A Brain Synaptosomal Adenylyl Cyclase of High Specific Activity Is Photolabeled with Azido-ATP†

Francis Castets,‡ Gilbert Baillat,‡ Salida Mirzoeva,‡ Kamel Mabrouk,§ Jérôme Garin,∥ Jacques d'Alayer,⊥ and Ariane Monneron*,‡

Département des Protéines de la Transduction, LCB, CNRS, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, Laboratoire de Biochimie, CNRS URA 1455, Faculté de Médecine secteur Nord, 13326 Marseille Cedex 15, Laboratoires de Biochimie et de Chimie des Protéines, CEA, 38041 Grenoble Cedex, and Laboratoire de Microséquencage des Protéines, Département des Biotechnologies, Institut Pasteur, 75015 Paris, France

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ABSTRACT: Partially purified adenylyl cyclase preparations of high specific activity (60 ± 10 μmol cAMP/(mg·min)) were obtained from rat brain synaptosomes (Orlando, C., d'Alayer, J., Baillat, G., Castets, F., Jeannequin, O., Mazié, J. C., & Monneron, A. (1992) *Biochemistry 31,* 3215–3222). Adenylyl cyclase activity was stimulated 4-fold by Ca^{2+}/calmodulin and 2-fold by forskolin or by Mn^{2+}. These preparations contained two major proteins of 140 and 110 kDa. The 140-kDa protein was identified as the neural cell adhesion molecule. The 110-kDa protein was specifically recognized by affinity-purified antibodies directed against a peptide corresponding to sequence 976–1013 of adenylyl cyclase type I. It was photolabeled by [α-^{32}P]8- and 2-N_3ATP in a light-dependent manner and was by far the most heavily labeled component of FC fractions. Saturation was obtained with 30 μM [^{32}P]8-N_3ATP. Photoinsertion of N_3ATP into the protein was largely prevented by ATP or adenylyl imidodiphosphate but not by ADP, AMP, or adenosine. A modest incorporation of N_3cAMP and photoinsertion of [α-^{32}P]N_3GTP into the 110-kDa protein were observed. Although some of the properties of the synaptosomal 110-kDa protein described here would match those expected from adenylyl cyclase type I, it appears that its specific activity is on the order of 1 mmol cAMP/(mg·min), about 200-fold that measured for brain adenylyl cyclases type I.

To date, eight distinct mammalian adenylyl cyclases, one enzyme from Drosophila, and one from Dictyostelium are described as members of the same family (Tang & Gilman, 1992). These enzymes are characterized by the following properties: (1) a same general transmembrane organization, comprising two hydrophobic regions and two cytoplasmic domains, C1 and C2; (2) extended sequence similarities within the latter two domains (the C1a and C2a regions have 50–92% sequence identities); (3) regulatory properties that vary from one type of enzyme to the other but always include stimulation by $G_{S\alpha}$ subunits and inhibition by adenosine; (4) comparable specific activities, in the 2–12 μmol cAMP/(mg·min) range, when determined for stimulated enzymes purified either from tissues (Coussen et al., 1986; Rosenberg & Storm, 1986; Smigel, 1986; Pfeuffer et al., 1989; Krupinski et al., 1989; Tang et al., 1991) or from Sf9 transfected cells (Taussig et al., 1993). The location of the ATP-binding site in these enzymes is not yet precisely known, although the importance for catalysis of a glycine residue conserved among many species and located in the C2a domain of the Drosophila adenylyl cyclase has been demonstrated (Levin et al., 1992). Bovine brain adenylyl cyclase I was the first and only one of this family of enzymes to be studied so far at the protein level and sequenced (Krupinski et al., 1989). The sequences of the cDNAs of all other known adenylyl cyclases of the same family (including those of flies and amoebae) have been obtained by the use of probes deduced from this original sequence.

In this paper, we show that a synaptosomal adenylyl cyclase of unusually high specific activity, purified by affinity chromatography (Orlando et al., 1992), is specifically photolabeled by both [α-^{32}P]8-N_3ATP[1] and N_3cAMP.

EXPERIMENTAL PROCEDURES

Materials. [α-^{32}P]8-N_3ATP (5–10 Ci/mmol; 1 Ci = 37 GBq), [γ-^{32}P]8-N_3ATP (5–15 Ci/mmol), [α-^{32}P]N_3GTP (7.3 Ci/mmol), and [^{32}P]N_3cAMP (50 Ci/mmol) were obtained from ICN (Irvine, CA). 2-N_3ATP (5–14 Ci/mmol) was prepared from 2-N_3AMP and PPi according to the method of Michelson (1964) as modified by Salvucci et al. (1992) and kindly donated by Boyd Haley (University of Kentucky, Lexington). Chemicals were of the highest grade available. Protein molecular weight prestained standards were from Sigma.

Preparation of Adenylyl Cyclase Enriched Fractions. The purification of synaptosomal adenylyl cyclase was as described (Orlando et al., 1992), with modifications. Briefly, rat brain synaptosomes were solubilized in cold 50 mM TEA, pH 7.5,

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‡ Département des Protéines de la Transduction, LCB, CNRS, 31 chemin Joseph Aiguier.

