EFFECTS OF IMIDO-ESTERS ON MEMBRANE-BOUND ADENYLATE CYCLASE

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1. Introduction

Crosslinking reagents are useful tools to stabilize purified proteins and to study their conformation and subunit assembly [1–3]. Yet their use to investigate protein organization in whole membranes inevitably leads to very complex data. A number of studies on erythrocyte membranes however suggest that most crosslinked products must arise from long-term protein associations rather than from random interactions [4,5]. This encouraged us to apply crosslinking reagents to adenylate cyclase containing membranes in an attempt to study the association of the several components of the enzyme. In [6], we analyzed how the exposure of rat cerebellar synaptosomes to aldehydes affected adenylate cyclase activity and regulation. We showed that aldehydes induced a loss of enzyme regulation by some effectors (GTP, guanylylimidodiphosphate (Gpp(NH)p), NaF and EGTA) but not by others (Ca^{2+}, adenosine). We also showed that aldehydes maintained the pre-established activated state of the enzyme in the particulate and soluble forms. Here, we describe the effects on adenylate cyclase activity and regulation of the exposure of rat brain synaptosomes to the monofunctional and bifunctional imidoesters methylacetimidate and dimethylsuberimidate (DMS).

2. Materials and methods

2.1. *Preparation of synaptosomes*

Cerebellar synaptosomes were prepared as in [6]. For cerebral synaptosomes, the homogenate was adjusted to 27% refractive index.

2.2. *Treatment of synaptosomes*

The pellets of washed synaptosomes were resuspended at 5–10 mg protein/ml final conc. in cold 50 mM triethanolamine (TEA) buffer (pH 8.1) containing 10% sucrose (w/v) and 5 mM $MgCl_2$, and divided into aliquots. They were incubated for 30 min at 36°C without further addition (control synaptosomes) or with 20 mM NaF or 0.2 mM Gpp(NH)p (preincubated synaptosomes). Each sample was then divided into aliquots which were further treated with DMS or methylacetimidate or buffer, in the dark, for 30 min at 4°C when the final pH was 8.1, or, in a few experiments, for 5 min at 20°C when the final pH was adjusted to 9.0. The fractions were then diluted 6-fold with 50 mM TEA buffer (pH 8.1) and centrifuged at 100 000 × *g*, 15 min, at 4°C. The pellets were resuspended in the same buffer and used at once or kept frozen in liquid nitrogen.

2.3. *Solubilization of synaptosomes*

Synaptosomes were solubilized as in [6] with minor modifications. The 50 mM TEA buffer (pH 8.1) contained 2 mM dithiothreitol (DTT), 1 mM EDTA, 2 mM $MgCl_2$ and 0.8% Lubrol-PX (Lubrol/protein ratio (w/w) ≥3). Homogenization was by hand in a Potter type-1 homogenizer, using 40 strokes. The suspensions were centrifuged at 40 000 × *g* for 10 min.

2.4. *Adenylate cyclase assays*

They were performed as in [7]. Linearity of the reaction with time was checked in all instances. When the effect of Gpp(NH)p or NaF was studied in soluble fractions preincubation was performed at 22°C for 3.5 h in the presence of either effector, or of GTP in the case of control samples. Aliquots were also preincubated at 4°C to ascertain that the observed changes were due to an activation rather than a protective effect of the effectors upon the enzyme.

2.5. *Source of materials*

This was as in [6] except for [α-^{32}P]ATP (25 Ci/

mmol) which was from ICN. Dimethylsuberimidate and methylacetimidate were from Sigma. 2′,5′-Dideoxyadenosine was from ICN.

3. Results

3.1. *Effects of the monofunctional imidoester on the activity of membrane-bound adenylate cyclase*

Treatment of cerebral synaptosomes with increasing concentrations of methylacetimidate caused a progressive increase in particulate adenylate cyclase activity which reached 1.6-fold the control value at 50 mM reagent (fig.1). The adenylate cyclase activity of samples preincubated in Gpp(NH)p or NaF was however unaffected by the addition of methylacetimidate. The regulation of the enzyme was comparable in methylacetimidate-treated and untreated synaptosomes. Gpp(NH)p and NaF added to the assay stimulated ⩾2-fold the enzyme activity (fig.2) except in the case of samples already preincubated with Gpp(NH)p or NaF, which no longer responded to further exposure

