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UNMASKING OF MEMBRANE ENZYME ACTIVITIES AND THE PROBLEM OF SUBCELLULAR LOCALIZATION OF ADENYLATE CYCLASE IN PIG LYMPH NODE LYMPHOCYTES

JEAN-CLAUDE BONNAFOUS, JACQUES DORNAND, JEAN FAVERO and JEAN-CLAUDE MANI

Laboratoire de Biochimie des Membranes, ER CNRS 228, Ecole Nationale Supérieure de Chimie, 8 rue de l'Ecole Normale, F-34075 Montpellier Cedex (France)

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Summary

A large-scale purification of plasma membranes from pig lymph node lymphocytes is described. Centrifugation on a discontinuous sucrose density gradient was performed in a zonal rotor. Adenylate cyclase activity of untreated fractions displayed a profile different from that of plasma membrane enzymatic markers and was maximal at higher density. However, when latent adenylate cyclase was unmasked by Lubrol PX treatment, its maximum was shifted to lower density and was no longer significantly different from that of plasma membrane markers. These results are discussed in terms of cell surface topography.

Introduction

The isolation and purification of lymphocyte plasma membrane enzymes [1—3] led us to prepare large amounts of plasma membrane-enriched fractions. The study of adenylate cyclase (EC 4.6.1.1), a very labile multicomponent system, requires the preparation of plasma membranes as rapidly as possible. We describe here a large-scale preparation of plasma membranes from pig mesenteric lymph nodes by sucrose density gradient centrifugation in a zonal rotor. The distribution of adenylate cyclase activity along the gradient, compared with the distribution of classical enzymatic markers, raised the problem of its subcellular localization. A few extensive studies of lymphocyte adenylate cyclase have already been carried out [4—7], but some discrepancies appear as to its distribution among subcellular fractions. We think that latent enzymatic

activities, as evidenced by detergent treatment [8], must be taken into account in order to estimate more accurately subcellular enzyme localization. Our results are discussed in terms of cell surface topography [9,10].

Materials and Methods

Pig lymph nodes were homogenized with a press, as previously described [11]. The homogenate was centrifuged twice at 1500 × *g* for 15 min and the supernatant at 30 000 × *g* for 1 h, the resulting pellet was resuspended in 50 ml of 10 mM Tris-HCl, pH 7.5, 0.25 M sucrose and introduced into a B XIV MSE zonal rotor filled with six layers of 10 mM Tris-HCl, pH 7.5, containing various sucrose percentages: 80 ml, 15%; 80 ml, 20%; 100 ml, 25%; 100 ml, 30%; 150 ml, 35%; 140 ml, 40%. After centrifugation for 2 h at 47 000 rev./min, equilibrium was reached for all particles. The rotor was unloaded using a 50% sucrose solution; 55-ml fractions were collected, diluted with sucrose-free buffer and centrifuged for 1 h at 30 000 × *g*. The pellet was resuspended in appropriate amounts of 10 mM Tris-HCl, pH 7.5, and stored in liquid N_2 before the determination of enzymatic activities.

Adenylate cyclase was assayed according to the method of Salomon et al. [12]: the reaction mixture contained 25 mM creatine phosphate, 2.5 mM $MgCl_2$, 0.1 mM EGTA, 50 μM GTP, 1 mM cyclic AMP, 0.5 mM [α-^{32}P]ATP ($1.5 \cdot 10^6$ cpm per assay).

Other enzymatic activities were determined under conditions reported earlier: 5′-nucleotidase (EC 3.1.3.5) [1], ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) [2], Ca^{2+}-ATPase (EC 3.1.3.5) [1], γ-glutamyltranspeptidase (EC 2.3.2.2) [13], alkaline phosphatase (EC 3.1.3.1) [14], phosphodiesterase I (EC 3.1.4.1) [15], acid phosphatase (EC 3.1.3.2) [16], glucose-6-phosphatase (EC 3.1.3.9) [17], succinate dehydrogenase (EC 1.3.99.1) [18], thiamine pyrophosphatase (EC 2.5.1.3) [19].

