Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochimica et Biophysica Acta 1613 (2003) 7–14
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Alkylation of sulfhydryl groups on $G\alpha_{s/olf}$ subunits by *N*-ethylmaleimide: regulation by guanine nucleotides

Laura Giusti[a], Simone Taddei[a], Francesca Ceccarelli[a], Silvio Chericoni[b], Gianni Bigini[a], Antonio Lucacchini[a], Maria R. Mazzoni[a,*]

[a] *Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Sezione di Neurobiologia e Famacologia, Via Bonanno 6, 56126 Pisa, Italy*
[b] *Dipartimento di Chimica Bioorganica e Biofarmacia, Via Bonanno 33, 56126 Pisa, Italy*

Received 18 February 2002; received in revised form 24 April 2003; accepted 2 May 2003

Abstract

In rat striatum A_{2A} adenosine receptors activate adenylyl cyclase through coupling to G_s-like proteins, mainly G_{olf} that is expressed at high levels in this brain region. In this study we report that the sulfhydryl alkylating reagent, *N*-ethylmaleimide (NEM), causes a concentration- and time-dependent inhibition of [^{3}H] 2-*p*-(2-carboxyethyl)phenylethylamino)-5′-*N*-ethylcarboxamido adenosine ([^{3}H]CGS21680) binding to rat striatal membranes. Membrane treatment with [^{14}C]*N*-ethylmaleimide ([^{14}C]NEM) labels numerous proteins while addition of 5′-guanylylimidodiphosphate (Gpp(NH)p) reduces labeling of only three protein bands that migrate in SDS-polyacrylamide gel electrophoresis with apparent molecular masses of ~ 52, 45 and 39 kDa, respectively. The 52- and 45-kDa labeled bands show electrophoretic motilities as $G\alpha_s$-long and $G\alpha_s$-short/$G\alpha_{olf}$ subunits. An anti-$G\alpha_{s/olf}$ antiserum immunoprecipitates two ^{14}C labeled bands of 44 and 39 kDa. The band density decreases by 21–26% when membranes are treated with NEM in the presence of Gpp(NH)p. An anti-A_{2A} receptor antibody also immunoprecipitates two ^{14}C labeled bands of 40 and 38 kDa, respectively. However, such protein bands do not show any decrease of their density upon membrane treatment with NEM plus Gpp(NH)p. These results indicate that in rat striatal membranes NEM alkylates sulfhydryl groups of both $G\alpha_{s/olf}$ subunits and A_{2A} adenosine receptors. In addition, cysteine residues of $G\alpha_{s/olf}$ are easily accessible to modification when the subunit is in the GDP-bound form. The 39- and 38-kDa labeled proteins may represent proteolytic fragments of $G\alpha_{s/olf}$ and A_{2A} adenosine receptor, respectively.

Keywords: A_{2A} adenosine receptor; G_s protein; Cysteine residue; Chemical modification; *N*-ethylmaleimide

1. Introduction

Adenosine modulates a variety of physiological functions both in the central and peripheral nervous system via stimulation of four G protein-coupled receptor (GPCR) subtypes, A_1, A_{2A}, A_{2B} and A_3 [1]. The nature of the responses to adenosine and other agonists depends on the selective coupling of the activated receptor to distinct G proteins. Whereas, A_1 and A_3 adenosine receptors are mainly coupled to G_i/G_o proteins, A_{2A} and A_{2B} adenosine receptors activate the adenylyl cyclase system through the interaction with G_s-like proteins [2,3]. Three distinct isoforms of $G\alpha_s$ subunits that share 88% identity in their amino acid sequences [4] have been biochemically characterized, $G\alpha_s$-short, $G\alpha_s$-long and $G\alpha_{olf}$ [5]. Whereas the $G\alpha_s$ subunits have a widespread distribution, the $G\alpha_{olf}$ subunit is distributed in a more restricted manner [6]. Originally, $G\alpha_{olf}$ was found expressed in olfactory epithelium, thus supporting the notion that it was exclusively involved in olfactory signaling [4]. Subsequently, $G\alpha_{olf}$ was also found in some brain regions and its expression is particularly high in striatum [6,7]. A_{2A} receptors are known to activate G_s but recent anatomical and biochemical evidences indicate that in

Abbreviations: Gα and Gβγ, the α and βγ subunit of heterotrimeric G proteins; G_s, a G protein linked with the activation of adenylyl cyclase; $G\alpha_s$, the α subunit of G_s; $G\alpha_i$, the α subunit of a G protein (G_i) linked with the inhibition of adenylyl cyclase; $G\alpha_t$, the α subunit of the G protein (G_t or transducin) present in rod outer segments; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); NECA, 5′-*N*-ethylcarboxamidoadenosine; Gpp(NH)p, 5′-guanylylimidodiphosphate; CGS 21680, 2-*p*-(2-carboxyethyl)phenylethylamino)-5′-*N*-ethylcarboxamidoadenosine; PMSF, phenylmethanesulfonyl fluoride; EDTA, [ethylenedinitrilo]tetracetic acid; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin

* Corresponding author. Tel.: +39-50-24092; fax: +39-50-503534.
E-mail address: mariarm@farm.unipi.it (M.R. Mazzoni).

0005-2736/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0005-2736(03)00133-0

striatum these receptors stimulate adenylyl cyclase and cAMP-dependent signal transduction by activating G_{olf} rather than G_s [8].

Similar to other GPCRs, adenosine receptors have the characteristic secondary structure of seven α-helical membrane spanning domains connected by three extra- and intracellular loops, respectively. Sequence analysis of A_{2A} adenosine receptors from various species has shown that this receptor subtype is particularly rich in cysteine residues in the extracellular loops and transmembrane domains [9,10]. Some of the cysteine residues in the extracellular loops may form disulfide bridges stabilizing receptor structural features [11,12]. In a previous work [12], we have shown that the thiol-alkylating reagent, *N*-ethylmaleimide (NEM), inhibits agonist binding to A_{2A} adenosine receptors in rat striatal membranes causing a two-fold reduction of receptor affinity for [^{3}H]CGS 21680. Since neither agonist nor antagonist ligands protect receptors from NEM effect, alkylated cysteine residues do not form part of the ligand binding site. On the other hand, guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) and 5′-guanylylimidodiphosphate (Gpp(NH)p) are able to prevent NEM effects, suggesting modification of cysteine residues that are involved in receptor G protein coupling and activation.

