EFFECTS OF CROSSLINKING AGENTS ON ADENYLATE CYCLASE REGULATION

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1. Introduction

Adenylate cyclases are supramolecular structures resulting from the association of several components: a catalytic subunit, a receptor for hormones or neurotransmitters, a guanine-nucleotide binding protein and, possibly, one or several additional subunits such as a coupling or transducing factor [1–6]. How the different components of the system interact is still largely unknown, although it has been suggested that the size of the adenylate cyclase complex increases with increasing complexity of regulation [7]. Crosslinking agents [8–10] might be useful tools to investigate these interactions, as recently done with the insulin-responsive glucose transport system in adipocytes [11]. Among the crosslinking agents, two simple aldehydes, formaldehyde and glutaraldehyde, have been widely used for fixation of tissues as well as for the study of subunits interactions in proteins [12–18]. The effects of these aldehydes on the adenylate cyclase activity of rat cerebellar synaptosomes are reported here. The exposure to these crosslinking agents is shown to alter the regulation of the particulate and soluble enzyme by GTP and by its non-hydrolysable analog guanylylimidodiphosphate (Gpp(NH)p), by NaF and by EGTA, but not by Ca^{2+} or adenosine and its analogs. Another interesting effect of aldehyde-treatment of membrane-bound adenylate cyclase is that it not only preserves the activated state of the particulate enzyme after exposure to NaF or Gpp(NH)p, but it also allows the maintenance of this activated state throughout solubilization. A preliminary account of this work has been presented [19].

2. Materials and methods

2.1. *Preparation of synaptosomes*

Cerebella from 3 week old Sprague-Dawley (male or female) rats were homogenized in an ice-cold solution (STM) containing 50 mM Tris–HCl (pH 7.3), 5 mM $MgCl_2$, 10% (w/v) sucrose and protease inhibitors, using a Potter glass–Teflon homogenizer (size B) and 4 strokes at 1500 rev./min. A 30% Ficoll solution in STM was then added to the homogenate so as to adjust it at a refractive index of 29% as measured in a Bleecker refractometer. This homogenate, overlayered by a sucrose–Ficoll solution of refractive index 22%, was centrifuged in a Spinco SW 27 rotor at 130 000 × *g* for 3 h. The synaptosomes floated at the interface between the 2 Ficoll–sucrose solutions (to be described elsewhere). This fraction was pelleted (100 000 × *g*, 20 min, 4°C) and the pellet washed in an ice-cold solution containing 50 mM sodium cacodylate (pH 7.3) and 10% sucrose (CS buffer) (1 mg protein/ml).

2.2. *Treatment of synaptosomes*

The pellets of washed synaptosomes were resuspended in CS buffer and incubated for 10 min at 20°C with or without glutaraldehyde or formaldehyde, at concentrations given in the text or legends. These fractions were then diluted 6-fold with cold CS buffer, pelleted, and washed in cold CS buffer. The pellets were resuspended in 50 mM Tris–HCl buffer (pH 8.1) and used at once or kept frozen. They were given the symbol 'A' for fractions treated by aldehydes, 'U' for untreated fractions.

In some cases, the synaptosomes (starting material) were incubated at 30°C for 30 min in CS buffer without addition, or with either 20 mM NaF, 0.1 mM Gpp(NH)p or 1 mM adenosine or 2′-deoxyadenosine. The fractions were then divided into 2 aliquots, one of which was treated for 10 min at 20°C with glutaraldehyde or formaldehyde as above.

2.3. *Adenylate cyclase assay*

Adenylate cyclase was assayed at 36°C or 30°C as

in [20]. Cyclic [^{32}P]AMP was measured according to [21]. The adenylate cyclase activity was strictly proportional to the amount of proteins added and remained stable for at least 6 min.

2.4. *Solubilization of synaptosomes*

Suspensions of synaptosomes in a 50 mM Tris–HCl (pH 8.1) buffer containing 2 mM DTT and 0.75% Lubrol-PX (Lubrol:protein ratio (w/w) $\geq$7) were homogenized by hand at 20°C using a Potter glass–Teflon homogenizer and 3–4 strokes. The suspensions were centrifuged at 40 000 × *g* for 10 min, 4°C. Supernatants and resuspended pellets were tested for adenylate cyclase activity at 30°C. Proteins were determined according to [22].

2.5. *Source of materials*

[α-^{32}P]ATP (0.5–10 Ci/mmol) and c[^{3}H]AMP (20 Ci/mmol) were from Amersham. Glutaraldehyde, stored frozen, was from Sigma. Formaldehyde solutions were freshly prepared from Merck paraformaldehyde by dissolving at 80°C. Nucleotides and analogs were from Sigma. All chemicals were reagent-grade.