[α-^{32}P]ATP was obtained from The Radiochemical Centre, Amersham.

Results and Discussion

Distribution of plasma membrane markers in sucrose density gradients

The purpose of this work was not to obtain the highest yield of plasma membranes, but to achieve a good and rapid purification. In order to reach a better resolution in the plasma membrane purification step, the lymph node homogenate was previously freed of most nuclei by two low-speed centrifugations (see Materials and Methods) and the 30 000 × *g* pellet was centrifuged on a discontinuous sucrose density gradient in a zonal rotor.

The specific activities of several enzymes usually considered as plasma membrane markers were determined in the 12 fractions collected from the gradient; Fig. 1 shows that the profiles of 5′-nucleotidase, ($Na^+ + K^+$)-ATPase, γ-glutamyltranspeptidase, alkaline phosphatase and phosphodiesterase I can be superposed, the maximal activities are found for sucrose density 1.12 g/cm^3 (fraction 6). This was in agreement with previously published works [13—15, 19—22] describing these enzymes as plasma membrane markers. Moreover, the profile of Ca^{2+}- (or Mg^{2+}-)ATPase is the same as those of the markers, which con-

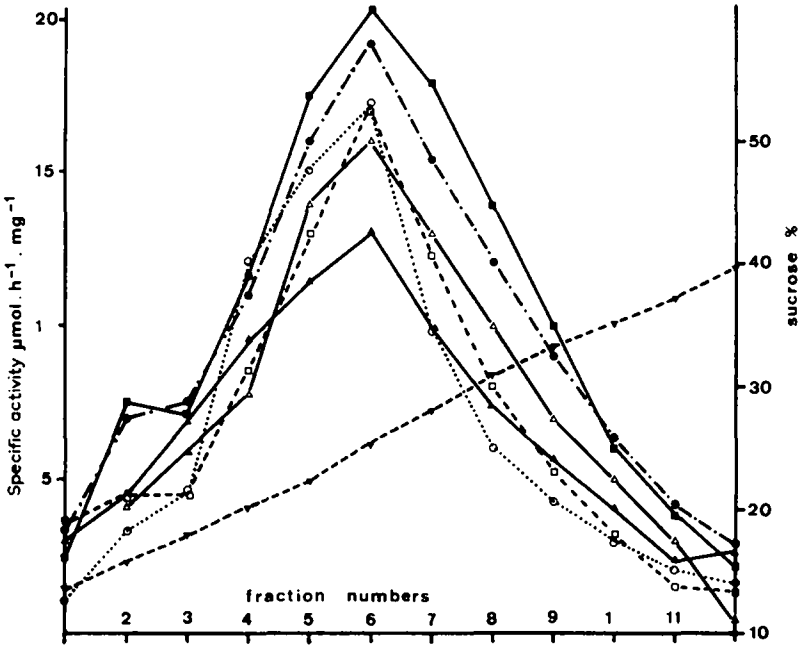

Fig. 1. Distribution of plasma membrane markers in sucrose density gradients (▼- - - - -▼, sucrose % of the fractions). Specific activities are expressed in µmol P_i/h per mg for 5′-nucleotide (□- - - - -□), (Na^++K^+)-ATPase (△————△), Ca^{2+}-ATPase (▲————▲), phosphodiesterase I (●— · — · —●), alkaline phosphatase (○· · · · · ·○) or in µmol *p*-nitroaniline/h per mg for γ-glutamyltranspeptidase (■————■).

firms the membrane localization of this enzyme which hydrolyses extracellular ATP [1]. These profiles were highly reproducible for different membrane preparations.

The extent of plasma membrane purification can be estimated from the 5′-nucleotidase specific activities in the homogenate, 30 000 × *g* pellet and fraction 6 : 0.8, 4.0 and 17 µmol P_i/h per mg protein, respectively.