In the present work, we have therefore searched whether [^{14}C]NEM modification occurred at cysteine residues of the A_{2A} adenosine receptor, $G\alpha_s$ subunits or both. By immunoprecipitation with specific antibodies, we show that [^{14}C]NEM alkylates sulfhydryl groups of 44 kDa $G\alpha_s$-short/$G\alpha_{olf}$ subunits and 40–38 kDa A_{2A} adenosine receptors. A labeled 39-kDa protein band is also immunoprecipitated by the anti-$G\alpha_s$/$G\alpha_{olf}$ antiserum. Addition of a GTP analog reduces alkylation of both 44- and 39-kDa immunoprecipitated proteins. The 39- and 38-kDa bands may represent proteolytic fragments of $G\alpha_s$/$G\alpha_{olf}$ subunit and A_{2A} adenosine receptor, respectively.

2. Materials and methods

[^{14}C]*N*-ethylmaleimide ([^{14}C]NEM) (40 mCi/mmol), [^{3}H] 2-*p*-(2-carboxyethyl) phenylethyl amino)-5′-*N*-ethylcarboxamidoadenosine ([^{3}H]CGS21680) (39.5 Ci/mmol) and EN^{3}HANCE™ were purchased from NEN Life Products (Boston, MA). Bacitracin, benzamidine, soyben trypsin inhibitor, phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, antipain, pepstatin, guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS), 5′-guanylylimidodiphosphate (Gpp(NH)p), Triton × 100, Tween-20, protein A Agarose, sodium orthovanadate (Na_3VO_4), biotinylated SDS molecular weight markers, high molecular weight standard mixture (SDS-6H), Dalton mark VII-L (SDS-7), rabbit IgG, anti-rabbit and anti-goat IgG peroxidase conjugate were from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate (SDS), glycine and β-mercaptoethanol were purchased from ICN Biomedicals Inc. (Aurora, OH). Adenosine deaminase was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Anti-A_{2A} adenosine receptor goat and rabbit polyclonal antibodies, and anti-$G\alpha_{s/olf}$ rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA). All other reagents were from standard commercial sources and of the highest grade available.

2.1. Membrane preparation

Striatal tissue was isolated by dissection from male Sprague–Dawley rat (250–300 g) brains. Membranes were prepared essentially as previously described [13]. Briefly, striatal tissue was homogenized in 20 vol. of ice-cold buffer A (50 mM Tris–HCl, pH 7.4, 1 mM EDTA) containing protease inhibitors (20 μg/ml soybean trypsin inhibitor, 200 μg/ml bacitracin and 160 μg/ml benzamidine). The membrane homogenate was centrifuged at 48,000 × *g* for 10 min at 4 °C. The resulting pellet was resuspended in ice-cold buffer B (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 10 mM $MgCl_2$) containing protease inhibitors (as above) and adenosine deaminase (ADA) (2 IU/ml) to 50 mg/ml of the original tissue weight. The suspension was incubated at 37 °C for 30 min and then centrifuged as described above. The final pellet was stored in aliquots at −80 °C until the time of assay. Protein concentration was determined by the method of Lowry et al. [14], using bovine serum albumin (BSA) as standard.

2.2. Membrane treatment with NEM and radioligand binding assay

NEM treatment of striatal membranes was performed as previously described [12]. Striatal membranes were resuspended (1.5 mg/ml of membrane proteins) in buffer B (pH 7.7) and incubated with various concentrations of NEM (0.5–5 mM) at 37 °C. At intervals, samples of the reaction mixture were removed, quenched by the addition of cysteine (10 mM final concentration) and centrifuged (13,000 × *g*) for 4 min at 4 °C. The resulting pellets were resuspended in buffer B and centrifuged for 4 min. This washing step was repeated twice. The final pellets were resuspended in buffer B at a protein concentration of 1 mg/ml and used in the binding assays. Routine binding assays were performed as previously described [13]. Briefly, incubation was for 90 min at 25 °C in glass tubes containing 0.5 ml of buffer B with striatal membranes (100 μg), 5 nM [^{3}H]CGS21680 and ADA (2 IU/ml). Binding reactions were terminated by filtration through Whatman GF/C filters under reduced pressure. Nonspecific binding was defined in the presence of 100 μM NECA and never exceeded 10% of total binding.

2.3. Membrane treatment with [^{14}C]NEM

Striatal membranes (750 μg of protein) and 2.5 mM [^{14}C]NEM (25 μCi) were incubated in the presence and

absence of 100 μM Gpp(NH)p in 500 μl of buffer B (pH 7.7) containing 0.1 mM PMSF, 1 mM benzamidine and ADA (2 IU/ml). After 3 h at 30 °C, the membrane suspension was diluted by the addition of 500 μl of buffer B and the sample was centrifuged as described above. The resulting pellet was resuspended in buffer B and recentrifuged. This washing step was repeated twice. Control samples were prepared as outlined above, except that 2.5 mM NEM was added to the membrane suspensions.