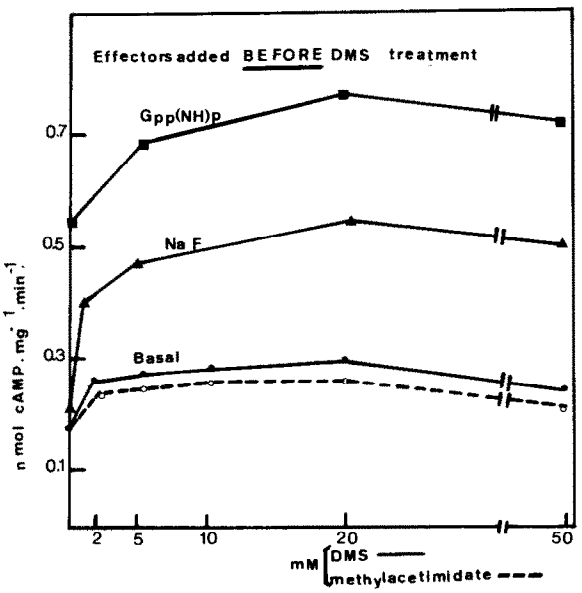

Fig.1. Aliquots of cerebral synaptosomes were incubated for 30 min in TEA–$MgCl_2$–sucrose buffer (see section 2), without addition (●,○), with 20 mM NaF (▲), and with 0.2 mM Gpp(NH)p (■). The cooled samples were then treated with DMS (—) or methylacetimidate (– – –) at various concentrations (abscissa), and processed as in section 2. The resuspended pellets were assayed for adenylate cyclase activity at 36°C in the presence of 0.5 mM ATP and 5 mM $MgCl_2$.

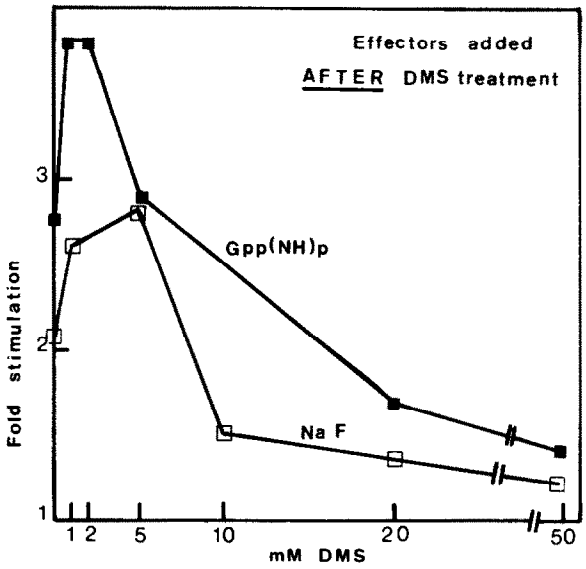

Fig.2. Aliquots of control cerebral synaptosomes were treated with increasing DMS concentrations (abscissa). The resuspended fractions were then incubated at 36°C for 30 min in TEA buffer containing 5 mM $MgCl_2$, without addition (basal activity), or in the presence of 10 mM NaF (□) or of 0.1 mM Gpp(NH)p (■), and assayed for adenylate cyclase. Ordinate: stimulation factor produced by each effector. (Basal activity taken for 1.)

to either effector. Adenosine (⩾100 μM) and its analogs, Ca^{2+} (⩾50 μM) and EGTA (⩾100 μM) inhibited adenylate cyclase in all cases, by a factor of 80%, 70% and 50%, respectively (fig.3).

3.2. *Effects of the bifunctional imidoester on the activity of membrane-bound adenylate cyclase*

Treatment of control synaptosomes with increasing concentrations of DMS caused a progressive increase in particulate adenylate cyclase activity, reaching 1.6-fold the value found in untreated samples at 25 mM reagent (fig.1). Above that concentration, the enzyme activity started to decline. The presence of the enzyme substrate, ATP, in the incubation medium did not affect the results. The adenylate cyclase of samples exposed to ⩽20 mM DMS retained the capacity to be further stimulated by NaF or Gpp(NH)p (fig.2). However, in samples treated with ⩾5 mM DMS, this stimulatory effect decreased as a function of increasing bifunctional reagent concentration. The inhibitory effects of adenosine and its analogs, and of Ca^{2+} were unaffected by the pre-exposure of samples to DMS (fig.3). The inhibitory effect of EGTA was retained in

