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HIGHLY ENRICHED, MINIMALLY DISRUPTED PLASMA MEMBRANE VESICLES FROM AORTIC MYOCYTES GROWN IN PRIMARY CULTURE

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A plasma membrane-enriched fraction (fraction 1B) has been obtained from rat aortic myocytes grown in primary culture. Plasma membrane markers, 5′-nucleotidase and ouabain-sensitive ($Na^+ + K^+$)-ATPase, are enriched 4.1- and 8.7-fold, respectively, in this fraction. Although endoplasmic reticulum marker NADPH-cytochrome *c* reductase is the most enriched in mitochondrial and heavy sucrose density gradient fractions, substantial enrichment of this marker is also observed in membrane fraction 1. This membrane preparation therefore contains a certain quantity of endoplasmic reticulum. Cytochrome *c* oxidase is de-enriched by a factor of 0.04 in fraction 1, indicating that it is essentially clear of mitochondrial contamination. Homogenization of aortic media-intima layers using a whole-tissue technique induces greater disruption of mitochondria and subsequent contamination of membrane fractions than does the procedure for cell disruption. Analysis of electrophoretic gels, vesicle density distribution and electron micrographs of enriched membrane fractions provide evidence that plasma membrane enriched from cultured myocytes is less traumatized than comparable fractions obtained from intact tissue. The potential value of such a highly enriched, minimally disrupted plasma membrane preparation is discussed.

Introduction

Exploration of the mechanisms underlying excitation-contraction coupling in muscle have turned increasingly over the past 10 years to the use of purified, subcellular membrane preparations as models for intact cell function. This has been particularly the case for studies concerning smooth muscle, where the cellular heterogeneity of whole tissue, and the presence of large quantities of extracellular material render experimental interpretation of gross function extremely complex. Characterization of smooth-muscle receptors [1–3], calcium transport [4–12] and affiliated control processes involving the action of cyclic nucleotides [5,13–22] or calmodulin [21,23–25] have been therefore carried out on selectively enriched membrane fractions.

But the whole tissue which serves as a source for enriched membrane material must, of necessity, be subjected to disruptive homogenization, during the course of which, membrane material from fibroblasts, fat cells and smooth muscle become mixed. Furthermore, careful examination of reported membrane-enrichment procedures also suggests the induction of substantial membrane damage.

We report for the first time the purification of a plasma membrane fraction from media-derived, rat aortic myocytes grown in primary culture. Hy-

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potonic disruption of these cells, with (procedure B) or without (procedure A) an additional gentle homogenization gave rise to subcellular fractions which are described here. These are compared with fractions arising from the mechanical homogenization of intact media-intima layers (procedure C). We discuss the value of this myocyte preparation as a source for minimally disrupted plasma membrane.

Experimental procedures

Cell culture

10–12-week-old female Wistar rats (Iffa-Credo, L'Arbresle, France) were killed by stunning and cervical dislocation. Thoracic aortae were excised under sterile conditions, cleaned free of fat and subjected to two successive digestive periods as described previously [26]. Myocyte suspensions were counted with a Coulter counter (Coultronics, France), cells were plated at a density of $5 \cdot 10^5$–$1 \cdot 10^6$ cells per dish on 5-cm plastic dishes (NUNC, Denmark) and incubated at 37 °C under 95% O_2/5% CO_2 conditions. All other methods for cell culture have been described previously [26]. Growth medium contained 5% fetal calf serum (Gibco Europe).

Purification of plasma membrane from primary myocytes

Procedures A and B. Membrane material was harvested from confluent dishes (5–7 days in culture) according to a modified method of Chang et al. [27]. Dishes were rapidly rinsed with a small volume of isotonic buffer (Isoton II, Coultronics, France), followed by a 3-min swelling period in the presence of hypotonic buffer K (1 mM Tris/1 mM $MgCl_2$/1 mM EGTA (pH 7.6)). These steps were carried out at room temperature. Swelled cells were transferred to a cold room (4 °C) where they were scraped free of the dish surface with a rubber policeman in the presence of buffer K. The final volume of this suspension was approximately 0.5 ml per dish. Thus, for a thirty-plate preparation, suspension volume was 15 ml. This suspension was either used directly (procedure A), or gently homogenized (procedure B) with a Teflon/mortar homogenizer (Kontos) turning at low speed (6 strokes/15 s). Homogenates from both of these procedures were subjected to the following series of sequential centrifugation steps: $2 \times 900 \times g$ for 10 min giving postnuclear supernatant (PNS); $9000 \times g$ for 10 min giving a pellet, which is resuspended and centrifuged at $5500 \times g$, giving a mitochondrial pellet and a supernatant. The latter is centrifuged for 30 min at $105\,000 \times g$ giving a microsomal pellet and supernatant (SOL). The microsomal pellet is resuspended and centrifuged for 90 min on a discontinuous sucrose density gradient (26.5, 32.5, 38.5 and 46.5%) at $105\,000 \times g$. Membrane material collected at sucrose interfaces are collected and respun for 1 h at $105\,000 \times g$. All membrane pellets were resuspended in 250 mM sucrose/10 mM imidazole (pH 7.5) and assayed for enzyme-marker activities.