2.4. SDS-polyacrylamide gel electrophoresis

Discontinuous gel electrophoresis was performed essentially as described by Laemmli [15], using homogeneous 11% (w/v) polyacrylamide resolving gels and 5% (w/v) polyacrylamide stacking gels. The final membrane pellet was solubilized in SDS sample buffer (120 μg protein/20 μl) and boiled for 3 min. Gels were run for 5 h at 30 mA. After electrophoresis, gel proteins were fixed in 10% (v/v) glacial acetic acid and 3% (v/v) methanol for 30 min. Then, gels were soaked in the EN^{3}HANCE™ solution for 1 h under gentle agitation. After this treatment, gels were dried, exposed to Kodak Biomax MR films with dual intensifying screens for 48–72 h at −80 °C and developed by an automatic film processor. The intensity of radiolabeled bands was quantified by densitometric scanning using the Bio-Rad Model GS-670 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Western blotting

SDS-polyacrylamide gel electrophoresis of membrane proteins (~ 120 μg/well) was carried out according to the method of Laemmli [15]. Proteins were electroblotted to nitrocellulose (0.2 μM, Sigma) which was then incubated in PBS (10 mM NaH_2PO_4, pH 7.5, 0.9% NaCl) containing 3% BSA for 45 min at room temperature (RT). Subsequently, nitrocellulose was incubated in PBS containing 1% BSA (PBS/BSA) and anti-$G\alpha_{s/olf}$ rabbit (1:100 dilution) or -A_{2A} receptor goat polyclonal antibodies (1:100 dilution) for 1 h at RT followed by two washes with PBS/0.05% Tween-20 (v/v). Afterwards, nitrocellulose was incubated in PBS/BSA containing peroxidase-labeled secondary antibody (1:8000 dilution) for 1 h at RT. The washing step was repeated as described above, followed by one wash with PBS. Immunoblots were incubated in a staining development solution prepared in PBS buffer by addition of 4-chloro-1-naphtol (2.8 mM), 15% methanol and 0.06% H_2O_2 (30%). The reaction was carried out under constant, gentle shacking until reaching the desired staining intensity. Biotinylated molecular weight markers were used as standards. Alternatively, after electroblotting nitrocellulose was incubated in PBS (as above) containing 3% milk (w/v), 0.2% Tween-20 (v/v) (PBS/milk buffer) for 30 min at RT. Subsequently, nitrocellulose was incubated in PBS/milk buffer containing either anti-$G\alpha_{s/olf}$ or -A_{2A} receptor rabbit polyclonal antibodies (1:100 dilution) for 1 h at RT followed by four washes with PBS/milk buffer. Then, nitrocellulose was incubated in PBS/milk buffer containing peroxidase-labeled secondary antibody (1:8000 dilution) for 1 h at RT. The washing step was repeated as described above, followed by one wash with PBS and one with water. Immunoblots were incubated in an enhanced chemiluminescent substrate for 1 min at room temperature and then exposed to a Kodak Biomax ML film for a few seconds. The intensity of immunoreactive bands was quantified by densitometric scanning using the Bio-Rad Model GS-670 Imaging Densitometer (Bio-Rad Laboratories).

2.6. Immunoprecipitation of $G\alpha_s/G\alpha_{olf}$ and A_{2A} receptor

For immunoprecipitation assays, radiolabeled membrane pellets (700 μg proteins) were resuspended in 175 μl of 1% SDS (1:20 w/v) and boiled for 3 min to solubilize membrane proteins. After cooling, 2.8 ml of solubilization buffer (1% Triton X-100, 10 mM EDTA, 10 mM NaF, 100 μM Na_3VO_4, 100 mM NaH_2PO_4, 50 mM HEPES, pH 7.2, at 4 °C) containing 1 mM PMSF, 3 mM benzamidine, 2 μg/ml soybean trypsin inhibitor, 10 μM leupeptin, 10 μg/ml aprotinin, 1 μg/ml antipain and 1μg/ml pepstatin A (buffer C) was added to each sample. After incubation in ice for at least 1 h, samples were centrifuged at 13,000 × *g* for 10 min at 4 °C. Supernatants (~ 1.4 ml each) were collected, mixed with 14 μl of anti-$G\alpha_{s/olf}$ or -A_{2A} receptor rabbit polyclonal antibodies (1:100 dilution) and incubated overnight at 4 °C under constant rotation. Nonspecific immunoprecipitation was assessed using nonimmune rabbit IgG (1.4 μg/14 μl). After overnight incubation, 80 μl of a 1:1 dilution of protein A-Agarose suspension in distilled water was added to each sample and incubation was extended for an additional 2 h at 4 °C as described above. Immunoprecipitates were separated by centrifugation in a microcentrifuge for 5 min and washed three times with 1.5 ml of 50 mM NaH_2PO_4, 50 mM HEPES, pH 7.2 at 4 °C, 1% Triton X-100, 100 mM NaCl, 100 mM NaF. Final pellets were resuspended in 35 μl of Laemmli sample buffer [15], boiled for 5 min and then centrifuged as described above. Sample proteins were resolved by SDS-polyacrylamide gel electrophoresis. After electrophoretic separation, gels were subjected to fixation and EN^{3}HANCE treatment as described above. After drying, gels were exposed to Kodak Biomax MR films as described above. Nonimmune rabbit IgGs were visualized by Coomassie blue staining.

2.7. HPLC separation of immunoprecipitate proteins

HPLC analyses were performed with a Waters W600E Multisolvent Delivery System, equipped with a Waters 996 DAD as detector. Data were acquired using the software Millennium v.3.2 (Waters). The column was a Chrompack Lichrosphere 10 RP18 (250 × 4.6 mm, Merck) and the run started with 100% water added with 0.2% of formic acid to

reach in 30 min 100% of acetonitrile (acidified with 0.2% of formic acid) with a linear gradient. The flow was of 1 ml/min and the chromatograms were extracted at 280 nm.

2.8. Analysis of data

Statistical analysis (Student's *t* test) and curve-fitting were performed using the GraphPad Prism Version 3.0 computer program (GraphPad Software, San Diego, CA, USA).

3. Results

In a previous paper we have shown inhibition of [^{3}H]CGS 21680 binding to A_{2A} adenosine receptors in rat striatal membranes by the sulfhydryl alkylating reagent, NEM [12]. In the present work, the possibility that one or more cysteine residues were implicated in mediating NEM effects was investigated. Such residues might be part of receptor and/or G protein sequences since they are not implicated in forming the receptor binding site [12] and G proteins modulate receptor affinity state.