Distribution of adenylate cyclase activity

In a typical experiment, the basal and fluoride-stimulated adenylate cyclase activities were (in pmol cyclic AMP/mg per 15 min); 246 and 704, 495 and 1201, 418 and 1793, for the homogenate, 30 000 × *g* pellet and fraction 6, respectively. It is not surprising that these values do not fit with those of 5′-nucleotidase and do not follow the purification pattern, because of the well known lability of adenylate cyclase and the possible loss of regulatory factors during the centrifugations.

Basal and fluoride-stimulated adenylate cyclase activities were reproducibly found to be maximal in fraction 8, the density of which was significantly higher (1.15—1.16 g/cm^3) than that of membranes (Fig. 2) and which contains plasma membrane vesicles as evidenced by electron microscopy and membrane enzymatic marker activities (Fig. 1). Besides the possibility that fractions 6 and 8 contain membrane vesicles from different cell subpopulations (a problem which can only be overcome by working with cultured cell lines), three hypotheses could account for this observation: (1) adenylate cyclase could be present not only in plasma membranes, but also in other subcellular structures,

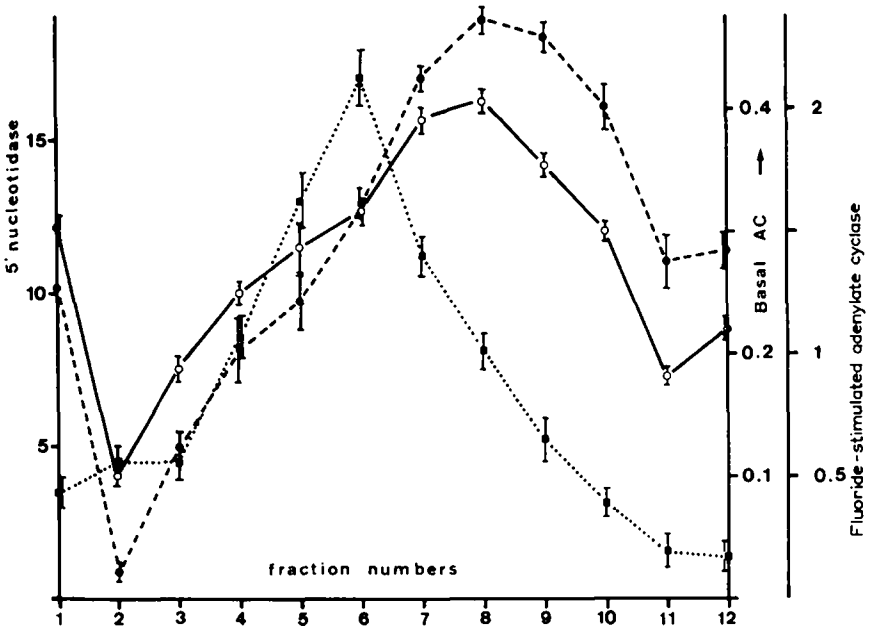

Fig. 2. Distribution of basal (●-------●) and fluoride-stimulated (○———○) adenylate cyclase in sucrose density gradients (untreated fractions), specific activity in nmol cyclic AMP/mg per 15 min, compared with the distribution of 5'-nucleotidase (■·····■), specific activity in μmol/h per mg.

as suggested by Snider and Parker [6] who detected adenylate cyclase activity in microsomal and mitochondria-rich fractions, and Wedner and Parker [5] who characterized a nuclear enzyme; (2) one could imagine that tissue homogenization leads to different types of plasma membrane vesicle with different densities and different enzyme distribution; such a possibility is strengthened by the data of Rode et al. [10] who suggested that lymphocyte plasma membranes display a 'functional mosaicism'; (3) during homogenization, some plasma membrane sheets or vesicles might artificially associate with heavier particles, leading to a stimulation of adenylate cyclase; the possibility of the involvement of proteolytic activities (e.g., of lysosomal origin) cannot be excluded, since proteolytic enzymes have been reported to stimulate adenylate cyclase [23].

