hansen [19]. A similar observation has been reported for cardiac muscle [20]. It does not contradict our previous data on rabbit

Table 2  
Radioligand binding to specific receptors in the fractions G1 and G2

Fractions	(+)-[ <sup>3</sup> H]PN200-110		[ <sup>3</sup> H]Ouabain				[ <sup>3</sup> H]en-TTX <sub>II</sub>			
	$B_{max}$ (pmol)	$K_d$ (nM)	High affinity		Low affinity		High affinity		Low affinity	
			$B_{max}$ (pmol)	$K_d$ (nM)	$B_{max}$ (pmol)	$K_d$ (μM)	$B_{max}$ (fmol)	$K_d$ (nM)	$B_{max}$ (fmol)	$K_d$ (nM)
G1	7.4	0.2	4.9	3	30	0.22	240	0.36	910	4.1
G2	7.2	0.1	2.0	4	13	0.33	87	0.22	223	2.6

Average of at least 2 determinations ( $B_{max}$  expressed in pmol or fmol per mg protein)

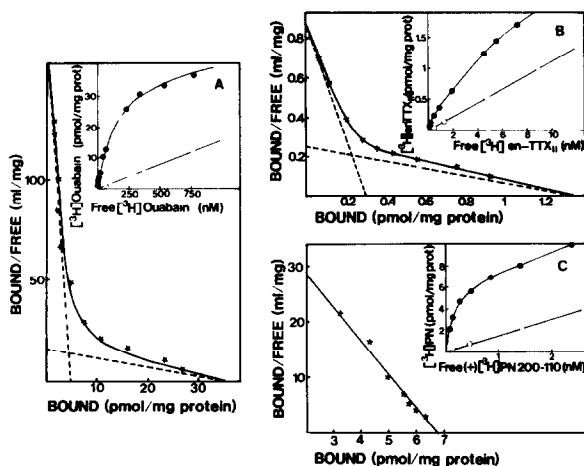


Fig.1. (A, inset) Typical example of  $[^3\text{H}]$ ouabain equilibrium binding data observed on membrane aliquots (15  $\mu\text{g}$  proteins) from fraction G1. Incubation was carried out for 1 h at 25°C in 1 ml triethanolamine-Cl buffer, pH 7.4, containing 150 mM NaCl 5 mM  $\text{MgCl}_2$ , 2 mM ATP, 0.1 mM PMSF, with (non-specific binding ( $\circ$ )) or without (total binding ( $\bullet$ )) 0.1 mM unlabeled ouabain. Radioligand concentrations were 1 nM to 3  $\mu\text{M}$ . (B, inset) Binding of  $[^3\text{H}]$ en-TTXII (0.1–10 nM) to isolated plasma membranes (80  $\mu\text{g}$  proteins) incubated for 20 min at 4°C in 1 ml of 50 mM Tris buffer containing 50 mM choline chloride at pH 7.8 in the presence ( $\circ$ ) or absence ( $\bullet$ ) of 2  $\mu\text{M}$  unlabeled TTX. (C, inset) Typical binding data of (+)-[methyl- $^3\text{H}$ ]PN200-110 (0.06–2.5 nM) on fraction G1. 10- $\mu\text{g}$  protein aliquots were incubated for 40 min at 20°C under dim light in 1 ml of 50 mM Tris-HCl buffer, pH 7.6, with ( $\circ$ ) or without ( $\bullet$ ) 2  $\mu\text{M}$  unlabeled nitrendipine. (A–C) Respective corresponding Scatchard plots leading to the  $B_{\text{max}}$  and  $K_d$  values listed in table 2.

[7] and human [2] muscle where only the low-affinity site ( $K_d$  about 1 and 0.5  $\mu\text{M}$ , respectively) was titrated at high ligand concentrations.

Similarly, our results are consistent with the existence in human skeletal muscle of voltage-dependent  $\text{Na}^+$  channels with high and low affinity for en-TTXII. Membranes of surface and T-tubule origin have been reported to differ in TTX receptor density [21], and the pharmacological properties [13,22] as well as the conductance kinetics [23] of surface membranes and tubular  $\text{Na}^+$  channel have been shown to differ. In the frog [13] use of en-TTXII showed that the low-affinity sites ( $K_d$  between 0.2 and 0.8 nM) are in the T-tubule system. Human skeletal muscle also possesses 2

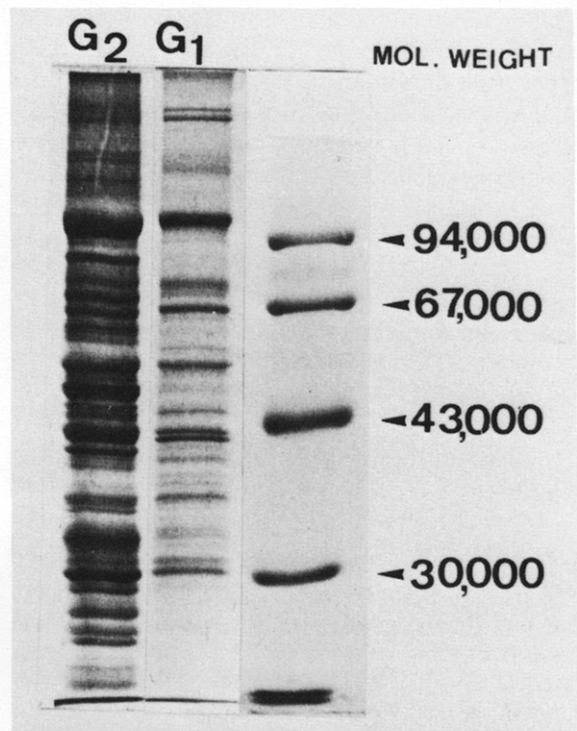


Fig.2. SDS gel electrophoresis patterns of the proteins in G1 and G2 (30–40  $\mu\text{g}$ ); Conditions as in [18]; Coomassie blue staining.

classes of en-TTXII-receptor complex with the same affinities for the ligand as in frog. Their co-existence in our experiment suggests that the preparations are a mixture of surface and T-tubule membranes.

On the other hand, the use of dihydropyridine derivatives has allowed the identification of  $\text{Ca}^{2+}$  blocker receptors associated with the voltage-dependent  $\text{Ca}^{2+}$  channels in skeletal muscle from various mammals [10–12]. Muscle tubular membrane preparations have been shown to possess a very high number of such receptors [10,12] and electrophysiological data have shown that  $\text{Ca}^{2+}$  channels are mainly localized in the T-tubules [24]. Human muscle plasma membrane binds (+)-[methyl- $^3\text{H}$ ]PN200-110 with the same affinity ( $K_d = 0.2$  nM) as in the rabbit [12]. This result confirms the presence of T-tubule in fraction G1. Surprisingly, we observed a similar concentration in G2 as in G1 of PN200-110 receptors. One possible explanation of these results is that dihydropyridine

binding sites might also be present in the sarcoplasmic reticulum membranes as claimed in [25].

Our first aim was to study some characteristics of ion translocation system properties in normal human skeletal plasma membranes. This study will shortly be extended to various pathological muscles.

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