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PARTIAL PURIFICATION OF AN INTERCALATED DISC-CONTAINING CARDIAC PLASMA MEMBRANE FRACTION

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The sarcolemma of cardiac muscle cells contains a specialised junctional region, the intercalated disc which includes three types of intercellular junction, the macula and fascia adherens and the nexus or gap junction. To facilitate the isolation of these junctions a procedure for the partial purification from mouse hearts of a subcellular fraction containing the intercalated disc region of the sarcolemma was developed. This involved investigating methods of tissue disruption that preserve the integrity of the intercalated disc and minimise myofibrillar entrapment of organelles. Examination of the distribution of marker enzymes showed that relative to the homogenate the intercalated disc fraction prepared by sucrose density centrifugation was only enriched 1.5- to 3-fold in 5′-nucleotidase and ($Na^+ + K^+$)-ATPase activities, whereas mitochondrial and sarcoplasmic reticulum marker enzymes were low. The properties of the intercalated disc-containing fraction were compared with the vesicular sarcolemmal fractions devoid of junctional complexes prepared by other methods.

Introduction

In heart tissue the cardiocytes are attached to each other by a specialised region of the sarcolemma termed the intercalated disc, that contains three types of intercellular junctions [1–4]. The fascia and macula adherens junctions of the disc attach cardiocytes and facilitate the transmission across the heart of the force generated by the cytoplasmic myofilaments. The third category of junctions, the gap junction or nexus, is generally accepted as a membrane specialisation providing pathways for direct intercellular communication [5]. In cardiac muscle, gap junctions are candidates for providing pathways for electrical coupling of the cardiocytes allowing rapid spread of current across the heart and leading to the orderly recruitment of contraction across the organ [6]. Studies with isolated beating cardiocytes suggest that the formation of gap junctions preceeds the acquisition of synchronous beating in cell aggregates [7,8].

To understand the mechanisms underlying cell-cell interactions in the heart, it is necessary to isolate the intercalated disc region of the plasma membrane and analyse at a molecular level the component parts of the junctional specialisations. Although there are many reports describing the isolation of cardiac sarcolemma [9–14], these preparations appear to consist predominantly of vesicular membranes derived in the main from non-junctional areas of the sarcolemma, and no reports of the isolation of the intercalated disc region of the sarcolemma have appeared. We now describe the development of procedures that yield inter-

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calated disc-containing plasma membranes from rodent hearts.

Materials and Methods

Animals. Mice (20–30 g) of either sex, fed ad libitum, were used.

Media and homogenisation conditions. Hearts (5–8 g) from 100 mice were washed in an ice-cold Krebs Ringer solution buffered with Tris-HCl (10 mM), pH 7.4 or a 'depolymerising buffer' (composition 10 mM Tris-histidine (pH 7.0), 20 mM sodium pyrophosphate and 0.1 mM EDTA). Hearts were homogenised in the same buffer using an Ultraturrax TP18/2 tissue disintegrator (Janke and Kunkel, Staufen im. Bresgau, F.R.G.) under various conditions, followed by loose or tight-fitting Dounce homogenisation steps.

Subcellular fractionation. The final fractionation procedure used is shown in Fig. 1. Differential centrifugation steps were carried out using a Sorvall GSA 6 × 300 ml rotor. Density-gradient centrifugation was carried out in a Beckman SW27 rotor.

Enzyme markers. Succinate dehydrogenase (EC 1.3.99.1) was determined by the method of Earl and Korner [18]. 5'-Nucleotidase (EC 3.6.1.5) was determined by the method of Avruch and Wallach [19] in the presence of 10 mM β-glycerophosphate. Ca^{2+}-activated and ouabain-sensitive $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) activities were determined by modification of the methods of Warren et al. [20] and Slack et al. [21], respectively. Protein was determined by the method of Lowry et al. [22].