3. Results

3.1. *Persistance of adenylate cyclase activity in aldehyde-treated synaptosomes*

Treatment of cerebellar synaptosomes by low concentrations of formaldehyde or glutaraldehyde caused a significant increase in adenylate cyclase activity. This increase reached as much as 130% and 150% of the control value with, respectively, 0.5 mM glutaraldehyde and 15 mM formaldehyde (fig.1). At higher aldehyde concentrations (>1 mM glutaraldehyde, 30 mM formaldehyde) a progressive decrease of adenylate cyclase activity took place.

3.2. *Loss of regulation of adenylate cyclase by GTP, NaF and EGTA, after aldehyde treatment*

Incubation of untreated synaptosomes with Gpp(NH)p or NaF resulted as expected [23,24] in a 1.5–3-fold increase in adenylate cyclase activity; with 50 μM GTP, the stimulation was 1.3–1.5-fold. On the other hand, after treatment of the synaptosomes by aldehydes the regulation of adenylate cyclase by these effectors was lost (fig.2). Incubation of aldehyde-treated synaptosomes with NaF or Gpp(NH)p caused a slight but reproducible decrease (20–40%) in adenylate cyclase activity.

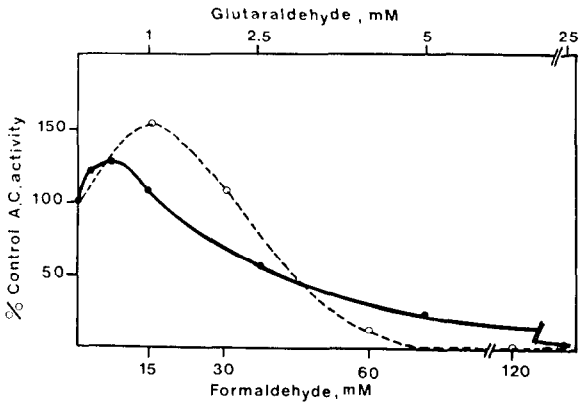

Fig.1. Aliquots of cerebellar synaptosomes were incubated for 10 min at 30°C in 50 mM cacodylate buffer (pH 7.4) and glutaraldehyde or formaldehyde at varying concentrations. They were diluted in cold buffer and pelleted. The pellets were resuspended in 50 mM Tris–HCl (pH 8.1) buffer, and tested for adenylate cyclase activity. The enzyme activity contained in untreated synaptosomes was taken as 100%. (—○——⊖—) Synaptosomes incubated in glutaraldehyde; (-⊖- - - -⊖-) synaptosomes incubated in formaldehyde.

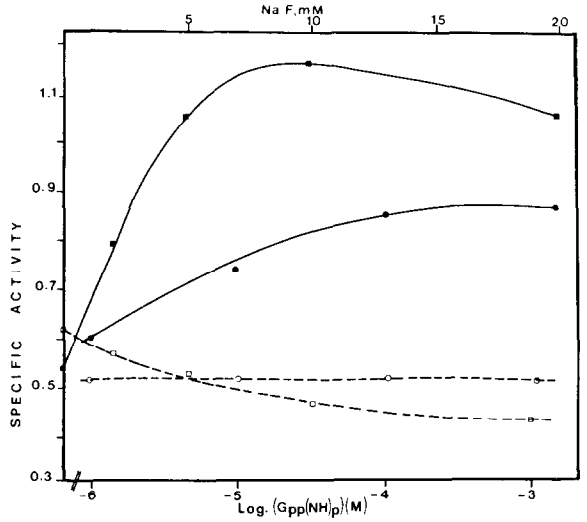

Fig.2. Aliquots of untreated (——) and 0.5 mM glutaraldehyde-treated (- - - -) synaptosomes were incubated for 20 min at 36°C in adenylate cyclase assay medium (pH 8.1, 5 mM Mg^{2+}) without [^{32}P]ATP, in the presence of varying concentrations of NaF (■, □) or Gpp(NH)p (●, ○). [^{32}P]ATP was then added, and adenylate cyclase activity assayed over 10 min. (The dose–response curve of particulate adenylate cyclase with respect to Gpp(NH)p appeared spread over several orders of magnitude of nucleotide concentration, and of rather low amplitude. This was probably due to the existence of an intrasynaptosomal pool of GTP).