§ Laboratoire de Biochimie, CNRS URA 1455, Faculté de Médecine secteur Nord.

∥ Laboratoires de Biochimie et de Chimie des Protéines, CEA.

⊥ Laboratoire de Microséquencage des Protéines, Département des Biotechnologies, Institut Pasteur.

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[1] Abbreviations: N_3ATP, azidoadenosine 5′-triphosphate; N_3GTP, azidoguanosine 5′-triphosphate; N_3cAMP, azidoadenosine 3′,5′-cyclic monophosphate; TEA, triethanolamine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; PA, polyacrylamide; PAGE polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate; TCA trichloroacetic acid; NCAM, neural cell adhesion molecule; $G_{S\alpha}$, the stimulatory, regulatory subunit of adenylyl cyclase; Gpp(NH)p, guanylyl imidodiphosphate; GTPγS, guanosine 5′-(3-*O*-thiotriphosphate).

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containing 20% glycerol, 0.9% Lubrol PX (Lubrol/protein w/w ratio of 2.7), 1 mM EDTA, 1 mM $MgCl_2$, 2 mM DTT. The 2-fold diluted Lubrol-soluble fraction was subjected to forskolin-agarose affinity chromatography, and the adenylyl cyclase activity was eluted as described. The eluate was subjected to calmodulin-Sepharose affinity chromatography. Calmodulin-bound material was eluted in a 50 mM TEA buffer, pH 7.5, containing 1 mM EDTA, 500 mM NaCl, 2 mM DTT, and 15 μg/mL of trypsin inhibitor. This eluate was named fraction FC (Orlando et al., 1992). Fractions to be used in photoaffinity labeling experiments were eluted in a buffer containing 20 mM Tris-HCl, pH 7.5, 250 mM NaCl, 1 mM EDTA, 15 μg/mL of trypsin inhibitor, and no DTT. Aliquots of the eluates were frozen in liquid nitrogen until use.

General Biochemical Procedures. The adenylyl cyclase assay medium contained, in a final volume of 0.2 mL, 50 mM TEA buffer, pH 7.5, 0.05% Lubrol-PX, 0.2 mM [α-^{32}P]ATP, 10 mM $MgCl_2$, 0.5 mM cAMP, 10 mM theophylline, 2 mM DTT, 6 mM NaCl, and the inhibitors of proteases carried by the enzyme sample. Added effectors are indicated in the text. The enzyme was preincubated for 3 min at 30 °C with the effectors and further incubated for 3 min in the presence of [α-^{32}P]ATP. Determinations of adenylyl cyclase activity at each purification step were done in a medium containing an ATP-regenerating system (Orlando et al., 1992). Protein measurements, SDS-PAGE, and Coomassie-blue- and silver-staining of the gels were as described (Orlando et al., 1992).

A preparative SDS-gel was loaded with pooled FC fractions obtained from 900 rat brains, containing an adenylyl cyclase activity of 14 μmol of cAMP/min. Pieces of the Coomassie-blue-stained gel corresponding to the 140- and 110-kDa proteins were cut out and subjected to electrophoresis with a gel-concentration device (protein recovery estimated at 40%). Pieces of the gel containing the 140- and 110-kDa proteins were cut out and digested with 2 μg/mL of endoproteinase Lys-C from *Lysobacter enzymogenes* (Boehringer) for 18 h at 30 °C. The digested material was injected onto a DEAE column linked to a C18 reversed-phase HPLC column. Eluted peptides were sequenced on an Applied Biosystems 470 gas-phase sequencer. The recovery of peptides was estimated as being 20% of the protein contained in the gel pieces (mol/mol) (as compared with yields obtained with known amounts of standard proteins treated in the same way).

Silver-stained gels containing photolabeled material were dried and either autoradiographed (Kodak X-Omat films) or cut into pieces and counted using a scintillation β-counter. Densitometry of silver-stained gels and of autoradiographs was conducted by image analysis, using digitized programs Biolab C and E with Macintosh Programming Workshop for Macintosh II (Dr. Rage, LNB, CNRS, Marseille, France). Thin-layer chromatography of the labeled nucleotides present in samples incubated with [α-^{32}P]ATP was performed on silica gel (600 F_{254} Merck plates) in dioxane/isopropanol/20% NH_4OH/50 mM EDTA (31:15:28:26) (Bronikov & Zakharov, 1983).

Peptide Synthesis. The 38 amino acid long peptide corresponding to sequence 976–1013 of the bovine brain adenylyl cyclase type I (FVLRVGINVGPVVAGVIGARRPQYDIWGNTVNVASRMD) was chemically assembled by the solid-phase method on an Applied Biosystems peptide synthesizer (ABI Model 430 A, Foster City, CA). Stepwise elongation of the peptide chain was carried out on 4-(((oxymethyl)acetamido)methyl)polystyrene resin (0.5 mM), using optimyzed *tert*-butyloxycarboxyl (Boc)/benzyl chemistry (Merrifield, 1986). Coupling was performed with hydroxybenzotriazole active esters in *N*-methylpyrrolidone. After anhydrous hydrogen fluoride cleavage, deformylation of tryptophan was achieved by 0.04 N NaOH treatment at pH 11.5 for 3 min followed by acidification with glacial acetic acid (Li et al., 1978). Crude peptide was purified by C18 HPLC. The purified peptide was further characterized by amino acid content determination after hydrolysis (6 N HCl, 72 h; 110 °C).