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out

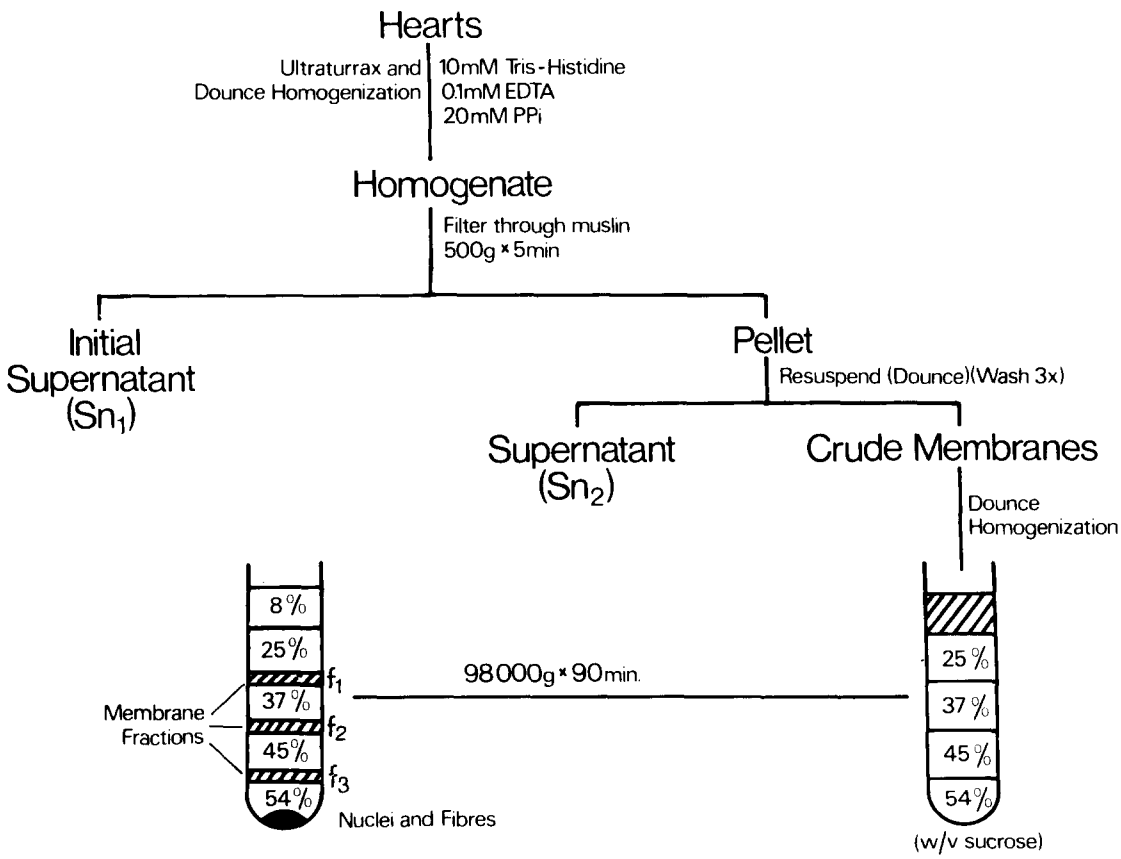

Fig. 1. Preparative procedure used for subcellular fractionation of hearts and the preparation of membrane fractions containing intercalated discs (fraction f_3).

in vertical slab gels using discontinuous buffer systems [23], routinely using 7.5% acrylamide gels. Molecular weight standards used were myosin (200 000), β-galactosidase (130 000), phosphorylase *b* (90 000), transferrin (78 000), ovalbumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (34 000), myosin light chain (25 000) and cytochrome *c* (12 500). Polypeptides were visualised by staining with Coomassie Blue. Glycoproteins in polyacrylamide gels were visualised by binding of ^{125}I-labelled concanavalin A, labelled using chloramine T [24] as described by Gurd and Evans [25].

Electron microscopy. Tissue blocks and subcellular fractions were fixed in 2% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.6) and post fixed in 2% osmium tetroxide in the same buffer. All samples were stained 'en block' with 1% uranyl acetate in cacodylate buffer and dehydrated in a graded series of ethanol. Samples were embedded in Araldite. Sections were stained with either uranyl acetate or uranyl acetate and lead citrate and examined in a Phillips 300 electron microscope.