In order to study the kinetic of receptor binding inhibition, rat striatal membranes were treated at 37 °C with different concentrations of NEM (0.5 to 5 mM) for different times. Inhibition of specific [^{3}H]CGS 21680 binding proceeded according to apparent first-order kinetics (Fig. 1A). Preincubation with 0.5 mM NEM caused an almost linear decrease of specific binding without reaching a plateau. On the other hand, specific binding decreased rapidly at 10 min of preincubation with 1 to 5 mM NEM and then it did not change up to 60 min of preincubation. When the percent of residual binding was plotted on a logarithmic scale (Fig. 1B), the decrease was linearly related to the preincubation time only at 0.5 mM NEM. The biphasic nature of the other plots suggested that at least two cysteine residues were alkylated when NEM concentration was increased over 0.5 mM.

To discriminate whether NEM modified sulfhydryl groups on A_{2A} adenosine receptors and/or $G\alpha_s$ subunits, we labeled cysteine residues of membrane proteins with [^{14}C]NEM. Rat striatal membranes were incubated with [^{14}C]NEM in the presence and absence of 100 μM Gpp(NH)p. Proteins were separated by SDS-polyacrylamide gel electrophoresis and radiolabeled bands visualized by EN^{3}HANCE fluorography. Densitometric scanning of autoradiograms revealed the presence of three protein bands with apparent molecular masses of 51.2 ± 1.5, 44.5 ± 0.7 and 39.2 ± 0.5 kDa (n = 3) that showed reduction of ^{14}C labeling of 19.1 ± 0.6, 33.0 ± 1.2 and 26.0 ± 2.9%, respectively, when membrane treatment was carried out in the presence of Gpp(NH)p (Fig. 2A and B). A similar reduction of such band intensity was also evident after treatment in the presence of GTPγS (data not shown). These results are consistent with guanine nucleotide protection by NEM effects as reported previously [12].

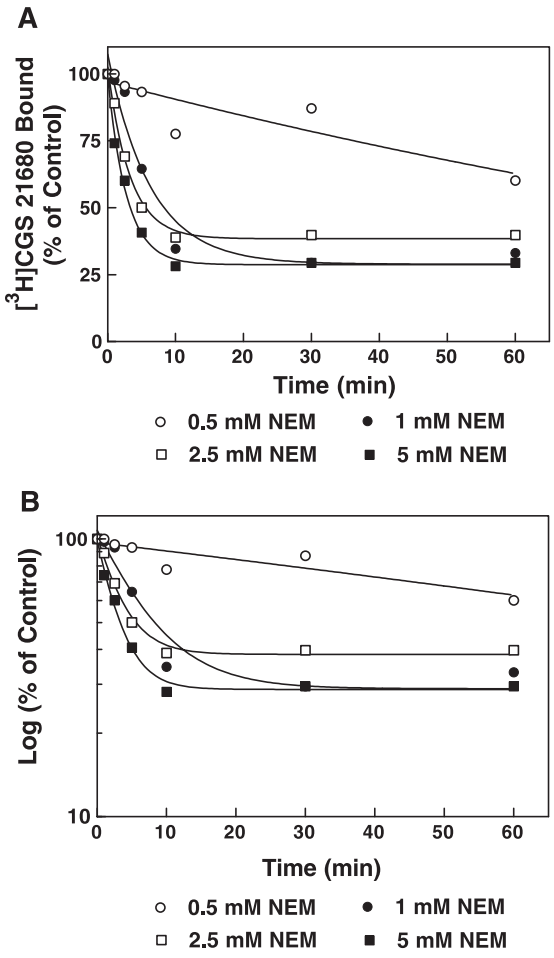

Fig. 1. Time-dependent inhibition of [^{3}H]CGS 21680 binding by NEM. (Panel A) Rat striatal membranes were preincubated with four different concentrations of NEM (0.5 to 5 mM) for various periods of time at 37 °C, washed three times with buffer and incubated with 5 nM [^{3}H]CGS 21680 as described under Materials and methods. Specific binding is presented as a function of preincubation time. Control binding refers to binding of tracer to membranes pretreated with only buffer B for each indicated time. Data points are the mean of triplicate determinations with S.E. less than 10%. These results are representative of experiments conducted two additional times. (Panel B) The percent of residual binding is plotted on a logarithmic scale as a function of preincubation time.

Western blotting analysis of rat striatal membranes was performed to define the relative mobility in SDS-polyacrylamide gels of A_{2A} adenosine receptor and $G\alpha_s$ subunits. Blots were probed with anti-A_{2A} or -$G\alpha_{s/olf}$ polyclonal antibodies. Anti-$G\alpha_{s/olf}$ and -A_{2A} receptor antibodies recognized protein bands of ~ 52 and ~ 46 kDa (Fig. 3A) and 39–40 kDa (Fig. 3A), respectively. Derived mean values of relative molecular masses were 51.3 ± 0.4, 46.4 ± 0.3 (n = 4) and 39.9 ± 0.4 (n = 3) kDa in accordance with the reported molecular masses of $G\alpha_s$-long, $G\alpha_{olf}$/$G\alpha_s$-short subunits [4,16–18] and the deglycosylated A_{2A} adenosine receptor [19]. However, other minor immunoreactive bands were also visible using the 4-chloro-1-naphtol staining detection system. To increase sensitivity, more membrane proteins (180 μg) were loaded on gels and a detection

