A Monoclonal Antibody Directed against the Catalytic Site of *Bacillus anthracis* Adenylyl Cyclase Identifies a Novel Mammalian Brain Catalytic Subunit†

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ABSTRACT: A brain adenylyl cyclase was shown to contain an epitope closely related to that specified by a conserved sequence containing a nucleotide-binding consensus sequence GXXXXGKS and located in the catalytic sites of bacterial, calmodulin-dependent adenylyl cyclases [Goyard, S., Orlando, C., Sabatier, J.-M., Labruyere, E., d'Alayer, J., Fontan, G., van Rietschoten, J., Mock, M., Danchin, A., Ullmann, A., & Monneron, A. (1989) *Biochemistry 28*, 1964–1967]. A monoclonal antibody, mab 164, produced against a peptide corresponding to this conserved sequence specifically inhibited the *Bordetella pertussis* adenylyl cyclase. It also specifically inhibited rat and rabbit brain synaptosomal adenylyl cyclases. The extent of inhibition depended upon the type of enzyme purification, reaching 90% for the calmodulin-sensitive species of enzyme and 20–35% for the forskolin–agarose-retained species. The extent of inhibition in a given fraction also depended upon the effector present. mab 164 reacted on Western blots of forskolin–agarose-retained fractions with a 175-kDa component and did not recognize the Gsα stimulatory subunit. Consequently, the 175-kDa protein was considered as a good candidate for an adenylyl cyclase catalyst. The adenylyl cyclase activity contained in forskolin–agarose-retained fractions was further purified on calmodulin–Sepharose. On Western blots of such fractions, mab 164 reacted with a 140-kDa protein, a component that appeared to derive from the 175-kDa protein enriched in the previous step. The k_{cat} of this 140-kDa presumptive adenylyl cyclase was estimated to be of the order of 600 s^{-1}. The cloned mammalian adenylyl cyclases of type I [Krupinski, J., Coussen, F., Bakalyar, H. A., Tang, W.-J., Feinstein, P. G., Orth, K., Slaughter, C., Reed, R. R., & Gilman, A. G. (1989) *Science 244*, 1558–1564] and type III [Bakalyar, H. A., & Reed, R. R. (1990) *Science 250*, 1403–1406] constitute a family which does not contain the GXXXXGKS sequence. The k_{cat} of the type I enzyme being lower than the one estimated for the catalyst described here, the possibility that the latter belongs to another family of adenylyl cyclases is discussed.

Mammalian adenylyl cyclase catalytic subunits are essential components of the signal transduction cascade. Many efforts have been devoted to their study, and particularly to their isolation (Coussen et al., 1985, 1986; Pfeuffer et al., 1985, 1989; Yeager et al., 1985). Yet, these proteins have not been purified to complete homogeneity. Several facts explain this situation. First, the adenylyl cyclase catalytic subunits (referred to as catalysts in this paper) seem to be exceedingly minor components of the cell. Second, since they are integral membrane proteins, their purification involves the use of detergents which, as a rule, produce micelles of heterogeneous composition. *Identifying* the catalytic subunits by the sole use of antibodies able to immunoprecipitate adenylyl cyclase activity is therefore not possible. Third, no specific ligand of these subunits has yet been discovered. Indeed forskolin, a very potent activator of adenylyl cyclase, is now known to have several other cellular targets (Laurenza et al., 1989). Finally, both calmodulin-sensitive and -insensitive forms of the enzyme are described, indicating that there are most probably several different catalysts (Brostrom et al., 1975; Lynch et al., 1976; Livingstone, 1985).

Molecular biology has come to the rescue. A cDNA able to elicit dose-dependent elevations of calmodulin-sensitive adenylyl cyclase activity when transfected into mammalian and insect cells has been isolated from a bovine brain library in Gilman's laboratory (Krupinski et al., 1989; Tang et al., 1991). The adenylyl cyclase catalyst encoded by this cDNA, designated as type I, corresponds, in bovine brain, to a 110-kDa protein. It contains two putative intracellular domains bearing a sequence homologous to a single cytoplasmic domain of several guanylyl cyclases. By making use of an oligonucleotide probe based on the type I sequence, used at low stringency, another cDNA was isolated from a rat olfactory library and was shown to code for an adenylyl cyclase catalyst, designated as type III, and corresponding to a 180-kDa glycoprotein (Bakalyar & Reed, 1990). This cDNA contains two duplicated sequences homologous to the bovine conserved domains. These domains are also homologous to an unduplicated sequence found in a bacterial adenylyl cyclase, that of *Rhizobium melilotti* (Beuve et al., 1990).

The existence of several different families of *bacterial* adenylyl cyclases has been established (A. Danchin, personal communication). One class, which comprises the *R. melilotti* enzyme and a few others, is characterized by a very low k_{cat} (10^{-3} s^{-1}) and shows sequence similarities with the *Saccharomyces cerevisiae* adenylyl cyclase, the eukaryotic adenylyl cyclases types I and III, and the eukaryotic guanylyl cyclases. Another family of prokaryotic adenylyl cyclases is composed of the calmodulin-sensitive enzymes characterized by a high

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k_{cat} (1000–2000 s^{-1}) and is secreted as toxins by, respectively, *Bordetella pertussis* (Leppla, 1982) and *Bacillus anthracis* (Wolff et al., 1980). These enzymes contain a well-conserved stretch of 17 amino acid residues (342–358 in *B. anthracis*, 54–70 in *B. pertussis*) (Escuyer et al., 1988; Glaser et al., 1988) which encompasses the GXXXXGK(T/S) consensus sequence found in many ATP- and GTP-binding sites, in a glycine-rich flexible loop able to undergo conformational changes (Fry et al., 1986). No sequence homology has been found between this class of adenylyl cyclases and the *R. melilotti* one (A. Danchin, personal communication).

Considering the possible existence of eukaryotic adenylyl cyclase belonging to families other than the one recently discovered in Gilman's laboratory, we undertook to compare our preparations of mammalian brain synaptosomal adenylyl cyclases to the bacterial, calmodulin-sensitive enzymes of high k_{cat} and found that an immunological relatedness existed among them (Monneron et al., 1988). We showed that a species of mammalian brain adenylyl cyclase catalyst contains an epitope closely related to that specified by the conserved sequence found in the bacterial, calmodulin-sensitive enzymes (Goyard et al., 1989).