Results

Drastic homogenisation is necessary for the efficient disruption of cardiac muscle to overcome the tensile strength of the myofibrillar apparatus. In the present work, the use of disruption methods producing high shear forces had to be reconciled with a requirement for preserving the morphological identity of the intercalated discs, especially since no biochemical markers have been established for this sarcolemmal specialisation. A variety of conditions for homogenising mouse hearts were examined. The emphasis on enzymic markers was confined to two widely used plasma membrane markers, 5′-nucleotidase and $(Na^+ + K^+)$-ATPase. The extent of contamination of the plasma membrane fractions by intracellular membranes and organelles was monitored using enzymic markers for the mitochondria (succinate dehydrogenase) and sarcoplasmic reticulum (Ca^{2+}-ATPase). Furthermore, the persistence of myofibrillar elements in the fractions was monitored morphologically using thin section electron microscopy and corroborated, semi-quantitatively, by using SDS-polyacrylamide gel electrophoresis to analyse the intensity of those polypeptide bands of electrophoretic mobilities corresponding to actin and myosin.

Tissue homogenisation and subcellular fractionation

The medium used for homogenisation 'depolymerising buffer' (see Materials and Methods) was selected because of its property of relaxing skeletal muscle [26]. When Tris-buffered Krebs Ringer was substituted or EDTA and pyrophosphate were excluded from the 'depolymerising buffer' a large amount of myofibrillar contamination of the subcellular fractions resulted. Mouse hearts and homogenates were left in 'depolymerising buffer' for 10 min or less, for after 20 min disintegration of the adherens-type junctions, separation of the intercalated discs and interiorisation of gap junctions occurred.

Various speed settings of the Ultraturrax followed by dispersion of pellets using Dounce homogenisers as well as a tissue press containing a perforated plate with holes 1 mm in diameter [16] were examined. The best condition, assessed by the recovery of 5′-nucleotidase and minimum recovery of succinate dehydrogenase in a low-speed pellet (Fig. 1) used the Ultraturrax homogeniser at the lowest setting (3 × 5 s bursts) followed by further dispersion of the tissue homogenate using a loose-fitting Dounce homogenizer. However, morphological examination of the low speed pellet (500 × *g* for 5 min) obtained under these conditions revealed insufficient myofibrillar disruption. Homogenisation of hearts in the Ultraturrax at setting 6 gave efficient tissue disruption but also caused breakdown of the intercalated discs and some nuclear damage. The final procedure selected involved homogenisation at setting 2.5 (3 × 5 s bursts) followed by further dispersion in a Dounce homogeniser with a loose-fitting pestle. The pellet collected after washing by low speed centrifugation was then fractionated on sucrose gradients (Fig. 1). The distribution of enzymic markers among the subfractions is shown in Table I. A large proportion of the total protein and enzymic activities applied to the sucrose gradients was recovered in the pellet that contained mainly nuclei and undisrupted myofibrillar fragments entrapping mitochondria and vesicular membranes.

TABLE I

SUBCELLULAR DISTRIBUTION OF PROTEIN AND MARKER ENZYMES IN MOUSE HEART SUBFRACTIONS

Mouse hearts were homogenised using 3×5 s bursts of the Ultraturrax at setting 2.5 followed by a Dounce homogenisation step with a loose-fitting pestle and filtration through two layers of musling to yield the homogenate. For further details see Fig. 1 and text. S.D. for three to five experiments given.

Fraction	Total protein (mg)	Specific activities of marker enzymes (μmol/h per mg protein)			
		5'-Nucleotidase	(Na^+/K^+)-ATPase	Ca^{2+}-ATPase	Succinate dehydrogenase
Homogenate	1525 ±302	4.4±1.6	11.2± 4.6	14.3± 6.1	4.6±2.2
Initial supernatant (Sn_1)	739 ±140	3.7±1.8	10.9± 5.6	11.2± 7.1	5.4±3.0
Supernatant (Sn_2)	295 ± 63	1.4±0.3	3.3± 0.9	9.6± 3.6	2.8±0.5
Crude membranes	339 ± 78	7.8±2.5	10.6± 3.1	27.3± 9.8	5.8±1.3
f_1	4.2± 2.0	14.4±4.9	30.6±10.0	19.3±10.0	3.0±1.2
f_2	6.3± 1.9	13.2±4.0	39.1±12.1	10.8± 4.3	3.8±1.4
f_3	7.4± 2.1	7.5±2.7	28.9± 9.1	8.3± 2.9	2.7±0.9
Pellet	282 ± 5.1	6.2±2.7	8.4± 3.33	29.5±15.3	4.1±1.4

