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CHARACTERIZATION AND SUBCELLULAR LOCALIZATION OF NUCLEOTIDE CYCLASES IN CALF THYMUS LYMPHOCYTES

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The role of cyclic nucleotides in the regulation of lymphocyte growth and differentiation remains controversial, as an adequate characterization of the key enzymes, adenylate cyclase and guanylate cyclase, in the plasma membrane of lymphocytes is still lacking. In this study, calf thymus lymphocytes were disrupted by nitrogen cavitation and various cellular fractions were isolated by differential centrifugation and subsequent sucrose density ultracentrifugation. As revealed by the chemical composition and the activities of some marker enzymes, the plasma membrane fraction proved to be highly purified. Nucleotide cyclases were present in the plasma membranes in high specific activities, basal activities of adenylate cyclase being 13.7 pmol/mg protein per min and 34.0 pmol/mg protein per min for the guanylate cyclase, respectively. Adenylate cyclase could be stimulated by various effectors added directly to the enzyme assay, including NaF, GTP, 5′-guanylyl imidodiphosphate, Mn^{2+} and molybdate. Addition of β-adrenergic agonists only showed small stimulating effects on the enzyme activity in isolated plasma membranes. Basal activity of adenylate cyclase as well as activities stimulated by NaF or 5′-guanylyl imidodiphosphate exhibited regular Michaelis-Menten kinetics. Activation by both agents only marginally affected the K_m values, but largely increased V_{max}. The activity of the plasma membrane-bound guanylate cyclase was about 10-fold enhanced by the nonionic detergent Triton X-100 and high concentrations of lysophosphatidylcholine, but was slightly decreased upon addition of the α-cholinergic agonist carbachol. Basal guanylate cyclase indicated to be an allosteric enzyme, as analyzed by the Hill equation with an apparent Hill coefficient close to 2. In contrast, Triton X-100 solubilized enzyme showed regular substrate kinetics with increasing V_{max} but unaffected K_m values. Thus the lymphocyte plasma membrane contains both adenylate cyclase and guanylate cyclase at high specific activities, with properties characteristic for hormonally stimulated enzymes.

Introduction

Nucleotide cyclases are well characterized enzymes in many cells. Their role in the regulation of lymphocyte growth and differentiation, however, has not yet been defined clearly [1,2], despite an overwhelming amount of literature dealing with intracellular levels of cyclic nucleotides under various conditions [3–10]. One of the major reasons is the lack of adequate characterization of the key

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Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; p[NH]ppG, guanosine 5′-[β, γ-imido]triphosphate (5′-guanylyl imidodiphosphate); G/F protein, regulatory component of the adenylate cyclase.

enzymes, adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1) and guanylate cyclase (GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2). Whereas the cellular localization of the adenylate cyclase has been relatively well established [11–15], surprisingly nearly no attempts have been made to define the cellular localization of the guanylate cyclase in lymphocytes. Thus, to our knowledge, only experiments measuring a soluble enzyme and/or an ill defined particulate enzyme have been reported so far [16–22]. In the present communication we therefore defined the distribution of both nucleotide cyclizing enzymes measured in identical cellular compartments. As a source of pure T-lymphocytes, calf thymocytes were used. The plasma membrane represents the organelle where antigens, mitogens or a number of regulatory substances such as hormones exert their actions. Hence, emphasis was given to the characterization of the plasma membrane-bound adenylate as well as the guanylate cyclase.

Materials and Methods

Materials. ATP, AMP, cAMP, GTP, p[NH]ppG, NADH, pyruvate, succinate, cytochrome *c*, coenzyme A, *p*-nitrophenylphosphate, *p*-nitrophenylglucuronide, creatine phosphate and creatine kinase were purchased from Boehringer, Mannheim. Oleoyl-CoA, ouabain, isobutylmethylxanthine, bovine serum albumin, epinephrine, norepinephrine, isoproterenol and carbachol were from Sigma, Munich. EDTA, EGTA, ammonium heptamolybdate and malachitegreenoxalate were obtained from Merck, Darmstadt. Triton X-100 was from Serva, Heidelberg and arachidonoylchloride was from NU-CHEK-PREP, Elysian, MN, U.S.A. The radiochemicals such as [α-^{32}P]-ATP, [α-^{32}P]GTP, [^{3}H]cAMP, [^{3}H]cGMP and [^{14}C]lysophosphatidylcholine were obtained from Amersham/Buchler, Braunschweig.

All other chemicals were of analytical grade and purchased from Merck, Darmstadt.