In the present study, we report that a monoclonal antibody, mab 164, directed against the conserved sequence of *B. anthracis* (designated as peptide B), inhibits the calmodulin-dependent adenylyl cyclase activity of mammalian brain synaptosomes. The specific activity of this catalyst was estimated to reach 200 μmol of cAMP·mg^{-1}·min^{-1}, an order of magnitude higher than that measured for the purified brain adenylyl cyclase of type I. mab 164 reacts, on Western blots of the corresponding purified fractions, with a high molecular weight protein which is different from the class I adenylyl cyclase catalyst.

Materials and Methods

Materials. Chemicals were of the highest grade available. Peptide B, a 17-residue synthetic peptide corresponding to the amino acid sequence 342–358 of the *B. anthracis* adenylyl cyclase, GVATKGLNVHGKSSDWG (Escuyer et al., 1988), was synthesized as described (Goyard et al., 1989). Peptide A corresponded to the amino acid sequence 54–70 of the *B. pertussis* adenylyl cyclase, GVATKGLGVHAKSSDWG (Glaser et al., 1988). An anti-clathrin heavy chain monoclonal antibody TD 1 was the generous gift of Dr. F. M. Brodsky (Brodsky, 1985). mab 97 was obtained in our laboratory, using purified rabbit brain adenylyl cyclase fractions (fractions Fs, see next paragraph) as antigen. It was screened on the basis of the recognition of 130–180-kDa proteins in Western blots of purified rabbit brain forskolin eluates. An antibody directed against a peptide specific to the adenylyl cyclase stimulatory subunit $G_s\alpha$, RM/1, was from NEN (Simonds et al., 1989). A truncated *B. pertussis* cya gene product, expressed in *Escherichia coli* (clone pDIA 5227) (Munier et al., 1991), and purified by affinity chromatography on VU-8 calmodulin–Sepharose (Haiech et al., 1988), was the generous gift of Dr. J. Haiech. This enzyme preparation was homogeneous, as analyzed by SDS gel electrophoresis, consisting of one 60-kDa component (not shown). Its specific activity, measured in the presence of 1 mM EGTA and 0.5 mM Ca^{2+}, was 300 μmol of cAMP·mg^{-1}·min^{-1}. (It is to be recalled that *B. pertussis* adenylyl cyclase activity is dependent upon the presence of calmodulin, regardless of the presence of free Ca^{2+}.)

Purification of Synaptosomal Adenylyl Cyclase. Synaptosomes were obtained from rat, rabbit, and bovine brain homogenates by flotation in a discontinuous Ficoll–sucrose gradient, as described (Monneron & d'Alayer, 1980). All buffers contained inhibitors of proteases (1 μg/mL leupeptin, chymostatin, aprotinin; 2 μg/mL trypsin inhibitor; 0.1 mM PMSF[1]). In some cases, synaptosomes were incubated for 15 min at 30 °C in a medium containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT,[2] and 100 μM GppNHp[3] in order to irreversibly activate the adenylyl cyclase stimulatory subunit $Gs\alpha$.[4] The treated synaptosomes and derived fractions were referred to as preactivated samples, and the untreated synaptosomes and derived fractions were referred to as control samples. Lubrol extracts were obtained by solubilizing synaptosomes or membranes in cold 50 mM TEA,[5] pH 7.5, 0.9% Lubrol PX (Lubrol/protein w/w ratio of 2 to 3), 1 mM EDTA, 1 mM $MgCl_2$, 2 mM DTT, and recovering the Lubrol-soluble fraction by centrifugation (150000*g*, 20 min). From this material, three types of adenylyl cyclase fractions were prepared.

One type of preparation, called fraction F, was based on *forskolin–agarose affinity chromatography*. Fractions prepared from control synaptosomes, in which the activity was in a basal state, were called fractions Fb, whereas fractions prepared from GppNHp-treated synaptosomes, in which the activity was stimulated, were called Fs. The synaptosomal Lubrol extracts, brought to 0.1 M NaCl and 0.5 M glucose, were incubated with a forskolin–agarose resin, for 2 h or overnight, at 4 °C (v/v ratio of 10) (Coussen et al., 1986). The resin was washed as described except that in some cases 0.5% CHAPS was substituted for Lubrol. Adenylyl cyclase activity was eluted in one step, in a buffer composed of 50 mM TEA, pH 7.5, 100 μM forskolin, 100 μM DTT, 250 mM NaCl, and either 0.05% Lubrol or 0.5% CHAPS.

A second type of adenylyl cyclase preparation consisted in *calmodulin–Sepharose eluates* or fractions C. In such cases, calmodulin-depleted synaptosomal membranes were prepared as described (Coussen et al., 1985). Calmodulin-depleted Lubrol extracts were either subjected to gel filtration on a Bio-Gel A-5m resin column (Coussen et al., 1985) or chromatographed on a DEAE-Sephacel resin (Yeager et al., 1985). The Bio-Gel pools 1 or the 150 mM KCl eluates from the DEAE column were subjected to calmodulin–Sepharose affinity chromatography (Coussen et al., 1985). The calmodulin-sensitive adenylyl cyclase activity was eluted from the washed resin in the presence of 1 mM EDTA.

A third type of adenylyl cyclase preparation resulted from the *combination* of the two latter procedures. (1) Fractions CF were obtained by subjecting calmodulin–Sepharose eluates to forskolin–agarose affinity chromatography. A total of 50–60% of the enzyme activity present in fractions C was recovered in fractions CF. (2) Alternatively, fractions FC were obtained by subjecting fractions F to calmodulin–Sepharose affinity chromatography (Guermah, 1986). In this case, 10–30% of the adenylyl cyclase activity present in fractions F was recovered in the final FC samples.

As a byproduct, we prepared fractions enriched in clathrin heavy chains and devoid of adenylyl cyclase activity by treating synaptosomes with 1 M Tris-HCl, pH 7.5 (Chuang et al., 1986).

