DEACTIVATION OF LYMPHOCYTE ADENYLATE CYCLASE BY AFFINITY CHROMATOGRAPHY ON Con A–SEPHAROSE

Its reactivation by a cytosolic factor

Jean-Claude BONNAFOUS, Jacques DORNAND and Jean-Claude MANI
Laboratoire de Biochimie des Membranes, Ecole Nationale Supérieure de Chimie, 8, rue de l'Ecole Normale, F-34075 Montpellier Cedex, France

Received 22 December 1978

1. Introduction

The adenylate cyclase system (ATP pyrophosphate lyase (cyclizing) EC 4.6.1.1.) appears more and more complex from the increasing number of studies devoted to the separation of its components and reconstitution of active systems from these components [1–5].

A study dealing with the effects of alamethicin on membrane-bound enzymes of pig lymph node lymphocytes [6] led us to prepare 'inside-out' vesicles by filtration of plasma membrane-enriched fractions on concanavalin A immobilized on Sepharose 4B (Con A–Sepharose) as in [7]; we got evidence that the fractions not retained on Con A–Sepharose were almost completely depleted of adenylate cyclase activity and that this effect was most probably due to the retention of (an) activating factor(s) on the affinity column. Here we develop the experiments which demonstrate:

(1) The deactivation of adenylate cyclase, while other enzyme activities, commonly considered as plasma membrane markers, are unaffected by filtration through Con A–Sepharose.
(2) The reactivation of adenylate cyclase by the cytosol of pig lymph node lymphocytes.
(3) Preliminary characteristics of this cytosolic activator and its comparison with stimulatory cytosol factors reported for the adenylate cyclase of other tissues [8–11].

2. Materials and methods

Creatine phosphate and creatine phosphokinase were obtained from Boehringer, Trysin-TPCK from Worthington, [α-^{32}P]ATP from Amersham Radiochemical Centre, cyclic [^{3}H]AMP from CEA (Saclay), Sepharose 4B and Con A–Sepharose from Pharmacia, soybean trypsin inhibitor type II S from Sigma.

Pig mesenteric lymph nodes were ground in 10 mM Tris–HCl buffer (pH 7.5), 0.9% NaCl, as in [12]. The so-called 'microsomal fraction' was obtained by successive centrifugations at 1500, 6000 and 30 000 × g, and by resuspending the final pellet in 10 mM Tris–HCl (pH 7.5). The cytosol was obtained by centrifuging the last supernatant at 100 000 × g for 1 h.

Microsomal fractions were filtered on Con A–Sepharose 4B or on Sepharose 4B for controls (1 mg microsomal protein/ml packed gel). About 30% microsomal proteins were retained on Con A–Sepharose.

Cytosol (1.5 ml) pre-heated and centrifuged to eliminate denaturated material (initial conc. 7 mg protein/ml) was filtered on columns containing 5 ml packed gel, either Con A–Sepharose 4B or Sepharose 4B.

5′-Nucleotidase [13] and ($Na^+ + K^+$)-ATPase [14] were assayed under the conditions reported. Adenylate cyclase was determined in 25 mM Tris–HCl (pH 7.5) containing 25 U creatine phosphokinase/ml, 15 mM creatine phosphate, 2.5 mM $MgCl_2$, 1 mM cyclic AMP (cAMP), 0.5 mM [α-^{32}P]ATP (1.5–3 × 10^6 cpm/

 Elsevier/North-Holland Biomedical Press

assay), 50 μM GTP and 100 μM EGTA. The reaction was initiated by adding to this mixture the enzyme source (and at the same time the cytosol when necessary). After 15 min incubation at 30°C the reaction was stopped by adding 150 μl 10 mM Tris–HCl (pH 7.5) containing 5 mM ATP, c[^{3}H]AMP (about 20 000 cpm/assay) and by heating the tubes for 3 min. c[^{32}P]AMP was purified as in [15].

Tryptic digestion: cytosol samples (7 mg protein/ml) were incubated 30 min at 37°C in the presence of trypsin (125 μg/ml), then soybean trypsin inhibitor was added (300 μg/ml); appropriate controls were carried out in the absence of cytosol and with trypsin inhibitor added at the same time as trypsin.