Analysis of the distribution of enzymic markers in the fractions collected from the sucrose gradients indicated that fractions f_1 and f_2 contained the highest specific activities and recovery of both 5'-nucleotidase and Ca^{2+}-ATPase, whereas $(Na^+ + K^+)$-ATPase activity showed a more even distribution in the fractions. Fig. 2 shows that fraction f_1 contained mainly small membrane vesicles, probably derived from the sarcolemma and the sarcoplasmic reticulum, whereas fraction f_2 contained vesicles of larger diameter than those in fraction f_1 and mitochondria (not shown). Fraction f_3 showed generally lower specific activities and recovery of the plasma membrane marker enzymes than fractions f_1 and f_2 with the exception of $(Na^+ + K^+)$-ATPase, and morphological examination showed it to be the fraction containing easily recognisable intercalated disc fragments as well as membrane vesicles, some mitochondria and amorphous material (Fig. 3). At higher magnification, some of the isolated intercalated discs contained all three intercellular junctional specialisations arranged adjacently (Fig. 3, inset).

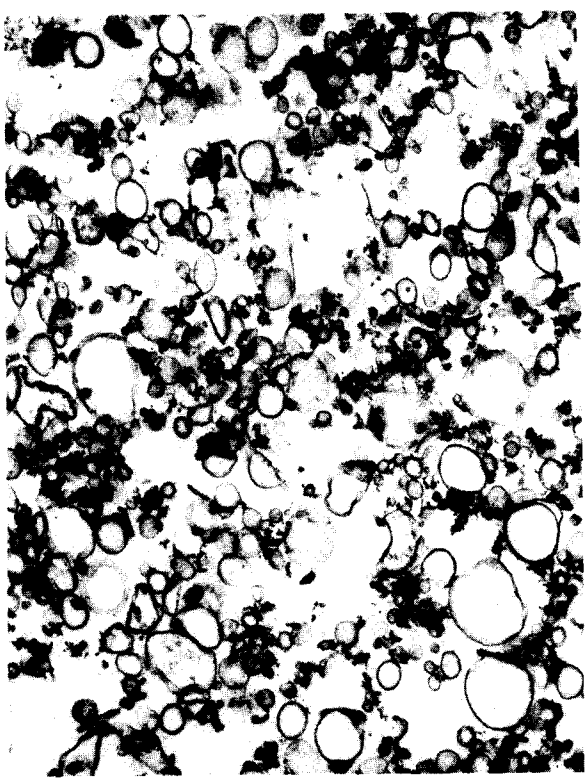

Fig. 2. Electron micrograph of membrane fraction f_1 collected from the sucrose gradient (see Fig. 1). (Magnification: ×14000).

Comparison of the protein composition of the three membrane fractions, f_1, f_2 and f_3 by SDS-polyacrylamide gel electrophoresis (Fig. 4) showed no major polypeptides unique to any one fraction. Fractions f_2 and f_3 contained major polypeptides corresponding in electrophoretic mobilities to myosin and actin, the major myofibrillar proteins. Autoradiography of gels exposed to iodinated concanavalin A (not shown) identified about seven glycoproteins in fractions f_2 and f_3, including a major glycoprotein of apparent molecular weight 82000 that stained poorly with Coomassie Blue.

