Biochimica et Biophysica Acta, 341 (1974) 388–395

BBA 67187

DETERGENT DISPERSION OF ADENYLATE CYCLASE FROM PARTIALLY PURIFIED RAT LIVER PLASMA MEMBRANES*

SIMON J. PILKIS and ROGER A. JOHNSON
Department of Physiology, Vanderbilt University, Nashville, Tenn. 37232 (U.S.A.)
(Received November 5th, 1973)

SUMMARY

Adenylate cyclase (EC 4.6.1.1) of partially purified rat liver plasma membranes was dispersed by a number of nonionic detergents. The presence of F^- stabilized adenylate cyclase activity during dispersion by detergent. Both dispersed and intact membrane adenylate cyclase preparations were more active in the presence of Mn^{2+} than Mg^{2+}. The specific activity of the dispersed enzyme was higher than either the basal or F^--stimulated cyclase activity of intact membranes, but the dispersed enzyme did not respond to glucagon or to further addition of F^-.

INTRODUCTION

From studies initiated by Sutherland and colleagues [1] and extended by others [2–4], it has become evident that adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) is bound to the plasma membrane of many cell types. However, the particulate nature and lability of the enzyme have made its purification and characterization difficult.

Nonionic detergents have been used to "solubilize" adenylate cyclase from a number of different sources. Sutherland et al. [1] first reported the dispersion of particulate adenylate cyclase of brain, muscle, and liver by Triton X-100, although with significant losses of enzyme activity. Subsequently, a number of workers have confirmed and extended this observation with cyclases prepared from heart muscle [5], corpus luteum [6], kidney [7], liver [8], and brain [9, 10]. Ray et al. [11] report that certain ionic and nonionic detergents have little effect on adenylate cyclase activity in liver plasma membranes, whereas Pohl et al. [3] report that they have been unable to "solubilize" the enzyme from liver plasma membranes.

It is evident from these reports that there are tissue differences which affect the dispersion of adenylate cyclase, though the inability of detergent to "solubilize" the enzyme from purified liver plasma membranes has been unexplained. In the present communication we report conditions for successful detergent-dispersion of rat liver plasma membrane adenylate cyclase and some properties of this dispersed enzyme as compared with the cyclase of intact membranes.

* Some aspects of this work have been presented at the Ninth International Congress of Biochemistry (ref. 8).

EXPERIMENTAL PROCEDURE

Membrane preparation

Plasma membranes were prepared by a modification of the method of Song et al. [12]. Rats weighing 175–230 g were used. In a typical preparation, four rat livers were homogenized in 2 vol of ice-cold 1 mM $NaHCO_3$, pH 7.4, using 20 strokes with a hand Teflon–glass homogenizer. The homogenate was diluted to 500 ml with 1 mM $NaHCO_3$ and filtered 3 times through 3 layers of surgical gauze. After centrifugation for 10 min at 1500 × *g*, the pellet was resuspended by homogenization (3 strokes) in a volume of $NaHCO_3$ equal to one-half the original liver weight. Then to this mixture exactly 5.5 vol. of 70.7% sucrose was added, mixed, and then distributed (16 ml) into Spinco 30 rotor centrifuge tubes. Over this suspension were layered 8 ml of 48.2% and 4 ml of 42.5% sucrose. After centrifugation at 66 000 × *g* for 60 min, the grayish-white material which accumulated at the 42.5%/48.2% sucrose interface was removed and washed 3 times with 40 ml of 1 mM $NaHCO_3$ buffer. Membrane adenylate cyclase activity was stable to storage in 1 mM $NaHCO_3$ at −70 °C for at least one month*.

Adenylate cyclase activity determination

Adenylate cyclase activity was determined with a 10-min reaction period at 37 °C, essentially as described by Johnson and Sutherland [9]. Cyclic AMP (adenosine-3′:5′-monophosphate) production was linear with respect to protein concentration and with time up to 10 min. The cyclic AMP formed was purified by cation exchange (BioRad AG50-X8 100–200 mesh, H^+ form) chromatography and the amount of cyclic nucleotide was determined by the protein kinase binding assay [13].

