Adenosine transporters in chromaffin cells

Quantification by dipyridamol monoacetate

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Chromaffin cells from bovine adrenal medulla are a useful model to approach adenosine transport and metabolism in neural cells. Dipyridamol has been shown to be an adenosine transport inhibitor with high affinity. To quantify the adenosine transporters a labelled dipyridamol analogue, [^{14}C]dipyridamol acetate, was synthesized. This compound had a K_i = 5.3 ± 0.43 nM according to the Dixon method, and 4.58 ± 0.46 nM when the receptor number molarity was taken into account showing, like dipyridamol, a non-competitive mechanism. The high-affinity receptors present in chromaffin cells showed a K_d = 6.8 ± 0.8 nM and the receptor number was 630000 ± 40000 per cell.

Adenosine transport *Chromaffin cell* *Dipyridamol*

1. INTRODUCTION

Chromaffin cells from bovine adrenal medulla are a useful model to approach basic neural problems [1–6]. Due to both the increasing importance of adenosine as a neural modulator [7,8] and the necessary purine ring recovery when ATP is released by exocytosis from these cells the transport, incorporation and metabolism of adenosine have been recently studied in this tissue [9–11]. Only one high-affinity adenosine transporter has been shown in recently isolated or cultured chromaffin cells; dipyridamol being a non-competitive transport inhibitor in the nM range [12]. This compound has been used extensively for clinical purposes and has also been shown to be a good inhibitor in other neural and non-neural cells [13,14]. The present possibility of quantifying adenosine transporters could prove to be the first step in studying the effects of hormonal or neural factors on subcellular distribution and its regulation, as is the case with glucose transporters [15,16]. Thus, a dipyridamol analogue, dipyridamol monoacetate, was synthesized, studied for its inhibitory capacity and, as a labelled derivative, used for binding in order to quantify the adenosine transporters in recently isolated chromaffin cells.

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2. MATERIALS AND METHODS

2.1. *Materials*

Collagenase (EC 3.4.24.3), Dulbecco's modified Eagle's medium and dipyridamol were purchased from Sigma; Percoll from Pharmacia; [2,5′,8-^{3}H]adenosine (45 Ci/mmol) from Amersham; [1-^{14}C]acetyl chloride (55 mCi/mmol) from New England Nuclear. Silicex 342 and silicex 334 were from Siliconas Hispania S.A.

2.2. *Synthesis of dipyridamol monoacetate*

Dipyridamol was acetylated at one of the alcohol functions using acetyl chloride in a 1:1 molar ratio. Both substances were dissolved in water-free chloroform, the mixture was then stirred at room temperture for 2 h and at the end of

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the reaction, HCl and chloroform were eliminated by evaporation.

Infrared spectra were obtained in Nujol emulsion and analyzed in a Nicolet FT-5DX.

When labelled derivative was necessary, [1-^{14}C]acetyl chloride (55 mCi/mmol) was used in the same reaction conditions.

The mass spectrum was recorded using a Hewlett-Packard 59993 GC/MS system. Thin layer chromatography was carried out in alumina precoated plastic sheets with benzene/ethanol 1:1 (by vol.).

2.3. *Isolation of chromaffin cells*

Chromaffin cells were isolated from bovine adrenal glands essentially according to Miras-Portugal et al. [9]. The cells were isolated by collagenase action and purified through a Percoll gradient, carefully collected and washed with Ca^{2+}, Mg^{2+} free Locke's solution. Finally, all cells were suspended in Dulbecco's modified Eagle's medium (DMEM). Only preparations with a cellular viability over 90% were employed.

2.4. *Adenosine transport studies*

Recently isolated chromaffin cells were always maintained in DMEM for at least 2 h before any experiment was performed, in order for the membranes to be restored; this being considered necessary due to other proteases present in commercial collagenases.