Adenylyl Cyclase Assays. The *B. pertussis* adenylyl cyclase activity was measured in a 50 mM TEA buffer, pH 7.7, containing 2 mM ATP ([α-^{32}P]ATP, 10^6 cpm per assay), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 10 μM calmodulin, with and

[1] Phenylmethanesulfonyl fluoride.
[2] Dithiothreitol.
[3] 5′-Guanylyl imidodiphosphate.
[4] The stimulatory, regulatory subunit of adenylyl cyclase.
[5] Triethanolamine.

without 1 mM EGTA, at 30 °C. *Brain* adenylyl cyclase activity was measured in a 50 mM TEA buffer, pH 7.7, containing 0.2 mM ATP ([α-^{32}P]ATP, (2–8) × 10^5 cpm per assay), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.05 % Lubrol PX, 5 mM DTT, 0.5 mM cAMP, 2.5 mM theophylline, 5 mM phosphocreatine, and 0.3 mg/mL creatine kinase. Incubation was for 3 min at 30 °C, with and without 100 μM forskolin. The method of Salomon et al. was used to quantify cAMP (Salomon et al., 1974).

SDS–PAGE and Immunoblotting Procedures. The samples were precipitated with 80% cold acetone overnight (–20 °C) or with 15% cold TCA[6] (15 min). The resulting pellets were boiled for 5 min in a buffer containing 6 M urea, 4% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.5, and 5% β-mercaptoethanol or 50 mM DTT. The solubilized proteins were separated by SDS–PAGE on 7.5% or gradient 7–12% acrylamide gels and stained with silver salts (Merril et al., 1984). Prestained Sigma molecular mass markers were used (180, 116, 84, 58, and 48.5 kDa). Proteins were also transferred onto nitrocellulose in a 20 mM Tris–glycine buffer, pH 8.5, containing 20% methanol and 0.02% SDS, for 16 h at 50 V. Proteins were stained with Ponceau red. Nitrocellulose strips were blocked by incubation in TBS-BT (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween, and 0.1% BSA) for 1 h at 37 °C. Antigens were probed with monoclonal antibodies or controls overnight at 4 °C. Alkaline phosphatase labeled anti-mouse immunoglobulins from Promega Biotec were used for immunodetection. In parallel, the luminol detection system was used (Amersham).

Immunization Protocol and mab 164 Obtention. Four BALB/c mice were subcutaneously injected with 200 μg of peptide B mixed with complete Freund's adjuvant. At 3-week intervals, they were given booster injections of 200 μg of peptide B mixed with incomplete adjuvant. The sera were screened both for the presence of anti-peptide B antibodies assayed by ELISA and for their ability to react with high molecular weight proteins in immunoblots of purified rabbit brain adenylyl cyclase samples (fractions Fs). Two mice were selected. Two days before fusion, they received an intravenous injection of 200 μg of peptide B. Spleen cells were fused with a nonsecretant myeloma cell line, AG8-X63-653, and hybridomas were grown and cloned as described (Köhler & Milstein, 1976). The presence of anti-peptide B antibodies was sought by ELISA. One fusion resulted in the presence of 4 positive wells out of 185. Clones were obtained; 5 × 10^6 cloned cells were injected into BALB/c mice pretreated with 0.5 mL of pristane (tetramethylpentadecane). Ascites fluids were collected and adjusted to 40% ammonium sulfate. Typing of the antibodies showed that three out of four of the monoclonal antibodies were IgM and one, mab 164, was an IgG_1. Only mab 164 was used in this study. It was verified by isoelectrofocusing that it was a monoclonal antibody. Ammonium sulfate precipitates were dissolved in water and extensively dialyzed against 10 mM TEA, pH 7.5, and chromatographed on a DE 23 Whatmann resin column. Antibodies eluted by 0.2 M NaCl were used as such (mab 164, crude fractions). Alternatively, the antibodies were chromatographed on a DEAE-Sephacel resin column (Pharmacia), as described (Harlow & Lane, 1988), and selected on the basis of electrophoretic purity (mab 164). Aliquots of the antibody solutions were frozen at –80 °C. Once thawed, they were used within the next 2 days.

Preparation of mab 164-Depleted Fraction. Peptide B was coupled to Sepharose CL-4B (Pharmacia) in the presence of 0.1 M bicarbonate buffer, pH 8.0 (15 mg of peptide bound/1 mL of wet gel). A total of 160 μg of mab 164 (crude fraction) was incubated with 0.5 mL of peptide B–resin for 16 h at 4 °C. The supernatant was decanted and mixed again with the same amount of resin for 1–6 h at 4 °C. It was verified by ELISA that this fraction was largely depleted (>85%) of mab 164. The supernatant, called mab-depleted fraction, was used as control.

ELISA Procedure. Falcon microtiter plates were coated for 2 h at 37 °C with 100 μL of a 50 μg/mL solution of peptide B (uncoupled or BSA-coupled) in phosphate-buffer saline (PBS, 10 mM Na_2/sodium phosphate, pH 7.4, 150 mM NaCl). The plates were blocked by incubation for 1 h at 37 °C with 3% BSA in PBS. A total of 100 μL/well of dilutions of mab 164 or mab-depleted fraction was incubated overnight at 4 °C. Bound antibodies were detected by addition of peroxidase-coupled anti-mouse IgG (Diagnostics Pasteur). The *o*-benzenediamine substrate (1 mg/mL) was prepared in 100 mM sodium citrate, pH 5.0, containing 0.015% hydrogen peroxide. Color development was stopped by addition of 4 N H_2SO_4 and read at 492 nm.

Characterization of mab 164. mab 164 purified from ascites bound the immobilized (on ELISA plates) or conjugated (to Sepharose-CL-4B) peptide B. The soluble, free peptide B did not compete for bound peptide in ELISA (data not shown), whereas BSA-bound peptide B did (Figure 1A). mab 164 equally well recognized peptide A in ELISA (not shown).

