Target size of the adenosine R_i receptor

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The adenosine receptor of rat cerebral-cortical membranes was examined by radiation inactivation. In control membranes the receptor is distributed between high- and low-affinity states, that can be preferentially expressed by Mg²⁺ ions and guanine nucleotides respectively. Upon exposure to increasing doses of radiation, the high-affinity receptor decayed linearly as a function of radiation dose. This decay rate corresponded to a target size of 63000 Da, when compared with the decay of the muscarinic cholinergic receptor that was also measured in these membranes.

INTRODUCTION

Adenosine exerts a neuromodulatory action in the brain, apparently by acting through specific membrane-bound receptors that are linked to adenylate cyclase. On the basis of these interactions, two classes of adenosine receptors have been described, termed R_i (or A₁) and R_a (or A₂), which respectively inhibit or stimulate adenylate cyclase activity (Londos *et al.*, 1980; Van Calcar *et al.*, 1979).

As with other neurotransmitter and hormone receptors that are associated with adenylate cyclase inhibition (Rodbell, 1980), the adenosine R_i receptor interacts with a guanine nucleotide regulatory complex (N_i). One measure of this interaction that is demonstrable *in vitro* is the regulation of receptor binding by guanine nucleotides and bivalent cations (Yeung & Green, 1983, 1984). Guanine nucleotides selectively decrease, whereas bivalent cations selectively increase, the overall affinity of agonists for the R_i receptor. The molecular basis and regulatory significance of these opposing effects requires an understanding of the sequence of the partial reactions involved in adenylate cyclase regulation, as well as the identification of the components involved and their organization in the membrane. Traditional purification procedures that involve detergent solubilization are an essential phase in the identification of regulatory components. However, such procedures may perturb the structure, activity, subunit stoichiometry and regulatory properties of the cyclase complex. Target-size analysis (radiation inactivation) provides a complementary non-invasive means to characterize the components *in situ* (Kepner & Macey, 1968; Houslay *et al.*, 1977; Kempner & Schlegel, 1979). In the present studies, the adenosine R_i receptor of rat brain cortex membranes was subjected to radiation inactivation. The high-affinity state of the R_i receptor decays as a simple exponential function of radiation dose, which is consistent with a molecular mass of 63000 Da.

EXPERIMENTAL

Chemicals

N⁶-Phenylisopropyl[2,8-³H]adenosine([³H]PIA, sp. radioactivity 49.9 Ci/mmol) was obtained from New England Nuclear. Adenosine deaminase (calf intestine, 1429 units/ml), GTP, creatine kinase (190 units/mg) and phosphocreatine were from Sigma Chemical Co. Unlabelled (–)-PIA was obtained from Boehringer Mannheim. Other reagents were analytical grade, or the best quality available.

Preparation of rat cortex membranes

Male Sprague–Dawley rats (180–230 g) were killed by decapitation, and the brain cortices were quickly removed and placed in ice-cold buffer (50 mM-Tris/HCl, pH 7.4, with 1 mM-dithiothreitol, 0.1 mM-diethylenetriaminopenta-acetic acid, 0.1 mM-benzamidine and 0.1 mM-phenylmethanesulphonyl fluoride) containing 0.3 M-sucrose. The tissue was homogenized by three to five strokes of a glass homogenizer (10 ml/g) and centrifuged (600 g, 10 min) to remove the nuclear fraction. The supernatant was centrifuged (12000 g, 10 min) to obtain the P₂ fraction. Pellets were resuspended in the above buffer containing 0.5 M-NaCl and left for 10 min at 4 °C. Centrifugation (12000 g, 10 min) yielded a pellet that was washed by resuspension in 20 vol. of buffer without NaCl. After a final centrifugation as above, pellets were resuspended in the buffer to a final protein concentration of 5–9 mg/ml and prepared for the irradiation procedure.

Irradiation procedure

Irradiation was performed essentially as described (Venter, 1983; Venter *et al.*, 1983), which was adapted from the method described by Lo *et al.* (1982). Samples (1 ml) of cortex membranes, prepared as described above, were layered to a depth of about 0.5 mm on open aluminium trays and quickly frozen either by immersion

Abbreviation used: [³H]PIA, N⁶-phenylisopropyl[2,8-³H]adenosine.

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of the trays in liquid N₂ or by contact with solid CO₂. The thin layers of membrane sample remained frozen throughout the irradiation procedure. The irradiation chamber was maintained in the range -45 to -52 °C. Samples were stored at -80 °C both before and after irradiation. The cortex membranes were irradiated with a 0.5 mm beam of 1.5 MeV electrons produced with a Van de Graaf generator. Calibration of the radiation dose was performed with dosimetry. Over the course of three radiation runs, samples were exposed to a number of different doses in the range 0.64–15.52 Mrad. Controls consisted of non-irradiated samples, otherwise treated identically.