The profiles of the activities of several organelle markers are shown in Fig. 3: succinate dehydrogenase for mitochondria, thiamine pyrophosphatase for Golgi apparatus, acid phosphatase for lysosomes and glucose-6-phosphatase for endoplasmic reticulum. From these profiles, it is difficult to attribute a specific localization to the adenylate cyclase activity of fraction 8. Electron-microscopic examination of this fraction showed the presence of a great amount of rough endoplasmic reticulum (Fig. 5). As for calf thymocytes [7], plasma membranes (fraction 6) were contaminated by Golgi membranes.

Since the profiles of six plasma membrane enzymes are well superposed (Fig. 1) hypothesis 2 seems unlikely: it would imply the existence of membrane areas enriched only in adenylate cyclase. We checked the effects of low concentrations of Lubrol PX on plasma membrane fraction 6 and on fraction 8

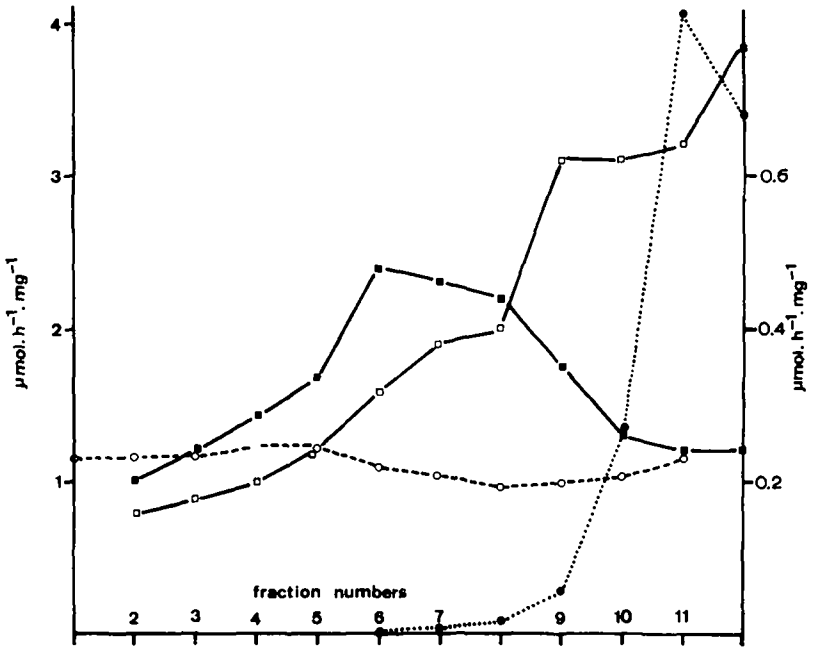

Fig. 3. Distribution of succinate dehydrogenase (●· · · · · ●), glucose-6-phosphatase (○- - - - -○), acid phosphatase (□————□) and thiamine pyrophosphatase (■————■) in sucrose density gradients. Specific activities are expressed in: (left scale) µmol P_i/h per mg for glucose-6-phosphatase and acid phosphatase or µmol oxidized succinate/h per mg for succinate dehydrogenase; (right scale) µmol P_i/h per mg for thiamine pyrophosphatase.

