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COMPLETE MONITORING OF THE PURIFICATION OF THE PLASMA MEMBRANE FROM RABBIT SKELETAL MUSCLE

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A fast and reproducible purification procedure for rabbit skeletal muscle plasma membrane is described. Each step was monitored by determination of tetrodotoxin, ouabain and insulin receptors. A ouabain-sensitive K^+-stimulated and a Ca^{2+}-dependent phosphatases, probably identical to, respectively the (Na^+-K^+) and Ca^{2+}-ATPases, were also evaluated. All plasma membrane receptors and the ouabain-sensitive activity accumulated in the lightest fraction separated by sucrose gradient centrifugation (peak at 18 % sucrose ; purification from crude homogenate, 30-fold).

Disorders of the plasma membrane (PM) in skeletal muscle cells is often associated with muscular pathology such as myotonia, periodic paralysis and muscular dystrophies. Therefore, it is of interest to purify the membrane and compare its properties in normal and pathological tissues. The purification procedure described below combines several features of already reported methods for the obtention of PM and sarcoplasmic reticulum membrane (SRM) from skeletal (1-5) and cardiac (6) muscle. Another original aspect is that each step of the procedure was monitored by determination of two characteristic components of the sodium translocation system in excitable membranes : the voltage-sensitive Na^+ channels (tetrodotoxin (TTX) receptors) and the (Na^+-K^+) ATPase (ouabain receptors). Insulin receptors, a specific marker of muscle PM were also determined. PM and SRM were further differentiated by their content in a ouabain-sensitive K^+-phosphatase and a Ca^{2+}-dependent phosphatase activities. The procedure led to a 30-fold purification of PM markers in the lightest fraction separated by sucrose gradient centrifugation.

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MATERIALS AND METHODS

Binding assays : Na^+ channel densities were measured using a highly tritium-labeled TTX derivative synthesized by Chicheportiche et al. (11) (specific radioactivity 27 Ci/mmol ; radiochemical purity 90 %). The assays (12) were done on fraction aliquots (0.1-0.5 mg protein) in the absence or presence of 5 µM unlabeled toxin (Sankyo Chemical Co) for total and non specific binding determination. Specific binding was calculated by difference. Likewise, fraction aliquots (0.1-0.5 mg protein) were incubated (13) with [^{3}H]-ouabain (NEN Europe ; specific radioactivity 11.6 Ci/mmol) in the absence or presence of 1 mM unlabeled ouabain (Sigma). Insulin receptors (14) were evaluated using 1-2 mg membrane proteins and a [^{125}I]-insulin sample prepared as in (15). (Specific radioactivity 480 Ci/mmol). Non specific binding was measured in the presence of 1.7×10^{-5} M of unlabeled insulin (Fluka). The concentration range of radioactive material was 0.1-5 nM for TTX, 0.1-10 µM for ouabain and 0.05-2 nM for insulin. Hill coefficients, maximal binding capacities (Bmax) and equilibrium dissociation constants (K_D) were calculated as in (16) with a Wang 2200 calculator to fit experimental points with theoretical curves.

Determination of enzymatic activities : All activities were linear with time and protein concentration. The ouabain-sensitive K^+-phosphatase was measured using p-nitrophenyl phosphate as substrate (7,8) in the absence or presence of 0.4 mM ouabain at pH 7.4 (ε_{410} of nitrophenolate ions after addition of two volumes of 1 M Tris base, 1.7×10^4 M^{-1} cm^{-1}). The Ca^{2+}-dependent phosphatase (9,10) was determined by difference in the presence of 2 mM $CaCl_2$ or 2 mM EGTA. Cytochrome oxidase was measured as in (17).

Purification of PM : The main steps of the procedure are (Fig. 1) :

(a) Removal of contractile proteins. All assays were done at 4°C with ice-cold reagents. Thinly minced rabbit thigh white muscle (30-40 g) was washed with buffer I (50 mM Triethanolamine-HCl pH 7.4, 20 mM $MgCl_2$), transferred into four volumes of buffer II (50 mM Triethanolamine-HCl pH 7.4, 0.7 M KCl) and homogenized for 5 sec in a Polytron homogenizer Model PT 20 SM (Brinckmann) at setting 6. The crude homogenate (Hg) was centrifuged 20 min at 40,000 g. The pellet was suspended in buffer II with a loose fitting Potter-Elvejhem homogenizer (five strokes) and washed three-times by homogenization in buffer III (50 mM Triethanolamine-HCl pH 7.4 10 mM sodium bicarbonate). The supernatants were pooled (S1) and the final pellet was rehomogenized for 3 x 30 sec with the Polytron homogenizer in 100 ml of buffer III (purified homogenate Ho).

(b) Second step : obtention of the microsomal fraction : Ho was centrifuged at 2,000 g for 20 min, 10,000 g for 20 min and then 105,000 g for 1 h. The final pellet is the microsomal fraction P4.