Immunological Techniques. Samples of a rabbit (no. 66) preimmune serum were collected. The synthetic peptide described (1.5 mg) was emulsified in complete Freund adjuvant and injected intradermally and in one popliteal lymph node of rabbit no. 66. The same amount of peptide (in incomplete adjuvant) was injected in the same way a month later. Blood was withdrawn after 3 weeks, twice a month over 6 months. Antibodies were purified as described (Anglade et al., 1993). A rabbit antiserum directed against NCAM was kindly supplied by G. Rougon (Rougon & Marshak, 1986). The antiserum directed against a peptide specific to the $G_{S\alpha}$ subunit, RM/1, was from NEN (Simonds et al., 1989). Elisa assays and immunoblotting procedures were as described (Orlando et al., 1992).

Photolabeling of FC Fractions. The protocols described by B. Haley were followed (Haley, 1991). Briefly, solvents of the photoprobes were evaporated, and the probes were dissolved in water (or 0.5 mM acetic acid in the case of 2-N_3-ATP). Samples of FC prepared in the absence of DTT were distributed in microcentrifuge tubes. They contained, in a final volume of 200 μL, 1 or 10 mM $MgCl_2$, 1 mM $MnCl_2$, 100 or 250 mM NaCl, 0.035% Lubrol, 10 mM Tris-HCl buffer, pH 7.6, 40 μM forskolin, and volumes of FC fractions containing various adenylyl cyclase activities (10–35 nmol of cAMP/(min·tube)). Tubes were kept on ice. Reactions were initiated by the addition of micromolar concentrations of the photoprobes, and unless otherwise indicated, the reaction mixtures were incubated for 1 min and irradiated for 2 min with a hand-held short-wavelength UV light (254 nm, 4500 μW/cm^2) (Ultraviolet Products Inc., San Gabriel, CA, or Spectroline, Spectronics Co., Westbury, NY). When N_3-ATP probes radiolabeled in the γ position were used, unirradiated aliquots were analyzed in parallel. For competition studies, the enzyme samples were preincubated on ice with various unlabeled nucleotides for 1 min prior to the addition of the photoprobe. Following photolysis, the samples were supplemented with 20 μg of soybean trypsin inhibitor and 5 mM DTT, 10-fold diluted with ice-cold 15% TCA, and kept on ice for 15 min. Precipitates were collected by centrifugation and dissolved in sample buffer as described (Orlando et al., 1992).

RESULTS

Analysis of FC Fractions by SDS-PAGE and Immunoblotting. FC fractions were prepared as described (Orlando et al., 1992), except that glycerol, a protein-stabilizing agent (Maloney & Ambudkar, 1989), was present upon solubilization of the synaptosomes and during the forskolin affinity chromatography step. This modification resulted in a 2-fold increase in specific activity (60 ± 10 μmol of cAMP/(mg·min)).

Coomassie-blue-stained gels of FC fractions contained an abundant protein of 140 kDa, as well as proteins of 58, 52, and 48 kDa (not shown). Only in the preparative gel (the loaded sample containing an enzyme activity of 14 μmol of cAMP/min) was a 110-kDa protein visualized. The amounts

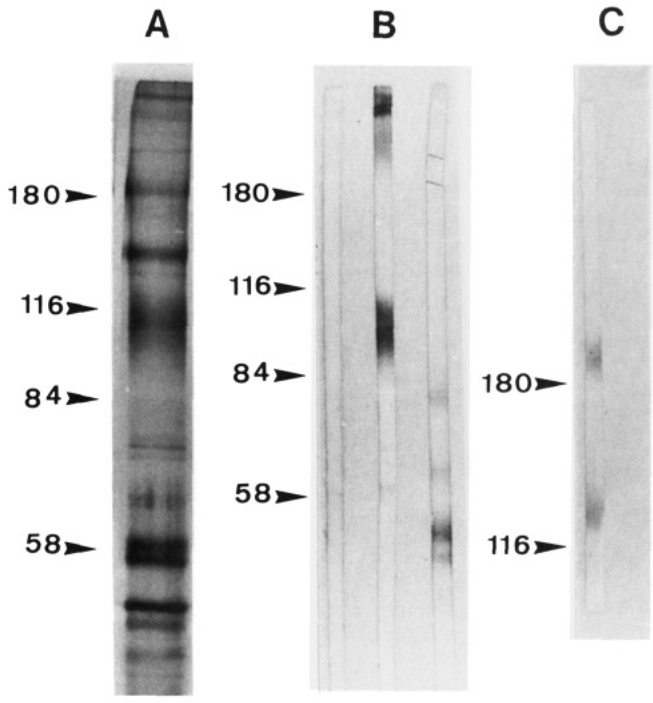

FIGURE 1: Protein pattern and Western blot analysis of FC fractions. (A) Silver-stained SDS-PA gel of a FC fraction (8% PA, adenylyl cyclase activity of 25 nmol of cAMP/min deposited on a 1-cm well). (B) Immunoblots of FC samples. Lanes 1 and 2 correspond to samples containing an adenylyl cyclase activity of 300 nmol of cAMP/min, and lane 3 corresponds to a sample containing 150 nmol of cAMP/min (activity deposited on a 1-cm well). Blots were reacted with proteins retained on a peptide-affinity resin from preimmune (lane 1) and immune serum no. 66 (lane 2), diluted 1:500, or with anti-$G_{S\alpha}$ antiserum (Simonds et al., 1989) (lane 3), diluted 1:50. (C) Immunoblot of an FC sample. The sample contained an adenylyl cyclase activity of 280 nmol of cAMP/(min·cm). The blot was reacted with an anti-NCAM antiserum (Rougon & Marshak, 1986) diluted 1:10 000.