Adenylate cyclase dispersion

In general, liver plasma membranes (protein concentration about 2 mg/ml) were incubated at 0 °C for 15 min with 10 mM NaF. Detergent (usually Lubrol-PX) was then added to a final concentration of 0.3%. This mixture was incubated for a further 10 min at 0 °C and then centrifuged at 38 000 × *g* for 60 min at 0–4 °C. The resulting supernatant fraction contained detergent-dispersed adenylate cyclase activity which was stable (decrease of 0 to 20%) if stored at 4 °C for 24 h. Variations in this procedure are described in the results.

ATPase activity and protein determination

Membrane ATPase activity was determined essentially according to Sugino and Miyoshi [14]. Protein was estimated by the method of Lowry et al. [15] with appropriate correction being made for the interference by reagents in the homogenizing medium, especially the detergents.

Materials

The protein kinase used in the binding assay for cyclic AMP was prepared

* This plasma membrane preparation has been characterized in terms of enzyme markers [23] and is similar to that of Song et al. [12]. The preparation contained an adenylate cyclase activity of about 0.2 to 0.5 nmole $(10\ min)^{-1}$ $(mg\ protein)^{-1}$ which was stimulated about 15-fold by 10^{-7} M glucagon, 5- to 10-fold by 10 mM NaF, and 2- to 3-fold by 0.1 mM GTP [23].

from beef muscle essentially according to published procedures [13, 16]. Lubrol-PX was a gift of ICI America, Stanford, Connecticut. Lubrol-WX was a gift of Dr E. J. Landon. The other detergents were obtained as follows: Triton X-100 from Research Products International, Triton X-114 from Rohm and Haas, sodium dodecylsulfate from Sigma, Brij-35 from Technicon, and deoxycholate from Mallinckrodt. Cyclic [G-^{3}H]AMP (24 Ci per mmole) was obtained from New Englamd Nuclear Corp. Dithiothreitol was from Calbiochem and glycylglycine buffer and crystallized-lyophilized bovine serum albumin were from Sigma.

RESULTS

The effect of F^- on cyclase stability and dispersion

From the work of others [7, 10, 17–19] it was apparent that F^- could exert a stabilizing effect on adenylate cyclase activity derived from several sources. In experiments of our own, when rat liver plasma membrane adenylate cyclase was preincubated at 37 °C for 40 min in 1 mM $NaHCO_3$, pH 7.4, greater than 90% of the initial activity was lost. This loss was partially prevented by the inclusion of 10 mM NaF in the preincubation medium and was completely prevented by the addition of both NaF and 5 mM $MgSO_4$ (Fig. 1). $MgSO_4$ alone was without effect. Since F^- exerted a protective effect against the heat-inactivation of membrane adenylate cyclase it was

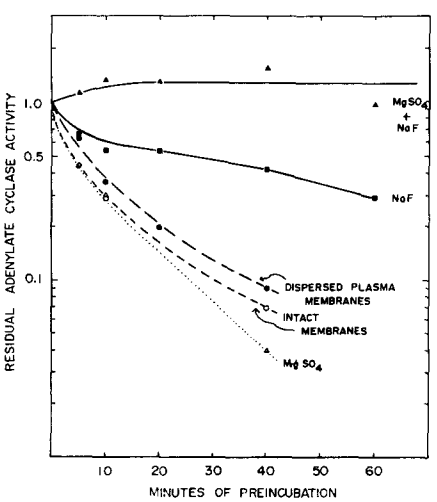

Fig. 1. Heat inactivation of adenylate cyclase of intact and detergent-dispersed liver plasma membranes. Intact membranes were prepared as described under Experimental Procedure. 1-ml aliquots of these membranes were preincubated at 37 °C, at a protein concentration of 2.2 mg/ml, alone (○------○), in the presence of 10 mM NaF (■—■), 5 mM $MgSO_4$ (△·····△), or both 10 mM NaF and 5 mM $MgSO_4$ (▲—▲). Detergent-dispersed plasma membranes were prepared as described under Experimental Procedure with 0.3% Lubrol-PX, 10 mM NaF, and 50 mM glycylglycine, pH 7.5. Dispersed membranes were preincubated without additions (●— —●) at 37 °C and a protein concentration of 1.6 mg/ml. At the times indicated 50-μl aliquots were taken from each preincubation tube and the adenylate cyclase activity determined. In each case, activity was determined in the presence of 4 mM ATP, 4 mM $MnSO_4$, 0.5 mM $MgSO_4$, and 1 mM NaF. The reaction was for 10 min at 37 °C and was initiated by the addition of the preincubated enzyme. Values represent the average of duplicate cyclase determinations.