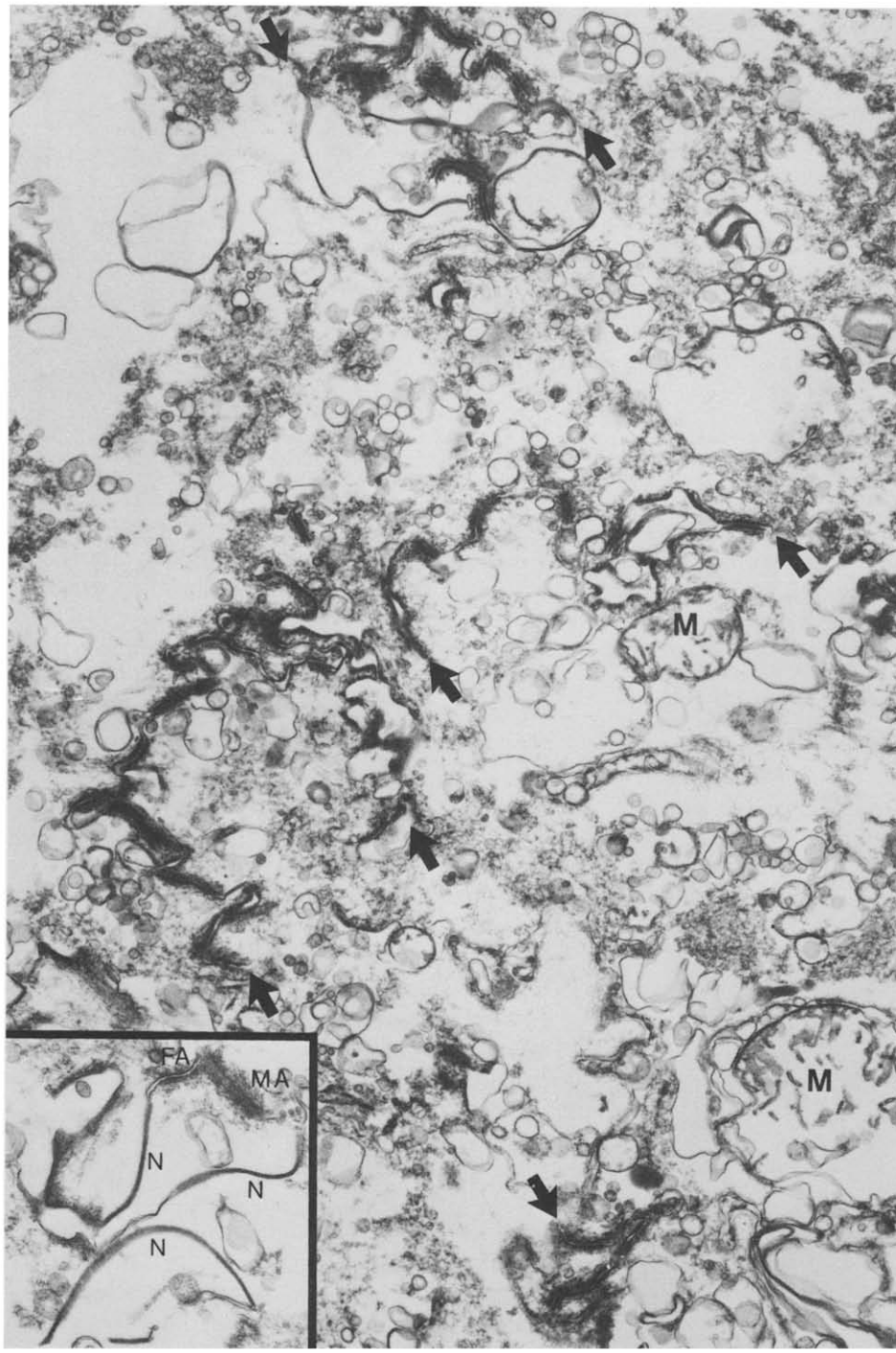

Fig. 3. Electron micrograph of membrane fraction f_3 from sucrose gradient (see Fig. 1) showing isolated intercalated discs (between arrows), mitochondria (M) and amorphous material. (Magnification: ×16600). Inset: An isolated intercalated disc showing nexus or gap junctions (N) and the two adherens-type junctions, the macula (M.A.) and fascia (F.A.) adherens. (Magnification: ×52000).