Assays of adenosine transport were carried out by the method of Paterson and co-workers [17] and modified by Miras-Portugal et al. as described [12]. Essentially, 2×10^6 cells were incubated in 200 μl DMEM with [2,5′,8-^{3}H]adenosine (1 μCi) and non-labelled adenosine at the required final concentration. Transport was stopped at the indicated times by the addition of 5 μM dipyridamol, the cells were immediately layered over a silicone oil mixture (200 μl, d = 1.04) and centrifuged at 10000 × g in a Beckman microfuge. Cells were pelleted at the end of the tube, resuspended in Triton X-100 (1%) and their radioactivity counted.

2.5. *Binding assay*

Binding experiments with [^{14}C]dipyridamol monoacetate (55 mCi/mmol) were carried out with 4×10^6 cells in a final volume of 1 ml. The ligand concentration ranged from 1.14 to 114 nM; equilibrium being reached after 15 min. The cells were then centrifuged over 300 μl silicone oil mixture as described, and the bound form (pellet) and free form (supernatant) were counted.

All values in the text are given as means ± SD. Linear regression equations were calculated by the least-squares method using an Olivetti M-20 computer with a linear regression program.

3. RESULTS

3.1. *Synthesis of dipyridamol monoacetate*

In fig.1, infrared spectra of dipyridamol (A) and its acetate derivative (B) are shown. A characteristic acetate band can be observed at 1740 cm^{-1}, the free hydroxyl groups having

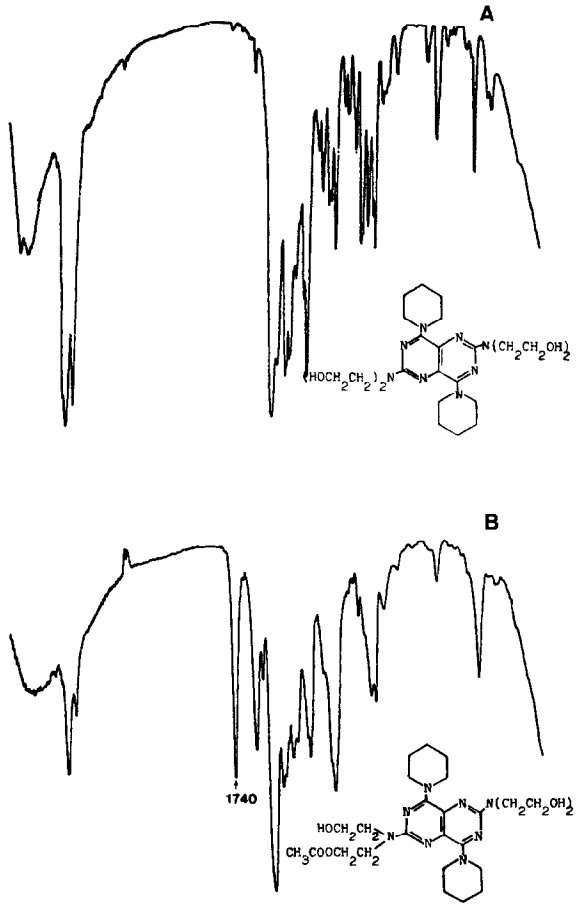

Fig.1. Infrared spectra of dipyridamol (A) and dipyridamol monoacetate (B). Infrared spectra were obtained in Nujol emulsion and analyzed in a Nicolet FT-5DX.

diminished. The mass spectrum (EI) confirmed the monoacetylated derivative by the presence of an expected molecular ion peak at m/z 546. The purity of the compound was checked by thin layer chromatography, only one molecular form being observed.