Adenylyl Cyclase Inhibition Assays. The 100-μL aliquots of *B. pertussis* adenylyl cyclase (2 ng, 650 pmol of cAMP·min^{-1}) were incubated overnight at 4 °C with gentle agitation in the presence of various amounts of mab 164, mab 97, or BSA, in the presence or absence of 20 μM calmodulin, in a medium containing 50 mM TEA, pH 7.5, 150 mM NaCl, 0.5 mM Ca^{2+}, and no or 1 mM EGTA. The 100-μL aliquots of the *brain* adenylyl cyclase fractions were incubated overnight at 4 °C, with gentle agitation, in the presence of various quantities of mab 164 or of various controls added in equivalent amounts: BSA, mab-depleted fraction, or mab 97, in a medium (buffer A) containing 50 mM TEA, pH 7.4, 150 mM NaCl, 0.05% Lubrol [or 0.5% CHAPS in the case of some fractions (F)], 0.1 mM DTT, 2 μg/mL inhibitors of proteases, 1 mM EDTA, 1 mM $MgCl_2$. When fractions C were studied, the incubation medium contained 50 μM $CaCl_2$, no or 10 μM calmodulin, and 1 mM diisopropyl fluorophosphate.

Analytical Procedures. Proteins were assayed by the method of Schaffner and Weissmann (1973). In the case of very dilute samples, large volumes were precipitated by 15% TCA in the presence of a known amount of a protein carrier (trypsin inhibitor) and hydrolyzed in 0.6 N NaOH, and the micro-BCA protein assay was used (Hill & Straka, 1988). For very dilute fractions, amino acids analysis was performed. Silver-stained gels containing known amounts of β-galactosidase (dissolved in the enzyme buffer and TCA-precipitated) and of samples corresponding to known amounts of brain adenylyl cyclase activity were scanned (Shimatzu densitometer CS 930), and the intensities of the protein bands were compared within each lane. In order to couple peptide B to BSA, a small quantity of peptide B was synthesized with a cysteine residue added at the N-terminal end. Coupling was achieved by use of (*m*-maleimidobenzoic acid) *N*-hydroxysuccinimide ester (Green et al., 1982). Unreacted BSA-coupled MB reagents were quenched by reduced glutathione.

Results

mab 164 Specifically Inhibits B. pertussis Adenylyl Cyclase Activity. To study the effect of mab 164 upon the enzyme

[6] Trichloroacetic acid.

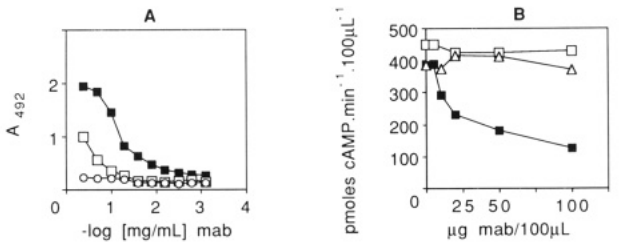

FIGURE 1: Characterization of mab 164. (A) Titration of mab 164. Binding of mab 164 to peptide B attached to a microtiter ELISA plate. (See Materials and Methods. A_{492} refers to the absorbance of the peroxidase reaction product.) mab 164 was incubated in the absence (■) or in the presence (□) of 40 µg/well soluble peptide B (BSA-bound). The mab-depleted fractions was also titrated (○) (mab 164, crude fraction). (B) Inhibition of *B. pertussis* adenylyl cyclase by mab 164. mab 164 (□, ■) and mab 97 (△) were incubated with 100-µL aliquots of *B. pertussis* adenylyl cyclase without calmodulin, in the presence of 1 mM EGTA and 0.5 mM Ca^{2+}, with (□) or without (■, △) 20 µg/100 µL peptide B (BSA-coupled). The enzyme assays were conducted in the presence of 10 µM calmodulin (mab 164, crude fraction).

Table I: Partial Purification of Synaptosomal Adenylyl Cyclase Catalytic Subunit[a]

material selected at each step	protein (µg)	act. (nmol·min^{-1})	sp. act. (nmol·mg^{-1}·min^{-1})	yield (%)	purification (fold)
solubilized synaptosomes	506000	3052	6	100	1
Lubrol extract	313000	2514	8	82.5	1.3
fraction Fs	226	952	4212	31.2	702
fraction FC	9	228	25300	7.5	4217

[a] The data in this table are from one typical experimental, in which the forskolin–agarose and calmodulin–Sepharose affinity chromatographies were sequential. Adenylyl cyclase activity was measured in the presence of 0.1 mM forskolin. The total activity is that of the Lubrol-solubilized sample, which is roughly 2.5 times that measured in the corresponding particulate fraction. Protein concentration was determined by the micro-BCA method, except for fraction FC, for which amino acid analysis was performed. (Experimental error here is of the order of ±50%.) The 140-kDa component represented 12% of the proteins of this fraction, as indicated by the scanning of silver-stained gels.

activity, the adenylyl cyclase preparation was incubated with the antibody in the presence as well as in the absence of calmodulin. In both cases, and regardless of the free Ca^{2+} concentration in the incubation or assay media, mab 164 inhibited the activity in a dose-dependent manner, up to 80% when the incubation was conducted in the absence of calmodulin (Figure 1B) and up to 40% in its presence (not shown). The specificity of this effect was shown by the following observations: (1) addition of BSA-coupled peptide B to the incubation medium in a molar excess of 20 over mab 164 prevented enzyme inhibition (Figure 1B); (2) mab 97 had no effect upon the enzyme activity (Figure 1B). These results encouraged us to study the effect of mab 164 on mammalian brain adenylyl cyclase activity.

Composition of the Various Synaptosomal Adenylyl Cyclase Fractions. The aim of this study being to characterize neuronal adenylyl cyclase catalytic subunits, the source of enzyme chosen consisted of synaptosomes-enriched, glia-depleted brain fractions. About two-thirds of the enzyme activity was calmodulin-dependent (Coussen et al., 1985). As described in Materials and Methods, three types of enzyme fractions were used.