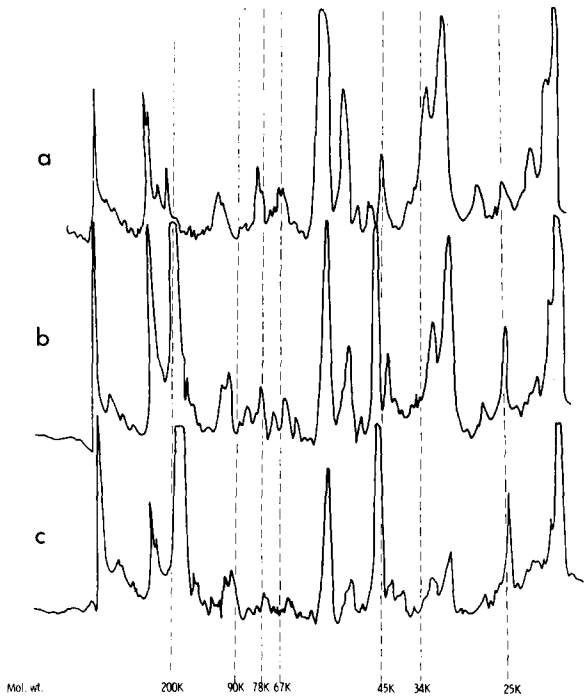

Fig. 4. SDS-polyacrylamide gel electrophoretic profiles of membrane fractions from mouse heart (see Fig. 1). (a) Fraction f_1 (146 μg); (b) fraction f_2 (240 μg); (c) fraction f_3 (210 μg). The myofibrillar proteins, actin and myosin were estimated to account for 8% of fraction f_1, 20% of fraction f_2 and 29% of fraction f_3. The anode is on the right-hand side.

Discussion

In muscle tissue, the presence of a sturdy myofibrillar apparatus makes it difficult to carry out efficient and controlled disruption [27]. Our goal required the preservation of the structural integrity of the intercalated discs to help identify them in the subcellular fractions. At the same time, the myofibrils had to be disrupted sufficiently to release organelles and dissociate them from the intercalated discs. Two main approaches have been used to minimise myofibrillar contamination of sarcolemmal fractions which deserve comment in context of the isolation of intercalated discs. In the first, intact myofibrils present in the tissue homogenate were removed by low speed centrifugation [28–30], but this could not be applied for the intercalated discs co-sedimented. In a second approach fractions were extracted with concentrated salt solutions [13,28,30–32]. In the present studies media containing EDTA and pyrophosphate, in combination with thorough filtration of the tissue homogenate were found to be sufficiently effective in minimising myofribillar contamination.

Almost half the total activities of the plasma membrane marker enzymes of the tissue homogenate were not sedimented at low centrifugal speeds and this observation is consistent with the isolation of vesicular sarcolemmal fractions from the supernatants of low speed centrifugation steps [13,14,28–30]. Although similar recoveries of plasma membrane marker enzymes were obtained in all three membrane fractions (collected from the sucrose gradients), the two lighter fractions f_1 and f_2 showed generally higher specific activities of the 5′-nucleotidase and Ca^{2+}-ATPase. This agrees with the higher specific activities of plasma membrane marker enzymes found in sarcolemmal fractions of low buoyant densities (< 1.16 g/cm^3) on sucrose gradients [13,14,29,30]. Sarcolemmal fractions isolated at higher buoyant densities in sucrose gradients (> 1.18 g/cm^3) showed generally lower specific activities of the plasma membrane marker enzymes [13,17,31,32] as did the heavy membrane fraction f_3 isolated by the present procedure. The $(Na^+ + K^+)$-ATPase appears to have a different distribution profile than 5′-nucleotidase among the three subfractions.

Two explanations may be advanced for the low specific activities of the two plasma membrane markers in sarcolemmal fraction f_3. The first is that the persistence of large amounts of myofibrillar proteins decreased the calculated values for the specific activities of the marker. However, when the contribution of actin and myosin to the total polypeptides separated on the polyacrylamide gels (Fig. 4) was taken into account, the specific activities of the plasma membrane enzymes were increased by only about 10–30%. We thus favour a second explanation that the low specific activities of te plasma membrane markers in sarcolemmal fraction f_3 is mainly a result of an uneven distribution of the 5′-nucleotidase and $(Na^+ + K^+)$-ATPase at the various regions of the cardiac plasma membrane. Indeed, vesicular sarcolemmal fractions of low buoyant densities, and assumed to

derive mainly from the 'free' surfaces of the cardiocytes have specific activities up to 50-fold higher than that of the homogenate [13,14,29,30]. Although Ca^{2+}-ATPase is a marker for the sarcoplasmic reticulum, some activity may also reside at the plasma membrane [14] making interpretation of subcellular fraction purity more difficult.

In conclusion, the results suggest that the intercalated disc region of the cardiocyte plasma membrane contains low amounts of two plasma membrane markers, especially when compared to the vesicular sarcolemmal membranes prepared by other procedures. This conclusion is, perhaps, in keeping with the large numbers of intercellular junctions present and which are postulated to function in cell-cell adhesion (macula adherens), the intercardiocytic transmission of contractile force (fascia adherens) and in intercellular communiation (gap junction or nexus). The isolation of fractions containing two of these junction specialisations has now been achieved commencing with the intercalated disc fraction described in the present work [33].

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