Vol. 131, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1985 Pages 115-121

SPECIFIC PHOTOAFFINITY LABELLING OF INHIBITORY ADENOSINE RECEPTORS

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Received June 27, 1985

SUMMARY. N^6(L-phenylisopropyl)adenosine (L-PIA) and N^6(3-iodo-4-azido benzyl)-adenosine (IAzBA) inhibit the adenylate cyclase activity in synaptic membranes of chick cerebellum via R_i adenosine receptors. [^{3}H]L-PIA and [^{125}I]AzBA bind to these membranes with K_d values of~1 nM and B_{max} values of~1000 fmol/mg protein. Photolysis of [^{125}I]AzBA bound to synaptic membranes results in the specific incorporation of radioactivity into a protein with M_r = 36,000. This photoincorporation is blocked by simultaneous exposure to L-PIA, theophylline, an adenosine receptor antagonist, or Gpp(NH)$_p$, but not by cytosine, suggesting that the 36,000 dalton protein is the R_i adenosine receptor or a subunit of the receptor that contains the adenosine binding site.

INTRODUCTION. It is now well established that brain tissue contains adenosine receptors that modulate adenylate cyclase activity (1-6). Adenosine receptors that mediate the stimulation of adenylate cyclase activity (R_a or A_2 receptors) are present in the highest concentrations in the striatum and in mesolimbic structures (5, 6), while the adenosine receptors that mediate inhibition of adenylate cyclase activity (R_i or A_1 receptors) are more evenly distributed throughout the central nervous system (6-8). The results reported herein show that synaptic membranes prepared from cerebella of newborn chicks contain R_i adenosine receptors. More importantly, we report on a new [^{125}I]radioligand that contains an azide group and can be used to selectively photoaffinity label the R_i adenosine receptor.

MATERIALS AND METHODS

Materials. [^{32}P]dATP and Na[^{125}I] (carrier free) were purchased from Amersham. N^6(p-aminobenzyl)adenosine (ABA) was a kind gift from Dr. Joel Linden (Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma), or was synthesized in our laboratories by the catalytic reduction of N^6(p-nitrobenzyl)adenosine. N^6(p-nitroben-

Abbreviations. ABA, N^6(p-aminobenzyl)adenosine; IABA, N^6(iodoaminobenzyl)adenosine; IAzBA, N^6(iodoazidobenzyl)adenosine; L-PIA, N^6(L-phenylisopropyl)adenosine.

0006-291X/85 $1.50

zyl)adenosine was prepared according to Dutta et al. (9). Electrophoresis supplies were from Bio-Rad. All other chemicals were from Sigma or other standard sources. Newborn chicks were purchased from Corn Belt Hatcheries (Forrest, IL.).

ABA was iodinated ([^{127}I] or [^{125}I]) following procedures described by Linden et al. (10) with the exception that sodium acetate buffer was employed (11). N^6(iodoaminobenzyl)adenosine (IABA) was purified by reverse phase HPLC with a mobile phase of methanol:water (3:2).

IABA was converted into N^6(iodoazidobenzyl)adenosine (IAzBA) by reacting the amine with sodium nitrite at 0°C followed by the addition of sodium azide at room temperature. The product was directly purified from the reaction mixture by HPLC. The azide had a greatly increased retention time and was eluted at 18-20 min when a mobile phase of methanol:H_2O, 1:1 was employed. The conversion of IABA to IAzBA was quantitative. The product was concentrated under vacuum and stored in 50% methanol at -20°C. The exposure of the azide to light was minimized by performing all procedures in the dark or in dim fluorescent lighting.

Membrane preparation. Chicks (3 to 5-day-old) were sacrificed by decapitation and the cerebella rapidly removed and placed in ice-cold saline. Synaptic membranes from 15-20 chicks were prepared as described by Jones and Matus (12) with the exception that the sucrose solutions were made in 10 mM HEPES (Tris), pH 7.4, and contained 1 mM dithiothreitol (buffer A). The lysis solution was 10 mM HEPES (K+), pH 8.1, 1 mM dithiothreitol. The membranes were resuspended at a concentration of 1-2 mg protein/ml in buffer A and stored at the temperature of liquid nitrogen. In selected experiments synaptic membranes were prepared in the presence of the following protease inhibitors (final concentrations) : benzamidine (0.1 mM), bacitracin (0.1 mg/ml), soybean trypsin inhibitor (0.01 mg/ml), phenylmethylsulfonyl fluoride (0.3 mM), EGTA (1 mM) and EDTA (5 mM). Identical results were obtained in membranes prepared with and without protease inhibitors. A crude membrane fraction of rat hippocampus was prepared as described previously (6).