In fractions F, obtained by a forskolin–agarose affinity chromatography, the adenylyl cyclase was purified 700-fold (Table I). Calculated on 50 Fs fractions, the yield in enzyme activity was 25% (Lubrol extract taken for 100%). The specific activity varied from 3 to 5 µmol of cAMP·mg^{-1}·min^{-1}. The

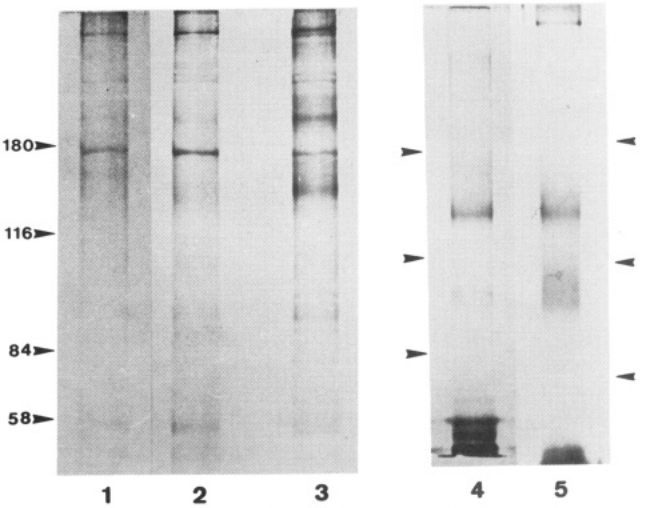

FIGURE 2: SDS–polyacrylamide gel electrophoresis of purified adenylyl cyclase fractions (7.5% polyacrylamide gel). The gels were silver-stained. Three gels are shown (lanes 1–3; lane 4; lane 5). The arrows correspond to the molecular mass standards of each gel (for the two gels on the right, 180, 116, and 84 kDa). Lanes 1 to 3: fractions Fs from (lane 1) rat, (lane 2) bovine, and (lane 3) rabbit (adenylyl cyclase activity was 1.5 nmol of cAMP·min^{-1} per lane). Lane 4: fraction CF (enzyme activity was 4 nmol of cAMP·min^{-1} per lane). Lane 5: fraction FC (enzyme activity was 4 nmol of cAMP·min^{-1} per lane).

electrophoretic pattern yielded by fractions Fs consisted of several bands or regions in the high molecular weight range: a major band at an apparent molecular mass of 175 kDa, a doublet at 230 kDa, a set of 3–4 very faint bands between 160 and 152 kDa and occasionally a band at 140 kDa (7% acrylamide gels) (Figure 2, lanes 1–3). Proteins of lower apparent molecular mass (76–60 kDa, 50 and lower) were abundant as seen in 7–12% gradient gels. This pattern was found in rat, rabbit, and bovine synaptosomal fractions F, with minor variations (Figure 2, lanes 1–3). Scanning of the silver-stained, gradient gels indicated that the 175-kDa component amounted to about 2–4% of the proteins seen in gradient gels. Sequence analysis of peptides derived from the major 175-kDa rat component indicated that it contained at least two proteins, one corresponding to the clathrin heavy chain, a protein not known to bind to forskolin–agarose, and at least another one encoding peptides not listed at this time in protein sequence data banks (in preparation). A monoclonal antibody directed against the clathrin heavy chain, TD 1, stained the 175-kDa component of the blots (Figure 5, lane 5).

The second type of adenylyl cyclase preparations, *fractions C* or calmodulin–Sepharose eluates, although of much lower adenylyl cyclase specific activity than fractions F, were interesting in that they contained an essentially calmodulin-dependent enzyme activity. Clathrin heavy chains were not detected by TD 1 antibody on the corresponding blots (not shown).

The third type of adenylyl cyclase preparations, resulting from the combination of the two latter procedures, was the purest. (1) The electrophoretic pattern of *fractions CF* consisted of a major, rather diffuse component centered on 140 kDa. A minor protein was seen at 106 kDa, as well as other proteins of lower molecular mass (Figure 2, lane 4). No component of 175 kDa was detected. On the basis of the scanning of 7–12% gradient gels, the 140-kDa protein corresponded to 20% of the proteins of the fraction.

(2) *Fractions FC* had a specific activity of 20–30 µmol of cAMP·mg^{-1}·min^{-1} (Table I). The electrophoretic pattern

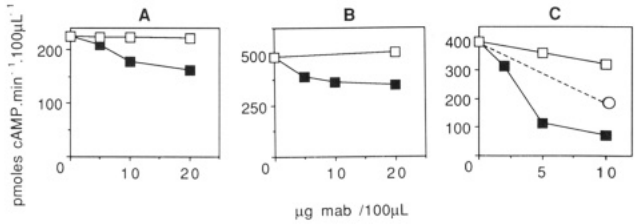

FIGURE 3: Inhibition of rat brain adenylyl cyclase activity by mab 164 in various fractions. A representative experiment is shown for three types of enzyme preparations. Forskolin-stimulated activities were measured in 100-μL samples containing (A) Lubrol extract (obtained from control synaptosomes), (B) fraction Fs, or (C) fraction C, incubated overnight at 4 °C with mab 164 (■, ○) or mab 97 (□). In (C) peptide B (○) was present during the incubation (10 μg, BSA-coupled).

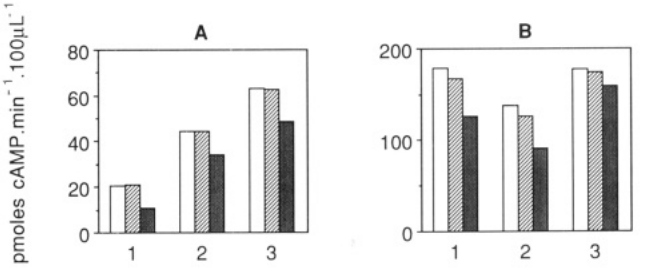

FIGURE 4: Inhibition by mab 164 of rat brain adenylyl cyclase activity in samples containing diverse effectors. (A) Adenylyl cyclase activities were measured in aliquots of a Lubrol extract (obtained from control synaptosomes) incubated overnight at 4 °C with mab 164 (shaded) or the mab-depleted fraction (cross-hatched), or BSA (open). In row 1, no forskolin was in the incubation or the assay: 48% inhibition. In row 2, forskolin was in the assay: 24% inhibition. In row 3, forskolin was in the incubation and the assay: 22% inhibition (mab 164 and BSA, 6 μg/100 μL). (B) Bars are as in panel A. Forskolin-stimulated adenylyl cyclase activities were measured in fractions F: Fb in rows 1 and 2, Fs in row 3. Samples were incubated overnight at 4 °C in the presence (1, 3) or absence (2) of forskolin. (In row 2, fraction Fb was depleted of forskolin by gel filtration on a PD 10 column.) Percentages of inhibition: (1) 29%, (2) 34%, and (3) 11% (mab 164 and BSA, 6 μg/100 μL).

