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Radiation inactivation analysis of the A_1 adenosine receptor of rat brain

Decrease in radiation inactivation size in the presence of guanine nucleotide

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Radiation inactivation analysis of the binding of the A_1 adenosine receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes yielded a radiation inactivation size of 58 kDa. In the presence of GTPγS this was reduced to 33 kDa, in good agreement with the size of the ligand-binding subunit detected after photoaffinity labelling. The data indicate that the structural association of A_1 adenosine receptors with G-protein components is altered in situ in the presence of guanine nucleotides.

Adenosine receptor, A_1; Radiation inactivation; Target size; G-protein; (Rat brain membrane)

1. INTRODUCTION

Adenosine is an important regulator of several biochemical and physiological processes in various tissues [1]. The effects of this nucleoside are mediated by extracellular receptors that have been classified into two groups, A_1 and A_2, according to their pharmacological and biochemical characteristics. In the central nervous system, the modulatory actions of adenosine on nerve cell activity are mediated via receptors of the A_1 type [2,3]. The emerging importance of adenosine as a neuromodulator has led to several investigations of its mechanisms of action and to molecular studies of the A_1 receptor. The determination of the molecular size of the receptor has been approached using several methods. In particular, photoaffinity labelling using several agonist and antagonist radioligands has shown that the ligand-binding polypeptide has an apparent molecular mass of approx. 35 kDa [4–6].

A further strategy for determining apparent molecular size is by the radiation inactivation technique, otherwise known as target size analysis [7]. The loss of biological activity, e.g. ligand-receptor binding, with increasing doses of ionising radiation allows determination of the radiation inactivation size (RIS). In addition, if destruction of a polypeptide can be monitored directly, this method gives a second structural parameter, the target size, which may or may not be equal to the functionally determined RIS [8]. This method has provided important information on the molecular sizes of several hormone and neurotransmitter receptors [9].

Recently, the RIS of the A_1 adenosine receptor was determined using the agonist ligand, *R*-[^{3}H]phenylisopropyladenosine [10]. The high-affinity binding site for this ligand was estimated

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Abbreviations: DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GTPγS, guanosine 5'-*O*-(3-thiotriphosphate); RIS, radiation inactivation size

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to be 63 kDa. Due to the apparent discrepancy between this result and that obtained using photoaffinity labelling techniques, together with recent observations indicating a different RIS for the agonist- and antagonist-binding sites for the D_1- and D_2-dopamine receptors [11,12], we have re-examined the RIS of the A_1 adenosine receptor in rat brain membranes using the recently developed, high-affinity antagonist ligand, 8-[^{3}H]cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX) [13,14]. The use of this antagonist has the advantage that binding appears to be largely independent of an interaction with the guanine nucleotide binding protein, G_i, thus allowing determination of the size of the target corresponding to the receptor alone.

2. MATERIALS AND METHODS

Experiments were performed using a crude, post-nuclear pellet from rat cerebral cortex, prepared as in [2]. The membranes were finally suspended in ice-cold 50 mM Tris-HCl (pH 7.4), at a concentration of 1 mg protein/ml. Where used, GTPγS was added 5 min before freezing to give a final concentration of 100 μM. Aliquots (5 ml) were frozen in solid CO_2 in aluminium pots and irradiated for various times with 2.5 MeV electrons generated by a Van der Graaf generator at the GSF, Munich, at -70°C exactly as described [15]. After irradiation samples were kept in solid CO_2 until assaying for ligand binding activity. The dosimetry calibration of the irradiation system used in this study has been described [15].

Binding of the antagonist, [^{3}H]DPCPX, to irradiated membranes was measured as in [14] at a protein concentration of 0.1 mg/ml. Saturation curves were obtained at ligand concentrations within the range 0.05–5 nM. Data were analysed using the curve-fitting program, LIGAND [16], to yield the K_d and B_{max} values. In all cases the best fit was observed with a one-site binding model.