Isolation of cellular fractions from calf thymus lymphocytes. Cellular fractions were isolated from calf thymus lymphocytes as described earlier [23,24]. Briefly, calf thymus, or in some experiments bovine lymph nodes, were removed within 10 min after slaughtering and put into cold phosphate-buffered saline. They were then freed from adherent tissue, cut into pieces and macerated gently in phosphate-buffered saline in a loosely fitting glass homogenizer to yield the lymphocytes. The cells were filtered through nylon wool (Leuko-Pak, Fenwall Lab., Travenol) in order to remove cell debris and tissue remnants, sedimented (10 min at $600 \times g$) and resuspended in 140 mM KCl/20 mM Hepes/0.25 mM $MgCl_2$ (pH 7.0). Cell disruption was achieved by the nitrogen cavitation method in an Artisan pressure homogenizer (Artisan Ind., Waltham, MA, U.S.A.) at 4°C and EDTA (final concentration 1 mM) was added to the homogenate. Various cellular fractions were obtained by several differential centrifugation steps as described in Ref. 24.

The following fractions were used for chemical determinations or enzyme assays:

(a) homogenate
(b) nuclear pellet: sediment after centrifugation at $600 \times g$ for 10 min
(c) large granules: sediment after centrifugation at $18\,000 \times g$ for 20 min (including mitochondria, lysosomes and aggregated plasma membranes)
(d) supernatant I: supernatant after centrifugation at $175\,000 \times g$ for 60 min
(e) microsomes: sediment of hypotonic shocked membranes (10 mM Hepes, pH 7.0) after centrifugation at $175\,000 \times g$ for 60 min
(f) supernatant II: supernatant after centrifugation at $175\,000 \times g$ for 60 min after the hypotonic shock treatment
(g) endoplasmic reticulum: sediment after sucrose density (35% (w/w)) ultracentrifugation of microsomes at $250\,000 \times g$ for 120 min
(h) plasma membranes: band at the interference between buffer and sucrose after sucrose density (35% (w/w)) ultracentrifugation of microsomes at $250\,000 \times g$ for 120 min

All sediments were resuspended in 140 mM KCl/20 mM Hepes (pH 7.0) and kept at 4°C.

Chemical determinations. Protein was determined using its native fluorescence as described earlier [25] with a SPF-500 spectrofluorometer (Aminco) using bovine serum albumin as standard. Phospholipids and cholesterol were extracted by a modification of the method of Ways and Hanahan [26]. Phospholipid phosphorus was determined as described by Ref. 27 and cholesterol

was measured according to Ref. 28 using a commercial test combination (Boehringer).

Enzyme assays. The activities of various enzymes were measured at least in duplicates according to the following quotations: lactate dehydrogenase (EC 1.1.1.27) [29], succinate dehydrogenase (EC 1.3.99.1) [30], γ-glutamyltranspeptidase(EC 2.3.2.2) [31], alkaline nitrophenylphosphatase (EC 3.1.3.1) [32], β-glucuronidase (EC 3.2.1.31) [23], 5′-nucleotidase (EC 3.1.3.5) [34], whereas free inorganic phosphate was measured according to Ref. 35 and acyl-CoA: lysophosphatidylcholine acyltransferase (EC 2.3.1.23) [32]. The synthesis of the substrate arachidonoyl-CoA was performed according to Refs. 33 and 36.

Adenylate cyclase and guanylate cyclase. The activities of adenylate cyclase (EC 4.6.1.1) and guanylate cyclase (EC 4.6.1.2) were measured by an assay originally designed by Salomon et al. [37] and modified by Jakobs et al. [38]. The incubation mixture contained in a total volume of 0.1 ml, 50 mM triethanolamine/HCl (pH 7.4), 1 mM isobutylmethylxanthine, 0.1 mM cAMP, 0.1 mM EGTA, 0.2% bovine serum albumin, 10 mM creatine phosphate and 0.4 mg/ml creatine kinase (final assay concentrations). As substrate for the adenylate cyclase 5 mM $MgCl_2$ and 0.2 mM [α-^{32}P]ATP (about 0.5–1 μCi/tube), in the case of guanylate cyclase 3 mM $Mn(CH_3COO)_2$ and 0.2 mM [α-^{32}P]GTP (about 0.5–1 μCi/tube) were used. The effects of hormone additions on adenylate cyclase were measured in the presence of 10 μM GTP. Optimal protein concentrations ranged between 20 and 50 μg protein/tube, the reaction was linear up to 45 min (data not shown). The reaction was stopped by the addition of $Zn(CH_3COO)_2$. Nucleotides were precipitated by $ZnCO_3$ with only cAMP and cGMP remaining in the supernatant after centrifugation (10 min at $16\,000 \times g$). The cyclic nucleotides were further purified by chromatography on acid alumina columns with a final recovery of 90% (cAMP) and 65% (cGMP). (α-^{32}P)-labelled cyclic nucleotides were determined by measuring Cerenkov radiation.

Because of the relatively low stability, especially of the adenylate cyclase, all nucleotide cyclase assays were conducted immediately after the isolation of the cellular fractions.

