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CHARACTERIZATION OF SPECIFIC BINDING SITES FOR [^{3}H]2-MeS-ADP ON MEGAKARYOCYTOBLASTIC CELL LINES IN CULTURE

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Abstract—Binding of [^{3}H]2-methyl thio-adenosine 5′ diphosphate ([^{3}H]2-MeS-ADP), a stable analogue of adenosine 5′ diphosphate (ADP) to DAMI and Meg-01, two megakaryocytoblastic cell lines, was time-dependent, reversible and saturable. Scatchard analysis of the saturation binding data indicated that [^{3}H]2-MeS-ADP bound to one class of specific binding sites with high affinity (dissociation constants $=45.3 \pm 13.4$ and 48.2 ± 17.7 nM, and maximum binding capacities $=341.2 \pm 31.1$ and 903 ± 98 fmole/10^6 cells for DAMI and Meg-01, respectively) (N = 3). Unlabelled 2-MeS-ADP competitively and selectively inhibited the specific binding of [^{3}H]2-MeS-ADP on DAMI and Meg-01 with inhibitory constant values of 118 ± 11 and 38 ± 11 nM, respectively (N = 3). ADP was 3 to 10 times less potent than 2-Mes-ADP in displacing [^{3}H]2-MeS-ADP from its binding sites on DAMI and Meg-01, whereas other ADP analogues, such as AMP, GDP, UDP, adenosine or FSBA, did not interfere with the binding of [^{3}H]2-MeS-ADP, suggesting that DAMI and Meg-01 contain ADP-specific receptors.

Key words: megakaryocyte; ADP; receptor; platelets; binding

Along with several other mediators, it has been proposed that ADP† is intimately involved in the etiology of thrombosis and it is anticipated that the development of potent and specific ADP receptor antagonists may offer a rational and novel therapeutic approach to this disease [1]. But, although one of the major impacts of ADP is the platelet, no ADP binding site has been identified so far on these cells. Among several works which tentatively described the binding characteristics of various ligands on platelets, this group, and others, recently showed that [^{3}H]2-MeS-ADP, a stable analogue of ADP, specifically bound to one class of receptor sites on the surface of these cells [2, 3]. This ligand, which showed high affinity and selectivity for ADP receptor sites on platelets, behaved as an invaluable experimental tool with which the exact mechanism of action of ticlopidine and clopidogrel, two well-known antiaggregating compounds, could be elucidated [2, 3]. Further to these studies the aim was to find out if megakaryocytes, the progenitor cells of platelets, also presented [^{3}H]2-MeS-ADP binding sites. In this paper the binding properties of [^{3}H]2-MeS-ADP and its use in demonstrating binding sites on cultured megakaryocytoblastic cells lines, are described.

MATERIALS AND METHODS

Chemicals. 2-MeS-ADP was purchased from RBI (Bioblock, Illkirsh, France). [^{3}H]2-MeS-ADP (6 Ci/mmol) was from Dositek (Orsay, France). FSBA was from Sigma (L'Ille d'Abeau, France). All other nucleotides were purchased from Boehringer Mannheim (Meylan, France).

Culture of megakaryocytoblastic cells. DAMI and Meg-01 cells were kind gifts from J.P. Caen and Z.C. Han (IVS, Paris). Cells were routinely cultured in RPMI-1640 culture medium supplemented with 10% foetal calf serum, glutamine (4 mM), penicillin (100 IU/mL), amphotericin B (0.5 μg/mL), nystatin (100 U/mL) and streptomycin (0.1 mg/mL) in 75 cm^2 culture flasks (Nunc, Denmark) which were incubated at 37° in a 95% air–5% CO_2 humidified atmosphere. Culture medium was removed every other day and cells were subcultured by centrifugation (200 *g*, 10 min).