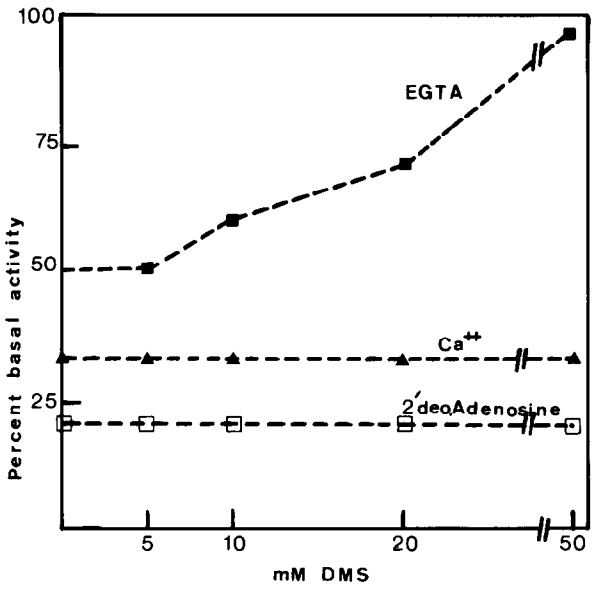

Fig.3. Aliquots of cerebral synaptosomes (NaF or Gpp(NH)p preactivated samples, or control samples) were treated with increasing DMS concentrations (abscissa). The resuspended fractions were preincubated for 3 min at 36°C without addition (basal activity), or in the presence of 1 mM 2'-deoxyadenosine (□), 1 mM Ca^{2+} (▲), or 1 mM EGTA (■), and then assayed for adenylate cyclase. Ordinate:percent of the basal activity (taken for 100%).

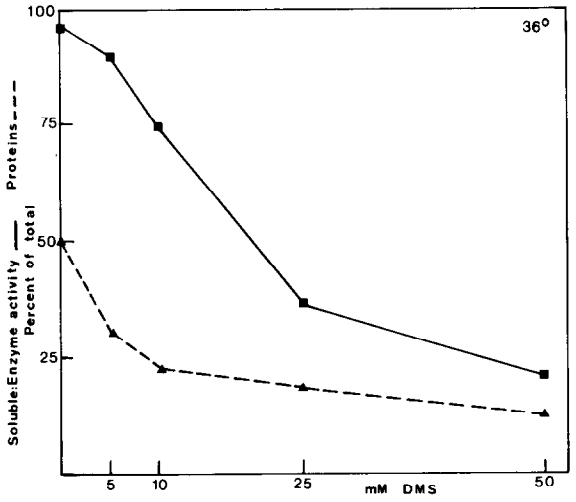

Fig.4. Gpp(NH)p preactivated cerebral synaptosomes were treated with increasing DMS concentrations (abscissa). The resuspended fractions were solubilized by homogenization in Lubrol-PX and centrifuged (see section 2). The percentage of soluble versus total proteins (▲—▲) and of soluble versus total adenylate cyclase activity (■—■) are plotted in the ordinates. The curves obtained for control or NaF preactivated cerebral synaptosomes were similar.

samples treated with up to 10 mM DMS, and progressively diminished in samples treated with higher DMS concentrations (fig.3).

Synaptosomes preincubated in Gpp(NH)p or NaF were also treated with increasing concentrations of DMS. This resulted in an additional increase in particulate adenylate cyclase activity with respect to synaptosomes pre-incubated in Gpp(NH)p or NaF but unexposed to DMS (fig.1). The increase in enzyme activity paralleled the increase in crosslinking reagent up to 25 mM. Above that value, the enzyme activity decreased. In such samples preactivated by Gpp(NH)p or NaF, the adenylate cyclase could no longer be activated by either effector. The effects of Ca^{2+}, adenosine and EGTA were comparable to those found in samples preincubated in buffer only and treated with DMS.

3.3. *Properties of adenylate cyclase solubilized from imidoester-treated synaptosomes*

Imidoester-treated and untreated synaptosomes were solubilized with Lubrol-PX. As expected from [6], the total adenylate cyclase activities in the Lubrol-treated fractions were ≥3-fold higher than in the corresponding particulate fractions. The percentage of solubilized proteins and of soluble versus total enzyme activity decreased with increasing DMS concentrations (fig.4) while exposure of synaptosomes up to 100 mM methylacetimidate did not affect the extent of enzyme solubilization. Whatever the history of the samples (control or preincubated with Gpp(NH)p or NaF), the soluble adenylate cyclase specific activities increased as a function of DMS up to 25 mM (table 1).