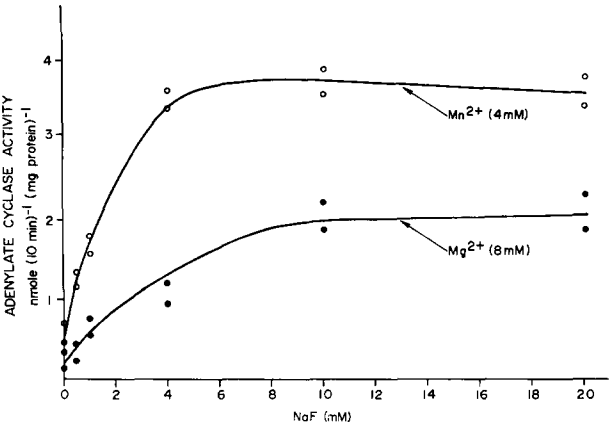

Fig. 2. Effect of NaF on the detergent-dispersion of plasma membrane adenylate cyclase. Plasma membranes were prepared and dispersed as described in Experimental Procedure with 0.3% Lubrol-PX in the presence of NaF at the concentrations indicated. The preparations were assayed with 8 mM $MgSO_4$ or 4 mM $MnSO_4$ and with 4 mM ATP. The enzyme preparation constituted 10% of the reaction volume, thereby diluting detergent and F^- 10-fold. Values were derived from duplicate incubation. Divalent cation was not present during the dispersion. NaF concentration was 2 mM in all cases during the assay for adenylate cyclase activity.

of interest to ascertain if it could exert a similar protective effect against the inactivation that was associated with attempts to solubilize the enzyme [3].

Fig. 2 illustrates the effect of NaF concentration on the Lubrol-PX-dispersion of rat liver plasma membrane adenylate cyclase. From other experiments (c.f. Fig. 3) it was determined that 4 mM Mn^{2+} or 8 mM Mg^{2+} resulted in maximal enzyme activity in the presence of 4 mM ATP. The activity of the dispersed enzyme assayed in the presence of Mn^{2+} was from 2 to 4 times that measured with Mg^{2+}. The F^- concentra-

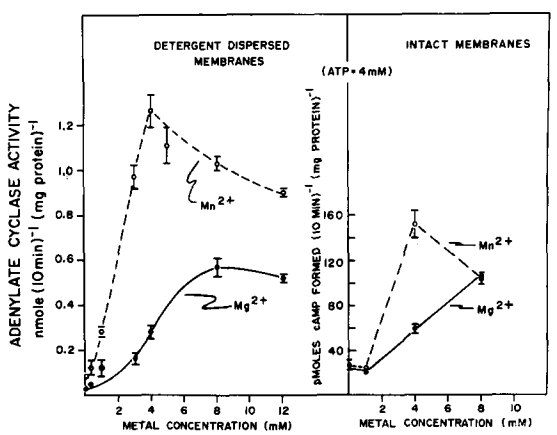

Fig. 3. Effect of cations on liver plasma membrane adenylate cyclase. Intact and detergent-dispersed membranes were prepared as described in Experimental Procedure and then assayed in the presence of 4 mM ATP at the cation concentrations indicated. Protein concentrations was 400 μg/ml for intact membranes and 120 μg/ml for dispersed membranes. Values represent the mean $\pm$S.E. from three incubations.

tion in the dispersing medium which elicited maximal activity of the dispersed cyclase was about 10 mM when assayed with Mg^{2+} and about 4 mM when assayed with Mn^{2+}.