Enzyme calibration

Standard enzymes (horse liver alcohol dehydrogenase and yeast pyruvate kinase) of known M_r values were irradiated simultaneously with the membrane preparation in order to confirm the irradiation doses. In addition, the decay of the muscarinic cholinergic receptor, which is present in these membranes, was monitored as a function of radiation dose (Venter, 1983). The muscarinic cholinergic receptor has previously been determined to demonstrate a target size of 80000 Da and an electrophoretic mobility corresponding to 80000 Da (Venter, 1983). The decay of the muscarinic cholinergic receptor was used to provide internal calibration of the doses and standardization of the target sizes obtained, as previously described (Venter, 1983; Venter *et al.*, 1983; Lo *et al.*, 1982).

Adenosine receptor binding

The method used was adapted from that described by Trost & Schwabe (1981) and Yeung *et al.* (1985). The incubation mixtures included [³H]PIA (see below), 30 mM-Tris/HCl, pH 7.4, 0.1 mg of creatine kinase/ml, 2 mM-phosphocreatine, 1 unit of adenosine deaminase/ml and 25–100 µg of freshly resuspended cortex membrane protein (as determined by the method of Lowry *et al.*, 1951) in a total volume of 100–200 µl. MgCl₂ (2 mM) and GTP (0.1 mM) were added where specified. For optimal definition of the affinity state(s) of the receptor, two [³H]PIA concentrations were used to generate each Scatchard plot; approx. 1 nM-[³H]PIA for total PIA concentrations of 1.5–40 nM, and 15–20 nM-[³H]PIA for total PIA concentrations up to 400 nM. Non-specific binding was defined in each case by a PIA concentration 150–200-fold in excess of the highest total PIA concentration used. Incubations were carried to equilibrium (2 h, 24 °C) and terminated by addition of 3 ml of ice-cold 30 mM-Tris/HCl (pH 7.4), followed by rapid filtration and two 4 ml rinses through glass-fibre filters (either Whatman GF/C or Schleicher and Schull no. 30). A Brandel Cell Harvester was used, which allowed simultaneous filtration of 24 samples. Between 15 and 25 concentrations of PIA were used to generate a typical Scatchard plot; each point was the average of duplicates. This design yielded an even distribution of points for subsequent curvilinear fitting and extraction of both high- and low-affinity binding parameters.

Data analysis and presentation

Binding parameters were computed by the non-linearizing iterative curve-fitting procedure LIGAND (Munson & Rodbard, 1980), on a Vax computer at the National Jewish Hospital (a copy of this program is

available from the authors). The control high- and low-affinity K_d and B_{max} values were established over at least six separate tissue preparations. Since receptors are progressively destroyed with increasing radiation dose, receptor number declines and the ability to discern accurately the relative contributions of high- and low-affinity receptor states and their binding parameters diminishes. The effect of imposing the K_d values determined in control tissue and then calculating the B_{max} values (by the LIGAND program) as a function of radiation dose was compared with the results obtained without constraining any of the parameters. The former method yielded more reliable parameter estimates in samples that had received high radiation doses, in which non-specific binding compromised the apparent stability of binding parameters. The decay in the binding of 1 nM-[³H]PIA (which would measure predominantly high-affinity binding) was compared with the decay in the high-affinity receptor number as determined by constraining the affinity parameters, as dose was increased. The same inactivation curve was obtained in both cases (results not shown).

The percentage of high-affinity receptors ($100 \cdot R_D/R_0$) remaining at a particular dose was plotted as log functions versus radiation dose. The dose at which 37% of the original receptor number remained (D_{37}) was determined by linear regression through these data (Kempner & Schlegel, 1979). A molecular mass (M_A) was determined from the empirical formula:

$$M_A = \frac{6.4 \times 10^{11}}{D_{37} \text{ (rads)}}$$

or by relating D_{37} of the adenosine receptor ($D_{37,A}$) to the D_{37} of the muscarinic cholinergic receptor ($D_{37,M}$; target size 80000 Da) in the same membranes, as previously described (Venter, 1983), by using the relationship:

$$\frac{D_{37,M}}{D_{37,A}} = \frac{M_A}{40000}$$

RESULTS AND DISCUSSION

Regulation of adenosine-receptor binding by GTP and Mg²⁺

Scatchard transformations of [³H]PIA-binding isotherms in the absence and presence of 0.1 mM-GTP and 2 mM-MgCl₂ are shown in Fig. 1. Addition of GTP decreased agonist binding, whereas MgCl₂ increased the binding. Non-linearizing curve fitting of these data (not shown) revealed that, whether in the absence or the presence of GTP, a two-site model fitted the data better than a one-site model ($P < 0.01$). In functional terms this would correspond to two affinity states of the R_i receptor being differentially promoted by GTP and Mg²⁺, since the total number of receptors in each case is similar (approx. 0.5 pmol/mg). These two affinity states might be expected to correspond to R_iN_i and free R_i forms of the receptor. From six similar experiments, the high- and low-affinity states of the R_i receptor yielded K_d values of 0.9 ± 0.2 nM and 24 ± 5 nM respectively. In control membranes, the receptors are distributed in a ratio of approx. 1:3 between high- and low-affinity states. The addition of GTP shifts the distribution such that approx. 90% of the receptors are in the low-affinity state. In contrast, 2 mM-MgCl₂ causes approx. 95% of the receptors to be in the

Fig. 1. Adenosine-receptor binding and regulation by GTP and Mg^{2+} ions

Adenosine-receptor binding to cerebral-cortical membranes (50–100 μg /assay) was determined as described in the Experimental section in the absence ($\bullet$) or presence of either 0.1 mM-GTP ($\triangle$) or 2 mM- $MgCl_2$ ($\square$). The data have been transformed into a Scatchard plot, and represent means of duplicate determinations.