In all fractions, the adenylate cyclase activity was stimulated by Gpp(NH)p, provided that a long preincubation time in the presence of the effector be allowed (see section 2). The extent of activation of the soluble enzyme depended upon the preincubation step and conditions of DMS treatment undergone by the synaptosomes (table 1). Adenylate cyclase solubilized from control synaptosomes not treated by DMS was stimulated 2.6-fold by Gpp(NH)p. The enzyme solubilized from control samples treated with DMS was also stimulated by Gpp(NH)p, the extent of activation decreasing as a function of increasing DMS concentration (table 1). In the case of synaptosomes (preincubated in NaF or Gpp(NH)p, although the par-

Table 1
Effects of DMS treatment of synaptosomes on the regulation of the solubilized adenylate cyclase

DMS (mM)	Basal enzyme (spec. act.)	Gpp(NH)p (0.1 mM)	NaF (10 mM)	EGTA (0.1 mM)	Ca^{2+} (1 mM)	2′,5′-dideoxy adenosine
0	674	× 2.7	× 0.90	× 0.4	× 0.14	× 0.07
5	740	× 2.2	× 0.96	× 0.55	× 0.14	× 0.07
10	854	× 2.1	× 1	× 0.60	× 0.14	× 0.06
20	1082	× 2.1	× 1	× 0.73	× 0.13	× 0.06
50	1216	× 1.8	× 0.96	× 0.88	× 0.12	× 0.06

Control cerebral synaptosomes were treated with various DMS concentrations, washed, and solubilized (see section 2). The soluble enzyme activities were measured at 30°C in the presence of 0.3 mM ATP. The samples were preincubated for 3.5 h at 22°C with 0.1 mM Gpp(NH)p or 10 mM NaF, in the presence of 5 mM $MgCl_2$, or 3 min with 0.1 mM EGTA or 1 mM Ca^{2+}, in the presence of 5 mM $MgCl_2$, or 0.5 mM 2′,5′-dideoxyadenosine in the presence of 5 mM $MnCl_2$; the samples were then assayed for adenylate cyclase. The basal specific activities (in the presence of 5 mM $MgCl_2$) are given in pmol cAMP . mg^{-1} . min^{-1}. Relative stimulations or inhibitions caused by incubation in the presence of various effectors are expressed as respect to the corresponding basal enzyme activities, taken for 1 for each sample

ticulate enzyme could no longer be activated by Gpp(NH)p, the enzyme prepared from such samples could still be stimulated 1.2–1.4-fold by Gpp(NH)p. In contrast with the Gpp(NH)p stimulation, activation of the soluble enzyme by NaF could not be demonstrated (table 1).

In all tested fractions, the adenylate cyclase activity was inhibited by adenosine and its analogs and Ca^{2+}. (table 1). The extent of inhibition by EGTA of the soluble enzyme dependent upon the DMS concentration, exactly as in the case of the particulate enzyme (table 1). Enzyme solubilized from 50 mM DMS-treated samples was practically insensitive to EGTA.

The apparent stability of the soluble adenylate cyclase activities were studied at 38°C (fig.5) and at 28°C. The enzyme activity sharply declined after 3 min incubation at 38°C and was no longer detected after 15–25 min in samples prepared from fractions incubated with or without methylacetimidate or DMS up to 10 mM (fig.5). On the contrary, the adenylate cyclase apparent inactivation was much slower in fractions solubilized from 50 mM DMS-treated samples. Two slopes were seen, the first one rather steep, during the first 10 min, the second very shallow, almost amounting to a plateau. Although the extent of apparent enzyme inactivation (measured at the plateau) somewhat varied from one experiment to the other, ~50% of the enzyme activity of 50 mM DMS-treated samples was still present after 30 min incubation at 38°C, whereas little activity was left in 20 mM DMS-treated samples, and none in untreated samples or samples treated with low DMS concentrations. The apparent adenylate cyclase stabilization observed in 50 mM DMS-treated samples was further enhanced by preactivation of the samples by Gpp(NH)p, a procedure which however had hardly any effect on the