Purification of plasma membrane from media-intima layers

Procedure C. Twenty thoracic aortae were cleaned free of fat, subjected to collagenase digestion and stripped of their adventitial layer [26]. These media-intima layers were cut into small pieces with a pair of scissors, placed in a 10-fold volume (w/v) of 250 mM sucrose/10 mM imidazole (pH 7.5) and homogenized twice for a total period of 30 s with an Ultra-Turrax homogenizer (Janke & Kunkel) adjusted to intermediate speed. The resulting homogenate was subjected to the same series of centrifugation steps outlined above for procedures A and B. In experiments where the three procedures were compared, aortae destined for cell suspension and those for mechanical homogenization were treated in parallel.

Enzyme assays

All subcellular fractions were assayed for plasma membrane marker enzymes, 5′-nucleotidase [28] and ouabain-sensitive $(Na^+ + K^+)$-ATPase [29], mitochondrial inner membrane marker cytochrome *c* oxidase [30] and endoplasmic reticulum marker NADPH-cytochrome *c* reductase [31]. All enzyme activities were linear with respect to time and protein. Assays were performed as soon as possible after isolation of subcellular fractions, with the exception of 5′-nucleotidase which was measured the day following fraction isolation. Mg^{2+}-ATPase activity was measured according to Ref. 29.

SDS-polyacrylamide gel electrophoresis

Subcellular fractions were prepared according to procedures A, B and C, in the presence of 0.1 mM phenylmethylsulfonyl fluoride, 6 mM 2-mercaptoethanol and 1 mM EGTA. 20 μg of each fraction were solubilized in sample buffer, achieving the following concentrations: 55 mM Tris (pH 6.8)/2.2% SDS/1.1% glycerol/2.7% 2-mercaptoethanol/0.002% Bromophenol blue. Solubilized suspensions were heated to 90°C for 5 min and electrophoresed according to the method of Laemmli [32]. Separating and stacking gels contained 12 and 5% acrylamide, respectively. Electrophoresis was carried out at a constant voltage of 150 V until the tracking dye reached the border of the separating gel, at which point the power was increased to 220 V for the remainder of the separation (5 h). Gels were stained with Coomassie blue, destained, and dried with a Bio-Rad model 220 gel drier. Lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin and phosphorylase *B* (Bio-Rad) were used as molecular weight standards.

Electron microscopy

Membrane pellets recovered by high-speed centrifugation were fixed in 5% glutaraldehyde/0.27 M cacodylate at a pH of 7.4. Fixation was carried out at room temperature, over a total period of 5 h. A 1-h postfixation in 1% osmic acid was carried out, followed by successive dehydration in 70, 95 and 100% alcohol, and finally in propylene oxide. Fixed pellets were impregnated with propylene oxide/Araldite (1 : 1) for 1 h at room temperature, followed by overnight treatment at 4°C in a 1/3 : 2/3 mixture of propylene oxide and Araldite. Pellets were embedded in Araldite capsules and sectioned with an ultramicrotome (Reichert). Ultra-thin sections were placed on copper grids and stained as follows: (1) saturated uranyl acetate in 50% alcohol for 20 min, followed by rinsing in 50% alcohol; (2) Reynolds stain (1 M lead nitrate/1 M sodium citrate/1 M NaOH) for 5 min, followed by rinsing in distilled water. Sections were viewed with the aid of Siemens Elmiskop 101.

Other methods

Protein determinations were carried out according to the method of Lowry et al. [33], using bovine serum albumin as a standard. All reagents were of the highest reagent grade. Water was deionized/double-distilled. Sucrose was obtained from Sigma.

Results

Purification of plasma membrane (procedure B)

Procedure B has been chosen as a standard method for obtaining enriched plasma membrane. The specific activity, percent recovery and fold-purification of four enzyme markers are presented for each procedure B subcellular fraction (Table I).

Ouabain-sensitive ($Na^+ + K^+$)-ATPase and 5′-nucleotidase are considered to be specific markers for plasma membrane [28,29]. They are enriched 8.7- and 4.1-fold, respectively, in the lightest (fraction 1) sucrose density gradient fraction . Diminished purification and recovery for both of these marker enzymes are observed with increasing sucrose density (i.e., in fractions 2 and 3).