Results

Localization of nucleotide cyclases in calf thymus lymphocytes

Table I shows the chemical composition of cellular fractions of calf thymus lymphocytes. About $(1.5–2) \times 10^8$ cells corresponded to 1 mg of protein in total. Resting T-lymphocytes contain large nuclei and therefore 40% of the protein was found in the nuclear fraction. The hypothesis that a considerable amount of protein is trapped in the membrane vesicles during homogenization was disproved as only less than 2% of the total protein was found in the supernatant II of hypotonic

TABLE I

COMPOSITION OF CELLULAR FRACTIONS FROM CALF THYMUS LYMPHOCYTES

Calf thymus lymphocytes were disrupted by nitrogen cavitation, the cellular organelles isolated by differential centrifugation and chemical determinations were made as described in Materials and Methods. Data given are means of at least three different preparations. Results in parenthesis are expressed as percentage distribution of protein, total phospholipid and cholesterol in each cellular fraction related to the homogenate. n.d., not detectable.

	Protein (mg/10^{10} cells)	Phospholipid (μmol/mg protein)	Cholesterol (μmol/mg protein)	Cholesterol/phospholipid ratio
Homogenate	57.0 (100)	0.15 (100)	0.06 (100)	0.40
Nuclear pellet	22.8 (39.9)	0.26 (48.6)	0.10 (46.7)	0.38
Large granules	6.0 (10.4)	0.41 (26.5)	0.16 (25.8)	0.39
Supernatant I	30.0 (52.5)	n.d.	n.d.	–
Supernatant II	0.9 (1.6)	n.d.	n.d.	–
Microsomes	3.2 (5.6)	0.62 (15.6)	0.28 (17.6)	0.45
Recovery	111%	91%	90%	

TABLE II

ENZYME ACTIVITIES OF CELLULAR FRACTIONS FROM CALF THYMUS LYMPHOCYTES

Cellular organelles were isolated as in Table I and the activities of various enzymes were measured as described under Materials and Methods. n.d., not detectable.

a	Lactate dehydrogenase [a]	Succinate dehydrogenase [a]	β-Glucuronidase [a]	Lysophosphatidyl-choline acyl-transferase [a]	γ-Glutamyl-transpeptidase [a]
Homogenate	809 (100) [d]	7.3 (100)	1.0 (100)	7.4 (100)	3.1 (100)
Nuclear pellet	305 (15.1)	7.7 (27.9)	0.8 (32.0)	11.0 (59.5)	1.9 (24.5)
Large granules	121 (1.6)	35.2 (92.2)	2.7 (28.4)	13.2 (18.8)	7.5 (25.5)
Supernatant I	1336 (86.9)	n.d. (0.0)	0.5 (26.3)	n.d. (0.0)	0.8 (13.6)
Supernatant II	437 (0.9)	n.d. (0.0)	0.4 (0.6)	n.d. (0.0)	1.8 (0.9)
Microsomes	n.d. (0.0)	0.1 (0.1)	0.4 (2.2)	22.2 (16.8)	12.2 (22.1)
Recovery	105%	120 %	90 %	95 %	87 %

b	Adenylate cyclase [b]		Guanylate cyclase [b]	
	Basal	NaF [c]	Basal	Triton X-100 [c]
Homogenate	11.2 (100)	46.6 (100)	11.3 (100)	77.9 (100)
Nuclear pellet	10.9 (38.9)	47.4 (40.9)	8.0 (28.3)	68.3 (35.1)
Large granules	21.2 (19.9)	144.8 (32.8)	5.5 (5.1)	130.1 (17.6)
Supernatant I	n.d. (0.0)	n.d. (0.0)	5.6 (26.0)	7.1 (4.8)
Supernatant II	n.d. (0.0)	n.d. (0.0)	15.1 (2.1)	17.9 (0.4)
Microsomes	10.8 (5.4)	121.4 (14.7)	15.4 (7.7)	140.0 (10.1)
Recovery	64%	88 %	69 %	68 %

[a] Enzyme activities are given in nmol/mg protein per min as means of three different preparations.
[b] Enzyme activities are given in pmol/mg protein per min as means of three different preparations.
[c] Assays were conducted in the presence of either 10 mM NaF or 0.1% Triton X-100.
[d] In parenthesis the percentage of total enzyme activity in each fraction related to the homogenate is expressed.

shocked membranes (Table I). The cholesterol content is one of the specific markers for membranes. The highest value of 0.28 μmol/mg protein, which means a 5-fold enrichment related to the cholesterol content of the homogenate, was detected in the microsomal fraction. No cholesterol could be measured in the supernatants.