When detergent-dispersed adenylate cyclase was incubated at 37 °C in 1 mM $NaHCO_3$ and 10 mM NaF for 40 min over 90% of the initial activity was lost (Fig. 1). If the detergent-dispersed enzyme was incubated at 37 °C for 20 min with $MgSO_4$ (5 mM) and NaF (10 mM) no activity was lost (data not shown).

The effect of detergents on the dispersion of adenylate cyclase

When the concentration of Lubrol-PX was varied, the most adenylate cyclase activity was dispersed from plasma membranes with about 0.3% detergent (Table I). Other detergents were compared at this concentration. The ionic detergents deoxycholate and sodium dodecylsulfate both were ineffective in dispersing activity in the

TABLE I

EFFECT OF DIFFERENT DETERGENTS ON DISPERSION OF PLASMA MEMBRANE ADENYLATE CYCLASE

50 μl of each preparation was assayed for adenylate cyclase activity in a 500-μl incubation system. The values in this table and Table II represent activity per tube. Each preparation was prepared as described in Experimental Procedure in the section on Adenylate Cyclase Dispersion. Lubrol-PX at high concentrations interfered with the Lowry protein determination. Detergent concentrations were w/v or v/v as appropriate. Membranes were dispersed either with or without NaF (10 mM). In all cases, the final concentration of NaF in the assay for cyclase was 1 mM.

Detergent	(%)	Cyclase activity (pmoles cyclic AMP per 10 min)		Protein (mg/ml)	
		−NaF	+NaF (10 mM)	−NaF	+NaF (10 mM)
Original membranes		18.2 ± 0.1	89.2 ± 12.1	1.81	–
Lubrol PX	0.03	3.0 ± 0.4	8.2 ± 1.1	0.63	0.48
	0.1	6.2 ± 0.4	69.2 ± 5.3	0.93	0.48
	0.3	5.7 ± 0.5	77.2 ± 6.1	1.05	0.55
	1.0	7.1 ± 0.4	61.1 ± 3.3	1.52	0.88
	3.0	4.3 ± 0.2	21.2 ± 2.3	–	–
Brij-35	0.3	0	0	1.05	0.87
Triton X-100	0.3	5.9 ± 0.6	44.2 ± 0.9	0.94	0.58
Triton X-114	0.3	6.9 ± 0.7	49.2 ± 0.9	0.95	0.67
Lubrol WX	0.3	7.0 ± 0.8	31.0 ± 2.1	1.07	0.61
Deoxycholate	0.3	0	15.1 ± 1.1	1.15	1.06
SDS	0.3	0.3	0	1.21	1.09

absence of NaF. Some activity was recovered in the supernatant fraction when deoxycholate plus F^- was used whereas sodium dodecylsulfate plus F^- was totally ineffective. The most cyclase activity was dispersed with the nonionic detergent Lubrol-PX. Triton-X100 and Triton-X114 were less effective. In contrast to the other nonionic detergents, no activity was recovered with Brij-35.

The inclusion of F^- during the dispersion not only protected adenylate cyclase activity, but also decreased the amount of "solubilized" protein (Table I). Accordingly, the enzyme specific activity was significantly enhanced by dispersion with F^-.

The recovery of adenylate cyclase activity following dispersion by Lubrol-PX is represented in Table II. Cyclase activity of intact membranes was stimulated slightly by preincubation with NaF. Preincubation with detergent alone caused significant loss of activity though some activity was dispersed. In the presence of NaF and Lubrol-PX more total activity was recovered (124 + 111) than was initially observed with F^- preincubation alone (107). 47% of the total activity was found in the supernatant fraction.

TABLE II

RECOVERY OF ADENYLATE CYCLASE ACTIVITY FOLLOWING DETERGENT DISPERSION

Preincubation, without or with 10 mM NaF as indicated, was at 0 °C for 15 min and was then followed by an additional 10 min preincubation at 0 °C without or with 0.3% Lubrol-PX as indicated. Enzyme constituted 10% of the reaction volume, thus diluting agents present during the preincubation by 10-fold. Pellet and supernatant derived from a 38 000 × *g* for 60 min centrifugation. The pellet was resuspended in 1 mM $NaHCO_3$ in the original volume.