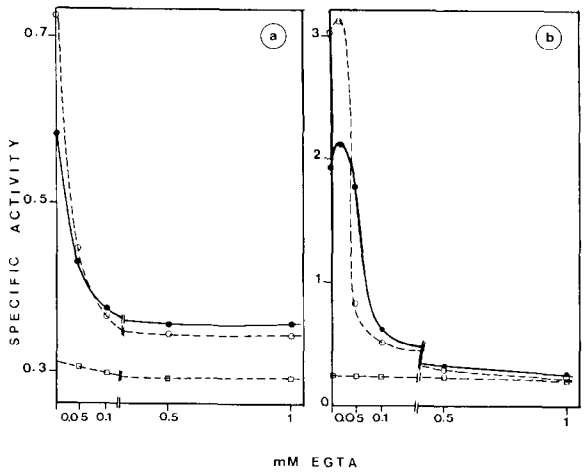

Fig.3. Aliquots of untreated (–●——●–), 0.2 mM glutaraldehyde-treated (-⊖- - -⊖-) and 2 mM glutaraldehyde-treated (□- - -□) cerebellar synaptosomes (a) and their corresponding Lubrol supernatants (b) were assayed for adenylate cyclase in the presence of 5 mM Mg^{2+}, and varying concentrations of EGTA. Assay conducted at 36°C for synaptosomes, 30°C for supernatants.

Similarly, the inhibitory effect of EGTA on adenylate cyclase occurring in the presence of Mg^{2+} was lost as a result of aldehyde treatment. However this loss of enzyme regulation by EGTA occurred only at >0.5 mM glutaraldehyde (fig.3a). By contrast, abolition of activation by NaF and Gpp(NH)p was obtained with much lower (0.05 mM) glutaraldehyde concentrations. Adenylate cyclase was not inhibited by EGTA in the presence of Mn^{2+} in untreated or aldehyde-treated fractions.

3.3. *Persistance of the inhibitory effects of Ca^{2+}, adenosine and its analogs on adenylate cyclase after aldehyde treatment*

In the presence of Mg^{2+}, the adenylate cyclase from both untreated and aldehyde-treated synaptosomes were inhibited by Ca^{2+} (table 1). Increasing Ca^{2+} from 0.05–1 mM caused a 90% inhibition of the adenylate cyclase activity in both cases. The enzymes from both sources were not inhibited by Ca^{2+} if Mn^{2+} was present.

Adenosine and some of its analogs modified in the ribose ring (2′-deoxyadenosine, 3′-deoxyadenosine) inhibited adenylate cyclase activity in aldehyde-treated and untreated synaptosomes in an identical manner at all concentrations tested (table 1).

3.4. *Solubilization of adenylate cyclase from aldehyde-treated synaptosomes*

3.4.1. Extent of solubilization

Exposure of untreated synaptosomes to 0.75% Lubrol resulted in the solubilization of 62% of the synaptosomal proteins. A 5–6-fold increase in total adenylate cyclase activity was observed under conditions where 95% of the enzyme activity was solubilized (table 2, data concerning fraction 1 U). The specific activity of the soluble enzyme was 8-fold higher than that of the particulate enzyme. These results are in accordance with those in [23] where solubilization was first shown to greatly increase brain adenylate cyclase specific activity. Exposure to 0.75% Lubrol of aldehyde-treated synaptosomes sobulized proteins to an extent that varied inversely with the aldehyde

Table 1
Effects of aldehyde-treatment on the regulation of particulate adenylate cyclase by various effectors

Synaptosomes	Basal enzyme spec. act.	Ca^{2+}	Ca^{2+}	2′-deoxyadenosine		NaF	Gpp(NH)p
		0.1 mM	1 mM	10 μM	1 mM	10 mM	0.1 mM
Untreated	528 ± 160	×0.63 (±0.08)	×0.14 (±0.03)	×0.5 (±0.1)	×0.16 (±0.05)	×2.1 (±0.3)	×1.5 (±0.2)
Glutaraldehyde-treated (0.5 mM)	686 ± 30	×0.62 (±0.1)	×0.20 (±0.05)	×0.5 (±0.2)	×0.25 (±0.1)	×0.7 (±0.05)	×0.9 (±0.05)

Cerebellar synaptosomes were suspended in cacodylate buffer, with or without 0.5 mM glutaraldehyde, for 10 min at 20°C. They were diluted in cold buffer and pelleted. The pellets suspended in 50 mM Tris–HCl (pH 8.1) buffer, 5 mM Mg^{2+}, were assayed for adenylate cyclase at 36°C. The aliquots assayed in the presence of NaF or Gpp(NH)p were preincubated with the effector for 20 min at 36°C. Basal adenylate cyclase activities (measured in 8 expt) are given in pmol cAMP . mg^{-1} . min^{-1}. The effects of various enzyme effectors are expressed with respect to the corresponding enzyme basal activity, taken for 1