The specific activities and also the percentage of total activities of some enzymes related to the total activities in the cell homogenate are demonstrated in the Tables IIa and IIb. The enzyme lactate dehydrogenase was almost exclusively found in the particle free supernatants (about 90% of the total activity), no lactate dehydrogenase activity could be measured in the microsomal fraction. The highest specific activity of the succinate dehydrogenase and the β-glucoronidase were measured in the large granules, a fraction containing intracelluiar components such as mitochondria and lysosomes. γ-Glutamyltranspeptidase, which is considered to be a marker enzyme for plasma membranes and lysophosphatidylcholine acyltransferase showed highest specific activities in the microsomes of T-lymphocytes. The nucleotide cyclases were measured under basal conditions as well as with the addition of sodium fluoride (NaF), a known stimulator of the adenylate cyclase [39], or Triton X-100, a stimulator of the membrane-bound guanylate cyclase [40]. As can be seen in Table IIb,adenylate cyclase was not found in the cytosol but in the various fractions containing membranes. High specific activities were measured in the large granules and in the microsomes. Guanylate cyclase is known to have two distinct forms in different cells [45], one being soluble, the other membrane-bound. In lymphocytes derived from calf thymus one third of the basal guanylate cyclase appeared in the cytosol, about two thirds

TABLE III

SUBFRACTIONATION OF MICROSOMES INTO PLASMA MEMBRANES AND ENDOPLASMIC RETICULUM

Microsomal membranes were subfractionated by sucrose density ultracentrifugation as described in Materials and Methods. Data given are means of three separate preparations.

	Microsomes	Plasma membrane	Endoplasmic reticulum
Phospholipid (μmol/mg protein)	0.62	0.58	0.32
Cholesterol (μmol/mg protein)	0.28	0.37	0.13
Cholesterol/Phospholipid ratio	0.45	0.64	0.41
γ-Glutamyltranspeptidase [a]	12.2	23.1	5.6
5'-Nucleotidase [a]	6.4	15.0	3.4
$(Na^+ + K^+)$ATPase [a]	11.1	44.5	7.0
Alkaline phosphatase [a]	240	510	130
Adenylate cyclase [b], basal	10.8	13.7	6.1
Adenylate cyclase [b], NaF [c]	121.4 (11.2) [d]	236.0 (17.2) [d]	38.2 (6.3) [d]
Guanylate cyclase [b], basal	15.4	34.0	16.4
Guanylate cyclase [b], Triton X-100 [c]	140.0 (9.1) [d]	327.0 (9.6) [d]	84.5 (5.2) [d]

[a] Enzyme activities are expressed in nmol/mg protein per min.
[b] Enzyme activities are expressed in pmol/mg protein per min.
[c] Assays were conducted in the presence of either 10 mM NaF or 0.1% Triton X-100.
[d] Degree of stimulation by NaF and Triton X-100, respectively.

were membrane-bound. The highest specific activity of guanylate cyclase was measured in the microsomes. As can be noticed, the basal specific activity of the guanylate cyclase in the microsomes was significantly higher than that of the basal adenylate cyclase.

The chemical composition and the marker enzyme activities as well as the nucleotide cyclases were also measured in cellular fractions of lymphocytes from bovine lymph nodes. The results found there were comparable to those from calf thymus (data not shown).

For further studies of the membrane-bound nucleotide cyclases the microsomal fraction was subfractionated into plasma membranes and a fraction largely consisting of endoplasmic reticulum by sucrose gradient ultracentrifugation (see Materials and Methods). The data given in Table III clearly show that highly purified plasma membranes were obtained. The cholesterol content and the cholesterol/phospholipid ratio were significantly increased in the plasma membrane and decreased in the endoplasmic reticulum compared to the microsomes. No activity of lactate dehydrogenase, succinate dehydrogenase or β-glucuronidase was measurable in the purified plasma membranes, whereas the specific activities of γ-glutamyltranspeptidase, 5'-nucleotidase, $(Na^+ + K^+)$-ATPase and alkaline phosphatase, which are all known as markers for plasma membranes, were predominately elevated compared to the microsomal fraction. Highest specific activities of the nucleotide cyclases, basal or stimulated, were also measured in the purified plasma membranes. Adenylate cyclase and guanylate cyclase are therefore present in plasma membranes in high specific activities. The basal activity of the guanylate cyclase is much higher than the one of the adenylate cyclase (34.0 to 13.7 pmol/mg protein per min). The degree of stimulation by NaF or Triton X-100 was higher in plasma membranes than in microsomes or endoplasmic reticulum.

Characterization of plasma membrane-bound adenylate and guanylate cyclase

For a detailed characterization of membrane-bound nucleotide cyclases only highly purified plasma membranes were used. The influence of some effectors added directly to the enzyme assays on the activities of the membrane-bound nucleotide cyclases are shown in Tables IV and V. The addition of GTP (10–100 μM) had a stimulating effect on the adenylate cyclase (about 2–3-fold) only in the plasma membrane fraction. The GTP

TABLE IV

EFFECTS OF VARIOUS SUBSTANCES ON THE ACTIVITIES OF PLASMA MEMBRANE-BOUND ADENYLATE CYCLASE

Adenylate cyclase was assayed in purified plasma membranes in the presence of effector concentrations as indicated.