of the 140- and 110-kDa proteins extracted from this gel were estimated to be respectively 250 and 80 pmol (see Experimental Procedures). The protein pattern of FC fractions revealed by silver-staining of the gels was different in that the protein of 110 kDa appeared to be the major component (30% of the total protein stain measured by densitometry in silver-stained gels) (Figure 1 and Figure 4, lane 1).

To be able to identify on Western blots any adenylyl cyclase belonging to the sequenced mammalian enzyme family (Tang & Gilman, 1992), we obtained several rabbit sera directed against peptides corresponding to sequences of C2a, a well-conserved domain of these enzymes. One of them, serum no. 66, immunized with the peptide corresponding to sequence 976–1013 of bovine adenylyl cyclase type I, yielded anti-peptide antibodies of high titer (>100 000). Applied to Western blots of FC fractions, the affinity-purified antibodies stained, besides some material aggregated at the top of the gel, a single protein band spreading from 95 to 110 kDa, not visualized with the preimmune antibodies (Figure 1B, lanes 1 and 2). Incubation of FC fractions with the affinity-purified antibodies did not result in any measurable change of the adenylyl cyclase activity, nor was it possible to immunoprecipitate adenylyl cyclase activity with these antibodies.

The blotted 140-kDa protein and a minor component of 200 kDa were recognized by a rabbit anti-NCAM antiserum applied to Western blots of FC fractions, indicating that these two proteins could be the NCAM species possessing a cytoplasmic domain (Figure 1C). The sequences of all peptides obtained from the 140-kDa protein were indeed found in the

Table 1: Adenylyl-Cyclase Activities Measured in FC Fractions in the Presence of Various Effectors

effector	% basal activity	sem
none	100	$n = 6$
forskolin	111	9 $n = 6$
Gpp(NH)p	97	5 $n = 2$
Gpp(NH)p + forskolin	106	14 $n = 3$
adenosine	42	16 $n = 2$
EGTA	50	8 $n = 3$
calmodulin	212	43 $n = 5$
Mn^{2+}	205	27 $n = 4$
Mn^{2+} + forskolin	264	21 $n = 4$

sequence of rat NCAM (Small et al., 1987) (data not shown). Treatment of Western blots of FC fractions with an antiserum directed against a peptide specific of $G_{S\alpha}$ indicated that the 48- and 52-kDa proteins were $G_{S\alpha}$ subunits (Figure 1B, lane 3).

Properties of the Adenylyl Cyclase Activity Contained in FC Fractions. As already mentioned, the $MnCl_2$- and forskolin-stimulated adenylyl cyclase specific activity measured in FC fractions was 60 ± 10 μmol of cAMP/(mg·min), a very high value compared to published data. Freezing and thawing decreased the adenylyl cyclase activity present in FC fractions by 20 to 40% without changing its properties. Several batches of FC were assayed in various conditions (Table 1). When $MgCl_2$ was the only divalent cation added to FC fractions, the so-called basal activity (no effector added) was twice that measured when 1 mM EGTA was added. In the latter assay, addition of 2 μM calmodulin did not affect the activity, whereas in the absence of EGTA, it resulted in a 4-fold stimulation. We believe that Ca^{2+} ions eluting from the calmodulin column upon EDTA treatment, as well as the small amount of calmodulin leaking from the column, were sufficient to stimulate some of the calmodulin-sensitive adenylyl cyclase present in FC fractions. Addition of Ca^{2+} up to 100 μM did not modify the effect of calmodulin. The activity measured in the presence of forskolin was comparable to the so-called basal activity, twice that measured in the presence of EGTA. When $MnCl_2$ (2 mM) was added to the basal medium, the adenylyl cyclase activity doubled. $MnCl_2$ and forskolin had synergistic effects. Adenosine (1 mM) was inhibitory (×0.5) in all conditions. No effect of GTP or Gpp(NH)p was detected in our assay conditions, in probable relation with the fact that synaptosomes were treated with Gpp(NH)p prior to solubilization. The K_m of adenylyl cyclase for ATP, measured in FC fractions in the presence of $MnCl_2$ and forskolin, was 9 μM. In synaptosomal Lubrol extracts, the K_m for ATP was comparable (7 μM). Forskolin-stimulated activity was assayed in synaptosomal Lubrol extracts with either [α-32P]ATP or [α-32P]8-N_3ATP. Identical V_{max} values were measured with both nucleotides; the K_m of the enzyme for 8-N_3ATP, 22 μM, was similar to the K_m for ATP, 7 μM (Figure 2). The effect of 40% DMSO on adenylyl cyclase activity was tested, since this agent is thought to modify the constrains of the ATP binding sites in certain ATPases (Al-Shawi & Senior, 1992). The adenylyl cyclase activity was diminished by 85%.