In contrast, the mitochondrial marker, cytochrome *c* oxidase, is de-enriched by a factor of 0.04 in fraction 1B, and is enriched 5.7-fold in the mitochondrial fraction.

Endoplasmic reticulum marker, NADPH-cytochrome *c* reductase, is enriched 2.1-fold in the sucrose density gradient fraction 1B, indicating that our plasma membrane fraction contains a certain quantity of endoplasmic reticulum. Table I shows that the greatest relative enrichment of this marker occurs in the mitochondrial and heavy sucrose density gradient fractions. Although the difference between 2.1- and 2.8-fold enrichment values is small, increasing enrichment of NADPH-cytochrome *c* reductase was observed in each individual experiment, as a function of sucrose density.

Comparing the standard preparative procedure (B) with others which are less (A) or more (C) brutal

Enrichment and recovery values for three membrane enzyme markers using procedures A, B and C are compared in Fig. 1, according to the representation of DeDuve et al. [34]. The five subcellular fractions which derive from PNS are essentially the same for each of these three procedures: enrichment of the two plasma membrane markers

TABLE I

SUBCELLULAR FRACTIONATION OF AORTIC MYOCYTES GROWN IN PRIMARY CULTURE – PROCEDURE B

Fraction abreviations are the same as those which are presented in Fig. 1. Values for enzyme and protein recovery and specific activity (S.A.) are presented as means ± S.E. Total number of separate preparations upon which data are based is presented at the bottom of each column. Recovery values and fold-purification (fold pure) are calculated with respect to PNS. Recovery is presented as a percentage. Units are as follows: ouabain-sensitive $(Na^+ + K^+)$-ATPase, nmol P_i (phosphate) liberated/mg membrane protein per min at 37 °C; 5′-nucleotidase, μmol P_i liberated from 5′-AMP/mg protein per h at 37 °C; cytochrome *c* oxidase, μmol cytochrome *c* oxidized/mg membrane protein per min at 37 °C; NADPH-cytochrome *c* reductase, μmol cytochrome *c* reduced/mg membrane protein per min at 37 °C. n.d., not detectable; PNS, postnuclear supernatant; MITO, mitochondrial fraction.

Fraction	Protein recovery	Oubain-sensitive $(Na^+ + K^+)$-ATPase			5′-Nucleotidase			Cytochrome *c* oxidase			NADPH-cytochrome *c* reductase		
		S.A.	Recovery	Fold pure	S.A.	Recovery	Fold pure	S.A.	Recovery	Fold pure	S.A.	Recovery	Fold pure
PNS	–	11.3 ± 2.5	–	–	4.7 ± 0.9	–	–	0.3 ± 0.02	–	–	1.7 ± 0.4	–	–
SOL	64.5 ± 2.2	n.d.	0	0	3.6 ± 0.4	41.8 ± 6.5	0.8	n.d.	0	0	0.9 ± 0.3	40.2 ± 5.7	0.5
MITO	11.5 ± 0.9	39.1 ± 8.6	35.7 ± 11.6	3.5	7.7 ± 1.9	18.0 ± 2.5	1.6	1.7 ± 0.1	64.9 ± 3.9	5.7	4.3 ± 0.5	30.4 ± 3.7	2.5
F-1	3.4 ± 0.5	98.7 ± 16.7	28.7 ± 7.5	8.7	19.1 ± 1.8	13.7 ± 3.1	4.1	0.013 ± 0.006	0.2 ± 0.1	0.04	3.6 ± 0.5	10.2 ± 4.7	2.1
F-2	2.9 ± 0.4	76.0 ± 23.2	15.9 ± 3.6	6.7	16.3 ± 2.7	8.3 ± 1.7	3.5	0.032 ± 0.008	0.3 ± 0.1	0.11	3.7 ± 0.4	7.2 ± 2.4	2.2
F-3	1.7 ± 0.3	34.5 ± 6.4	4.4 ± 0.9	3.1	14.2 ± 3.1	3.9 ± 0.5	3.0	0.13 ± 0.02	0.8 ± 0.2	0.43	4.7 ± 0.5	5.3 ± 2.4	2.8
Total	84.3 ± 3.1		91.0 ± 18.7 7			85.8 ± 10.6 7			68.8 ± 3.8 11			77.2 ± 18.2 6	