Effector	Stimulation [a] index	Optimal concentration	Concentration tested
GTP	2 - 3	10 -100 μM	
NaF	10 -20	10 mM	
p[NH]ppG	10 -20	0.1- 1.0 mM	
Mn^{2+}	3	1.0 mM	
Molybdate (MoO_4^{2-})	6	50 mM	
Vanadate (VO_3^{2-})	1		10 mM
Triton X-100	0.4		0.1%
Lysophosphatidylcholine	0.5		250 μM
Carbachol	0.7		1 - 10 μM
Epinephrine	1.0- 1.4		1 -100 μM
Norepinephrine	1.0- 1.4		1 -100 μM
Isoproterenol	1.0- 1.4		1 -100 μM

[a] Enzyme activities in the presence of the effector/enzyme activities in the absence of the effector.

analogue p[NH]ppG (0.1–1 mM), NaF (10 mM), molybdate (50 mM) and Mn^{2+} (1 mM) caused a significant stimulation of the adenylate cyclase activity. Addition of β-adrenergic agonists such as epinephrine, norepinephrine or isoproterenol had only marginal stimulating effects. The cholinergic agonist carbachol led to a decrease of the adenylate cyclase activity. Vanadate was without any measurable effect and Triton X-100 inhibited the adenylate cyclase. Plasma membrane-bound guanylate cyclase was stimulated by the nonionic detergent Triton X-100 (0.1–1%) and by high concentrations of lysophosphatidylcholine (500 μM) (Table V). Other detergents like deoxycholic acid, lubrol WX (Table V), lubrol PX and cholic acid, however, had no influence on the activity of the enzyme (data not shown). Carbachol, NaF and sodium nitroprusside depressed the activity of the plasma membrane-bound guanylate cyclase (Table V). On the other hand the soluble guanylate cyclase found in the cytosol could be strongly stimulated by sodium nitroprusside directly added to the enzyme assay (data not shown).

The substrate dependence of basal or stimulated (10 mM NaF, 0.1 mM p[NH]ppG, 0.1% Triton X-100) adenylate or guanylate cyclase from

TABLE V

EFFECTS OF VARIOUS SUBSTANCES ON THE ACTIVITIES OF PLASMA MEMBRANE-BOUND GUANYLATE CYCLASE

Guanylate cyclase was assayed in purified plasma membranes in the presence of effector concentrations as indicated.

Effector	Stimulation [a] index	Optimal concentration	Concentrations tested
Triton X-100	8 -10	0.1–1.0%	
Lysophosphatidylcholine	10	500 μM	
Deoxycholate	1.0		0.01%
	0.0		1.0%
Lubrol WX	1.0		0.01%
Sodium nitroprusside	0 -0.2		0.1 mM
NaF	0.0		10 mM
Carbachol	0.8		1 -10 μM

[a] Enzyme activities in the presence of the effector/enzyme activities in the absence of the effector.

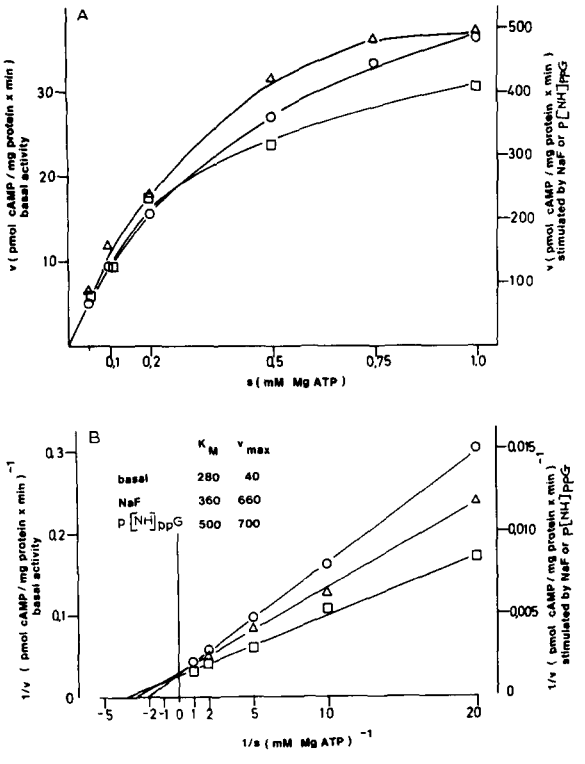

Fig. 1. Substrate dependence of plasma membrane-bound adenylate cyclase. Adenylate cyclase was assayed either under basal conditions (□) or in the presence of 10 mM NaF (△), respectively, 0.1 mM p[NH]ppG (○) as described in Materials and Methods with various concentrations of MgATP. Apparent K_m values are given in μM and V_{max} values are expressed in pmol/mg protein per min. Data given are means of two different preparations.