Table 2
Effects of pre-exposure to NaF and/or glutaraldehyde-treatment of synaptosomes on the solubilized adenylate cyclase

Fractions		Particulate enzyme (spec. act.)	Increase in enzyme activity caused by Lubrol (-fold)	Proteins solubilized by Lubrol (%)	Enzyme solubilized by Lubrol (%)	Soluble enzyme (spec. act.)
Preincubation without NaF	U1	317 ± 122	5.55 ± 0.45	62.5 ± 3.5	93.3 ± 3.5	1810 ± 110
	A1	316 ± 169	5.45 ± 0.35	31.5 ± 0.5	60.5 ± 0.5	2870 ± 93
Preincubation with NaF	U2	475 ± 63	5.5 ± 0.3	61.5 ± 6.5	92 ± 2	2832 ± 70
	A2	570 ± 80	5.5 ± 0.4	33.5 ± 2.5	63 ± 8	4917 ± 143

Cerebellar synaptosomes were incubated at 30°C for 30 min in cacodylate buffer, with (fraction 2) or without 20 mM NaF (fraction 1). Each fraction was divided into 2 aliquots, one of which was treated with 1 mM glutaraldehyde for 10 min at 20°C, (fractions A) and the other was not treated (fractions U). The fractions were diluted and pelleted. The pellets, resuspended in 50 mM Tris–HCl buffer (pH 8.1), 5 mM Mg^{2+}, were assayed for adenylate cyclase activity at 30°C. The enzyme basal specific activities (spec. act.), expressed in pmol . mg^{-1} . min^{-1}, are given in the first column (mean of 3 expt). Aliquots were homogenized by hand in 50 mM Tris–HCl buffer (pH 8.1) containing 2 mM DTT and 0.75% Lubrol-PX (Lubrol : protein ratio, 9 ± 2). The adenylate cyclase activities of the unfractionated Lubrol suspensions, and of the supernatants and pellets obtained by centrifugation at 40 000 × *g* for 10 min, were measured at 30°C in the presence of 5 mM Mg^{2+}. The increase in enzyme activity caused by Lubrol treatment is given with respect to the particulate enzyme activity, taken for 1. (The differences in adenylate cyclase specific activities, between table 1 and table 2, are due to the differences in the temperature of the assays, and to the 30 min preincubation at 30°C in the case of table 2)

concentration. The increase in total adenylate cyclase activity caused by Lubrol also depended on the aldehyde concentration. At low glutaraldehyde concentration (≤0.5 mM), 60% of the enzyme activity was solubilized, and its specific activity was 9-fold that of the particulate enzyme (table 2, data concerning fraction 1 A). At higher glutaraldehyde concentrations, the amount and specific activity of the soluble enzyme decreased (not shown). Adenylate cyclase solubility was however less affected than that of average membrane proteins.

3.4.2. Properties of aldehyde-treated soluble adenylate cyclase

For convenience, the adenylate cyclase solubilized from aldehyde-treated synaptosomes (fractions A) and from untreated synaptosomes (fractions U) will be referred to as aldehyde-treated and untreated soluble adenylate cyclases. The affinity of the aldehyde-treated enzyme for ATP appeared to be significantly higher than that of the untreated enzyme; however proper K_m values could not yet be determined safely because of the interference of an ATPase at low ATP concentrations (in preparation).

In contrast with the untreated particulate enzyme, the untreated soluble enzyme was slightly inhibited by NaF (20–30%), as already shown [23]. NaF also inhibited the aldehyde-treated soluble enzyme. GTP and Gpp(NH)p slightly activated the untreated soluble enzyme (0–20%) whereas they slightly inhibited the aldehyde-treated soluble enzyme. Solubilization did not alter the effects of EGTA (fig.3b), Ca^{2+} and adenosine (or its analogs) on aldehyde-treated or untreated enzymes.

3.5. *Effects of synaptosomes exposure to NaF or Gpp(NH)p prior to aldehyde treatment*

In the previous sections, we considered the situation where the regulation of adenylate cyclase was studied after treatment of the synaptosomes by aldehyde. Here we report on the effects of aldehyde treatment upon the adenylate cyclase activity of synaptosomes pre-exposed to Gpp(NH)p or NaF.