Photoaffinity labelling of membranes was performed according to [6] using the photolabile agonist ligand, [^{125}I]AHPIA. Labelled membranes were irradiated at 0.2 mg/ml in the presence of 1 mg/ml bovine serum albumin. After irradiation, membranes were recovered by centrifugation at 100000 × g for 1 h, dissolved in sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The ^{125}I-labelled A_1 receptor band was excised and radioactivity determined in a gamma counter. The radioactivity in each band was expressed as a percentage of that in a frozen but unirradiated sample for constructing inactivation plots.

Acetylcholinesterase was determined radiometrically [17] and protein by the method of Lowry et al. [18].

The inactivation profiles obtained by plotting ln(% control) vs radiation dose were linear in all cases reported here. RIS was calculated using the empirical formula: $6.4 \times 10^{11}/D_{37}$ where D_{37} is the dose (in rad) at which 37% of the control binding remains [19]. Since this relationship was originally derived at room temperature, a further empirical factor of two was employed to allow for the sensitivity of protein inactivation to temperature [20].

3. RESULTS

Irradiation of membranes from rat brain led to a decrease in the B_{max} values for [^{3}H]DPCPX binding (fig.1A). No significant effect was observed on binding affinity, K_d values being in the range 0.3–0.6 nM with no clear trend on increase in irradiation dose. Incubation in the presence of GTPγS led to reduction in the sensitivity of [^{3}H]DPCPX-binding sites to irradiation (fig.1A) and therefore a decrease in RIS. The RIS values calculated in the absence and presence of GTPγS were 58 ± 2.5 and 33 ± 3 kDa, respectively. Direct measurement of the loss of previously photoaffinity-labelled receptor gave a target size of 35 ± 2 kDa (fig.1B). The endogenous acetylcholinesterase of cerebral membranes had an RIS of 62 ± 2.5 kDa, in good agreement with

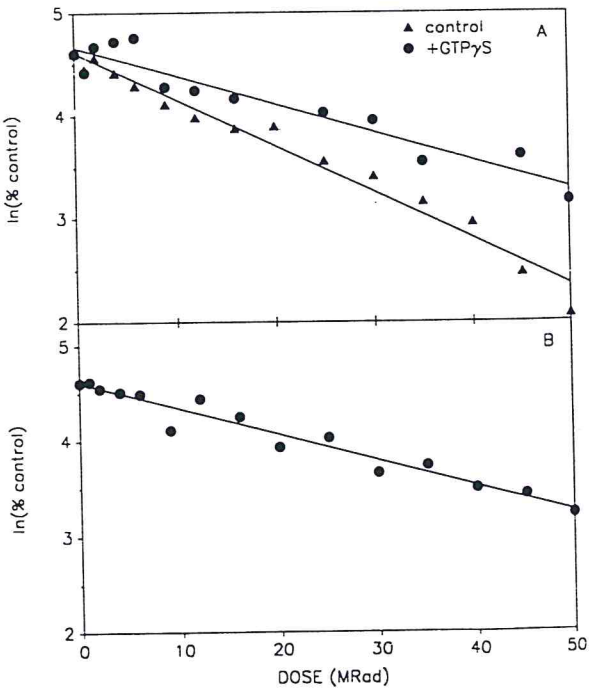

Fig.1. Inactivation profiles of (A) [^{3}H]DPCPX-binding sites in the absence (▲) or presence (●) of 100 μM GTPγS (B_{max} values derived from saturation analysis) and (B) photoaffinity-labelled cerebral membranes. Membranes were irradiated for different times to give the total doses shown.

previously reported values of 61 kDa [15] and 65 kDa [21].

4. DISCUSSION

The RIS of ~58 kDa for the antagonist-binding component of the cerebral A_1 adenosine receptor found here is in good agreement with the value of 63 kDa reported by Frame et al. [10] for the high-affinity agonist-binding state as measured in the presence of Mg^{2+}.