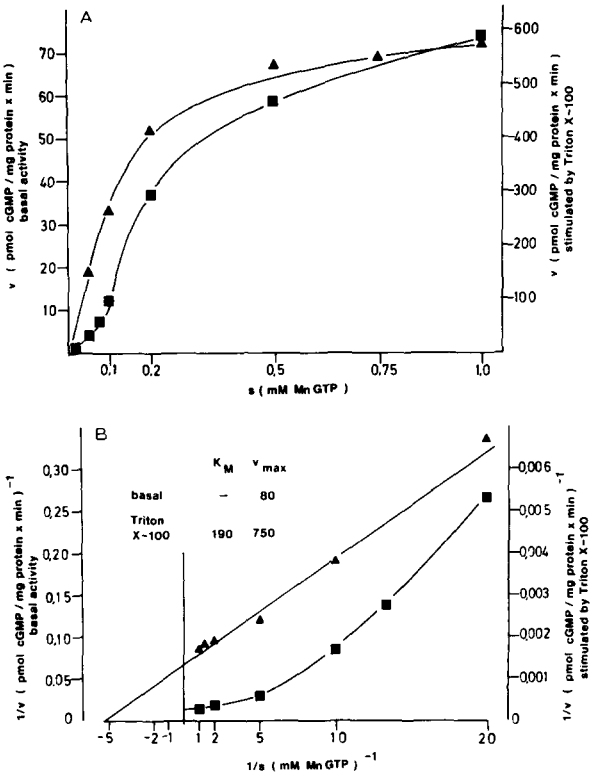

Fig. 2. Substrate dependence of plasma membrane-bound guanylate cyclase. Guanylate cyclase was assayed either under basal conditions (■) or in the presence of 0.1% Triton X-100 (▲) as described in Materials and Methods with various concentrations of MnGTP. Apparent K_m values are given in μM and V_{max} values are expressed in pmol/mg protein per min. Data given are means of three separate experiments.

purified plasma membranes is shown in the Figs. 1 and 2. As the true substrates for the cyclases are MgATP or MnGTP and not the unchelated nucleotide triphosphates, the chelated forms and the free metal concentrations were computed by a cubic equation (see Appendix). As the total metal concentrations are much higher than the concentrations of the nucleotide triphosphates (5 mM Mg^{2+}, 3 mM Mn^{2+} compared to ATP and GTP 0.02–1.0 mM), in all cases the nucleotide triphosphates are converted completely into the chelated form. The free Mg^{2+} concentration ranges from 4.2 mM (at MgATP 0.05 mM) to 3.4 mM (at MgATP 1.0 mM), the free Mn^{2+} concentration ranges from 1.5 mM (at MnGTP 0.02 mM) to 1.0 mM (at MnGTP 1.0 mM). Adenylate cyclase showed a kinetic behaviour according to the classical theory of Michaelis-Menten in all cases. Through the addition of NaF, respectively, p[NH]ppG, the maximal velocity of the enzyme reaction was enhanced to a large extend (about 17-fold from 40 to 700 pmol/mg protein per min), the affinity of the substrate to the enzyme was only marginally decreased (K_m increase, Fig. 1B). Plasma membrane-bound guanylate cyclase showed a different substrate dependence (Fig. 2). When the substrate concentration was plotted against the initial enzyme velocity in the case of the basal guanylate cyclase a sigmoid graph was obtained with an apparent $s_{0.5}$ value of about 200 μM. Activated by Triton X-100 membrane-bound guanylate cyclase showed regular Michaelis-

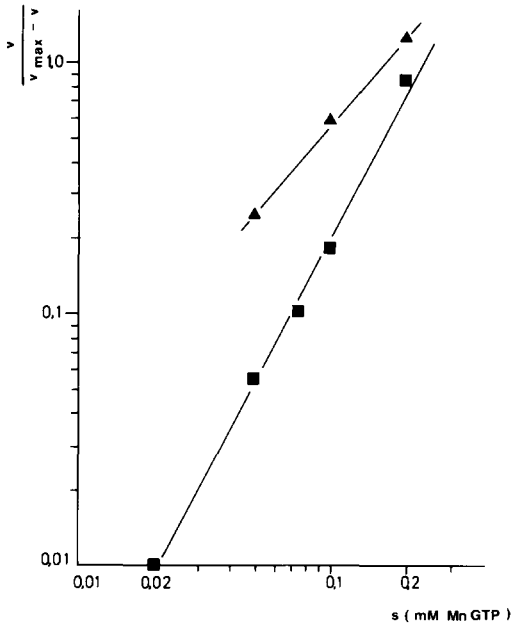

Fig. 3. Basal or Triton X-100 stimulated membrane-bound guanylate cyclase plotted according to Hill. Experimental details were as those in Fig. 2.