Preincubation conditions	Assay conditions (pmoles cyclic AMP/10 min)			
	Pellet			Supernatant
	Basal	1 mM NaF	10 mM NaF	1 mM NaF
Control membranes (no additions)	71 ± 3	80 ± 2	235 ± 4	N.D.
Membranes + NaF	—	107 ± 4	—	N.D.
Membranes + Lubrol-PX	—	27 ± 2	—	16 ± 2
Membranes + NaF + Lubrol-PX	—	124 ± 5	—	111 ± 5

N.D., not determined.

Effects of substrate and cation on dispersed adenylate cyclase

The divalent cation dependency of detergent-dispersed and intact membrane adenylate cyclase was compared and is shown in Fig. 2. In both preparations, with 4 mM ATP as substrate, maximal activity was obtained with 8 mM Mg^{2+} or 4 mM Mn^{2+}, and half-maximal activity with about 4 mM Mg^{2+} or 2 mM Mn^{2+}. At concentrations of Mn^{2+} in excess of 4 mM, activity decreased whereas the activity with 8 and 12 mM Mg^{2+} were comparable. Invariably the maximal activity observed with Mn^{2+} was greater, usually about 2-fold, than the maximal activity with Mg^{2+}.

Both intact and detergent-dispersed membrane adenylate cyclase preparations were inhibited 50% by about 0.3 mM $CaCl_2$ in the presence of 4 mM ATP and 8 mM $MgSO_4$ (data not shown).

The apparent K_m for ATP of the intact membrane or dispersed enzyme was about 0.1–0.4 mM in the presence of 8 mM $MgSO_4$. These values are in agreement with those reported by Rosselin and Freychet [27] for intact liver plasma membranes. ATP hydrolysis by ATPase activities present in these preparations, was significantly less in the dispersed membranes than in intact membranes. With 8 mM $MgSO_4$ and 4 mM ATP, hydrolysis of ATP ranged between 25 and 35% of the initial concentration in intact membranes but was less than 1% in the dispersed preparation. At lower ATP

concentrations (e.g. 0.1 mM), intact membranes hydrolyzed greater than 70% of the ATP and the detergent-dispersed preparation hydrolyzed about 20 to 25%.

While the adenylate cyclase from intact membranes was stimulated by glucagon, low concentrations of GTP, and also by NaF, the detergent-dispersed enzyme was unresponsive to the addition of either glucagon or GTP or to the further addition of F^- (data not shown).

DISCUSSION

Adenylate cyclase was dispersed from purified rat liver plasma membranes by a number of nonionic detergents. While the detergent-dispersed enzyme exhibited some properties similar to the intact membrane enzyme, the characteristic stimulatory effect of glucagon was not observed in the dispersed preparation. The loss of hormone sensitivity upon dispersion of the enzyme by detergent is consistent with the observations of others [1, 4, 5, 20, 21] but it is not clear why this occurs. It may be that detergent disrupts glucagon binding to its receptor, although this appears less likely in view of recent reports [22, 24], indicating that glucagon binds to proteins in detergent dispersed preparations. Perhaps the more likely explanation is that in the presence of F^- the detergents disperse and cause the physical separation of both the "receptor" and the active catalytic moiety of adenylate cyclase.

The dispersion of adenylate cyclase from the partially purified liver plasma membranes was facilitated by the stabilization of the enzyme by F^- in the presence of detergent. This requirement for fluoride is apparently unique to the solubilization of kidney and liver plasma membrane cyclase [7, 8] and has not been observed by others working with other preparations [1, 5, 6, 9, 10]. The stabilization by F^- was also associated with an increase in the enzyme specific activity which was greater than either the basal or F^--stimulated activity of intact membranes. F^- acts in two apparently distinct manners in this regard. First, it diminished the amount of protein dispersed by the detergents. Second, it increased the activity of the enzyme in the absence of ATP or cation in a manner which was unaffected by the subsequent addition of detergent. This latter observation would be consistent with the idea that F^- acts by an essentially irreversible deinhibition of the enzyme. It remains to be determined whether or not the "inhibited" form of the enzyme exists as a "phospho"-form [25] and also if F^- stimulates adenylate cyclase by a direct action on the enzyme or by an action at some other interrelated site (or sites) [9, 26].