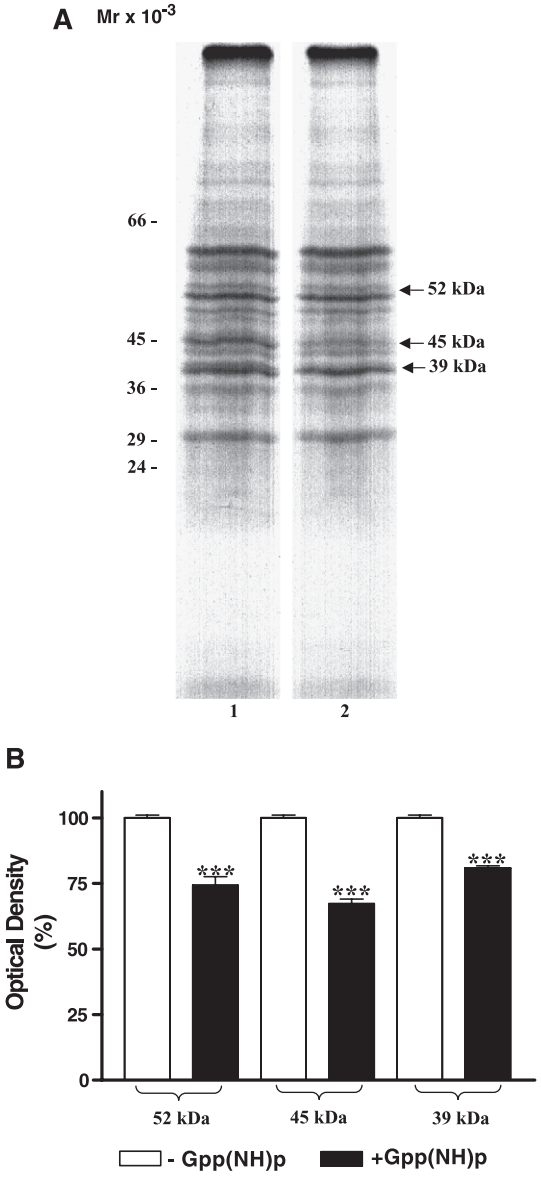

Fig. 2. [^{14}C]NEM alkylation of rat striatal membrane proteins. (Panel A) Rat striatal membranes were incubated with 2.5 mM [^{14}C]NEM for 3 h at 30 °C in the presence and absence of 100 μM Gpp(NH)p. After three washes, membrane pellets were solubilized in SDS sample buffer, proteins separated by SDS-polyacrylamide gel electrophoresis and radiolabeled bands detected by gel fluorography as described under Materials and methods. Lane 1: [^{14}C]NEM treated membranes (120 μg of proteins). Lane 2: [^{14}C]NEM treated membranes in the presence of 100 μM Gpp(NH)p (120 μg of proteins). The illustrated results are from a representative experiment repeated two additional times. (Panel B) Quantitative analysis of 52-, 45- and 39-kDa ^{14}C labeled bands. The density of radiolabeled protein bands was measured using the Bio-Rad Model GS-670 Imaging Densitometer. Bars represent the integrated area of 52-, 45- or 39-kDa band divided by the integrated areas of all bands present in the sample. Values are mean ± S.E. of three independent experiments. Values that are significantly different from corresponding control (no Gpp(NH)p), as determined by Student's *t* test, are indicated (***$P<0.001$; **$P<0.01$; *$P<0.05$).

system based on chemiluminescence was used. In these conditions, probing blots with anti-G$\alpha_{s/olf}$ antibodies revealed five intense immunoreactive bands of apparent molecular masses ranging between ~50 and ~35 kDa (Fig. 3B) while in immunoblots probed with anti-A_{2A} receptor antibodies, two bands of ~43 and ~40 kDa showed the most intense density (Fig. 3B). The relative mobility of A_{2A} and Gα_s immunoreactive bands was very similar to that of ^{14}C labeled bands that showed a decrease of density upon alkylation in the presence of GTP analogs.

In order to determine an unequivocal identification of ^{14}C labeled bands, we performed immunoprecipitation of [^{14}C]NEM alkylated membrane proteins using anti-G$\alpha_{s/olf}$ or -A_{2A} receptor antibodies. The anti-G$\alpha_{s/olf}$ antibody was able to immunoprecipitate 44- and 39-kDa radiolabeled proteins (Fig. 4A) while nonimmune rabbit IgG did not show this pattern of immunoprecipitation (data not shown). In addition, 52- and 33-kDa alkylated proteins which migrate with similar electrophoretic mobility than rabbit IgG heavy and light chains were present in immunoprecipitated samples (Fig. 4A). When membrane treatment with [^{14}C]NEM was carried out in the presence of 100 μM Gpp(NH)p, the intensity of 44- and 39-kDa immunopreci-

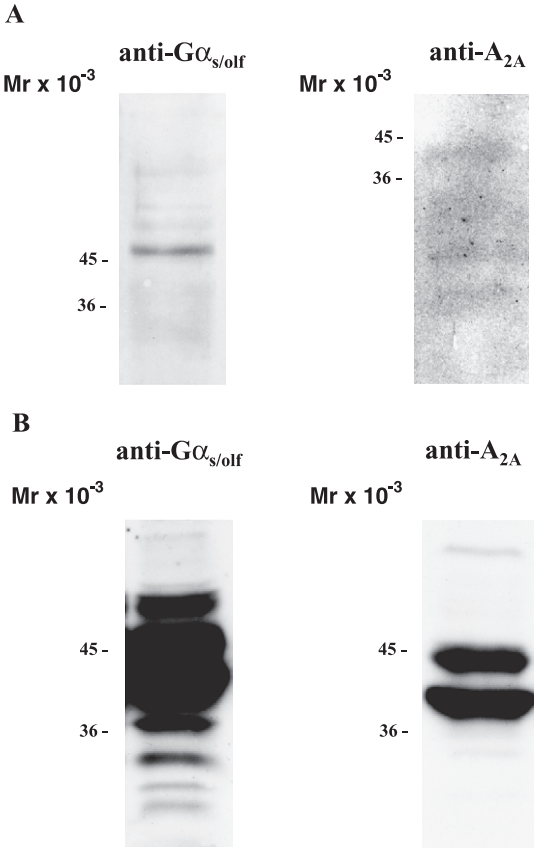

Fig. 3. Western blot analysis of anti-G$\alpha_{s/olf}$ and -A_{2A} receptor antibody binding to rat striatal membrane proteins. Membrane proteins were solubilized in SDS sample buffer, separated (120 μg/lane, panel A; 180 μg/lane, panel B) by SDS-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose as described under Materials and methods. Antibody binding to proteins was revealed using either the 4-chloro-1-naphtol staining (panel A) or a chemiluminescent (panel B) detection system as described under Materials and methods. These are representative immunoblots of two to four independent experiments.