(c) Third step : disruption of P4 and subfractionation in a sucrose gradient : After suspension (15 mg protein/ml) in buffer IV (50 mM Triethanolamine-HCl pH 7.4 0.3 M KCl and 50 mM potassium pyrophosphate (18)), P4 was treated in a pre-cooled French Press at 6,800 psi (3) and layered on the top of a discontinuous sucrose gradient (18 ml of 23 % and 10 ml of 46 % (w/w) sucrose in buffer IV) prepared in a tube of a Beckman SW 27 swinging bucket rotor. After centrifugation at 113,000 g for 14 h, 1.2 ml fractions were withdrawn. The particles banding at the 0 %-23 % and 23 %-46 % interfaces were separately pooled to give fractions GI and GII (peak at 18 % and 30 % sucrose, respectively). The fractions and the heaviest pellet (Gp) were diluted to 8 % sucrose in buffer I, centrifuged at 105,000 g for 1 h and re-suspended in 4 ml of buffer I for further analysis.

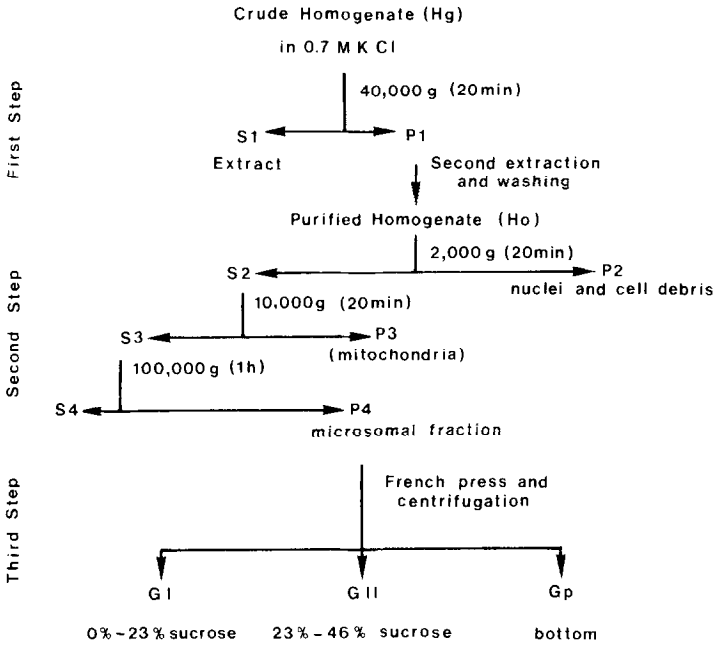

Fig. 1 : Flow sheet of PM purification from rabbit skeletal muscle. S stands for supernatant and P for pellet. GI, GII and Gp are particulate fractions separated in the sucrose gradient.

RESULTS

Receptors : A typical example of total, non specific and specific TTX binding to GI is given in Fig. 2. The specific binding curve showed a plateau

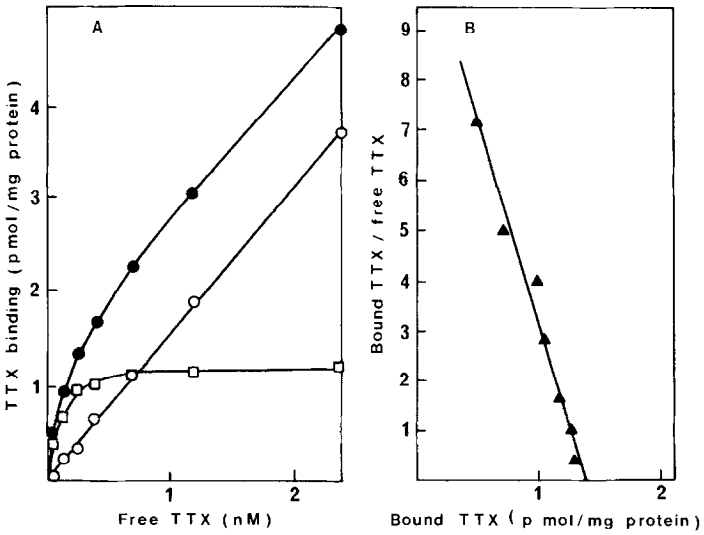

Fig. 2 : $[^3H]$-TTX binding to the GI fraction. (A):total (filled circles), non specific (open circles) and specific (open squares) binding versus free ligand concentration. (B) : Scatchard plot of specific binding data.

Table I. Binding to specific receptors in GI.

Fraction	[^{3}H]-TTX		[^{3}H]-Ouabain		[^{125}I]-Insulin	
	Bmax	Purif. (-fold)	Bmax	Purif. (-fold)	Bmax	Purif. (-fold)
Hg	45	1	0.4	1	3.0*	1
Ho	90	2.0	0.9	2.2	6.1	2.0
P4	240	5.3	1.9	4.8	15.0	5.0
GI	1380	30	10.5	26	57	19
GII	230	-	0.9	-	9.2	-

* Tentative value due to the very low specific radioactivity in Hg.
Bmax (average of two assays) are expressed in fmole/mg protein for TTX and insulin, in pmol/mg protein for ouabain.

for free ligand concentrations higher than 0.8 nM. The corresponding Scatchard plot was linear. Comparable results were obtained with ouabain and insulin with a good reproducibility. Bmax values for the three ligands in all fractions are given in Table I. Both TTX and ouabain receptor densities increased in parallel from Hg to G1 with a 30-fold overall purification. The densities of the two receptors in G II were only 16 % and 8.5 % of those in GI. Insulin receptors varied in nearly the same manner. As expected, the dissociation constants of the receptor-ligand complexes varied little during purification. They were 1.3-1.8 x 10^{-9} M for TTX, 0.8-1.1 x 10^{-6} M for ouabain and 0.3 x 10^{-10} M for insulin.