resembled the previous one, with a major component of 140 kDa, a fainter one at 106 kDa, and a few proteins of smaller size (less than 100 kDa) (Figure 2, lane 5). On the basis of the scanning of silver-stained, gradient gels, the 140-kDa component amounted to 12% of the proteins of the final fraction. On immunoblots, none of the transferred proteins reacted with the anti-clathrin TD 1 antibody (not shown). To summarize, in the purest enzyme fractions FC and CF, a 140-kDa protein appeared to be the major, constant component of high molecular mass.

mab 164 Inhibits Synaptosomal Adenylyl Cyclase Activity at Various Stages of Enzyme Purification. The effect of mab 164 on the enzyme activity was tested at each step of the purification procedures described. Rat brain fractions from more than 30 independent purifications were used, with the same results.

mab 164 inhibited adenylyl cyclase activity (assayed in the presence of forskolin) in all fractions. Whatever the adenylyl cyclase samples incubated with mab 164, the percentage of inhibition of a fixed amount of enzyme activity increased with increasing amounts of mab until a plateau was reached (Figure 3A,B), except in the case of fractions C, where nearly complete inhibition was obtained (Figure 3C). The activity measured in samples incubated in the absence of mab 164 being taken for 100%, the percentages of inhibition (calculated on at least eight separate experiments per sample) were the following: 17 (±3) in control synaptosomes; 33 (±9) in control Lubrol extracts; 26 (±6) in Lubrol-containing fractions Fb; 31 (±13) in CHAPS-containing fractions Fb; 40 (±11) in calmodulin-depleted Lubrol extracts; 69 (±13) in Bio-Gel pools 1; in the case of fractions C, nearly complete inhibition was obtained. Complementation of fractions C with 10 μM calmodulin prior to the addition of mab 164 did not change the level of inhibition (not shown). The enzyme fractions FC proved to be unstable during the overnight incubation with mab 164 or 97, losing much of its activity (65%). The effect elicited by mab 164 on the residual adenylyl cyclase activity was minimal (20% inhibition or less).

The same type of study was conducted with rabbit brain synaptosomal fractions, yielding comparable results.

The following experiments demonstrated that the enzyme inhibition was due to mab 164. (1) The mab-depleted fraction did not affect, or only very slightly inhibited (<10%), adenylyl cyclase activity (not shown). When present, the minor inhibition noticed was correlated with a small amount of mab 164 not retained by the peptide B–resin column (see Materials and Methods). (2) mab 97 had no or only a very slight effect upon adenylyl cyclase activity (Figures 3 and 4). (3) Heat-denatured mab 164 did not inhibit adenylyl cyclase activity (not shown). (4) The inhibitory effect of mab 164 on adenylyl cyclase activity was diminished by a factor of 50% when the incubation medium contained BSA-coupled peptide B (added in a molar excess of 100 with respect to mab 164) (Figure 3C). However, free, uncoupled peptide B did not modify the inhibitory effect of mab 164 on the enzyme activity.

The presence of forskolin, either in the incubated samples or merely in the assays, decreased the inhibitory effect of mab 164, as seen in Figure 4 which shows representative experiments conducted with a Lubrol extract (Figure 4A) and fractions F (Figure 4B). It is to be noticed, however, that the *amount* of inhibited adenylyl cyclase activity was roughly the same, in control and in forskolin-containing aliquots, although the percentage of inhibition differed.

All these results were obtained with fractions prepared from control synaptosomes. In parallel, we studied the effect of mab 164 on enzyme fractions prepared from preactivated synaptosomes, in which both Gsα and the adenylyl cyclase catalytic subunits were irreversibly activated by GppNHp. The inhibitory effect of mab was lower in preactivated samples than in control fractions: 11% (±4%) in fractions Fs (versus 29%) (Figure 4B). Here again, the *amount* of inhibited adenylyl cyclase activity was about the same in comparable control and preactivated samples.

mab 164 Detects, on Western Blots of Purified Adenylyl Cyclase Samples, 175- and 140-kDa Proteins. On blots of fractions F, mab 164 reacted with two regions: the lower edge of the major 175-kDa band and a diffuse region centered on 60 kDa (Figure 5, lanes 1 and 2). The patterns obtained with the alkaline phosphatase method and with the luminol method (not shown) were the same. The mab-depleted fraction did not reveal any component (Figure 5, lane 3). The signal strength was directly proportional to the amount of adenylyl cyclase run in the gels (Figure 5, lanes 1 and 2) and depended upon the amount of mab 164 used in the assay (not shown). mab 97 gave a strong signal in the 60-kDa region but none at 175 kDa. mab TD 1, directed against the clathrin heavy chain, intensely stained a sharp band at 175 kDa (Figure 5, lane 5). Since clathrin represented a major component in the blotted samples, it might be unspecifically stained by mab 164. However, when preparations of clathrin heavy chains devoid of adenylyl cyclase activity were subjected to immunoblot analysis with mab 164, no reaction was detected at 175 kDa (not shown).