Photolabeling of FC Components with [32P]N_3ATP, N_3-cAMP, and [32P]N_3GTP. ATP hydrolysis in FC fractions could be attributed to adenylyl cyclase rather than to ATPases or protein kinases, as shown by TLC analysis of the nucleotides present in fractions incubated with 0.2 mM [α32P]ATP (Figure 3). [32P]cAMP was the only nucleotide readily detected in FC fractions by this method. [32P]ADP and [32P]AMP were detected in Lubrol extracts and, when using long exposure

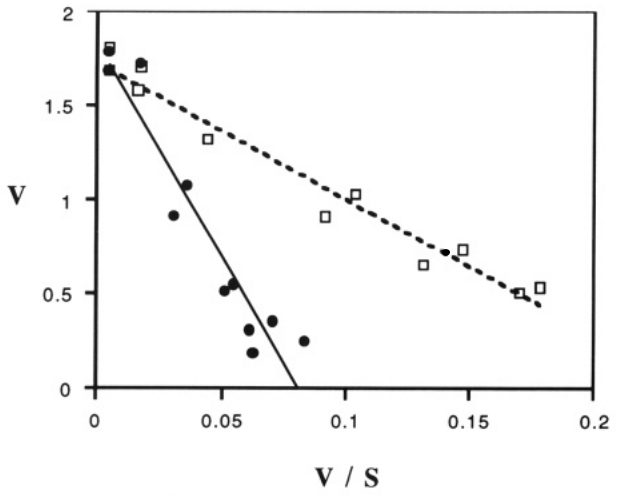

FIGURE 2: Determination of the K_m of synaptosomal Lubrol extract adenylyl cyclase for ATP and N_3ATP. Adenylyl cyclase activity, expressed as pmol of cAMP/s, was measured in synatosomal Lubrol extract using either [α-^{32}P]ATP (□) or [α-^{32}P]8-N_3ATP (μM) (●) as substrate, in the presence of 0.5 mM cAMP, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM DTT, and 10 mM theophylline, at 30 °C. The K_m values for [α-^{32}P]ATP and [α-^{32}P]8-N_3ATP were calculated using Hofstee plots.

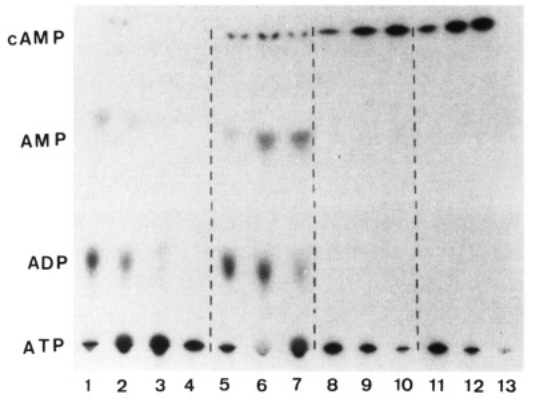

FIGURE 3: Autoradiograph of a TLC plate showing that cAMP is the only nucleotide detected upon incubation of FC fractions with [α-^{32}P]ATP. Samples of synaptosomal Lubrol extract (adenylyl cyclase activity: 10 nmol of cAMP/(min·mL)) (lanes 1–3 and 5–7), forskolin eluate (5 nmol of cAMP/(min·mL)) (lanes 8–10), and FC fraction (23 nmol of cAMP/(min·mL)) (lanes 11–13) incubated at 30 °C in the presence of 20 μM [α-^{32}P]ATP, 0.1 mM forskolin, 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.05% Lubrol. The Lubrol extract corresponding to lanes 5–7 contained 10 mM theophylline while the extract used for lanes 1–3 did not. A 2-μL sample of each tube was taken at 1, 5, and 15 min and spotted on the TLC plate, which was processed as described and autoradiographed: (lane 4) unincubated probe.

times, in forskolin eluates. The enzyme most likely to bind ATP in FC preparations was thus adenylyl cyclase. Consequently, we searched for one (or several) component in which photoinsertion of [^{32}P]N_3ATP would occur.

Photolabeling experiments were conducted using different FC batches and either [^{32}P]8-N_3ATP or [^{32}P]2-N_3ATP. To lower consumption of the probes, which are substrates of adenylyl cyclase, incubation as well as photolysis took place on ice and 40% DMSO was occasionally added. The major component specifically photolabeled by [α^{32}P]8-N_3ATP was the 110-kDa protein (Figure 4, lane 2). Labeling depended upon UV irradiation. The possibility that the 110-kDa protein might have been phosphorylated by [γ^{32}P]N_3ATP was ruled out, since labeling absolutely depended upon UV irradiation. Autoradiographs obtained from samples incubated with [γ^{32}P]8- or [γ^{32}P]2-N_3ATP were identical. Photoinsertion