3. Results

3.1. *Loss of adenylate cyclase activity by filtration of lymphocyte microsomes on Con A–Sepharose columns*

Samples of microsomal fractions from pig lymph node lymphocytes were filtered either on Sephadex 4B as controls or on Con A–Sepharose 4B. We measured ($Na^+ + K^+$)-ATPase, 5′-nucleotidase and adenylate cyclase activities in the fractions unretained on these columns (table 1). While 5′-nucleotidase and ($Na^+ + K^+$)-ATPase activities remained unaffected by filtration on Con A–Sepharose, the basal and fluoride-stimulated adenylate cyclase activities were completely lost, since they represented only 1% and 1.6% of the control activities, respectively; the microsomes filtered on Con A–Sepharose will be referred to as 'deactivated microsomes'.

3.2. *Restoration of adenylate cyclase activities by cytosol*

The suppression of adenylate cyclase activity in fractions not retained on Con A–Sepharose was not the result of enzyme retention on the column since this activity could be restored by adding cytosol to the assays. Moreover the ability to restore the cyclase activity was entirely preserved in cytosol which had been heated for 5 min at 90°C and centrifuged to eliminate the precipitated material. Figure 1 shows the reactivation of deactivated microsomes by various amounts of heated cytosol. In the typical experiment described, basal activity increased from 3 ± 2 to 77 ± 1 pmol cAMP. mg prot.$^{-1}$. 15 min^{-1}, and fluoride-stimulated activity from 22 ± 5 to 381 ± 10 pmol cAMP. mg prot.$^{-1}$. 15 min^{-1}.

3.3. *Loss of stimulatory capacity of cytosol by filtration on Con A–Sepharose*

Equal amounts of heated cytosol were filtered through a control column of Sepharose 4B and through a Con A–Sepharose 4B column; the unretained fractions were pooled and their ability to reactivate the adenylate cyclase tested on pre-deactivated microsomes. While the control cytosol stimulated adenylate cyclase, the cytosol filtered on Con A–Sepharose was no longer able to induce any stimulation (table 2). This suggests that the stim-

Table 1
Effects of filtration on Con A–Sepharose on some enzymatic activities of microsomal fractions from pig lymph node lymphocytes

Enzyme spec. act.	Fractions excluded from Sepharose 4B columns (controls)	Fractions unretained on Con A–Sepharose 4B
5′-Nucleotidase μmol P_i. h^{-1}. mg prot.$^{-1}$	4.3 ± 0.2	4.3 ± 0.3
($Na^+ + K^+$)-ATPase μmol P_i. h^{-1}. mg prot.$^{-1}$	3.2 ± 0.1	2.9 ± 0.2
Adenylate cyclase, pmol cAMP. mg prot.$^{-1}$. 15 min^{-1}		
basal	284 ± 14	3 ± 2
10 mM NaF-stimulated	1397 ± 23	22 ± 5

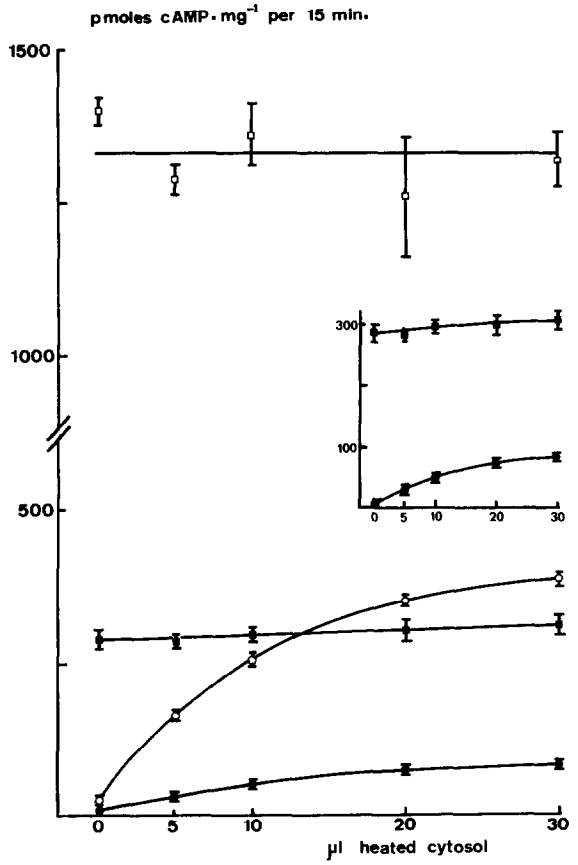

Fig.1. Reactivation by heated cytosol of the adenylate cyclase from deactivated microsomes. Microsomal fractions from pig lymph node lymphocytes were filtered on a Sepharose 4B column (control) or on a Con A–Sepharose 4B column as in section 2 and the effects of heated cytosol tested on the adenylate cyclase activities: basal (■ – ■) and NaF-stimulated (□ – □) activities of control microsomes; basal (● – ●) and NaF-stimulated (○ – ○) activities of microsomes filtered on Con A–Sepharose 4B. The values are the mean of 3 determinations ± SEM.