Adenylate cyclase assay. Adenylate cyclase was assayed by the method of Cooper and Londos (13) using [α-^{32}P]dATP as the subsrate. The reaction mixture (100 µl) contained : 50 µM [α-^{32}P]dATP (50-80 cpm/pmol), 10 mM HEPES (Tris), pH 7.4, 0.5 mM $MgSO_4$, 50 µM dcAMP, 100 µM papaverine, 100 µM GTP, 5 mM creatine phosphate (disodium salt), 40 mM NaCl, 100 µM EGTA, 30 units/ml creatine phosphokinase, 5 units/ml adenosine deaminase, 0.01% digitonin and 10-20 µg protein of synaptic membranes. The low concentration of digitonin was included because it increased basal activity 4 to 5-fold with proportionate increases in the inhibitory effects of the adenosine analogs. This is presumably due to a permeabilizing effect of digitonin that increases the access of the reagents to the adenylate cyclase system. The reactions were started by adding substrate after a 5-min preincubation at 24°C and were run for a period of 15 min. Product formation was linear with time and protein concentration under these conditions.

Ligand binding assays. Synaptic membranes were preincubated with 1 mM EDTA and 5 units/ml adenosine deaminase for 10 min at 37°C before they were added to either the binding or photoaffinity labelling reactions. Ligand binding reactions (50 µl) contained 10 mM L-histidine, 10 mM $MgSO_4$, 1 mM EDTA, 0.01% digitonin, the radioligand as specified, and either 10-20 µg ([^{125}I]AzBA) or 50-100 µg ([^{3}H]L-PIA) of synaptic membrane protein. The binding reactions were incubated 15 min at 37°C to attain equilibrium and bound and free ligand were separated by filtration through Whatman GF/A filter paper presoaked in 0.3% polyethylenimine (14). Filtration was performed on a modified cell harvester (Brandel, Gaithersburg, MD) and employed three 5-ml washes with ice-cold wash buffer (1 mM glycylglycine pH 7.0, 1 mM $MgSO_4$). L-PIA (100 µM) was used to define non-specific binding.

Photoaffinity labelling experiments. Photoaffinity labelling reactions (150 µl total volume) were as described above except they contained 200 µg membrane protein. After 30-min incubations at room temperature the reactions were diluted with ice-cold buffer B (5 mM L-histidine, 1 mM EDTA, 10 mM $MgSO_4$) and centrifuged 20 min at 25,000 x g (4°C). The pellets were resuspended in 1 ml of buffer B and photolyzed for 5 min at 0°C with a mineral light (model R52) positioned approximately 5 cm above the 1.5-ml microfuge tubes containing the samples. The samples were diluted with an additional 3-4 ml of buffer B and centrifuged for 30 min at 15,000 x g (4°C). The samples were dissolved in SDS-PAGE sample buffer (15) and heated for 5 min in a boiling water bath. The tubes were counted before loading onto the SDS-PAGE gels to

determine the amount of radioactivity applied to each lane of the slab gel. SDS-PAGE was performed according to Laemmli (15) using 4% polyacrylamide stacking gels and 10% polyacrylamide resolving gels. Gels were stained, destained, and dried prior to autoradiography. Autoradiography was performed at -80°C with Kodak XAR-2 film and one intensifying screen (Cronex Lighting Plus, Dupont).

RESULTS

The effects of the adenosine analogs, L-PIA and IAzBA, on adenylate cyclase activity in three synaptic membrane preparations were determined (Fig. 1A). Both adenosine analogs produced dose-dependent inhibition of adenylate cyclase activity with maximal inhibitions of approximately 30%. The IC_{50} values for L-PIA and IAzBA were 102 ± 24 nM and 161 ± 4 nM, respectively. Adenylate cyclase activities were assayed under numerous conditions (variations in GTP, monovalent cations, temperature, $MgSO_4$) to determine if stimulation of adenylate cyclase activity by these or other analogs could be demonstrated (data not shown). Adenosine receptor-mediated stimulation of the adenylate cyclase activity in these preparations could not be demonstrated.

Scatchard plots of the binding of [^{3}H]L-PIA and [^{125}I]AzBA to the same type of preparation are shown in Fig. 1B. The K_D values for [^{3}H]L-PIA and [^{125}I]AzBA from this and an identical experiment on a different membrane preparation were 1.46 ± 0.21 and 1.11 ± 0.12 nM, respectively. The corresponding B_{max} values were 936 ± 45 and 967 ± 33 fmol/mg protein.