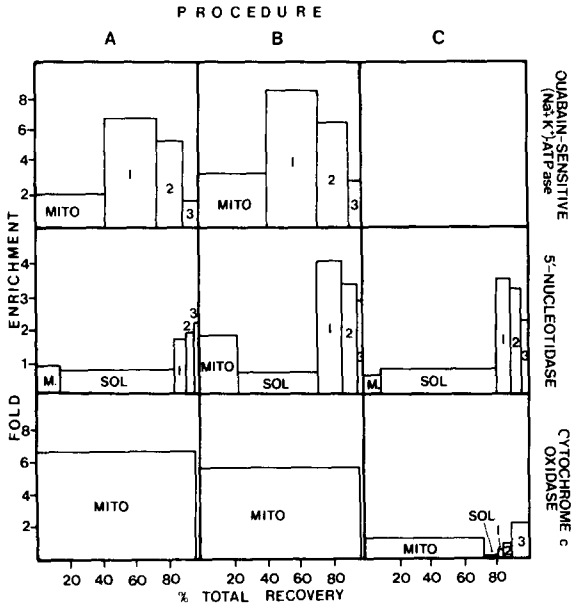

Fig. 1. Fold-purification and percent total recovery of three membrane marker enzymes – a comparison between procedures A, B and C. Data for cytochrome *c* oxidase, 5′-nucleotidase and ouabain-sensitive ($Na^+ + K^+$)-ATPase are presented for each of the three membrane preparative procedures. Fold-enrichment is the ratio of specific activity in a given fraction divided by specific activity in the PNS fraction. Percent total recovery is calculated as the total activity in a given fraction divided by the total activity of that marker recovered in all fractions subsequent to the PNS. SOL, soluble fraction; MITO, mitochondrial fraction; 1, sucrose density gradient fraction 1; 2, sucrose density gradient fraction 2; 3, sucrose density gradient fraction 3. This representation is according to DeDuve et al. [34].

occurs in fraction 1, parallel to a de-enrichment of the mitochondrial marker, cytochrome *c* oxidase.

Nonetheless, some noteworthy differences between the three procedures can be observed. Firstly, ouabain-sensitive ($Na^+ + K^+$)-ATPase is undetectable in the PNS fraction of procedure C. Enrichment and recovery values in subsequent procedure C fractions are therefore indeterminate. The specific activity of ($Na^+ + K^+$)-ATPase is 60.2 ± 15.6 nmol P_i/mg protein per min at 37 °C in fraction 1C, and descends in heavier sucrose density gradient fractions.

Secondly, selectively lower enrichment of 5′-nucleotidase does not occur in heavy sucrose density gradient fractions of procedure A, with respect to its enrichment in fraction 1A. Greatest relative enrichment of this marker appears to occur in fractions 2A and 3A. Although such a reversal in the enrichment profile of ouabain-sensitive ($Na^+ + K^+$)-ATPase does not occur, the difference between ($Na^+ + K^+$)-ATPase enrichment in fractions 1A and 2A appears slightly smaller than the difference between its enrichment in fractions 1B and 2B. These observations have led us to consider procedure B as the method of choice for obtaining maximal plasma membrane enrichment in one fraction (fraction 1).

Thirdly, 98% of total recovered cytochrome *c* oxidase is found in the mitochondrial fractions of procedures A and B, whereas only 72% of its total recovered activity is found in the mitochondrial

TABLE II

DISTRIBUTION OF CYTOCHROME *c* OXIDASE ACTIVITY IN SUBCELLULAR FRACTIONS – PROCEDURES A, B AND C

The three preparative procedures are outlined in Fig. 1. All data are presented as means ± S.E. The number of individual preparations upon which data are based is presented at the bottom of each column. Specific activity is expressed as μmol cytochrome *c* oxidized/mg membrane protein per min at 37 °C. n.d., not detectable; S.A., specific activity; PTR, percent total recovery. PTR values are calculated as the total recovery of cytochrome *c* oxidase in each individual fraction divided by the total activity recovered in all fractions subsequent to PNS.

Fraction	A		B		C	
	S.A.	PTR	S.A.	PTR	S.A.	PTR
PNS	0.26 ± 0.05	–	0.30 ± 0.02	–	0.43 ± 0.01	–
SOL	n.d.	0	n.d.	0	0.02 ± 0.02	8.1 ± 4.6
MITO	1.76 ± 0.24	98.4 ± 0.39	1.70 ± 0.10	98.1 ± 0.39	0.74 ± 0.23	72.0 ± 8.8
F-1	0.02 ± 0.01	0.27 ± 0.15	0.01 ± 0.01	0.22 ± 0.11	0.26 ± 0.09	2.5 ± 0.4
F-2	0.02 ± 0.01	0.44 ± 0.25	0.03 ± 0.01	0.48 ± 0.13	0.43 ± 0.21	5.5 ± 1.9
F-3	0.07 ± 0.01	0.93 ± 0.20	0.13 ± 0.02	1.23 ± 0.29	1.00 ± 0.44	7.8 ± 2.8
	3		6		3	

fraction of procedure C. Cytochrome *c* oxidase is undetectable in the SOL fractions A and B, whereas 8% of its activity emerges in the SOL fraction C. Specific activity and percent total recovery values for cytochrome *c* oxidase in the three sucrose density gradient fractions of procedure C are approx. 10-times higher than in corresponding fractions from procedures A and B. Data concerning cytochrome *c* oxidase for the three procedures are presented in detail in Table II.