Binding of [^{3}H]2-*MeS-ADP to megakaryocytoblastic cells.* Experiments studying the specific binding of [^{3}H]2-MeS-ADP to DAMI or Meg-01 cells, and its inhibition by ADP-receptor antagonists were performed on non-adherent cells. Cells were incubated in a 96-well microplate ($\sim 5 \times 10^6$ cells/well) in a total 0.2 mL volume of culture medium which contained [^{3}H]2-MeS-ADP (0–80 nM for saturation experiments, 100 nM for competition studies). Other substances were added in saline as indicated. Triplicate incubations were carried out at 37° for 60 min and were terminated by the addition of 3 mL of ice-cold assay buffer, followed by rapid vacuum filtration over glass-fibre filters (Filtermats 11734, Skatron Instruments Inc., Sterling, U.S.A.). Filters were then washed with 5 mL ice-cold incubation buffer, dried and the radioactivity measured by scintillation counting. Non-specific

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† Abbreviations: B_{max}, maximum binding capacity; BSA, bovine serum albumin; K_D, dissociation constant; K_i, inhibitory constant; ADP, adenosine 5′ diphosphate; 2-MeS-ADP, 2-methyl thio-adenosine 5′ diphosphate; FSBA, 5′-*p*-fluorosulphonylbenzoyl-adenosine.

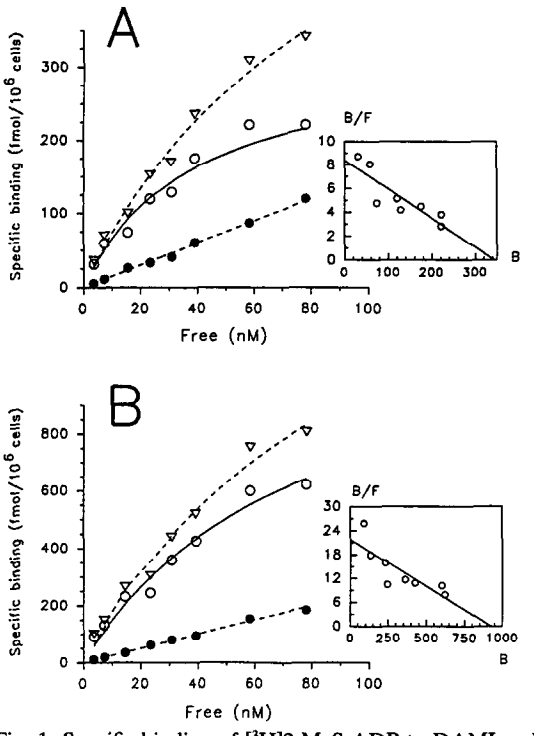

Fig. 1. Specific binding of [^{3}H]2-MeS-ADP to DAMI and Meg-01 cells. DAMI (A) and Meg-01 (B) cells (5×10^6 cells/well) were incubated for 60 min at 37° with increasing concentrations of [^{3}H]2-MeS-ADP (0–80 nM). Specific binding (○) was given by the difference between total (▽) and non-specific binding (●) determined in the presence of 1 mM of ADP. Insets: Scatchard plots of the specific binding of [^{3}H]2-MeS-ADP calculated from saturation isotherms. Each point is the average of results from at least three independent experiments performed in triplicate.

binding was defined as the total binding measured in the presence of excess unlabelled ADP (1 mM) and specific binding was defined as the difference between total binding and non-specific binding. The percentage inhibition was expressed as: $\%I$ = (total binding − total binding with antagonist)/specific binding × 100. The apparent K_D and B_{max} were calculated by using Scatchard representation of the experimental data [4]. Data from saturation, competition and association studies were analysed using a non-linear regression program [5].