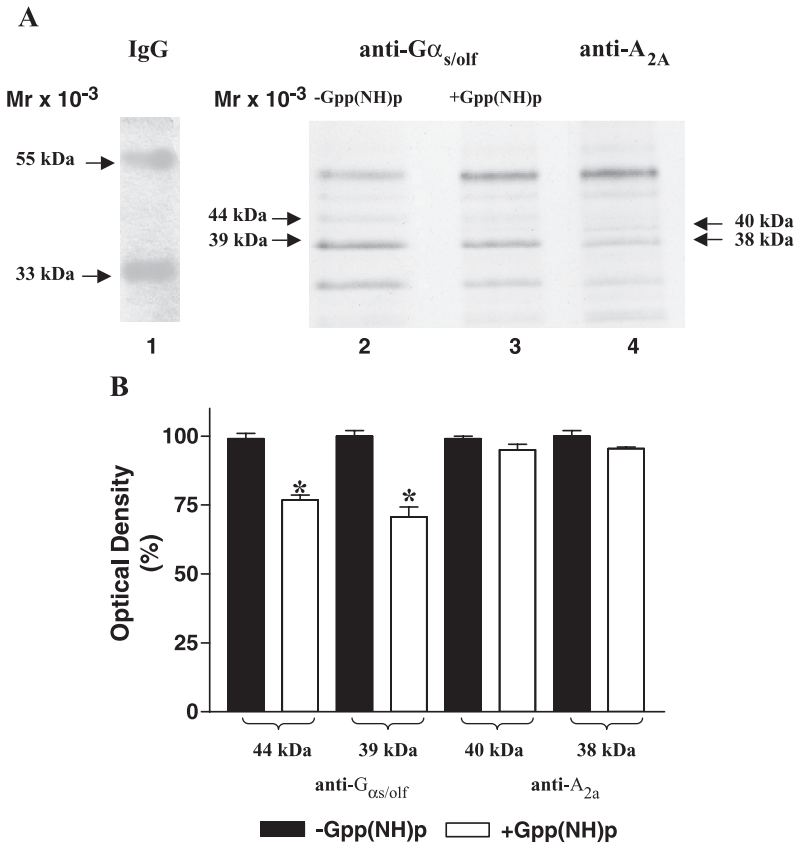

Fig. 4. Immunoprecipitation of ^{14}C labeled bands by either an anti-$G\alpha_{s/olf}$ or -A_{2A} receptor antiserum. Rat striatal membranes were treated with [^{14}C]NEM, protein solubilized, immunoprecipitated using either anti-$G\alpha_{s/olf}$ or -A_{2A} receptor polyclonal antibodies and separated by SDS-polyacrylamide gel electrophoresis as described under Materials and methods. Gels were treated with EN^{3}HANCE and subjected to fluorography as described under Materials and methods. (Panel A) Lane 1: 14 μg/lane of non-immune rabbit IgG stained with Coomassie Blue; lane 2: membrane treated with [^{14}C]NEM and protein immunoprecipitated using 1 mg/ml anti-$G\alpha_{s/olf}$ rabbit polyclonal antibodies; lane 3: membrane treated with [^{14}C]NEM in the presence of 100 μM Gpp(NH)p and protein immunoprecipitated using 1 mg/ml anti-$G\alpha_{s/olf}$ rabbit polyclonal antibodies; lane 4: membrane treated with [^{14}C]NEM and protein immunoprecipitated using 1 mg/ml anti-A_{2A} receptor rabbit polyclonal antibodies. The illustrated results are from a representative experiment repeated one or two additional times. (Panel B) Quantitative analysis of ^{14}C labeled bands. The density of radiolabeled protein bands was measured using the Bio-Rad Model GS-670 Imaging Densitometer. Bars represent the integrated area of 44-, 39-, 40- or 38-kDa band divided by the integrated areas of all bands present in the sample. Values for immunoprecipitation with anti-$G\alpha_{s/olf}$ antibodies are means ± S.E. of three independent experiments while values for immunoprecipitation with anti-A_{2A} antibodies are averages of two experiments.

pitated bands decreased by approximately 21–25% (Fig. 4A and B). The density of the 33-kDa band was also reduced while that of the 52-kDa band increased. These results suggest that treatment of rat striatal membranes with NEM causes alkylation of sulfhydryl groups of $G\alpha_s/G\alpha_{olf}$ proteins (44 kDa) but modification of IgG cysteine residues also occurs during the immunoprecipitation reaction. The radiolabeled 39-kDa band may represent a proteolytic fragment of $G\alpha_{s/olf}$ subunits. Indeed, the presence of a GTP analog during the alkylation reaction decreased labeling of both 44-($G\alpha_s$-short/$G\alpha_{olf}$) and 39-kDa protein bands (Fig. 4A and B). Immunoprecipitates obtained with anti-A_{2A} receptor antibodies showed 52- and 33-kDa ^{14}C labeled bands corresponding to IgG heavy and light chains and two other labeled proteins migrating with apparent molecular masses of 40 and 38 kDa, respectively (Fig. 4A). Such protein bands show mobility as deglycosylated and proteolyzed forms of the A_{2A} adenosine receptor. The density of these bands does not change upon treatment of membranes with [^{14}C]NEM plus Gpp(NH)p (Fig. 4B).