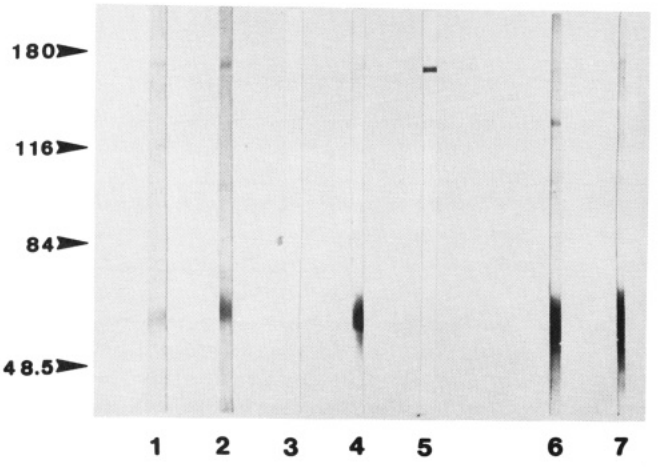

FIGURE 5: Immunoblotting of fractions F and FC (prepared from rat brain synaptosomes): lanes 1–5, fraction Fs; lanes 6 and 7, fraction FC. The nitrocellulose strips were probed with various primary antibodies or controls. Lanes 1, 2, and 6: mab 164. Lane 3: mab 164-depleted fraction. Lanes 4 and 7: mab 97. Lane 5: anti-clathrin heavy chain antibody TD 1. [For Fs, the material loaded per lane corresponded to an adenylyl cyclase activity of 14 nmol of cAMP·min^{-1}, except for lane 2 (28 nmol of cAMP·min^{-1}); for FC, activity was 100 nmol of cAMP·min^{-1}] (mab 164 and 97 (crude fractions), 80 μg/mL; TD, 1, 1 μg/mL).

On blots of fractions FC, mab 164 stained the 140-kDa component and a diffuse region spreading from 65 to 40 kDa. The latter was also labeled by mab 97, but not the 140-kDa component (Figure 5, lanes 6 and 7). As noticed in silver gels, there was no component detectable at 175 kDa. This finding raised the questions of (1) whether the 140-kDa protein was produced by proteolysis of the 175-kDa protein, when bound to the calmodulin–Sepharose resin, in the presence of Ca^{2+} or (2) whether the amount of the 140 kDa protein in fractions "F" may have been too low to allow detection by immunoblotting. Therefore, we applied mab 164 to overloaded blots of fraction F from which the 175-kDa region was excised. No reaction could be detected at 140 kDa (not shown).

Gsα subunits were revealed by an antibody directed against a peptide specific for Gsα on blots of fractions F (49 kDa, not shown). mab 164 did not recognize proteins in the same molecular weight range.

To summarize, a high molecular weight component was recognized by mab 164 in all adenylyl cyclase containing fractions. In Lubrol extracts and fractions F, the detected component was at 175 kDa. In more purified enzyme fractions (preparations involving a calmodulin–Sepharose affinity chromatography step), a 140-kDa component was revealed.

Discussion

Monoclonal antibodies directed against peptides representing portions of a protein sequence are very precise and useful tools (Bouhnik et al., 1987; Nunnally et al., 1987; Milne et al., 1989; Edwards et al., 1990). In the present study, a monoclonal antibody, mab 164, directed against peptide B, corresponding to a conserved bacterial adenylyl cyclase sequence, was shown to inhibit rabbit and rat brain synaptosomal adenylyl cyclases as well as the prokaryotic *B. pertussis* adenylyl cyclase. Several controls indicated that the inhibition was specific. Neither the mab 164-depleted fraction nor an unrelated monoclonal antibody affected the adenylyl cyclase activities. BSA-coupled peptide B competed with the enzymes for mab 164. However, free, uncoupled peptide B, did not affect the extent of enzyme inhibition by mab 164. The screening procedure used to select mab 164 may have been determining in selecting an antibody directed against an epitope present in brain adenylyl cyclase and resembling one given, stabilized conformation of peptide B. If the glycine-rich peptide B lacks a stable secondary structure in solution, which seems plausible given the flexibility of the P loop (Fry et al., 1986), it would indeed be unable to compete significantly for mab 164.

mab 164 not only recognized the native enzyme but also specifically reacted with denatured, blotted proteins, most probably identifying them as adenylyl cyclase catalytic subunits. It should be recalled here that some degree of protein renaturation occurs during electrotransfer of proteins. Consequently, it is not known whether mab 164 recognizes a conformational or a sequential epitope.

mab 164 was raised against a peptide which corresponds to a highly conserved domain of 17 amino acid residues shared by both *B. anthracis* and *B. pertussis* adenylyl cyclases (Escuyer et al., 1988; Glaser et al., 1988). This peptide contains one of the established nucleotide-binding consensus sequences, GXXXXGKS (Fry et al., 1986). Both bacterial enzymes have been expressed in *E. coli*, and site-directed mutagenesis allowed assignment of their catalytic sites to a region containing the conserved sequence. In particular, it has been shown that point mutations of the lysines present in the conserved sequences resulted in almost inactive enzymes (Glaser et al., 1989; Xia & Storm, 1990; Labruyère et al., 1991) and in the severe impairment of substrate binding (Labruyère et al., 1990). The specific inhibition of *B. pertussis* adenylyl cyclase by mab 164 shown in this study was therefore expected. Our results also show that the catalytic domain is more accessible to the antibody in the absence of calmodulin than in its presence, indicating a calmodulin-dependent change in its 3D structure.

More importantly, mab 164 was shown to inhibit a *mammalian synaptosomal* adenylyl cyclase. Can this property be attributed to the specific binding of mab 164 to a well-defined region of the enzyme *catalyst*?

The GXXXXGKS motif is present in the regulatory subunit Gsα (Gilman, 1987). This sequence is thought to play a key role in catalyzing hydrolysis of bound GTP (Bourne et al., 1989). Should mab 164 bind this region (although the four X amino acid residues differ from those present in peptide B), it would provide a possible explanation for our results. However, this seems unlikely for two reasons. One is that the Gsα subunits present in our preparations were recognized on Western blots by an anti-Gsα antibody but not by mab 164. (Denatured proteins present on blots may not, however, be recognized by an antibody reacting with the native protein). The second reason is that the percentage of inhibition of adenylyl cyclase activity was *always lower* in enzyme samples in which the association of Gsα with the catalytic subunit had been favored. If mab 164 would exert its inhibitory effect through Gsα, the reverse results would be expected.

The possibility that part of peptide B, or a given spatial arrangement of its residues, might be present in a brain adenylyl cyclase catalytic subunit, as it is in the calmodulin-dependent bacterial toxins, was then considered.