Enzymatic activities : The substrate p-nitrophenyl phosphate (7-10) led to better results than ATP for evaluation of the ouabain-sensitive phosphatase activity in impure fractions except Hg. The variations of the Ca^{2+}-phosphatase, cytochrome oxidase and total proteins from Hg to Ho is given in Table 2. As shown, the two short extractions with 0.7 M KCl removed 77 % of the proteins initially present in crude Hg. The enzymes were purified two-fold. The variations of the ouabain-sensitive and Ca^{2+}-dependent activities from Ho to GI and GII are summarized in Table 3. Both co-purified up to P4 and then diverged. The former followed the PM markers in GI while the latter accumulated in GII and Gp. Taking into account the two-fold activity increase noted in Table 2, the overall purification is 25-fold for the first enzyme in GI and 40-fold for the

Table 2. Variation of enzyme markers and total proteins from Hg to Ho.

Fraction	Ca^{2+}-stimulated phosphatase.		Cytochrome oxidase		Total proteins
	SA	Yield (%)	SA	Yield (%)	(%)
Hg	31	100	33	100	100
P1	66	63	57	66	38
S1	-	-	18	33	61
Ho	70	62	88	61	23

Specific activities (SA) are expressed in nmol/min/mg protein. Average of six determinations.

second in GII. The specific activity of the ouabain-sensitive enzyme in GII did not exceed 3 % of that in GI whereas a significant Ca^{2+}-phosphatase activity was present in GI (15 % of that in GII). The latter could not be separated by centrifugation in a continuous shallow sucrose gradient. GI and GII were slightly contaminated by mitochondria debris as judged by their cytochrome oxidase content

Electron microscopy and SDS electrophoresis : Results obtained by electron microscopic observations on ultrathin sections of GI and SDS-electrophoresis after dissolution of GI and GII in SDS were similar to those already reported by others (1,3,5).

Table 3. Variation of enzymes and total proteins from Ho to GI, GII and Gp.

Fraction	Ouabain-sensitive phosphatase			Ca^{2+}-dependent phosphatase			Total proteins (%)
	SA	Purif. (-fold)	Yield (%)	SA	Purif. (-fold)	Yield (%)	
Ho	3.5	1	100	70	1	100	100
S2	6.0	1.7	63	135	1.9	79	34
S3	16	4.7	52	415	5.9	71	11
P4	26	7.4	38	776	11.0	56	3.5
GI	43	12.3	12	48	0.7	0.5	0.5
GII	6.4	1.8	3	1442	20.6	33	1.9
P2	0.9	-	19	19	-	10	70
P3	1.4	-	15	42	-	8	28
Gp	-	-	-	1317	-	19	1

Average of six determinations. SA, specific activity expressed in nmol/min/mg protein.

DISCUSSION

The procedure described above can be discussed as follows : (a) Muscle contractile proteins are as efficiently removed by short extraction with 0.7 M KCl than by prolonged incubation at high ionic strength (4) with less risk of altering functional elements of the membrane. The vesicles thus obtained were carefully washed at low ionic strenght to eliminate outer and inner protein contaminants. (b) In agreement with (3), PM and SRM were more easily separated after treatment of P4 in a French Press to break membrane aggregates and any remaining triad junctions. Mechanical disruption does not alter the capacity of muscle PM vesicles to transport sodium and chloride (1). The occasional obtention of several SRM fractions with different densities (1) may arise from incomplete separation from PM when disruption is omitted. (c) Sucrose concentration in the discontinuous gradient layers were adjusted according to preliminary assays in a continuous gradient. The lightest fractions GI (peak at 18 % sucrose) contained the bulk of the excitable membrane receptors and the ouabain-sensitive K^+-phosphatase which can probably be identified to the (Na^+-K^+) ATPase. Therefore, it is clear that GI is strongly enriched in functional PM. The 30-fold purification common to all markers is in good agreement with the value recently reported for dog heart muscle (6).

On the other hand, the density of the GII fraction (30 % sucrose) and its strong enrichment in the Ca^{2+}-dependent enzyme (probably identical to Ca^{2+} ATPase) suggest that the fraction is mostly composed of SRM. A more aggregated form of SRM is also present in Gp. Therefore, the technique appears to cleanly separate muscle PM and SRM with no need of previous loading of the latter with calcium ions (1). Assays to ascertain the sidedness of our vesicles (5,6) and its possible effect on the determination of receptors and activities are presently in progress. The procedure was recently applied to normal and pathological human muscle (13).

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