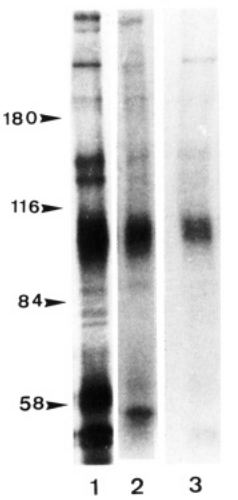

FIGURE 4: Silver-stained SDS-PA gel (8% PA) and autoradiographs of gels on which proteins of FC fractions photolabeled with [α-^{32}P]8-N_3ATP and [α-^{32}P]N_3GTP were separated: (lane 1) an FC sample (25 nmol of cAMP/min) was incubated for 2 min with 20 μM [α-^{32}P]8-N_3ATP and photolyzed for 2 min; silver-staining of the gel; (lane 2) autoradiograph of the same gel lane; (lane 3) an FC sample (16 nmol of cAMP/min) was preincubated for 15 min on ice with 400 μM AMP, incubated for 2 min with 40 μM [α-^{32}P]N_3GTP, and photolyzed for 2 min.

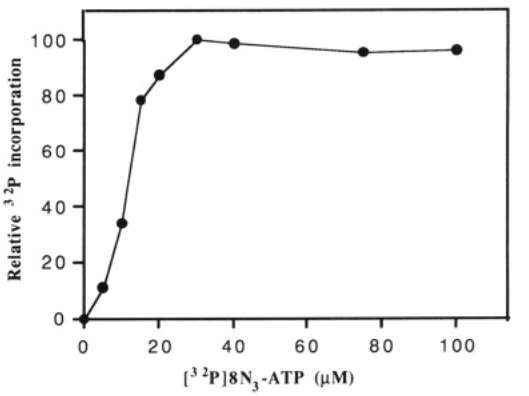

FIGURE 5: Saturation of [γ-^{32}P]8-N_3ATP photoinsertion into the 110-kDa protein. Aliquots of an FC fraction (10 nmol of cAMP/min) were incubated with 5–100 μM [γ-^{32}P]8-N_3ATP for 1 min, photolyzed for 2 min, and subjected to SDS-PAGE. ^{32}P incorporation was detected by autoradiography and quantified by densitometry.

of the probe into the 110-kDa protein saturated at 30 μM (Figure 5). Selective protection against photoinsertion of 8-N_3-ATP (Figure 6) or of 2-N_3ATP (Figure 7) was obtained using an appropriate concentration of ATP (Figure 6, lanes 5–7; Figure 7, lane 2) or of AMP-PNP (Figure 6, lane 4; Figure 7, lanes 1 and 4), the presence of 40% DMSO favoring reduction of photolabeling (Figure 7, compare lanes 1 and 4). AMP-PNP was found to be a better competitor than ATP. As measured by scintillation counting, addition of ATP in a 10-fold elevated concentration compared to the azidoprobe decreased the labeling of the 110-kDa protein by 25% while a 25-fold concentration decreased it by 42%. On the contrary, photoinsertion of N_3ATP into the 110-kDa protein was not affected by the presence of AMP, adenosine (Figure 6, lane 2), and GMP (Figure 6, lane 3; Figure 7, lanes 3 and 5). Photolabeling of the 110-kDa protein by N_3ATP was decreased if 1 mM Ca^{2+} was present, or if the ionic strength was raised above 0.25 M NaCl, and was totally prevented by the presence of 10 μM DTT (not shown).

Although the 110-kDa protein was the major photolabeled component, other proteins in FC fractions also bound N_3-ATP. A protein of 56 kDa was specifically labeled by 8-N_3-

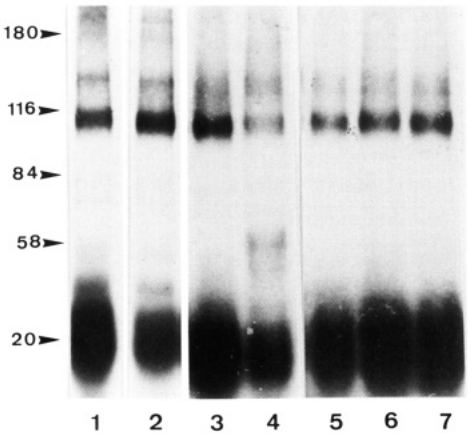

FIGURE 6: Autoradiograph of an SDS-PA gel (12% PA) on which proteins of FC fractions photolabeled with [32P]8-N3ATP were separated. FC samples (adenylyl cyclase activity: 15 nmol of cAMP/min) were preincubated for 1 min with water (lane 1), 500 μM adenosine (lane 2), 500 μM GMP (lane 3), 500 μM AMP-PNP (lane 4), 500, 200, and 100 μM ATP (lanes 5, 6, and 7), incubated with 20 μM [32P]8-N3ATP for 1 min, and photolyzed for 2 min. The intensely labeled protein at the bottom of the gel is trypsin inhibitor, added as a carrier at the end of the photolabeling experiment, before addition of DTT and TCA.