Reverse-phase HPLC analyses of immunoprecipitated complexes obtained with either anti-$G\alpha_s/G\alpha_{olf}$ or -A_{2A} receptor antibodies revealed the presence of two separate peaks with retention time of 24.7 ± 1.1 and 29.2 ± 0.7 min. Elution profiles were similar with both antibodies and in all conditions of membrane treatment. Control rabbit IgGs were eluted at a retention time of 15 min, suggesting that during immunoprecipitation cysteine residues of IgG were alkylated.

4. Discussion

The present data indicate that in rat striatal membranes cysteine residues of $G\alpha_s/G\alpha_{olf}$ subunits and A_{2A} adenosine receptors are alkylated by [^{14}C]NEM. Alkylation of $G\alpha_s$/

$G\alpha_{olf}$ subunits is also negatively affected by the presence of a GTP analog.

In a previous paper [12], we have shown that NEM treatment of rat striatal membranes causes a dose- and time-dependent inhibition of [^{3}H]CGS 21680 binding, which is prevented by either Gpp(NH)p or GTPγS. Neither agonist and antagonist ligands nor GDP has any protective effect. Here, we present kinetic data suggesting that at NEM concentrations between 1 and 5 mM, more than one cysteine residue is alkylated. After membrane treatment with [^{14}C]NEM, several protein bands are labeled but the density of only three bands, ~ 52, 45 and 39 kDa, decreases in the presence of Gpp(NH)p. The 45- and 52-kDa proteins show an electrophoretic mobility as $G\alpha_s$-short/$G\alpha_{olf}$ and $G\alpha_s$-long subunits, respectively, while the 39-kDa protein displays a similar migration pattern as the rat A_{2A} adenosine receptor in its deglycosylated form [16].

Two ^{14}C labeled protein bands with apparent molecular masses of 44 and 39 kDa are immunoprecipitated by an anti-$G\alpha_{s/olf}$ antiserum. Membrane treatment with Gpp(NH)p reduces alkylation of both proteins. The 44-kDa protein shows an electrophoretic mobility as $G\alpha_s$-short/$G\alpha_{olf}$ subunits while the 39-kDa band may represent a proteolytic fragment of the subunit. In immunoblots, this same anti-$G\alpha_{s/olf}$ antiserum recognizes at least five bands with molecular masses ranging between 52 and 35 kDa, suggesting the presence of $G\alpha_s$/$G\alpha_{olf}$ fragments in our membrane preparation. Miki et al. [20] have also shown that anti-$G\alpha_s$ and -$G\alpha_{olf}$ antibodies detect at least three specific proteins in immunoblots of rat striatal membranes. In immunoprecipitation experiments, the 52-kDa $G\alpha_s$-long isoform is not visible since the IgG heavy chain migrates with similar mobility. Two ^{14}C labeled protein bands with apparent molecular masses of 40 and 38 kDa are also immunoprecipitated by the anti-A_{2A} receptor antiserum. The 40-kDa protein represents the deglycosylated form of the A_{2A} adenosine receptor while the 38-kDa protein band is likely a proteolytic product of the deglycosylated receptor. However, the intensity of these bands as well as that of IgG chain bands does not change upon membrane incubation with [^{14}C]NEM plus Gpp(NH)p, indicating that the guanine nucleotide triphosphate does not protect cysteine residues of A_{2A} receptors from alkylation.

Stimulatory Gα subunits contain numerous cysteine residues ($G\alpha_s$, eight residues; $G\alpha_{olf}$, nine residues) but a cysteine in switch region II (helix α2) can be the main target for alkylation. In fact, this region assumes a tight α-helical conformation upon GTP binding while its conformation is more relaxed when GDP is bound [21–23]. Thus, the change of conformation induced by GTP can explain the partial protection in the presence of Gpp(NH)p since the sulfhydryl group becomes less accessible. When Gα subunits are in the GDP-bound form this same cysteine residue is part of an interaction site for Gβγ [24,25]. Irreversible modification of this cysteine residue by alkylation disrupts Gα interaction with Gβγ, thus affecting G proteins ability to interact with activated receptors and stabilize the high-affinity state for agonist ligands. GTP binding to Gα subunits also interrupts this interaction, but in a reversible dynamic fashion, while alkylation induces an irreversible effect. Therefore, we are able to observe a reduction of receptor affinity for agonist ligands since heterotrimeric G proteins cannot effectively couple to activated receptors.

For β_1-adrenergic receptors in turkey erythrocyte membranes, an effect of NEM treatment on ligand binding has been reported [26,27]. Inactivation of β_1-adrenergic receptors occurs after membrane treatment with NEM in the presence of an agonist ligand. The alkylating reagent has no effect on the free or antagonist-bound forms of the receptor while guanine nucleotides prevent inactivation. Therefore, it has been suggested that the reactive sulfhydryl groups may not reside on receptors but on G_s proteins [27,28]. Here, we have reported evidences that sulfhydryl groups of $G\alpha_s$ subunits are alkylated by NEM in a fashion that is dependent on the presence of guanine nucleotide triphosphate.

Acknowledgements

This was supported by a joined grant from University of Pisa and Ministero dell'Università e della Ricerca Scientifica e Tecnologica (to A. Lucacchini) and a Human Capital Mobility grant from European Community (CHRX-CT94-0689) (to M.R. Mazzoni).