Analysis of receptor structure using agonist radioligands has been complicated by two factors. Firstly, addition of GTP or its analogues to convert the receptor into the low-affinity agonist-binding state results in the loss of agonist binding and consequently greater errors in determining binding at higher irradiation doses. Secondly, attempts to determine the low-affinity state after saturation analysis of agonist-binding curves [22] or RIS analysis of cerebral membranes in the absence of exogenous Mg^{2+} [23] result in non-linear radiation inactivation profiles, with an increase in agonist binding occurring at low doses. These difficulties could be overcome in the present study by using a high-affinity antagonist radioligand, [^{3}H]DPCPX, which does not distinguish between the agonist high- and low-affinity states [14]. Thus, [^{3}H]DPCPX binding could readily be measured in the presence of the GTP analogue, GTPγS. Under these conditions, the RIS estimated for the antagonist-binding component was found to decrease from 58 kDa for control membranes to 33 kDa, which is in excellent agreement with the value of approx. 35 kDa for the ligand-binding polypeptide determined after SDS-polyacrylamide gel electrophoresis [4–6].

The reduction in RIS for [^{3}H]DPCPX binding in the presence of GTPγS indicates the dissociation of a G-protein such as G_i from the ligand-binding component and provides a structural analogy to the changes in agonist affinity observed in the presence of guanine nucleotides in binding studies [24]. The difference in RIS of about 25 kDa in the presence and absence of GTPγS is, however, insufficient to allow identification of the associated G-protein subunit. Indeed, caution should be exercised when interpreting RIS data in terms of molecular structure. As discussed by Beauregard et al. [8] the original interpretation of target size was based upon the assumption that a single ionisation results in the physical breakdown of a protein with consequent loss of its biological activity. In the case of oligomeric proteins, however, examples have been found where a differential loss of specific functional domains occurs, resulting in RIS values less than the size of the polypeptide [25,26]. The 25 kDa difference in RIS observed in this study may therefore be an underestimate of the true molecular size of the associated component. On this assumption, either the α- or β-subunit or the $\beta\gamma$ complex of the associated G-protein could account for the 25 kDa difference in RIS after GTPγS treatment.

A further complication in the interpretation of the RIS in terms of a radiation-induced breakdown of protein structure is revealed by the data obtained with the covalent agonist-receptor complex. Irradiation of photoaffinity-labelled membranes yielded a target size of 35 kDa based upon the destruction of a 35 kDa ^{125}I-labelled band after polyacrylamide gel electrophoresis. This coincidence between the target size and the electrophoretically derived molecular size indicates that loss of the 35 kDa ligand-binding component occurs independently of the transfer of destructive energy from the associated G-proteins which would lead to a greater target size. On the other hand, measurement of the loss of [^{3}H]DPCPX binding activity in the absence of GTPγS yielded an RIS of 53 kDa. Since the membranes were irradiated in the absence of adenosine deaminase and the A_1 receptors were therefore presumably occupied by endogenous adenosine, it seems reasonable to suppose that, as in the case of the covalent agonist-receptor complex, no destructive energy transfer occurred from G-protein to receptor. This would seem to indicate, as suggested by Venter [9], that changes in tertiary structure of the receptor as a result of destruction of the G-protein might be sufficient to influence the RIS based upon measurement of biological activity.

In conclusion, the reduction of the RIS for [^{3}H]DPCPX binding in the presence of GTPγS strongly suggests the structural association of A_1 adenosine receptors with G-protein components in brain membranes in situ. The nature of the G-protein subunit cannot be inferred from the GTPγS-induced shift in RIS. On the basis of current models of receptor-G protein interactions, the

association with an α-subunit seems most likely [27]. However, solely on consideration of the RIS values, the possibility of the β-subunit being involved cannot be ruled out. Clearly, however, incubation of brain membranes with guanine nucleotide leads to changes in the structure and function of the A_1-receptor/G-protein complex.

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