RESULTS

Characteristics of [^{3}H]2-*MeS-ADP binding to DAMI and Meg-01 cells*

Specific binding of [^{3}H]2-MeS-ADP to both cell types at 37° was time-dependent and reached an equilibrium 40–60 min after the beginning of the incubation period (not shown). The specific binding of [^{3}H]2-MeS-ADP was totally reversible, since the addition of excess unlabelled ADP (1 mM) or 2-MeS-ADP (1 μM) dissociated [^{3}H]2-MeS-ADP from the cells. On the basis of this finding, equilibrium binding experiments were performed by setting the incubation time at 60 min. Figure 1 shows the dose-dependent binding of [^{3}H]2-MeS-ADP to DAMI and Meg-01 cells. The non-specific binding, as measured in the presence of an excess of unlabelled ADP (1 mM), was linearly-dependent on the concentration of [^{3}H]2-MeS-ADP. The specific binding, defined as the total amount of [^{3}H]2-MeS-ADP bound minus the non-specific binding, was saturable. The specific binding reached a maximum around 60 nM. At 25 nM of [^{3}H]2-MeS-ADP, the non-specific binding to both cell types varied between 5 and 10% of the total binding. A Scatchard analysis [4] of the bound/free ratio of the radiolabelled [^{3}H]2-MeS-ADP vs bound [^{3}H]2-MeS-ADP revealed the presence of only one class of binding sites (Fig. 1: insets). The population of sites exhibited high affinity with apparent K_D values of 45.3 ± 13.4 and 48.2 ± 17.7 nM, and B_{max} of 341.2 ± 31.1 fmol/10^6 cells (204,500 ± 38,300 sites/cell) and 903 ± 98 fmol/10^6 cells (567,000 ± 137,000 sites/cell) for DAMI and Meg-01, respectively. It is noteworthy that, under these experimental conditions, no degradation of the unlabelled ligand occurred. Indeed, incubation

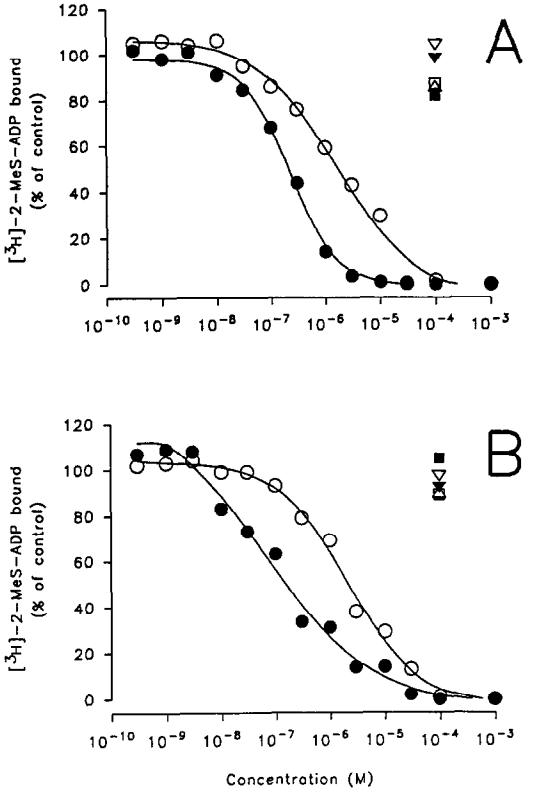

Fig. 2. Effect of ADP analogues on [^{3}H]2-MeS-ADP binding megakaryocytoblastic cells. DAMI (A) and Meg-01 (B) cells (5×10^6 cells/well) were incubated with 100 nM of [^{3}H]2-MeS-ADP for 60 min at 37° with increasing concentrations of 2-MeS-ADP (●), ADP (○), AMP (▼), GDP (▽), UDP (□), adenosine (▲) or FSBA (■). Each data point is the average of results from at least three independent determinations performed in triplicate.

Table 1. Comparative abilities of various ADP analogues to inhibit [^{3}H]2-MeS-ADP binding to DAMI and Meg-01 cells

Compounds	K_i (μM) DAMI	K_i (μM) Meg-01
2-MeS-ADP	0.118 ± 0.011	0.038 ± 0.011
ADP	0.333 ± 0.075	0.420 ± 0.073
AMP	>100	>100
GDP	>100	>100
UDP	>100	>100
Adenosine	>100	>100
FSBA	>100	>100

Values are means ± SD obtained from at least three independent experiments performed in triplicate.

at 4° with or without sodium azide (0.1%) did not significantly modify the binding characteristics of [^{3}H]2-MeS-ADP for either cell type (not shown).