The use of purified membrane preparations from which the adenylate cyclase can be dispersed by detergent should aid in the purification of the enzyme and may aid in the elucidation of those mechanisms by which F^- and hormones modulate its activity.

ACKNOWLEDGEMENTS

The authors would like to thank Dr E. W. Sutherland and Dr C. R. Park for their encouragement and support.

We are grateful to Ms Janette Welden and Mr Tom Baumann for their excellent technical assistance.

This work was supported by Grants HE-08332 and AM-07462 from the United States Public Health Service.

S.J.P. is a Career Investigator of the Howard Hughes Medical Institute.

REFERENCES

1 Sutherland, E. W., Rall, T. W. and Menon, T. (1962) J. Biol. Chem. 237, 1220–1227
2 Davoren, P. R. and Sutherland, E. W. (1963) J. Biol. Chem. 238, 3016–3023
3 Pohl, S. L., Birnbaumer, L. and Rodbell, M. (1971) J. Biol. Chem. 246, 1849–1856
4 Birnbaumer, L., Pohl, S. L. and Rodbell, M. (1971) J. Biol. Chem. 246, 1857–1860
5 Levey, G. S. (1970) Biochem. Biophys. Res. Commun. 38, 86–92
6 Stansfield, D. A. and Franks, D. J. (1971) Biochim. Biophys. Acta 242, 606–616
7 Forte, L. R. (1972) Biochim. Biophys. Acta 266, 524–542
8 Johnson, R. A., Pilkis, S. J. and Sutherland, E. W. (1973) 9th Int. Congr. Biochem., Abstr 258, Aktiebolaget Egnellska Boktryckeriet, Stockholm
9 Johnson, R. A. and Sutherland, E. W. (1973) J. Biol. Chem. 248, 5114–5121
10 Swislocki, N. I. and Tierney, J. (1973) Biochemistry 12, 1862–1866
11 Ray, T. K., Tomassi, V. and Marinetti, G. V. (1970) Biochim. Biophys. Acta 211, 20–30
12 Song, C. S., Rubin, W., Rifkind, A. B. and Kappas, A. (1969) J. Cell Biol. 41, 124–132
13 Gilman, A. G. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 305–312
14 Sugino, Y. and Myoshi, Y. (1964) J. Biol. Chem. 239, 2360–2366
15 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
16 Miyamoto, E., Kuo, J. F. and Greengard, P. (1969) J. Biol. Chem. 244, 6395–6402
17 Schramm, M. and Naim, E. (1970) J. Biol. Chem. 245, 3225–3231
18 Lin, M. C. (1971) Fed. Proc. 30, 1206
19 Perkins, J. P. and Moore, M. M. (1971) J. Biol. Chem. 246, 62–68
20 Levey, G. S. (1971) J. Biol. Chem. 246, 7405–7407
21 Rodbell, M., Krans, H. M. J., Pohl, S. L. and Birnbaumer, L. (1971) J. Biol. Chem. 246, 1861–1871
22 Georgio, N. A., Johnson, C. B. and Blecher, M. (1973) Fed. Proc. 37, 567
23 Pilkis, S. J., Exton, J. H., Johnson, R. A. and Park, C. R. (1974) Biochim. Biophys. Acta, 343, 250–257
24 Klein, L., Fletcher, M. A. and Levey, G. S. (1973) J. Biol. Chem. 248, 5552–5555
25 Constantopoulos, A. and Najjar, V. A. (1973) Biochem. Biophys. Res. Commun. 53, 794–799
26 Layne, P., Constantopoulos, A., Judge, J. F. X., Rauner, R. and Najjar, V. A. (1973) Biochem. Biophys. Res. Commun. 53, 800–805
27 Rosselin, G. and Freychet, P. (1973) Biochim. Biophys. Acta 304, 541–551