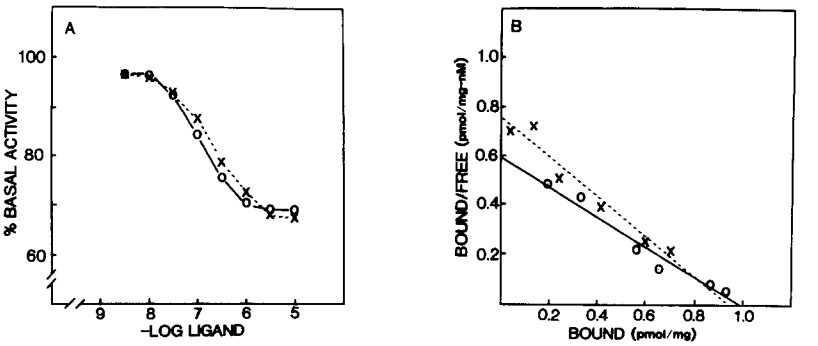

Fig. 1 A. Effects of L-PIA (O) and IAzBA (X) on adenylate cyclase activity in cerebella synaptic membranes. Each point is the average derived from three different synaptic membrane preparations, each assayed in triplicate. The basal activity was 31.4 ± 7.8 pmol/min/mg protein. **B.** Scatchard plots of binding of [^{3}H]L-PIA (O) and [^{125}I]AzBA (X) to cerebellar synaptic membranes. The concentration ranges for [^{3}H]L-PIA and [^{125}I]AzBA were 0.5-20 nM and 0.1-4 nM, respectively. The specific binding ranged between 87 and 93% for [^{3}H]L-PIA and between 87-90% for [^{125}I]AzBA.

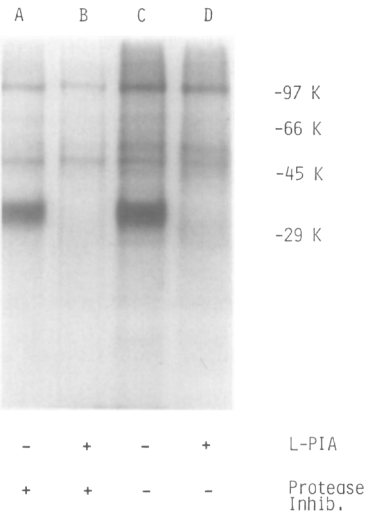

Fig. 2 Autoradiogram of SDS-PAGE gel showing photoincorporation of [^{125}I]AzBA into cerebellar synaptic membranes. Lanes A and B : preparations made in presence of protease inhibitors. Lanes C and D : preparations made in absence of protease inhibitors. The amounts of radioactivity (dpm) applied to lanes A-D were 30,000, 19,000, 38,000 and 15,000, respectively.

Synaptic membranes were photolabelled with [^{125}I]AzBA in the absence and presence of L-PIA and subjected to SDS-PAGE (Fig. 2, lanes C and D). The most prominent band labelled in the absence of L-PIA was not labelled when L-PIA was present and had a molecular weight of 36,000 daltons. Identical results were obtained when a preparation of membranes prepared in the presence of protease inhibitors was studied simultaneously (Fig. 2, lanes A and B). These results suggest that the 36,000-dalton protein is not a product of proteolysis during the preparation of the membranes.

Fig. 3 shows densitometer tracings of the autoradiogram from a similar photoaffinity labelling experiment in which the effects of Gpp(NH)p and cytosine were tested. Panel A shows the blockade of the labelling of the 36,000-dalton peak by L-PIA. Panel B shows that the 36,000-dalton peak is labelled in the presence of a high concentration of cytosine but is not labelled when Gpp(NH)p is present in the reaction mixture. In a separate experiment (data not shown) theophylline, an adenosine receptor antagonist, blocked the incorporation of radioactivity into the 36,000-dalton protein.

Adenosine receptors in rat hippocampus have been widely studied by both biochemical (16, 17) and electrophysiological (18, 19) techniques. Fig. 4 shows an experiment in which [^{125}I]AzBA specifically photoaffinity labelled a M_r = 36,000