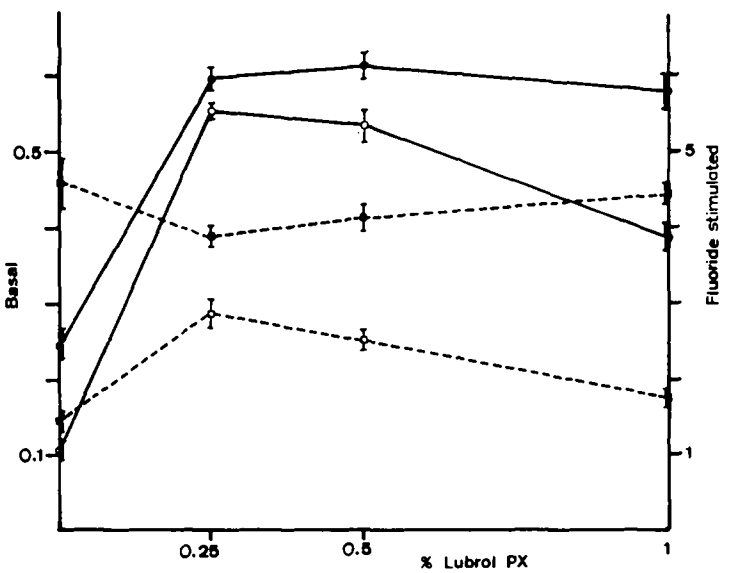

Fig. 4. Effect of Lubrol PX on adenylate cyclase specific activities of sucrose gradient fractions: basal (●————●) and fluoride-stimulated (○————○) activities of fraction 6; basal (■- - - - -■) and fluoride-stimulated (□- - - - -□) activities of fraction 8. Fractions 6 and 8, obtained as described in Materials and Methods, were preincubated for 12 min at 0°C with the indicated Lubrol PX concentrations before adenylate cyclase determination. (Specific activities are expressed in nmol cyclic AMP/mg per 15 min.)

corresponding to maximal adenylate cyclase activity. After Lubrol PX treatment, the basal and fluoride-stimulated adenylate cyclase activities of fraction 6 are higher than the corresponding activities of fraction 8 (Fig. 4). The ratio of fluoride-stimulated activities in fractions 6 and 8, measured in the presence of Lubrol PX, does not noticeably differ from the ratios of plasma membrane marker activities (the fluoride-stimulated activity can be considered to reflect the amount of adenylate cyclase). The unmasking action of the detergent which has already been reported [8,24] is more important for fraction 6 than for fraction 8. The ratio of specific adenylate cyclase activities in fractions 6 and 8 was also inverted after treatment with alamethicin (data not shown), a channel-forming antibiotic which was shown to exhibit detergent-like properties in our preparations from pig lymph node lymphocytes [8], and the action of which is not relevant to the problem of plasma membrane vesicle orientation.

While isoproterenol stimulated the adenylate cyclase of homogenates from pig lymph node lymphocytes [25], PGE_1 was the only hormonal effector which stimulated the enzyme in purified membrane preparations, in agreement with the data of Monneron and d'Alayer [7] for calf thymocytes; the extent of PGE_1 stimulation (maximum 100%) was not significantly different for fractions 6 and 8.

It is impossible to rule out the possibility of the existence of two adenylate cyclase enzymes localized on different types of membrane or on different plasma membrane areas, since both enzymes display different sensitivity to detergent treatment due to a differing lipid environment. However, an alternative explanation for this differential detergent or alamethicin unmasking is that plasma membrane vesicles of fraction 8 are artificially associated with other subcellular components and that this association leads to unmasking of adenylate cyclase through either mere physical contact or enzymatic activities (i.e., proteolytic activities [23]) associated with these components, which would make any subsequent detergent treatment less efficient.

The fact that plasma membranes were obtained from whole lymph nodes instead of isolated lymphocytes does not appear to modify the enzyme distributions as Allan and Crumpton [22] showed that lymph nodes contain at least 93% small lymphocytes, which was confirmed by the comparison of plasma membranes from lymph node and purified lymphocytes [26]. On the other hand, such apparent anomalies of adenylate cyclase profiles were observed for fractions from isolated lymphocytes [7].

The importance of the unmasking of membrane enzymatic activities

In the preceding section, we have emphasized the difficulty of finding standard conditions to measure adenylate cyclase activity and the need to take into account the unmasking of latent activities in the study of its subcellular localization.

Our studies dealing with the role of cyclic AMP in lymphocyte stimulation led us to determine cyclic AMP-phosphodiesterases in pig lymph node lymphocytes [25]. As for many other cells, we found at least two forms of cyclic AMP-phosphodiesterase (EC 3.1.4.17): a high K_m enzyme and a low K_m (0.17 μM) enzyme [25]; the latter has been reported to be membrane bound in