ulatory factor which is present in the cytosol is identical to the entity which is lost when microsomes are filtered on Con A–Sepharose.

3.4. *Some characteristics of the adenylate cyclase stimulatory factor*

It is likely that this factor contains a carbohydrate moiety since it binds to Con A–Sepharose. Attempts to reverse this binding by methyl-α-D-mannopyranoside

Table 2
Effects of filtration on Con A–Sepharose on the ability of cytosol to restore the adenylate cyclase activity of deactivated microsomes

Additions to microsomes	Adenylate cyclase spec. act. pmol cAMP. mg prot.$^{-1}$. 15 min^{-1}	
	Basal	10 mM NaF-stimulated
None	4 ± 1	21 ± 1
Cytosol filtered on Sepharose 4B (control)	107 ± 3	358 ± 14
Cytosol filtered on Con A–Sepharose 4B	4 ± 2	26 ± 6

were unsuccessful but this problem has often been encountered in affinity chromatography with Con A–Sepharose. As mentioned, this factor is heat-stable. It is completely lost by dialysis or by ultrafiltration through PM 10 Diaflo membranes, which is indicative of < 10 000 mol. wt. Its activity is not lowered by tryptic digestion. This factor is not GTP alone, since all experiments were carried out in the presence of 50 µM GTP; moreover Gpp(NH)p was unable to restore the stimulatory capacity of dialysed cytosol. However, we cannot exclude the possibility that this factor is a dialysable molecule acting with GTP as cofactor, as described for rat liver [5,8]. NAD does not appear to be involved in the process of deactivation and reactivation since it did not enhance the cytosol effect and did not restore activation by dialysed cytosol. The participation of NAD was checked since a NAD-dependent ADP-ribosylation of components of the adenylate system has been reported [16], which is reminiscent of the mechanism of action of cholera toxin [2,4].

4. Discussion

This report demonstrates that lymphocyte adenylate cyclase can be deactivated by affinity chromatography of plasma membrane-enriched fractions on Con A–Sepharose, while other plasma membrane marker enzymes, such as 5′-nucleotidase and ($Na^+ + K^+$)-ATPase, are not affected. The membrane fractions not retained on Con A–Sepharose are 'inside-out' vesicles [7], but this problem is not relevant with the

experiments described here since we showed that these vesicles were permeable to ATP, 5′-AMP (and even Con A, in agreement with [7]) and had the same 5′-nucleotidase and ($Na^+ + K^+$)-ATPase activities as a 50:50 mixture of 'right-side out' and 'inside-out' vesicles (table 1 and [6]). The basal and fluoride-stimulated (and also PGE_1-stimulated, data not shown) activities can be restored in a dose-dependent manner, by adding cytosol to the assays. The extent of restoration reached 27%, which can be considered as satisfactory for reconstitution experiments. This stimulatory factor contained in the heated cytosol can be retained on Con A–Sepharose columns, which means that this factor is most probably identical to the entity which is lost from microsomal fractions filtered on Con A–Sepharose.

Cytosolic factors have recently been reported to activate the adenylate cyclase of rat liver plasma membranes [10], of rat osteosarcoma [11] and to restore the hormone responsiveness of the adenylate cyclase of particulate fractions from rat liver [5,8] or rat cardiac muscle [9]. It is impossible to find unique properties shared by these various factors and our lymphocyte factor. Our lymphocyte factor is insensitive to trypsin, while all other described factors are destroyed. The lymphocyte factor is heat-stable, as that of rat liver [8,10] while those reported for heart [9] and osteosarcoma [11] are deactivated upon heating.

The factor described [8] is dialysable as the lymphocyte one, but the factors [9–11] are non-dialysable. It is noticeable that there is no consensus for the rat liver factor, since the results in [8,10] are opposite in this respect.