3.2. *Dipyridamol monoacetate inhibition of adenosine transport*

Dipyridamol monoacetate is a good inhibitor of adenosine transport, as shown in fig.2, its effects being greater than those obtained by the same dipyridamol concentration. Adenosine transport was measured at short periods of time (5–60 s) because, at these times, no significant synthesis of adenine nucleotides from external adenosine took place in these cells [12]. The high affinity of dipyridamol acetate for adenosine transporters is shown in fig.3, where a classical Dixon plot can be seen; the K_i obtained was 5.3 ± 0.43 nM, showing a non-competitive mechanism at the adenosine transport level. The K_i value was also determined

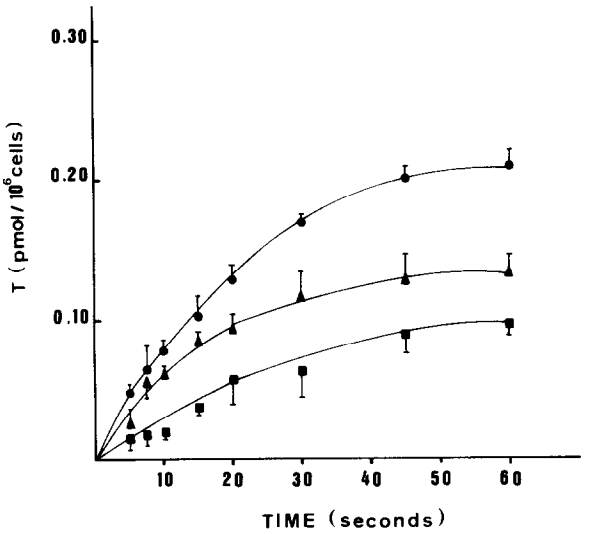

Fig.2. Effect of dipyridamol and dipyridamol monoacetate on adenosine transport by recently isolated chromaffin cells. 2 × 10^6 cells were incubated in the absence (●) or presence of 10 nM dipyridamol (▲) and 10 nM dipyridamol monoacetate (■) in a final volume of 200 μl DMEM for 5 min. Adenosine transport was measured by adding 0.1 μM [2,5′,8-^{3}H]adenosine (1 μCi essay). T represents the adenosine transport expressed in pmol/10^6 cells. The data points are means (± SD) of 3 experiments in triplicate.

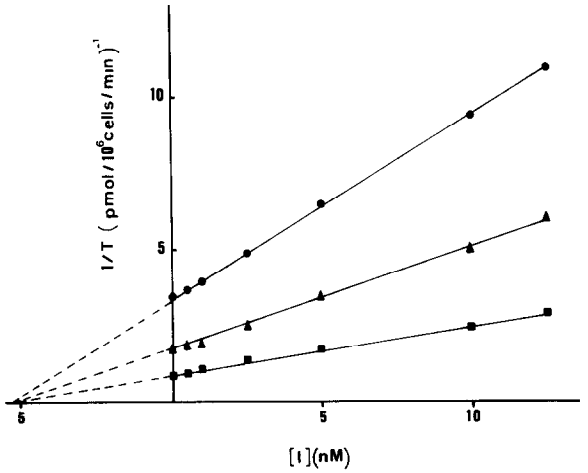

Fig.3. Dixon plots of dipyridamol monoacetate inhibitory effect on adenosine transport by recently isolated chromaffin cells. As before, cells were preincubated with variable concentrations of dipyridamol monoacetate for 5 min. 1 μCi [2,5′,8-^{3}H]adenosine and non-labelled adenosine were then added to reach 0.1 μM (●), 0.5 μM (▲) and 1 μM (■), this being the final concentration. T represents the adenosine transport measured over a linear range period and expressed in pmol/10^6 cells per min. [I] represents the nM concentration of dipyridamol monoacetate. This figure is typical of results obtained from 3 different experiments in triplicate, the correlation coefficients being $r = 0.999$ for 0.1 μM adenosine, $r = 0.996$ for 0.5 μM adenosine, and $r = 0.985$ for 1 μM adenosine.

taking into account the total transporter number ($AdoT_T$) obtained from fig.4 and resolving the Henderson equation [18] for reversible non-competitive inhibitors with high affinity, $[I_T]/[1 - (t_i/t_o)] = K_i(t_o/t_i) + (AdoT_T)$, t_o and t_i being the transport values from fig.2. The K_i obtained was 4.58 ± 0.46 nM, without significant changes during a 20–60 s period, once transport had started.