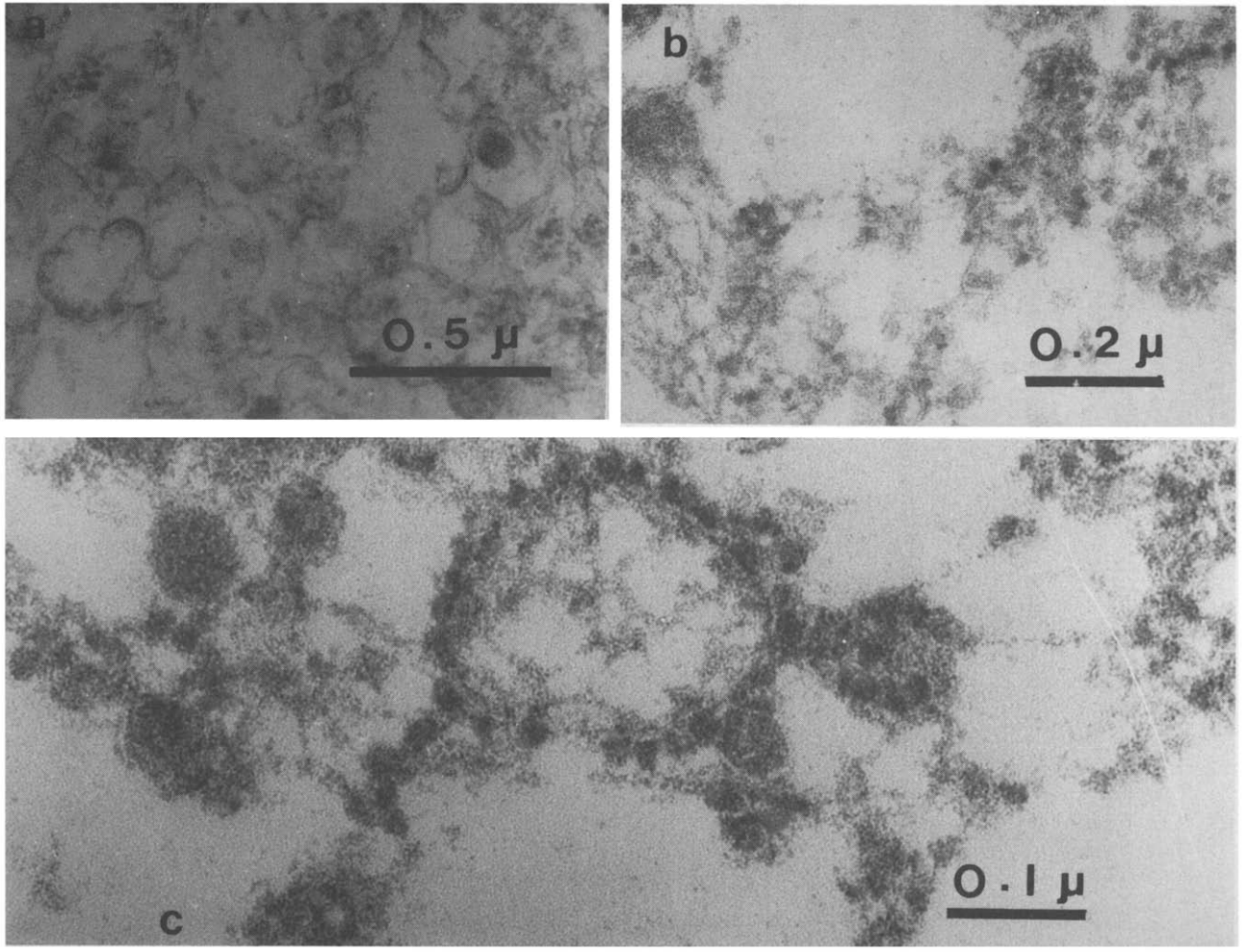

Fig. 5. Electron-microscopic study of sucrose gradient fractions 6 (a) and 8 (b, c). While fraction 6 was homogeneous, fraction 8 contained both plasma membrane vesicles and rough endoplasmic reticulum.