References

[1] B.B. Fredholm, M.P. Abbracchio, G. Burnstock, J.W. Daly, T.K. Harden, K.A. Jacobson, P. Leff, M. Williams, Nomenclature and classification of purinoreceptors, Pharmacol. Rev. 46 (1994) 143–156.
[2] J. Linden, Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection, Annu. Rev. Pharmacol. Toxicol. 41 (2001) 775–787.
[3] T.V. Dunwiddie, S.A. Masino, The role and regulation of adenosine in the central nervous system, Annu. Rev. Neurosci. 24 (2001) 31–55.
[4] D.T. Jones, R.R. Reed, Golf: an olfactory neuron specific-G protein involved in odorant signal transduction, Science 244 (1989) 790–795.
[5] D.T. Jones, S.B. Masters, H.R. Bourne, R.R. Reed, Biochemical characterization of three stimulatory GTP-binding proteins. The large and small forms of G_s and the olfactory-specific G-protein, G_{olf}, J. Biol. Chem. 265 (1990) 2671–2676.
[6] D. Herve, M. Levi-Strauss, I. Marey-SemperVerney, C. Tassin, J.P. Glowinski, J.A. Girault, G_{olf} and G_s in rat basal ganglia: possible involvement of G_{olf} in the coupling of dopamine D_1 receptor with adenylyl cyclase, J. Neurosci. 13 (1993) 2237–2248.
[7] S.L. Drinnan, B.T. Hope, T.P. Snutch, S.R. Vincent, G_{olf} in the basal ganglia, Mol. Cell. Neurosci. 2 (1991) 66–70.
[8] B. Kull, P. Svenningsson, B.B. Fredholm, Adenosine A_{2A} receptors are colocalized with and activate G_{olf} in rat striatum, Mol. Pharmacol. 58 (2000) 771–778.
[9] K.A. Jacobson, P.J.M. Van Galen, X.-D. Ji, V. Ramkumar, M.E. Olah, G.L. Stiles, Molecular characterization of A_1 and A_{2a} adenosine receptors, Drug Dev. Res. 28 (1993) 226–231.
[10] A.P. Ijzerman, E.M. Van der Wenden, P.J.M. Van Galen, K.A. Jacobson, Molecular modeling of adenosine receptors. The ligand binding

site on the rat adenosine A_{2A} receptor, Eur. J. Pharmacol. 268 (1994) 95–104.
[11] K.A. Jacobson, G.L. Stiles, X.-D. Ji, Chemical modification and irreversible inhibition of striatal A_{2a} adenosine receptors, Mol Pharmacol. 42 (1992) 123–133.
[12] M.R. Mazzoni, L. Giusti, E. Rossi, S. Taddei, A. Lucacchini, Role of cysteine residues of rat A_{2a} adenosine receptors in agonist binding, Biochim. Biophys. Acta 1324 (1997) 159–170.
[13] M.R. Mazzoni, C. Martini, A. Lucacchini, Regulation of agonist binding to A_{2A} adenosine receptors: effects of guanine nucleotides (GDP[S] and GTP[S]) and Mg^{2+} ion, Biochim. Biophys. Acta 1220 (1993) 76–84.
[14] O.H. Lowry, N.J. Rosebrought, A.L. Farr, R.J. Randall, Protein measurement with folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[15] U.K. Laemmli, Cleavage structural proteins during the assembly of the heat of bacteriophage T4, Nature 227 (1970) 680–685.
[16] J.D. Robishaw, M.D. Smigel, A.G. Gilman, Molecular basis for two forms of the G protein that stimulates adenylate cyclase, J. Biol. Chem. 261 (1986) 9587–9590.
[17] D.T. Jones, R.R. Reed, Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium, J. Biol. Chem. 262 (1987) 14241–14249.
[18] J.R. Hepler, A.G. Gilman, G protein, Trends Biochem. Sci. 17 (1992) 383–387.
[19] D.L. Rosin, A. Robeva, R.L. Woodard, P.G. Guyenet, J. Linden, Immunohistochemical localization of adenosine A_{2A} receptors in the rat central nervous system, J. Comp. Neurol. 401 (1998) 163–186.
[20] M. Miki, T. Hamamura, H. Ujike, Y. Lee, T. Habara, M. Kodama, K. Ohashi, Y. Tanabe, S. Kuroda, Effects of subchronic lithium chloride treatment on G-protein subunits (G_{olf}, $G_{\gamma 7}$) and adenylyl cyclase expressed specifically in the rat striatum, Eur. J. Pharmacol. 428 (2001) 303–309.
[21] J.P. Noel, H.E. Hamm, P.B. Sigler, The 2.2 A crystal structure of transducin-α complexed with GTP γS, Nature 366 (1993) 654–663.
[22] D.G. Lambridght, J.P. Noel, H.E. Hamm, P.B. Sigle, Structural determinants for activation of the α-subunit of a heterotrimeric G protein. Nature 369 (1994) 621–628.
[23] R.K. Sunahara, J.J.G. Tesmer, A.G. Gilman, S.R. Sprang, Crystal structure of the adenylyl cyclase activator $G_{\alpha s}$, Science 278 (1997) 1943–1947.
[24] I. Garcia-Higuera, T.C. Thomas, F. Yi, E.J. Neer, Intersubunit surfaces in G protein αβγ heterotrimers. Analysis by cross-linking and mutagenesis of βγ. J. Biol. Chem. 271 (1996) 528–535.
[25] D.G. Lambridght, J. Sondek, A. Bohm, N.P. Skiba, H.E. Hamm, P.B. Sigler, The 2.0 A crystal structure of a heterotrimeric G protein, Nature 379 (1996) 311–319.
[26] G. Vauquelin, S. Bottari, C. Andre, B. Jacobson, A.D. Strosberg, Interaction between β-adrenergic receptors and guanine nucleotide sites in turkey erythrocyte membranes, Proc. Natl. Acad. Sci. U. S. A. 77 (1980) 3801–3805.
[27] G. Vauquelin, S. Bottari, A.D. Strosberg, Inactivation of β-adrenergic receptors by *N*-ethylmaleimide: permissive role of β-adrenergic agents in relation to adenylate cyclase activation, Mol. Pharmacol. 17 (1980) 163–171.
[28] S.E. Pedersen, E.M. Ross, Functional activation of β-adrenergic receptors by thiols in the presence or absence of agonists, J. Biol. Chem. 260 (1985) 14150–14157.