3.3. *Adenosine transporter quantification*

Adenosine transporter quantification was accomplished by the labelled ligand technique, using [^{14}C]dipyridamol monoacetate, as described in section 2. In fig.4, the Scatchard analysis of equilibrium is shown. A value of 630000 ± 40000 transporters/cell was obtained and the K_d was 6.8 ± 0.8 nM; this being very close to the K_i values obtained from the Dixon plot and the Henderson method [18].

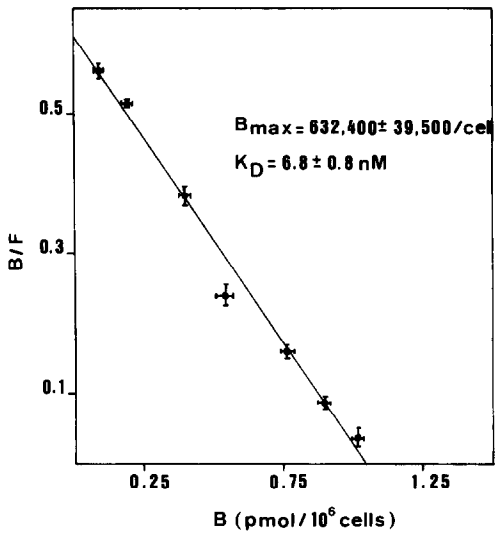

Fig.4. Scatchard analysis of equilibrium of [^{14}C]-dipyridamol monoacetate binding to recently isolated chromaffin cells. 4 × 10^6 cells were incubated in the presence of variable concentrations of [^{14}C]dipyridamol monoacetate (55 mCi/mmol) as described in section 2. This figure is the result obtained from 3 different experiments in triplicate.

4. DISCUSSION

The work reported here shows that adenosine transport in recently isolated chromaffin cells can be inhibited by a dipyridamol derivative, dipyridamol monoacetate, with the same or similar effectiveness (K_i = 5 nM) and a non-competitive mechanism as described for the non-modified compound. In other neural or non-neural cells, dipyridamol is also a good inhibitor of adenosine transport, although its effectiveness depends on the tissue under study [12–14,19–21]. It is, however, extensively used for clinical purposes, essentially in the cardiovascular system. Recently, a new adenosine transport inhibitor, nitrobenzylthioinosine, has been used to quantify these transporters, this presenting a high affinity and specificity for some adenosine transporters [8,22,23]. It could also prove interesting for later comparison of the transporter number obtained with these two inhibitors in the same cellular model. Dipyridamol monoacetate binding studies could furthermore be a tool used to understand the pharmacological action of this widely employed product. The high-affinity binding sites of dipyridamol monoacetate were about 600000/cell which corresponds to 9.5 pmol/mg protein, this value being about 70-times greater than that found by other authors in whole brain preparations employing nitrobenzylthioinosine [22]. This high adenosine transporter number could explain the effectiveness of these cells to transport adenosine [12] in order to (i) replenish the exocytotically released ATP [24,25], or (ii) avoid the inhibitory effects of adenosine upon secretion in chromaffin cells [26]. In chromaffin tissue, ectonucleotidases [26,27] can produce adenosine from the ATP released. In this way, transport can eliminate the inhibitory effects and also ATP recovery via a salvage pathway [10–12]. Nevertheless, we have no information at present on the distribution of the nucleoside transporters between plasma and internal membranes. Perhaps, as occurs with glucose transporters, a dynamic equilibrium exists between them [28–30], a neural or hormonal control of this equilibrium being possible [31]. Chromaffin cells could, thus, be a useful model for a neurobiological approach to adenosine action on neural tissues and also to evaluate the pharmacological action of dipyridamol.

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