Inhibition of [^{3}H]2-*MeS-ADP binding to DAMI and Meg-01 cells*

As shown in Fig. 2, unlabelled 2-MeS-ADP displaced, in a dose-dependent manner, [^{3}H]2-MeS-ADP specifically bound to high affinity receptor sites on both cell types. The concentrations required to reach IC_{50} were 642 ± 62 and 197 ± 58 nM (N = 3) for DAMI and Meg-01, respectively. When calculated from the Cheng and Prusoff equation [6], the K_i values for the specific binding of [^{3}H]2-MeS-ADP were 118 ± 11 and 38 ± 11 nM (N = 3) (Table 1). Under the same experimental conditions, ADP dose-dependently inhibited the specific binding of [^{3}H]2-MeS-ADP to both cell types with the K_i values shown in Table 1. In this respect, ADP was 3–10-fold less potent than 2-MeS-ADP in its ability to interfere with the binding of [^{3}H]2-MeS-ADP to both cell types. For 2-MeS-ADP and ADP, the Hill slope factors (nH) were near unity (data not shown), suggesting a bimolecular reaction and indicating that the inhibition of [^{3}H]2-MeS-ADP binding by these compounds was due to an interaction with the receptor itself. Other ADP analogues, including AMP, GDP, UDP, adenosine and FSBA, showed no effect on the specific binding of [^{3}H]2-MeS-ADP to DAMI and Meg-01 (Fig. 2, Table 1).

DISCUSSION

In the past few years the biochemical characteristics of specific ADP binding sites on platelets have been described by using radioligand techniques [7–12] but, to date, no ADP binding sites on megakaryocytes, the progenitor cells, have been identified. In this paper the specific binding of [^{3}H]2-MeS-ADP, a stable analogue of ADP to DAMI and Meg-01, two megakaryocytoblastic cell lines in culture, is described. These cells are known to express megakaryocytic phenotype characteristics such as glycoprotein GpIIb–IIIa complex, GpIb and CDW-14 antigens and not to be recognized by various antibodies specific for epitopes present on myeloid or lymphoid cells [13, 14]. Although intact cell preparations appear to be more suited for the physiological characterization of radiolabelled ADP binding sites, ADP can activate the cells, undergo degradation to inactive metabolites, such as AMP or adenosine, or be taken up into the cells. The radiolabelled-ADP binding procedure is therefore characterized by highly non-specific binding and intersubject variability. For these reasons, specific ADP analogues such as [^{3}H]2-MeS-ADP, [β^{32}P]-2-MeS-ADP or [β^{32}P]AzPET-ADP have been described as useful markers of ADP receptor sites on intact platelets [2, 9, 10, 12].

The binding of [^{3}H]2-MeS-ADP to both megakaryocytoblastic cell lines showed high affinity, specificity and saturability. These ADP receptors appeared to be of a single class and exhibited high affinity and high binding capacity. The K_D values were higher than those reported for the ligand on platelets [2]. Similarly, the binding capacity was 200–500-fold higher, but this can easily be explained by the difference in size between both cell types. This observation might be related to previous results showing that megakaryocyte cell lines are not equally responsible to agonists as platelets [15].

In conclusion, the presence of high-affinity ADP binding sites on the surface of megakaryocytoblastic cell lines have been demonstrated for the first time. These sites could be labelled by [^{3}H]2-MeS-ADP, which is considered one of the most potent agonists of ADP receptors on platelets yet described [2]. Its unique feature of having a higher affinity for ADP receptors than unlabelled ADP itself makes it a very interesting tool for furthering the understanding of the mediating role of ADP in certain physiological and pathological situations. Nevertheless, work is now needed to determine if megakaryocytes within the bone marrow also exhibit such binding sites.

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