As expected for NaF activation, which is known to withstand washing of the fractions [24], the adenylate cyclase activities of washed fractions were higher if they had been preincubated with NaF (table 2, fraction 2 U) than in the absence of NaF (table 2, fraction 1 U). Similarly, the adenylate cyclase activities from fractions treated with aldehydes were higher if they had been preincubated with NaF (table 2, fraction 2 A) than without NaF (table 2, fraction 1 A). As expected for Gpp(NH)p activation [24], the same results were found in the case of fractions preincubated with Gpp(NH)p. Once established, the activated state of the enzyme was thus maintained by aldehyde treatment. Contrarily to adenylate cyclase activation by NaF or Gpp(NH)p which was not affected by washes

and/or aldehyde treatment, inhibition of adenylate cyclase by 2′-deoxyadenosine did not persist after washes or exposure to aldehydes.

The adenylate cyclases from fractions preincubated with NaF or Gpp(NH)p, and either washed, or aldehyde-treated, could no longer be 'super' activated with NaF or Gpp(NH)p, but were still susceptible to inhibition by Ca^{2+} or by adenosine or its analogs.

Initial exposure of synaptosomes to NaF did not modify the extent of protein or adenylate cyclase solubilization by Lubrol with respect to that found in the fraction incubated without NaF (table 2, compare fractions 1 U and 2 U, and fractions 1 A and 2 A). Adenylate cyclase solubilized from fractions preincubated with NaF had much higher specific activities than those solubilized from fractions preincubated in the absence of NaF (table 2, compare fractions 1 U and 2 U, and fractions 1 A and 2 A). Once established the activation of the enzyme was thus maintained in the soluble state, regardless of the aldehyde treatment.

4. Discussion

Treatment of cerebellar synaptosomes by low aldehyde concentrations preserved the particulate and soluble adenylate cyclase basal activity and resulted in the complete loss of activation of particulate enzyme by GTP, Gpp(NH)p or NaF. Other compounds, such as filipin, have been reported to abolish the enzyme stimulation by GTP or Gpp(NH)p but not by NaF [26]. Aldehydes also caused the loss of enzyme inhibition by EGTA, although this phenomenon appeared at higher aldehyde concentration than that observed for the loss of enzyme stimulation by Gpp(NH)p or NaF.

In contrast with this loss of enzyme regulation, no change in adenylate cyclase inhibition by Ca^{2+}, adenosine and its analogs could be observed under the influence of aldehyde treatment.

Our results lead to the distinction of at least two categories of sites, or structures within the adenylate cyclase complex: (i) those which are not affected by aldehydes and may be closely related to the catalytic site (Ca^{2+} site, adenosine site or sites); (ii) those which are affected by aldehyde treatment and become functionally uncoupled from the catalytic site. The loss of adenylate cyclase regulation by GTP, Gpp(NH)p, NaF and EGTA strongly resembles the 'desensitization' described in the case of allosteric enzymes [27]. As a consequence of the aldehyde treatment, the function of the regulatory subunits responding to Gpp(NH)p and to NaF, and/or their association with the catalytic subunit, could be altered. Several possibilities should be explored.

(1) We do not yet know the effects of aldehyde treatment upon the adenylate cyclase associated GTPase [28].

(2) The catalytic site of the enzyme could be modified in such a way as to prevent any subsequent conformational change. If activation of the enzyme by Gpp(NH)p or NaF takes place after aldehyde treatment, then the conformational change of the catalytic subunit normally associated with ligand binding and resulting in activation would be prevented. If activation of the enzyme by Gpp(NH)p or NaF takes place before aldehyde treatment, then the conformational charge in the catalytic subunit induced by the ligand and leading to the activated state would be permanently preserved, although any subsequent heterotropic regulation by NaF, Gpp(NH)p or eventually EGTA would be abolished. Indeed, aldehydes have been shown to fix the active conformation of allosteric enzymes in the crystalline [29] or in the soluble state [18]. Other crosslinking agents also stabilize conformational states of purified enzymes [30,31].

(3) The state of association of components of the adenylate cyclase complex prevailing at the time of aldehyde treatment could be stabilized by this treatment. A rather large set of data is in favour of a parallel increase in solubilized adenylate cyclase catalytic activity and molecular weight [5,32,33], possibly due to a close association of the enzyme catalytic subunit with the guanine-nucleotide binding protein and/or other structures. Discrepant data have been reported however some of them favouring the concept of adenylate cyclase being most active when existing as a fully dissociated catalytic subunit [34,35]. Finally, in the case of adenylate cyclase regulation by EGTA, the possibility should also be explored that Mn^{2+} would become permanently trapped inside the protein–EGTA is indeed thought to inhibit the brain enzyme by chelating Mn^{2+} [36].

Distinction between these alternatives must await further purification of the components of the ade-

nylate cyclase complex. In this respect, the use of chemical crosslinkers should open new possibilities.

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