The extent of adenylyl cyclase inhibition caused by mab 164 was shown in this study to depend upon the functional state of the enzyme: the percentage of mab 164-related inhibition was always higher when the enzyme was in the basal state rather than in the Gsα-activated state or in the presence of forskolin. This observation can be interpreted in two ways. (1) When the enzyme is activated, the recognized epitope may be less accessible or the catalytic site may become less susceptible to structural changes elicited by mab binding. (2) Alternatively, it is possible that mab 164 inhibits only one given species of adenylyl cyclase. Indeed, in all experiments the

amount of inhibited activity did not grossly vary when enzyme aliquots were compared that differed only by the nature of the effectors present. Besides, the enzyme present in fractions C was almost fully inhibited by mab 164, as if this preparation contained essentially one species of catalyst.

mab 164 was able to recognize a 175-kDa protein on Western blots of fractions F. Although the 175-kDa region is known to be composite, notably containing clathrin heavy chains, several controls were in favor of the specificity of the immunoreaction. We thus postulate that this 175-kDa protein is a brain adenylyl cyclase catalytic subunit. Pfeuffer and co-workers have characterized a 180-kDa protein in adenylyl cyclase fractions purified from rat olfactory cilia and suggested that it might be the catalyst (Pfeuffer et al., 1989).

Calmodulin–Sepharose affinity chromatography yielded adenylyl cyclase preparations cleared of clathrin heavy chains. When both types of affinity chromatography (forskolin and calmodulin) were used sequentially, the final fractions were enriched in adenylyl cyclase activity: the specific activity of fractions FC reached 20 ± 5 μmol of cAMP·mg^{-1}·min^{-1} (Table I). Their analysis in SDS gels showed a major component of 140 kDa as well as the absence of any component at 175 kDa. Considering that the protein of 140 kDa was recognized by mab 164 on Western blots of fractions FC (but not of fractions F), we postulate that it most probably was a proteolytic product of the adenylyl cyclase catalyst. The 140-kDa protein amounting to 10–12% of the proteins in fractions FC, its specific activity would then be of the order of 200 μmol of cAMP·mg^{-1}·min^{-1}.

A 135–155-kDa protein has been previously proposed as a brain adenylyl cyclase catalyst by our laboratory (Coussen et al., 1985, 1986) and by Storm's laboratory (Yeager et al., 1985; Rosenberg & Storm, 1987). Pfeuffer and co-workers identified a brain 115-kDa protein, but they did not exclude that it could be a "nicked" enzyme (Pfeuffer et al., 1985b); they also prepared several monoclonal antibodies against a partially purified brain adenylyl cyclase preparation, with which they precipitated, depending upon the antibody, several components of 160, 115, 45, and 38 kDa, together with an adenylyl cyclase activity (Mollner et al., 1991).

As already discussed, the GXXXXGKS sequence is not present in the family of mammalian brain adenylyl cyclases described in the laboratories of Gilman and Reed (Krupinski et al., 1989; Bakalyar & Reed, 1990; Tang et al., 1991). Since we do not know, at the moment, whether mab 164 recognizes a sequence or a spatial arrangement of residues in the target enzyme, the possibility remains that the catalyst described in this study belongs to the same family as the one identified by Gilman. Its specific activity appears however much higher than that of the purified, recombinant type I enzyme (8 μmol of cAMP·mg^{-1}·min^{-1}; Tang et al., 1991). The silver-stained gels of fractions FC or CF occasionally showed a 110-kDa band (Figure 2). Would this protein correspond to adenylyl cyclase type I, it should have a specific activity of at least 400 μmol of cAMP·mg^{-1}·min^{-1} to account for the enzyme activity contained in the corresponding sample.

Polyclonal antibodies directed against a synthetic peptide located in the duplicated, conserved domain of the class I and III family of enzymes reacted, on Western blots of our preparations, with a protein other than that recognized by mab 164 (manuscript in preparation). This finding is not in favor of the hypothesis of an alternate splicing of a gene coding for both the catalyst described here and the adenylyl cyclases of types I or III. The possibility that two or several families of brain adenylyl cyclase catalysts are present in eukaryotic cells has thus to be taken into account. Sequencing of peptides obtained from the protein recognized by mab 164 is in progress to clarify this point.

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Scaffold-Attached Regions (SAR Elements) Mediate Transcriptional Effects Due to Butyrate†

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ABSTRACT: The expression of certain genes has been reported to respond positively to sodium butyrate. This study demonstrates the same feature for two marker genes under the control of five different promoters. In all examples, the stimulatory effect is largest if one or especially two scaffold/matrix-attached regions (SAR/MAR elements) are present adjacent to the gene, and in one case, the stimulation depends entirely upon this situation. These results are observed with several SAR sequences including those obtained by oligomerizing short stretches of DNA surrounding a core motif. It is suggested that butyrate exerts important actions at the level of the chromatin structure.

Recent evidence suggests that the eukaryotic genome is organized into topologically constrained domains enabling a differential gene expression (Gasser & Laemmli, 1987; Gross & Garrard, 1987; Kellum & Schedl, 1991). In *Drosophila*, it has been demonstrated that the size of a domain is inversely related to its transcriptional activity (Gasser & Laemmli, 1987). Following this rationale, various groups have tried to isolate the DNA sequences mediating the attachment to the nuclear scaffold or matrix (called scaffold- or matrix-attached regions, SARs or MARs)[1] and to use them alone or in combination in order to create artificial domains with improved transcriptional properties (Klehr et al., 1991; Phi-Van & Strätling, 1988; Phi-Van et al., 1990; Stief et al., 1989).

Some domain borders are constituted by a class of (A + T)-rich DNA elements which were initially detected by differential melting of samples prepared for electron microscopy (Moreau et al., 1981, 1982; Scherrer & Moreau, 1985). It is probably the same class of elements which have been

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[1] Abbreviations: SAR, scaffold-attached region; MAR, matrix-attached region; IFN, interferon; huIFN-β, human interferon-β; muIFN, murine interferons; muHMG, murine hydroxymethylglutaryl-CoA reductase; neo^r, neomycin-resistance gene (Tn5 aminoglycoside 3′-phosphotransferase); LIS, lithium 3,5-diiodosalicylic acid; PCR, polymerase chain reaction.

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