We wondered whether the displacement of markers such as cytochrome *c* oxidase and 5′-nucleotidase towards lighter membrane density might occur in direct relation to homogenization force. Distribution of these markers in procedures A and B could conceivably differ from their distribution in procedure C as a result of the difference between media-intima layers, and cells in primary culture. In order to isolate homogenization force as the sole factor in this regard, we built two continuous 20–50% sucrose density gradients, and centrifuged procedure A and B microsomal fractions (Fig. 2). Distributions for protein, Mg^{2+}-ATPase and ouabain-sensitive ($Na^+ + K^+$)-ATPase subsequent to the centrifugation of procedure B microsomes are slightly displaced towards the lighter region of the sucrose gradient with respect to corresponding distributions for procedure A microsomes. Maximum ($Na^+ + K^+$)-ATPase activity for procedure B is recovered at approx. 25% sucrose. For procedure A, ($Na^+ + K^+$)-ATPase activity is recovered over a broader sucrose density range, with maximal recovery occurring at approx. 30% sucrose.

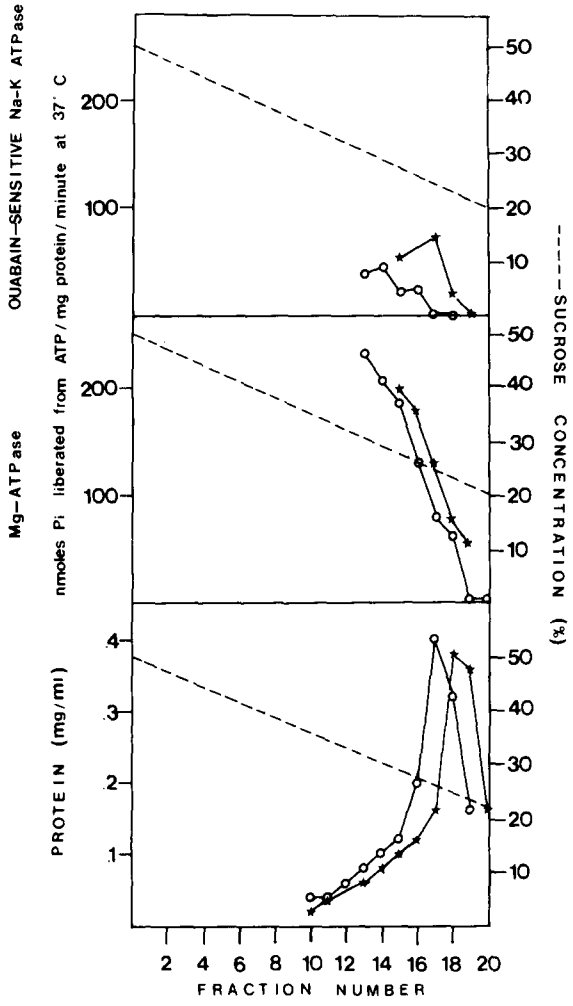

Fig. 2. Distribution of protein and two membrane enzyme markers on a continuous sucrose density gradient – procedures A and B Microsomal pellets arising from procedures A and B were centrifuged on continuous 20–50% sucrose density gradients, as described in Experimental procedures. ------, Sucrose density gradient profile; O ——— O, procedure A; * ——— *, procedure B; protein (bottom), Mg^{2+}-ATPase (middle), and ouabain-sensitive ($Na^+ + K^+$)-ATPase (top) were measured also as described.

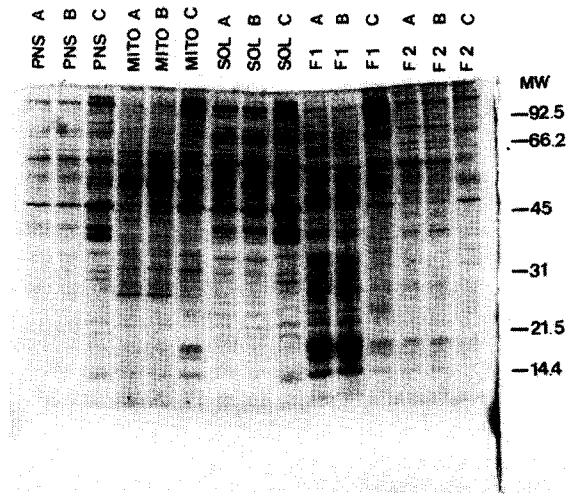

Fig. 3. SDS-polyacrylamide gel electrophoresis of subcellular fractions – procedures A, B and C. Fraction abbreviations above each channel refer to those which are presented in Fig. 1. 20 μg protein from each fraction were electrophoresed, as described in Experimental Procedures. The position of standard molecular weight markers are presented along the right-hand side of the plate. This gel is typical of others which were obtained.