The activation of adenylate cyclase by the lymphocyte factor does not require calcium, since EGTA was present in all our adenylate cyclase measurements, and therefore cannot be compared to the activation of adenylate cyclase by the calcium-dependent regulator protein from brain [17,18].

It has been proposed that the protein factor [5] from rat liver is GTP-dependent and that the GTP–protein complex acts as a transducing component in the coupling process of the hormone receptor with adenylate cyclase. It has been shown [19] that the adenylate cyclase of pigeon erythrocytes could be deactivated by treating the membranes with a GTP–Sepharose derivative, and that the activity could be restored by the guanylnucleotide-binding protein removed from GTP–Sepharose. Since GTP was present at saturating amounts in all our assays, we cannot rule out the involvement of GTP in the action of the lymphocyte factor, although the molecular weight of the GTP-binding protein of pigeon erythrocyte (42 000 mol. wt [2,19]) is not consistent with our dialysis experiments; this problem is now under study.

The factor we found in pig lymph-node lymphocytes possesses most probably a carbohydrate moiety, since it binds to Con A. Although methyl-α-D-mannopyranoside failed to reverse the binding of this factor to Con A–Sepharose, other methods will be applied to try to recover this factor.

The almost complete deactivation (much greater than that reported for filtration on GTP–Sepharose [19]) of adenylate cyclase by filtration of membrane vesicles on Con A–Sepharose provides a convenient new tool for the study of activating factors and restoration experiments. It seems reasonable to speculate that this method might be applied to other cell types than lymphocytes.

Acknowledgements

This work received financial support from the Centre National de la Recherche Scientifique and the Fondation pour la Recherche Médicale Française. The authors acknowledge skillful technical assistance of R. Larguier and N. Bernad.

References

[1] Hebdon, M., Le Vine H., iii, Sahyoun, N., Schmitges, C. J. and Cuatrecasas, P. (1978) Proc. Natl. Acad. Sci. USA 75, 3693–3697.
[2] Cassel, D. and Pfeuffer, T. (1978) Proc. Natl. Acad. Sci. USA 75, 2669–2673.
[3] Ross, E. M., Howlett, A. C., Ferguson, K. M. and Gilman, A. G. (1978) J. Biol. Chem. 253, 6401–6412.
[4] Johnson, G. L., Kaslow, H. R. and Bourne, H. R. (1978) Proc. Nat. Acad. Sci. USA 75, 3113–3117.
[5] Pecker, F. and Hanoune, J. (1977) FEBS Lett. 83, 93–98.
[6] Bonnafous, J. C., Dornand, J. and Mani, J. C. (1979) submitted.
[7] Walsh, F. S., Barber, B. H. and Crumpton, M. J. (1976) Biochemistry 15, 3557–3562.
[8] Pecker, F. and Hanoune, J. (1977) J. Biol. Chem. 252, 2784–2786.

[9] Sanders, R. B., Thompson, W. J. and Robison, G. A. (1977) Biochim. Biophys. Acta 498, 10–20.
[10] Doberska, C. A. and Martin, B. R. (1977) FEBS Lett. 82, 273–277.
[11] Egan, J. J., Majeska, R. J. and Rodan, G. A. (1978) Biochem. Biophys. Res. Commun. 80, 176–182.
[12] Dornand, J., Mani, J. C., Mousseron-Canet, M. and Pau, B. (1974) Biochimie 56, 1425–1432.
[13] Dornand, J., Réminiac, C. and Mani, J. C. (1977) Biochimie 59, 425–432.
[14] Dornand, J., Réminiac, C. and Mani, J. C. (1978) Biochim. Biophys. Acta 509, 194–200.
[15] Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541–548.
[16] Moss, J. and Vaughan, M. (1978) Proc. Nat. Acad. Sci. USA 75, 3621–3624.
[17] Brostom, C. O., Brostrom, M. A. and Wolff, D. J. (1977) J. Biol. Chem. 252, 5677–5685.
[18] Lynch, T. J., Tallant, E. A. and Cheung, W. Y. (1977) Arch. Biochem. Biophys. 182, 124–133.
[19] Helmreich, E. J. M. and Pfeuffer, T. (1978) in: Cell membrane receptors for drugs and hormones: A multidisciplinary approach (Straub, R. W. and Bolis, L. eds) pp. 119–127, Raven Press, New York.