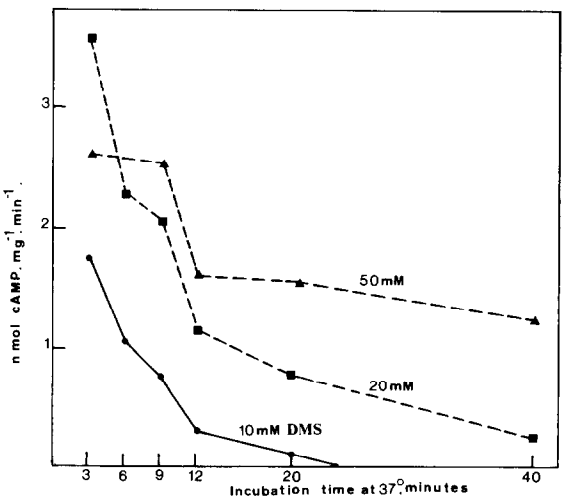

Fig.5. Gpp(NH)p preactivated cerebral synaptosomes were treated with buffer or 10 mM DMS (●–●), 20 mM DMS (■–■) or 50 mM DMS (▲–▲) and solubilized (see section 2). The soluble enzyme activities (ordinate, nmol cAMP . mg^{-1} . min^{-1}) were measured after various incubation times at 36°C, according to [7].

enzyme stability of samples treated with lower DMS concentrations. Preincubation of the samples with NaF did not noticeably affect the enzyme apparent stability. Comparable results were observed at 28°C, the apparent inactivation of adenylate cyclase being however much slower in all samples.

4. Discussion

Treatment of brain synaptosomes by the monofunctional imidoester methylacetimidate and by the bifunctional reagent dimethylsuberimidate DMS resulted in an increase in the particulate and the solubilized adenylate cyclase activities. A direct effect on enzyme activation of the imidination of some sites of adenylate cyclase is thus likely, but appears however insufficient to account for the increase observed in the enzyme activity of DMS-treated fractions. DMS was indeed able to increase the adenylate cyclase activity of fractions pre-stimulated with Gpp(NH)p and NaF, whereas methylacetimidate had no effect on such fractions. The regulation of adenylate cyclase was affected by DMS, but not methylacetimidate, treatment.

Important similarities exist in the effects of aldehydes and of DMS upon adenylate cyclase properties and regulation. In DMS-treated fractions, however, the enzyme basal activity was enhanced even at high crosslinking reagent concentrations, whereas treatment with $\geqslant$1 mM glutaraldehyde quickly led to enzyme inactivation [6]. Using rather low ($\leqslant$10 mM) DMS concentrations, it was also possible to obtain an adenylate cyclase with enhanced basal activity (as compared to the untreated enzyme), while still being susceptible to activation by NaF and Gpp(NH)p. In aldehyde-treated fractions, on the contrary, complete desensitization of the enzyme towards NaF and Gpp(NH)p was seen at the lowest tested aldehyde concentrations [6]. Yet the two categories of crosslinking agents allow the distinction of two classes of regulatory sites within the adenylate cyclase complex:

(i) Those which are unaffected by the crosslinking agents, Ca^{2+} site, adenosine site, presumably the P site [8], and may be closely related to the catalytic site;

(ii) Those which are affected by these agents and become functionally uncoupled from the catalytic site. Among those sites, two, the NaF site and the GTP site of action, are now known to be located on the so-called N subunits [9]. Crosslinking agents might then affect the association state of the catalytic and of the regulatory N subunits, or they might alter any one of the components.

The DMS treatment of synaptosomes produced intermolecular crosslinks, since there was a sharp decrease in the extent of protein, and of adenylate cyclase solubilization. Whether intermolecular crosslinks also form within the soluble adenylate cyclase entities is currently being studied. Preliminary data indicate that the apparent *S* values of enzymes solubilized from Gpp(NH)p or NaF preactivated, DMS-treated fractions are larger than those from corresponding samples not treated by DMS [10]. The presence of physiologically relevant molecules (such as regulatory subunits, or receptors) in the soluble complexes will be sought.

Intramolecular crosslinks have been implied in the enhancement of conformational stability, and used to prevent or retard denaturation [11]. Since the fraction of 50 mM DMS-treated adenylate cyclase which could be solubilized had gained increased apparent thermal stability, DMS may have been freezing the enzyme catalytic subunit in an active conformation, but however not in such a rigid way as to prevent the enzyme from being inhibited by Ca^{2+} and adenosine. Using a related compound, 3,3′-dithiobispropionimidate, on a different system, an apparent stabilization of adenylate cyclase to dispersion in detergent was reported [12,13]. Finally, the progressive loss of EGTA inhibition occuring upon aldehyde or DMS treatment, presumably related to the permanent trapping of Mn^{2+} inside the complex, might be related to either inter- or intra-molecular crosslinks. Purification of the crosslinked entities should thus help to dissect the complex adenylate cyclase architecture.

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