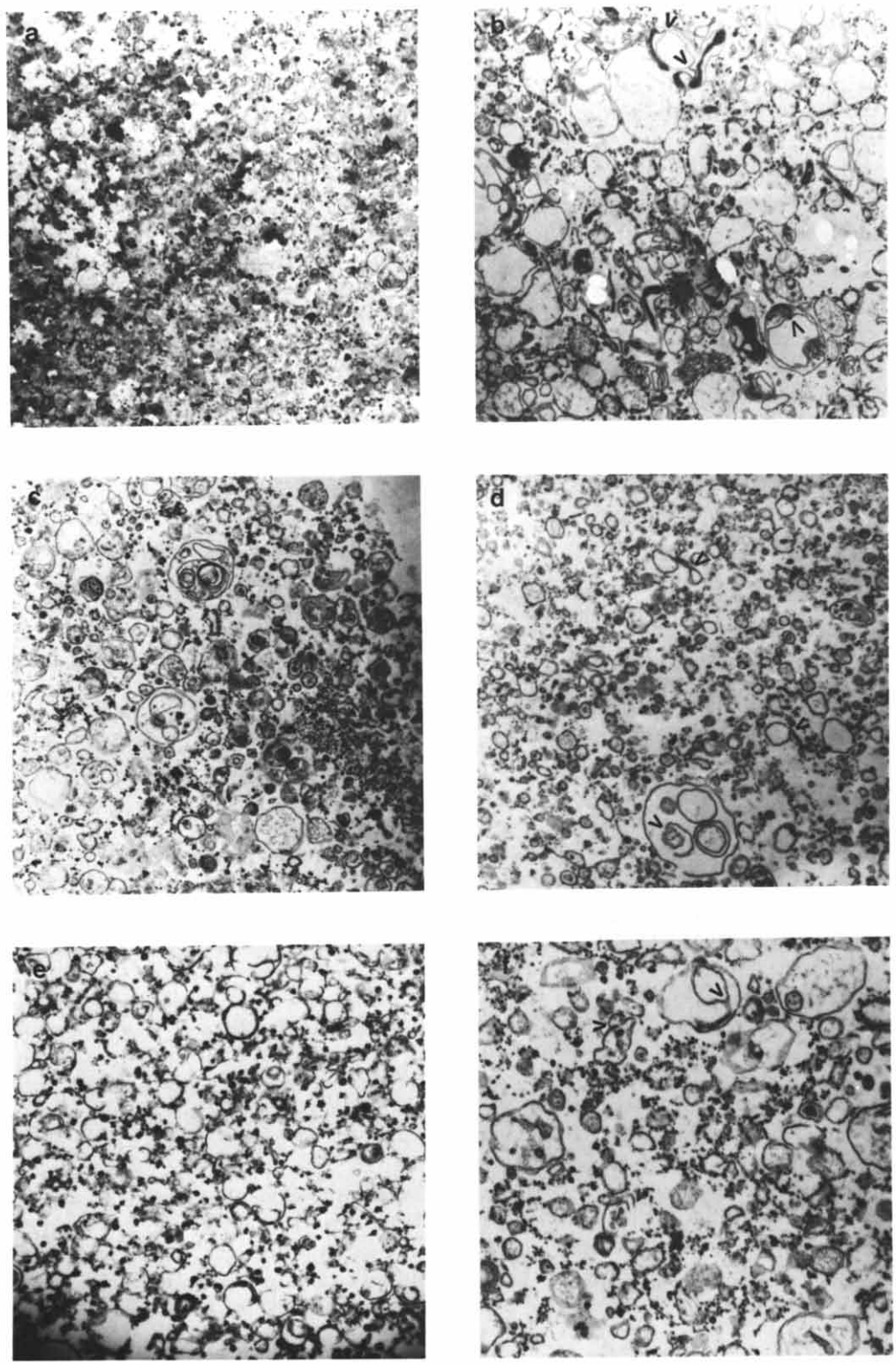

Fig. 4. Electron photomicrographs of individual membrane fractions. (a) PNS fraction C; (b) PNS fraction A; (c) fraction 1A; (d) fraction 1B; (e) fraction 1C; (f) fraction 3A. Vesicles in f are magnified 25000×. All others are of 16000× magnification. Putative membrane attachments are indicated by arrows in each micrograph. The images here are typical of other sections.