TABLE I

EFFECT OF LUBROL PX ON CYCLIC AMP-PHOSPHODIESTERASE SPECIFIC ACTIVITY OF SUCROSE GRADIENT FRACTIONS

Fractions 6 and 8 were pre-incubated for 12 min at 0°C with the indicated detergent concentrations; cyclic AMP-phosphodiesterase was determined as previously described [25], using 0.45 μM cyclic AMP as substrate.

Lubrol PX concentration (%)	Cyclic AMP-phosphodiesterase specific activity (nmol cyclic AMP hydrolysed/min per mg)	
	Fraction 6	Fraction 8
0	0.060	0.083
0.05	0.112	0.136
0.25	0.131	0.133
1.25	0.124	0.130

numerous cell types [26]. We found low K_m cyclic AMP-phosphodiesterase activities in fractions 6 and 8, which confirms that this enzyme is particulate. The effect of Lubrol PX (0—1.25%) was tested on both fractions: detergent concentrations as low as 0.05% induced unmasking of cyclic AMP-phosphodiesterase activities (Table I).

Another example of membrane enzyme unmasking was provided by the action of SDS [2], Lubrol or alamethicin [8] on pig lymphocyte (Na^++K^+)-ATPase. Membrane enzyme activities can also be unmasked by simpler procedures than detergent action. The (Na^++K^+)-ATPase activity of lymph node lymphocyte plasma membranes (measured in the absence of SDS which is usually included in the assay medium [2]) increased from 0.96 to 2.31 μmol P_i/h per mg protein after 2 h of agitation at 4°C with magnetic stirring and to 3.85 μmol P_i/h per mg protein after agitation in the presence of Sepharose. The latter result appears to conflict with the data of Rode et al. [10] concerning the (Na^++K^+)-ATPase activity of thymocyte membrane subfraction adsorbed on Con A-Sepharose and recovered by magnetic stirring: the high value reported for (Na^++K^+)-ATPase activity might well be due to mechanical unmasking and does not necessarily support the concept of the existence of specialized plasma membrane areas with high (Na^++K^+)-ATPase activity.

Conclusion

Monneron and d'Alayer [7] recently reported that the conditions used for subcellular fractionation of calf thymocytes greatly influence the apparent distribution of adenylate cyclase activity. The main parameter investigated in their study was the preservation of the enzyme by ATP and dithiothreitol. In the present study on pig lymph node lymphocytes, we demonstrated that the apparent distribution of adenylate cyclase in sucrose density gradients greatly depends upon the extent of unmasking of the enzyme activity present in the various fractions: the profile of adenylate cyclase activity measured in the absence of detergent was not superposed on those of plasma membrane markers, its maximum being observed at higher density. However, low concentrations of Lubrol PX (0.25%) induced differential unmasking of adenylate

cyclase activity so that its maximum was shifted towards lighter fractions and was no longer significantly different from that of plasma membrane markers.

Our experiments do not allow us to draw definitive conclusions concerning the subcellular localization of adenylate cyclase in pig lymph node lymphocytes. However, they are not inconsistent with the unique localization on the plasma membrane and, as pointed out by Monneron and d'Alayer [7], the lymphocyte enzyme distribution might well be similar to that of other cell types, in contrast to the findings of Parker and coworkers [5,6]. Enzymatic activities, fully unmasked by detergent treatment, might well be the best means of evaluating the amount of plasma membranes in the various fractions.

Moreover, our data raise a more general problem, i.e., the difficulty of finding standard conditions for the measurement of membrane enzymatic activities, since the latter can be unmasked by detergent or alamethicin treatment [2,8, 24], by mechanical action, or even by mere storage at −20°C (unpublished observations for $(Na^+ + K^+)$-ATPase activity). As a consequence, these phenomena should be taken into account in any study involving the comparison of different subcellular fractions or membrane subpopulations, especially if these fractions have been subjected to different treatments or if they are artificially associated with different contaminant structures. For this reason, we believe that it remains to be demonstrated that, if intact lymphocytes display a functional mosaicism [10], the resulting biochemical heterogeneity can be shown after cell disruption and classical procedures for separation of plasma membrane vesicles and other subcellular components.

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