Menten substrate kinetics like the adenylate cyclase. The maximal enzyme velocity was increased about 10-fold whereas the apparent K_m value (about 190 μM) was very close to the calculated $s_{0.5}$ value of the basal enzyme (Fig. 2B). When the experimental data were plotted according to Hill (Fig. 3) an apparent Hill coefficient of 1.9 (basal activity) or 1.1 (Triton X-100 stimulated activity) could be computed.

Discussion

The major problem in analyzing nucleotide cyclases in lymphocytes resides in their relative low stability, especially of adenylate cyclase, known as a labile multicomponental system [14]. In purified plasma membranes the activity of adenylate cyclase decreased within hours at 4°C or being frozen at −80°C. Kept at 37°C or room temperature without substrate, the enzyme activity was lost within minutes, which is in accordance with results reported from human lymphocytes [41]. In our hands, recovery of adenylate cyclase in cellular fractions, however, was always 60% (basal) to 90% (NaF stimulated) (Table IIb), provided enzyme activities were measured in the cell homogenate and all cellular fractions immediately after isolation of the membranes, which was about 8 h after cell disruption in our preparative scheme (see Materials and Methods). During this time loss of enzyme activity in the cell homogenate was negligible when kept at 4°C (data not shown). The somewhat higher recovery after activation with 10 mM NaF most probably was due to the fact that the measured activities were higher than the basal activities (Table IIb) and thus could be measured more correctly. Addition of 2-mercaptoethanol (up to 15 mM) to all buffers had no effect on adenylate cyclase activity (data not shown). Lymphocyte membrane-bound guanylate cyclase was more stable than adenylate cyclase. In cell homogenates and in isolated cell fractions the enzyme activity was not decreased within 24 h at 4°C. Accordingly, the recovery of guanylate cyclase in cellular fractions of thymus lymphocytes was about 70% for both basal and Triton X-100-stimulated activities (Table IIb).

Adenylate cyclase

As in other studies [11–15], no activity of adenylate cyclase was found in the cytosol of calf thymocytes, pointing out that this enzyme exists only in a membrane-bound form (see Table IIb). Although the majority of the enzyme is present in the nuclear pellet, the highest specific activities are present in the large granules and the plasma membrane. As indicated by the chemical composition and the activities of some plasma membrane-bound enzymes, our plasma membrane fraction is highly purified (Table III). Thus adenylate cyclase is a constituent of the outer membrane of lymphocytes. In most cases, adenylate cyclase is sufficiently stable in homogenates and crude membrane preparations, whereas enzyme activity becomes progressively labile when membranes are further purified. This usually leads to low recoveries of the activity originally present in the cell homogenate. Thus Monneron and D'Alayer [14] only recovered totally about 40% of the basal activity of adenylate cyclase in fractions prepared without stabilizing agents. The reason for the relatively high recovery of adenylate cyclase in our preparations may be due to the cell disruption procedure applied: Nitrogen cavitation disrupts

the cells under isosmotic conditions strictly in the cold, without the danger of local heating. These conditions may be sufficiently mild to preserve the enzyme activities during the isolation procedure.

Stimulation by NaF led to a more than 17-fold increase of the specific activity of adenylate cyclase in plasma membranes, compared to a much lower effect (5–7-fold) in other cell membranes. This stimulation is consistent with the presence of the regulatory G/F protein of the adenylate cyclase [42]. Stimulators of the hormone sensitive enzyme (Mn^{2+}, molybdate, NaF, GTP or its nonmetabolized analogue p[NH]ppG) activated the plasma membrane-bound adenylate cyclase (Table IV and Fig. 1). In the case of NaF and p[NH]ppG this only marginally affected the apparent K_m values, but largely increased the maximal enzyme velocity V_{max}. On the other hand, hormones known to stimulate adenylate cyclase, such as β-adrenergic agents, only had marginal effects on enzyme activity over a wide dose range even in the presence of 10 μM GTP in the incubation mixture (Table IV). Lymphocytes have been shown to possess β-adrenergic receptors [43] coupled to the adenylate cyclase. The failure of the isolated plasma membranes to respond to these agent may be due to physicochemical reasons. Nitrogen cavitation disrupts plasma membranes into small fragments which form vesicles of about 70 nm diameter in the mean [24]. Small membrane vesicles have been shown to fail to respond to hormonal stimulation. Alternatively, a regular coupling of hormone receptors to the regulatory and catalytic subunit may be impaired during plasma membrane vesiculation.