Subcellular fractions arising from the three preparative procedures were electrophoresed, and protein banding patterns were compared (Fig. 3). Clear differences can be observed between the range of proteins present in fractions arising from procedures A and B, and those arising from procedure C. In the PNS fraction C, for example, protein bands of M_r 93 000, 90 000, 45 000, 38 000 and 14 000 are clearly more predominant than in PNS fractions A and B. Fractions 1 and 2 from procedures A and B possess a wider range of protein constituents in the low molecular weight range than fractions 1C and 2C. In contrast, other high molecular weight proteins predominate in the latter two. Of particular interest are the protein profiles for the three soluble fractions, where quite a few bands predominate for procedure C.

A selection of membrane fractions arising from the three preparative procedures has been examined electron microscopically (Fig. 4). PNS fractions C and A are presented in Fig. 4a and 4b, respectively. One clearly sees a relatively large quantity of rough endoplasmic reticulum in PNS fraction A, and many 'vesicle-within-vesicle' structures. Structures which appear to be membrane attachments are indicated with arrows in Fig. 4b. None of the structures mentioned above appears in the micrograph of PNS fraction C (Fig. 4a). A large quantity of extracellular debris is present in the section of PNS fraction C shown here. Vesicle structure though, is typical of other sections which we have observed.

Fraction 1 membrane vesicles from procedures A, B and C are shown in Fig. 4c, d and e, respectively. In contrast to fraction 1A, fraction 1B contains very few vesicle-within-vesicle structures. Putative membrane attachments are observed in both micrographs, though (indicated with arrows), and vesicles are closed. On the other hand, fraction 1C vesicles (Fig. 4e) possess a decidedly disrupted appearance. Most are unsealed, and few attached structures are apparent.

Higher magnification (25 000 ×) of fraction 3A in Fig. 4f shows a wide assortment of putative membrane attachments and vesicle shapes, which might contribute to the high density of these vesicles.

Discussion

Enriched plasma membrane preparations from large and small blood vessels [1,2,5,7,8,10–12,16, 17,23], uterus [3,9,18,20,52], gastrointestinal tissue [29,35] and trachea [16,21,24,36] have been reported. Plasma membrane of varying purity has also been obtained from individual non-muscle cells [53–57] and a skeletal muscle cell line [58]. To our knowledge, this is the first time that plasma membrane has been selectively enriched from smooth muscle cells grown in primary culture. Confluent dishes serving as a source for membrane material contain an essentially pure population of smooth muscle cells, derived exclusively from the aortic media.

A non-traumatic homogenization procedure would appear to be the method of choice when working with individual cell preparations. Nonetheless, the studies cited above have resorted to traditional whole-tissue techniques: sonication, pressure disruption and extensive mortar/pestle homogenization. Chang et al. [27] and Fortier et al. [59] have both reported the use of gentle homogenization procedures – similar to our own – based on the hypotonic disruption of cells. The latter study involved rat myometrial cell cultures, but did not attempt to extensively purify plasma membrane as we have.

Enrichment of the plasma membrane markers, 5′-nucleotidase and ouabain-sensitive ($Na^+ + K^+$)-ATPase is the same, or slightly higher in fraction 1B than in comparable fractions obtained from intact smooth muscle tissue [3,9,11,12,20].

NADPH-cytochrome *c* reductase is the only confirmed marker for smooth muscle endoplasmic reticulum [42]. We observe somewhat greater enrichment of this enzyme in the mitochondrial pellet, and in heavy sucrose density gradient fractions, than in plasma membrane fraction 1B. In spite of the fact that consistently greater enrichment of this membrane marker occurs in fractions 2B and 3B than in fraction 1B, the latter fraction clearly contains endoplasmic reticulum. Partial contamination of plasma membrane by endoplasmic reticulum has been reported elsewhere [3,35,42]. The close structural similarity of these two membrane types may preclude what many seek: an enriched plasma membrane fraction con-

taining no endoplasmic reticulum whatsoever. In our particular case, the high degree of cellular protein synthesis known to occur in primary cultures [43] would lead to a relatively high proportion of rough endoplasmic reticulum in the PNS fraction, rendering the selective enrichment of plasma membrane even more difficult.

In contrast to this limitation, our plasma membrane preparation distinguishes itself from others which have been reported, in than it contains essentially no mitochondrial contamination. The presence of cytochrome *c* oxidase activity in enriched plasma membrane fractions is considered to be evidence for mitochondrial disruption. Many studies have skirted this issue [7,18,35,52] by stating that contamination is minimal, even when its activity amounts to almost one-quarter of that found in the PNS fraction [35]. Cytochrome *c* oxidase activity in heavy sucrose density gradient fractions – presumed to contain predominantly endoplasmic reticulum – may amount to an enrichment superior to that in the mitochondrial fraction itself [35].