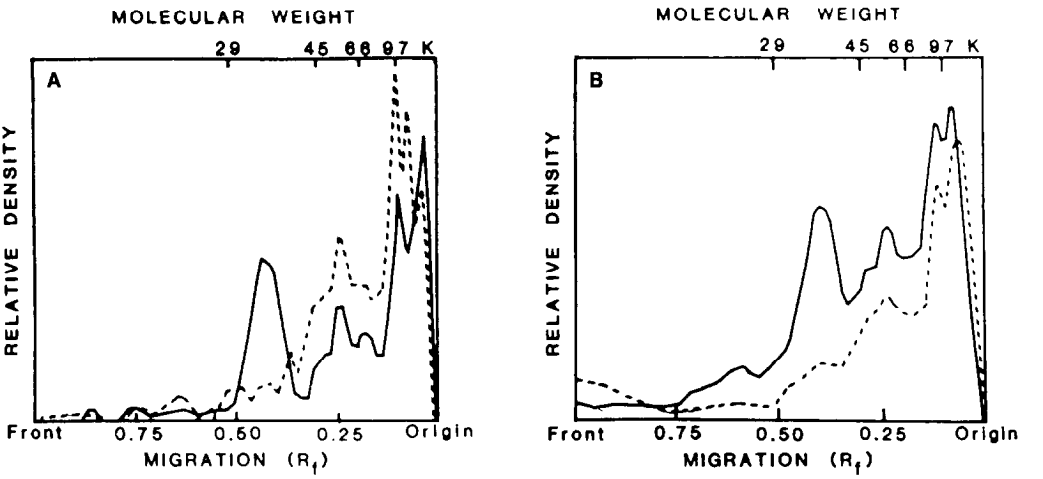

Fig. 3 Densitometer tracings of autoradiogram of SDS-PAGE gel showing photoincorporation of [^{125}I]AzBA into cerebellar synaptic membranes. Panel A : control, ——; L-PIA, 100 μM, -----. Panel B : cytosine, 100 μM, —— ; Gpp(NH)p, 100 μM, -----. The amounts of radioactivity (dpm) applied to each lane were : control, 30,000; L-PIA, 7000; cytosine, 34,000; Gpp(NH)p, 10,000.

protein in a crude membrane preparation of rat hippocampus. This incorporation was protected by the presence of L-PIA.

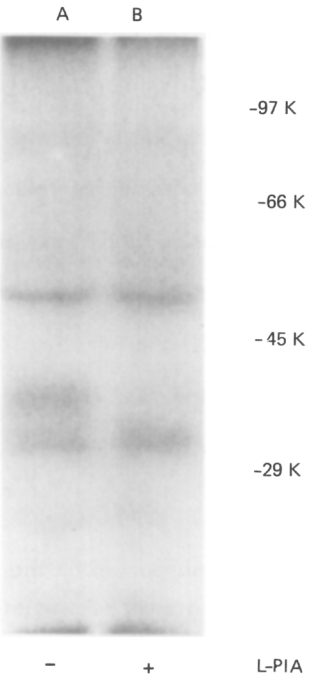

Fig. 4 Autoradiogram showing photoincorporation of [^{125}I]AzBA into rat hippocampus membranes. The amounts of radioactivity (dpm) applied to lanes A and B were 44,000 and 19,000, respectively.

DISCUSSION

[^{3}H]L-PIA has been widely used to study R_i adenosine receptors in the central nervous system (3, 20, 21). Recent attempts to develop a [^{125}I]radioligand for adenosine receptors led to the development of [^{125}I](hydroxyphenylisopropyl)adenosine (22) and [^{125}I]ABA (10). The latter radioligand is the superior of the two for studies on heart membranes as the percent specific binding is higher (10, unpublished results). [^{125}I]ABA has the additional advantage in that it can be easily converted to [^{125}I]AzBA.

We have synthesized both [^{127}I]AzBA and [^{125}I]AzBA. The compound containing the stable iodine isotope (^{127}I) inhibited the adenylate cyclase activity in chick cerebellum membranes with a K_i similar to L-PIA (Fig. 1 A). When the photosensitive radioligand ([^{125}I]AzBA) was studied in the dark to prevent photolysis, it bound to cerebellum synaptic membranes with high affinity and labelled the same number of sites as [^{3}H]L-PIA. This binding was inhibited in a concentration-dependent manner by L-PIA (data not shown).

When cerebellar membranes were photolyzed with [^{125}I]AzBA bound to the receptors the majority of the covalently bound radioactivity was localized to a 36,000-dalton peptide detected in SDS-PAGE gels. Similar results were obtained with a crude membrane preparation of rat hippocampus. The labelled peptide is most likely <u>not</u> the nucleoside transport protein as the incorporation of radioactivity was unaffected by the presence of cytosine, another nucleoside which one expects to bind to the same transport system. We conclude that the 36,000-dalton protein is the R_i adenosine receptor or a subunit thereof because : (1) the labelling is blocked by L-PIA and the adenosine receptor antagonist theophylline, and (2) incorporation is blocked by the GTP analog Gpp(NH)p which destabilizes the high activity binding of agonists to R_i adenosine receptors (12, 21).

ACKNOWLEDGEMENTS. This work was supported by the NIH (HL 33106). MMH is an Established Investigator of the American Heart Association. The expert secretarial assistance of C. Roulinat-Bettelheim is gratefully acknowledged.

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