Fig. 2. Effect of irradiation on adenosine-receptor binding

Cerebral-cortical membranes were subjected to various irradiation doses as described in the Experimental section. Subsequently binding was determined in the presence of 2 mM- $MgCl_2$. For clarity, only the doses (in Mrad) indicated on the Figure (0, $\bullet$; 2.57, $\triangle$; 6.22, $\square$; 9.88, $\blacktriangledown$; 14.1, $\circ$) are displayed.

high-affinity state, as determined by constraining the data to fit a two-site model ($K_{d1} = 0.9$ nM; $K_{d2} = 24$ nM). However, a two-site model does not fit the data significantly better than a one-site model, because of the weighting caused by the preponderance of high-affinity receptors. Thus guanine nucleotides and bivalent cations have opposing effects in promoting the low- and high-affinity receptor states, respectively, as is the case for many receptors that are associated with adenylate cyclase (Yeung & Green, 1983, 1984).

Radiation inactivation of cortex membrane adenosine receptors

For this initial characterization of the adenosine R_1 receptor by irradiation, the high-affinity state of the receptor was examined. Consequently, to maximize the initial representation of these sites, $MgCl_2$ was included in the incubation buffer. For each of 14 doses, including the non-irradiated controls, comprehensive binding isotherms were performed and binding data were analysed by a two-site model. For clarity, only five doses (transformed into Scatchard plots) are shown in Fig. 2. The overall decrease in high-affinity binding with increasing radiation dose (right to left) is readily apparent. Non-linearizing curve-fitting of such data is used to determine the rate of decay in receptor number. The high-affinity receptor population declines by 75% over this radiation dose range.

The logarithm of the high-affinity R_1 receptor remaining ($100 \times R_D/R_0$) is plotted as a function of radiation dose in Fig. 3. A simple exponential decay curve is readily apparent, with a D_{37} of approx. 10.2 Mrad. This D_{37} value yields a molecular mass of approx. 63 000 Da (see the Experimental section). If, instead of the decay of the B_{max} , the amount bound with 1 nM- $[^3H]PIA$ (which presumably reflects binding predominantly to high-affinity receptors) is plotted as a function of radiation dose, a similar slope is obtained (not shown), corresponding to a molecular mass of 62 000 Da. This result was observed in three separate radiation series.

Swillens (Simon *et al.*, 1982; Swillens, 1984) has pointed out that non-linear inactivation curves indicate the contribution of subunits of different sizes to the function being measured. Linear decay curves, however, as in the present case, indicate that, if inhibitors of different composition contribute to the function being measured, then they must be of the same size, and therefore do not distort the apparent target size.

The target size obtained by these methods for the high-affinity state was 63 000 Da. This is a very similar value to that obtained for β -adrenergic receptors, but smaller than that seen with dopaminergic D_2 and muscarinic cholinergic receptors (Venter, 1983; Lilly *et al.*, 1983; Benovic *et al.*, 1984). A receptor size of approx. 60 000 Da may represent a minimal size that conveys adequate surface recognition area, membrane insertion

Fig. 3. Decay of high-affinity adenosine-receptor binding as a function of radiation dose

In this experiment membranes were exposed to 14 irradiation doses. The percentage of high-affinity receptors ($\circ$) (calculated by non-linear curve-fitting) at each dose is indicated. Linear regression through these points, which is used in the calculation of the D_{37} , is indicated. Also shown is the decay in muscarinic cholinergic receptor binding, detected by [3 H]quinuclidinyl benzilate (82 pM) binding, as described in the Experimental section.

domains and GTP-regulatory-protein interacting domain.

Two reports have appeared that describe photolabelling studies of adenosine R_i receptors. In both studies the mobility of the major photolabelled band on SDS/polyacrylamide-gel electrophoresis corresponded to a particle of 36000–38000 Da (Stiles *et al.*, 1985; Choca *et al.*, 1985). This size is somewhat smaller than might have been expected on the basis of studies of other receptors that are coupled to adenylate cyclase. However, adenosine receptors may be dimeric or composed of subunits other than those that can be photolabelled by adenosine analogues.

The high-affinity state of the receptor is presumably most important in determining the initial association of a neuromodulator with its target. However, achievement of the low-affinity state is a complementary measure of the interaction of the receptor with the signalling system. For this reason the target size of both the high-affinity and

the low-affinity form of the receptor might provide insights into the association of the receptor and the GTP regulatory protein. However, determination of the size of the low-affinity state of the receptor is complicated by technical and systematic difficulties. The relatively low affinity of the GTP-induced state of the receptor for agonists, coupled with an increasing background of non-specific labelling as radiation dose is increased, requires that additional measures be taken in future studies for the accurate determination of the target size of the low-affinity form of the receptor.

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