Guanylate cyclase

In the light of the abundant data on effects of stimulation of lymphocytes on cGMP levels [3–10] it is amazing that no data have been published so far about the distribution of the guanylate cyclase in cellular fractions. In a first attempt recently Coffey and co-workers [21] differentiated a soluble guanylate cyclase from a particulate one, without efforts to purify membranes. In addition, as their membranes contained a high contamination by the cytoplasmic marker enzyme lactate dehydrogenase, interpretation of their data is rather difficult. In our experiments, guanylate cyclase was present in lymphocytes in a soluble cytoplasmic form as well as in isolated membranes. With respect to basal activities, about 30% of the enzyme activity was present in the cytoplasm (see Table IIb). Although membrane-bound guanylate cyclase was found to be present in all cellular membranes, the highest specific activities were found in the plasma membrane. The basal activity of guanylate cyclase in plasma membranes was more than 2-fold higher than that of adenylate cyclase. Solubilization of plasma membranes by Triton X-100 (or high concentrations of lysophosphatidylcholine) increased the specific activity of the membrane-bound guanylate cyclase but as expected was without effect on the soluble enzyme, indicating the cytoplasmic nature of this enzyme and excluding a contamination by putative membrane fragments not sedimented during ultracentrifugation. In contrast to the regular Michaelis-Menten kinetics exhibited by the basal or stimulated (NaF/p[NH]ppG) adenylate cyclase, the basal activity of membrane-bound guanylate cyclase did not follow Michaelis-Menten kinetics (Fig. 2). When analysed by the Hill equation (Fig. 3), a Hill coefficient close to 2 (1.9) was apparent, suggesting that the plasma membrane-bound guanylate cyclase is an allosteric enzyme. The simplest explanation is the assumption of two different substrate binding sites showing positive cooperativity. In contrast Triton X-100 solubilized enzyme showed regular substrate kinetics. The maximal enzyme velocity V_{max} was increased by a factor of about ten while the apparent K_m value is of the same order of magnitude as the apparent $s_{0.5}$ value determined from the Hill plot of the basal activity. These results are in accordance with those of Garbers [44] from experiments with sea urchin sperm cells. Similar to adenylate cyclase plasma membrane-bound guanylate cyclase was not stimulated when isolated membranes were exposed to cholinergic stimulants, although lymphocytes have been shown to possess such receptors coupled to guanylate cyclase [46]. The reasons for this failure may be identical to those discussed above for adenylate cyclase.

Alterations in cyclic nucleotide levels have been described in lymphocytes following activation with mitogens or stimulation with various hormones. Experiments are in progress to analyse the effects

of mitogen activation or hormonal stimulation at the level of plasma membrane-bound nucleotide cyclases.

Appendix

The concentrations of the chelated substrates MgATP and MnGTP and the concentrations of the free metals Mg^{2+} and Mn^{2+} in the kinetic studies of the adenylate and guanylate cyclase were calculated according to the following formula (Schultz, G., Department of Pharmacology, Free University, Berlin, F.R.G., personal communication)

$$M + N + CP \rightleftharpoons MN + CP$$

$$[MN] = K_N \times [M] \times [N]$$

$$[MCP] = K_{CP} \times [M] \times [CP]$$

$$[M] = [M]_{tot} - [MN] - [MCP]$$

$$[N] = [N]_{tot} - [MN]$$

$$[CP] = [CP]_{tot} - [MCP]$$

where M, free Mg^{2+} or free Mn^{2+}; N, free ATP or free GTP; CP, free creatine phosphate; MN, MCP, complexes; $[X]_{tot}$ = total concentrations; K_X = molar stability constants (association constant).

EGTA as third chelator was negligible, as its final assay concentration was 0.1 mM (compared to 5 mM Mg^{2+}_{tot} and 3 mM Mn^{2+}_{tot}).

Cubic equation:

$$aM^3 + bM^2 + cM + d = 0$$

where

$$a = 1$$

$$b = 1/K_N + 1/K_{CP} - [M]_{tot} + [N]_{tot} + [CP]_{tot}$$

$$c = 1/K_N \times K_{CP} + [N]_{tot}/K_{CP} + [CP]_{tot}/K_N - [M]_{tot}(1/K_N + 1/K_{CP})$$

$$d = -[M]_{tot}/K_N \times K_{CP}$$

After computing [M] from this equation the concentrations of the chelated nucleotide triphosphates were calculated according to:

$$[MN] = [N]_{tot} \times [M]/(1/K_N) + [M]$$

where $(1/K_N)$ is the dissociation constant (mM). The stability constants ($\log_{10}$) used were: 4.74 for MgATP [47], 1.30 for MgCP [47], 5.54 for MnGTP [48] and 2.04 for MnCP [48].

All concentrations are given in mM. Mg^{2+}_{tot} was 5 mM, Mn^{2+}_{tot} was 3 mM, creatine phosphate total concn. was 10 mM.

ATP_{tot}	MgATP	Mg^{2+}_{free}	GTP_{tot}	MnGTP	Mn^{2+}_{free}
0.05	0.0498	4.18	0.02	0.01996	1.54
0.1	0.0996	4.14	0.05	0.0499	1.52
0.2	0.199	4.05	0.075	0.0749	1.51
0.5	0.498	3.80	0.1	0.0998	1.49
0.75	0.746	3.59	0.2	0.1996	1.44
1.0	0.995	3.37	0.5	0.499	1.27
			0.75	0.748	1.14
			1.0	0.997	1.00

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