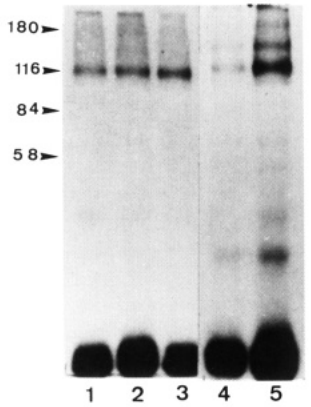

FIGURE 7: Autoradiograph of an SDS-PA gel (12% PA) on which proteins of FC fractions photolabeled with [32P]2-N3ATP were separated. FC samples (adenylyl cyclase activity: 12.5 nmol of cAMP/min) were prepared in a medium containing (lanes 4 and 5) or lacking (lanes 1–3) 40% DMSO. They were preincubated for 1 min with 500 μM AMP-PNP (lanes 1 and 4), ATP (lane 2), and GMP (lanes 3 and 5) prior to addition of 40 μM [32P]2-N3ATP: incubation, 1 min; photolysis, 2 min. The labeled protein at the bottom of the gel is trypsin inhibitor.

ATP, although to a much lesser extent than the 110-kDa protein (Figure 4, lane 2; Figure 6, lane 4). In a few experiments, the light-dependent labeling of the 140-kDa protein (NCAM) was noticed (Figure 6) and will be discussed elsewhere. The light-independent labeling of a very minor, 200-kDa component and of two proteins of about 50 and 54 kDa could be observed when using [γ32P]8-N3ATP (not shown); consequently this labeling was attributed to a phosphorylation event.

Incubation of FC fractions with [32P]N3cAMP, in the presence of DMSO, led to its photoinsertion in one protein, the 110-kDa component, although in modest quantity. Photoinsertion was largely prevented by the presence of AMP-PNP or ATP (Figure 8). Interestingly, addition of cAMP reproducibly increased photoinsertion (Figure 8). When FC fractions were incubated in the presence of [α32P]8-N3GTP, the 110-kDa protein was the major photolabeled component (Figure 4, lane 3); labeling was light-dependent and was decreased by the presence of GTP or AMP to the same extent.

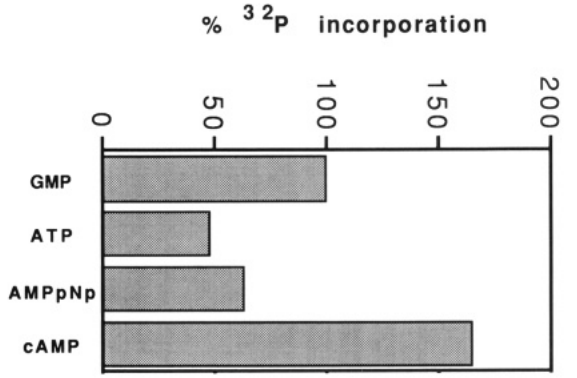

FIGURE 8: Photoincorporation of [32P]N3cAMP into the 110-kDa protein. FC fractions (adenylyl cyclase activity: 35 nmol of cAMP/min) were complemented with 40% DMSO, preincubated for 1 min with 100 μM GMP, or ATP, or AMP-PNP, or cAMP, incubated for 1 min with 1 μM [32P]N3cAMP, and photolyzed for 2 min, and proteins were separated on an SDS-PA gel. Each lane of the corresponding autoradiograph was analyzed by densitometry. Bars represent, for each lane, the densities measured in arbitrary units at the level of the 110-kDa protein. The density measured in the lane corresponding to the sample preincubated with GMP was taken for 100%.

DISCUSSION

Synapses contain a variety of molecules devoted to neurotransmission and its regulation. The abundance of some of these components, like the brain-specific forms of Ca^{2+}/calmodulin-, Ca^{2+}/phospholipid-, and cAMP-dependent protein kinases, reflect the importance of Ca^{2+} and cAMP in the control of synaptic mechanisms (Schwartz & Greenberg, 1987). Thus, it would not be surprising to find specialized isoforms of signaling enzymes in synapses. We have proposed that this might be the case for adenylyl cyclase (Orlando et al., 1992). Adenylyl cyclase highly enriched fractions, termed FC, obtained from brain synaptosomes, had a specific activity on the order of 60 μmol of cAMP/(mg·min). This value suggests the presence in these fractions of an enzyme endowed with an unusually high turnover number, on the order of 3000 s^{-1}. By comparison, the turnover numbers of the $GTP_\gamma S$-activated olfactory and myocardial adenylyl cyclases have been shown to be 37 and 127 s^{-1} (Pfeuffer et al., 1989). The latter enzymes had K_m values for ATP of respectively 130 and 160 μM (Pfeuffer et al., 1989), whereas that measured in FC fractions was 1 order of magnitude lower (9 μM). A specialized isoform of adenylyl cyclase is therefore likely to exist in synapses.