In our study, the specific activity of cytochrome *c* oxidase in fraction 1 membrane is less than one-tenth of its activity in the PNS fraction. 98% of total recovered cytochrome *c* oxidase activity is found in the mitochondrial fraction of procedures A and B. The recovery and distribution of cytochrome *c* oxidase activity, using intact media-intima layers as source material (procedure C), are comparable to those reported in the literature. The presence of only 75% of the total recovered cytochrome *c* oxidase activity in the mitochondrial fraction C strongly supports our contention that mechanical disruption of intact tissue induces mitochondrial fragmentation, and subsequent contamination of lighter membrane fractions.

If mechanical homogenization of intact tissue induces disruption of mitochondrial membrane, then the disruption of plasma membrane may also occur, along with concomitant reduction in its functional properties. The experiments summarized in Figs. 1 and 2 suggest that this may indeed be the case. In Fig. 1, we see a clear displacement of 5′-nucleotidase activity towards the lighter end of a discontinuous sucrose density gradient, when a gentle homogenization is carried out subsequent to cell lysis (thus, procedure B). In the absence of this gentle homogenization (procedure A), 5′-nucleotidase seems to distribute itself evenly down the discontinuous gradient.

Analysis of the distribution of protein, Mg^{2+}-ATPase, and ouabain-sensitive $(Na^+ + K^+)$-ATPase subsequent to the centrifugation of microsomes on continuous sucrose density gradients (Fig. 2) reveals a slight shift in average vesicle density as a result of increased homogenization force. The distribution profile for these three markers shifts slightly towards lighter sucrose density when procedure A is modified to procedure B.

The second, gentle homogenization used in procedure B may either eliminate extrinsic proteins through shearing force, creating a vesicle population of slightly reduced density, or induce vesicle disaggregation. The latter situation might be viewed as a reduction in the average size of vesicles. We have attempted to evaluate the extent of homogenization-induced vesicle disruption through SDS-polyacrylamide gel analysis, and electron microscopic analysis of individual membrane fractions.

In Fig. 3, a relatively greater quantity of certain protein bands is observed in PNS fraction C and SOL fraction C than in procedure A and B counterparts. This may result from force-induced displacement of membrane proteins. Likewise, the relative lack of certain protein bands in sucrose density gradient fractions arising from procedure C could be explained by the loss of these proteins during the course of homogenization.

Interpretation of these findings is limited by the fact that cultured cells are more synthetic than cells in situ [43]. One might therefore expect certain proteins to predominate in SDS-polyacrylamide gels of procedure A and B subcellular fractions. Likewise, we cannot be certain that plasma membrane derived from media-intima layers (procedure C) is of the same nature as membrane purified from primary cultures – containing exclusively media-derived smooth muscle cells.

The photographs presented in Fig. 4, however, suggest that homogenization force – not simply the difference between media-intima layers and cells in culture – is responsible for membrane disruption and marker enzyme displacement. The presence of variably shaped, closed vesicles, vesicle-within-vesicle structures and putative mem-

brane attachments in procedure A and B fractions strongly suggests that these vesicles are more structurally intact than those which have been enriched from media-intima layers. In certain instances (Fig. 4f), structures as detailed as caveoli appear to be retained. Electron micrographs of other enriched plasma membrane preparations do not reveal structures of such a variety [3,6,9,11,44, 45]. Plasma membrane vesicles prepared according to procedure C (Fig. 4e) do not appear to be as intact as those prepared from cell cultures.

Two pieces of evidence suggest that plasma membrane-enriched fraction 1B possesses functional characteristics worthy of future study. Firstly, preliminary studies conducted in our laboratory [38] have shown that fraction 1B membrane contains an endogenous type II cyclic AMP-dependent protein kinase, and a phosphoprotein of M_r 16000, which is phosphorylated either in the presence of endogenous kinase or exogenous catalytic subunit.

Secondly, the specific activity of ouabain-sensitive ($Na^+ + K^+$)-ATPase in fraction 1B (98.1 $\pm$ 16.7 nmol P_i/mg protein per min at 37°C) is substantially higher than its activity in other smooth muscle membrane preparations [29,46–50]. It is similar to the activity reported recently for carotid cells grown in vitro [51]. Ouabain-sensitive ($Na^+ + K^+$)-ATPase has been historically difficult to measure in smooth muscle, because of the high backround level of Mg^{2+}-ATPase [35]. We have also found its activity to be rather low in membrane fractions derived from media-intima layers (procedure C) and undetectable in cruder fractions (e.g., PNS fraction C). Either primary cell cultures synthesize the enzyme more actively, or our method for preparing membrane from cultured myocytes is less detrimental to its activity than the procedures used in the above studies.

In any case, further studies on the functional capacity of our plasma membrane-enriched fraction 1B may contribute significantly to present knowledge concerning the role of plasma membrane in smooth muscle regulation.

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