Several arguments stand in favor of the 110-kDa protein being the adenylyl cyclase present in FC fractions. Firstly it is, besides the 140-kDa protein, now identified as NCAM, and $G_{S\alpha}$ subunits, the major component of FC fractions. Any other component in FC fractions, being less represented, would have, were it an adenylyl cyclase, an even higher specific activity. We have calculated, on the basis of protein determinations in FC fractions and quantitation of peptides extracted from the preparative gel, that the specific activity of the 110-kDa protein is 1 ± 0.5 mmol of cAMP/(mg·min), close to that of the *Bordetella pertussis* enzyme, considered to be very high (Ladant et al., 1986). Secondly, the 110-kDa protein is the only labeled component in Western blots of FC fractions treated by an antibody directed against a peptide corresponding to a sequence largely conserved among the mammalian adenylyl cyclases sequenced so far. Thirdly, the 110-kDa protein is specifically labeled by both N_3ATP and N_3cAMP. Cyclic AMP being the single nucleotide abundantly produced in FC fractions upon incubation with ATP, the

likelihood that adenylyl cyclase, rather than any other ATP-hydrolyzing enzyme, was the labeled component was great. At first sight, however, achieving the specific photolabeling by azido-nucleotides of one among several components of a highly diluted (0.5–1 μg of protein/mL) suspension of mixed detergent–protein micelles could appear as an unrealistic goal. The concentration of the azido-probe was indeed necessarily high with respect to that of the target protein(s), a condition favoring nonspecific binding (Haley, 1991). Furthermore, photolabeling an enzyme with its substrate requires the hydrolysis of the probe to be slow enough to ensure sufficient time for photolysis. Given the high specific activity reported here for adenylyl cyclase, conditions had to be found such that a decrease in the enzyme velocity would be compatible with the unimpaired affinity of the protein for nucleotides. Thus all steps were carried out on ice, a condition which generally increases the apparent affinity of nucleotide-binding proteins for nucleotides (Haley, 1991). DMSO was added in order to reduce the velocity of the enzyme without denaturing the protein. In such conditions, the photolabeling by [α-^{32}P]N_3-ATP of one component, the 110-kDa protein, was reproducibly obtained in a saturable, UV-dependent manner. Photoinsertion of the probe into the 110-kDa protein saturated at 30 μM, indicating high affinity. This value is consistent with that determined for the adenylyl cyclase K_m for ATP. Selective protection was demonstrated using low concentrations of ATP and AMP-PNP.

The sequence and location of the putative P site, considered as being responsible for the direct inhibition of mammalian adenylyl cyclases by adenosine, are not yet identified in the so-far-sequenced adenylyl cyclases. Strong arguments that the P site and the catalytic site are distinct domains have been obtained by Johnson et al. (Johnson & Shoshani, 1990; Yeung and Johnson, 1990). In agreement with these results, our photolabeling study shows that adenosine (1 mM) does not affect the labeling of the 110-kDa protein by N_3ATP, indicating that the N_3ATP-binding site and the putative P site appear to be different.

N_3cAMP also photolabeled the 110-kDa protein. This observation is in favor of the 110-kDa protein being the adenylyl cyclase present in the fraction. Labeling occurred in the presence of DMSO and was partially prevented by ATP or AMP-PNP. N_3cAMP can probably be trapped in the catalytic site, since changes in the properties of this site are likely to occur in assay conditions involving low temperature and presence of DMSO. Surprisingly, an excess of cAMP in the assay enhanced photoinsertion of N_3cAMP. A similar observation has been reported in the case of the cAMP-dependent protein kinase by Schoff et al. (1982), who showed that cAMP increases photoinsertion when the cAMP sites are not saturated. They proposed that this is due to the cooperative, allosteric interactions between the two R subunits. In the case of the labeling of the 110-kDa protein, further studies are needed. Finally, the 110-kDa protein is the only protein in FC fractions to bind N_3GTP in the assay conditions described. ($G_{S\alpha}$ subunits were not expected to be labeled by N_3GTP, since they had been activated by Gpp(NH)p in the synaptosomes.) Binding of N_3GTP to the 110-kDa protein might take place either at the level of an authentic GTP-binding site or at the level the ATP-binding site. Such findings are of common occurrence. In that case, discrimination between nucleotide triphosphates by the catalytic site would be relatively loose. It has to be recalled that the *Rhizobium meliloti* adenylyl cyclase is inhibited by GTP (Beuve et al., 1990). The DNA sequence of this enzyme contains a region of great homology with the peptide against which the antibodies used in this study were obtained (Beuve et al., 1990). These antibodies inhibit the bacterial enzyme activity (Beuve, A., personal communication). It may be that the peptide-containing sequence, presumed to be present in the 110-kDa protein (on the basis of its immunoreactivity), retains the possibility of loosely binding GTP. Apart from the 110-kDa protein, another protein of FC fractions, NCAM, was *occasionally* photolabeled by 8-N_3ATP. This phenomenon is currently under investigation.

In conclusion, we have demonstrated, in a brain synaptosomal fraction, that a 110-kDa protein identified as an adenylyl cyclase by immunochemical methods is specifically photolabeled with both N_3ATP and N_3AMP. This enzyme is however endowed with an unusually high specific activity and with a K_m value for ATP lower than expected from the literature. Besides, a partial cDNA coding for the 110-kDa protein has been obtained, which contains sequences not present in data banks (manuscript in preparation). It is therefore likely that this protein is a new adenylyl cyclase isoform specific for synaptosomes. This hypothesis, however, does not preclude another one, namely that an adenylyl cyclase activating factor present in synapses copurified with the enzyme in FC fractions.

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