12. Symington J, Electrophoretic transfer of proteins from two-dimensional gels to sheets and their detection. In: *Two-Dimensional Gel Electrophoresis of Proteins; Methods and Applications* (Eds. Celis JE and Bravo R), pp. 127–168. Academic Press, Orlando, 1984.
13. Johansson I, Ekström G, Scholte B, Puzycki D, Jörnvall H and Ingelman-Sundberg M, Ethanol-, fasting-, and acetone-inducible cytochromes P-450 in rat liver: Regulation and characteristics of enzymes belonging to the IIB and IIE gene subfamilies. *Biochemistry* **27**: 1925–1934, 1988.
14. Ingelman-Sundberg M and Jörnvall H, Induction of the ethanol-inducible form of rabbit liver microsomal cytochrome P-450 by inhibitors of alcohol dehydrogenase. *Biochem Biophys Res Commun* **124**: 375–382, 1984.
15. Elshourbagy NA and Guzelian PS, Separation, purification and characterization of a novel form of hepatic cytochrome P-450 from rats treated with pregnenolone-16α-carbonitrile. *J Biol Chem* **255**: 1279–1285, 1980.
16. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
17. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. *J Biol Chem* **239**: 2370–2378, 1964.
18. Ingelman-Sundberg M and Johansson I, The mechanism of cytochrome P-450-dependent oxidation of ethanol in reconstituted membrane vesicles. *J Biol Chem* **256**: 6321–6326, 1981.
19. Johansson I and Ingelman-Sundberg M, Carbon tetrachloride-induced lipid peroxidation dependent on an ethanol-inducible form of rabbit liver microsomal cytochrome P-450. *FEBS Lett* **183**: 265–269, 1985.
20. Wrighton SA, Thomas PE, Ryan DE and Levin W, Purification and characterization of ethanol-inducible human hepatic cytochrome P-450HLj. *Arch Biochem Biophys* **258**: 292–297, 1987.
21. Lasker JM, Raucy J, Kubota S, Bloswick BP, Black M and Lieber CS, Purification and characterization of human liver cytochrome P-450-ALC. *Biochem Biophys Res Commun* **148**: 232–238, 1987.
22. Ingelman-Sundberg M and Johansson I, Mechanisms of hydroxyl radical formation and ethanol oxidation by ethanol-inducible and other forms of rabbit liver microsomal cytochromes P-450. *J Biol Chem* **259**: 6447–6458, 1984.
23. Wrighton SA, Thomas PE, Molowa DT, Haniu M, Shively JE, Maines SL, Watkins PB, Parker G, Mendez-Picon G, Levin W and Guzelian PS, Characterization of ethanol-inducible human liver *N*-Nitrosodimethylamine demethylase. *Biochemistry* **25**: 6731–6735, 1986.
24. Raucy J, Fernandes P, Black M, Yang SL and Koop DR, Identification of a human liver cytochrome P-450 exhibiting catalytic and immunochemical similarities to cytochrome P-450 3a of rabbit liver. *Biochem Pharmacol* **36**: 2921–2926, 1987.
25. Morgan ET, Koop DR and Coon MJ, Comparison of six rabbit liver cytochrome P-450 isozymes in formation of a reactive metabolite of acetaminophen. *Biochem Biophys Res Commun* **112**: 8–13, 1983.

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Adenosine-A_1 receptors are not coupled to Ca^{2+} uptake in rat brain synaptosomes

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Adenosine (ADO) and analogues inhibit the release of neurotransmitters in the central nervous system and depress neuronal activity in a number of experimental models [1–4]. Adenosine exerts its effects via membrane bound receptors, which are generally divided into two subtypes, that are coupled to adenylate cyclase in an inhibitory (A_1 receptor) or a stimulatory (A_2 receptor) way. The relationship, however, between A_1 or A_2 adenosine receptor mediated modulation of adenylate cyclase and these calcium sensitive processes, is not clear. It has been proposed that the inhibition of presynaptic inward calcium currents is involved [3, 4].

From the structure activity relationships for the neurodepressing effects of ADO analogues, it can be deduced that an A_1-receptor-like subtype is involved [1]. As reports on this subject are not very consistent, the existence of a third receptor subtype was proposed, which is coupled to a calcium channel [5].

$^{45}Ca^{2+}$ uptake by synaptosomes can be measured for the investigation of calcium dependent effects of ADO. Wu *et al.* [6] and Ribeiro *et al.* [7] reported, that $^{45}Ca^{2+}$ uptake by synaptosomes is inhibited by ADO and analogues. Barr *et al.* [8], in contrast, were unable to demonstrate any influence of ADO on $^{45}Ca^{2+}$ uptake. However, it was not confirmed, whether the ADO analogues that were tested, actually bound to synaptosomes under the experimental conditions. In neither study the presence of endogenous ADO was taken into account. As synaptosomes are metabolically active [9], the ATP content of these preparations is high. Williams *et al.* [10] demonstrated that ATP interferes with binding of A_1 receptor ligands, due to metabolic breakdown of ATP to ADO. Consequently, endogenous ADO and ATP may compete with the effects of externally added ADO or analogues. Moreover, the incubation media contain ions, like Mg^{2+}, Ca^{2+}, Na^+ and K^+, that may interfere with ADO receptor binding [11, 12]. The availability of the tritiated, highly A_1-selective antagonist [^{3}H]DPCPX (1,3-dipropyl-8-cyclopentyl-xanthine) [13] enabled us to compare the effects of ADO analogues on $^{45}Ca^{2+}$ uptake with their binding to the A_1-receptor, represented by displacement of [^{3}H]DPCPX binding. When virtually identical preparations are used for both tests, differences in tissue penetration can be avoided and effects on $^{45}Ca^{2+}$ uptake and radioligand binding can be directly compared.

Methods

All solutions were made in distilled water and buffered with 10 mM Hepes, adjusted to pH 7.4 at the required temperature with Tris. The incubation medium contained (in mM) NaCl 140, KCl 5, $MgCl_2$ 1.2, Na_2HPO_4 1.2, $CaCl_2$ 0.1 or 1.0 and glucose 10. The wash buffer contained (in mM) NaCl 140 and KCl 5, the stop buffer NaCl 140, KCl 5 and EGTA 5. $^{45}Ca^{2+}$ uptake solutions had the same composition as the incubation medium, but contained *ca.* 10^6 cpm $^{45}Ca^{2+}$ (NEN, F.R.G.). For basal uptake the final KCl concentration was 5 mM, for KCl-stimulated uptake 30 mM.

Synaptosomes were prepared by a combination of differential and density gradient centrifugation [14]. Rat

(Wistar, CPB, Zeist, The Netherlands) brain was homogenized in icecold 0.3 M sucrose. The homogenate was centrifuged for 10 min at 1000 *g*. The P_2 fraction, obtained by centrifugation of the supernatant for 20 min at 9000 g, was resuspended in 0.3 M sucrose. This suspension was used as the top layer of a discontinuous sucrose gradient (1.2 (3 ml), 0.8 (5 ml) and 0.3 M (5 ml)). The gradient was spun for 30 min at 9000 g, the synaptosomes were harvested from the 0.8 M layer and the 0.8/1.2 M interface, and were slowly (in 30 min) diluted with a 10-fold volume of incubation medium. The synaptosomes were pelleted by centrifugation for 15 min at 9000 g, and kept on ice until use. Essentially the same results were obtained at the beginning and the end of an experiment.

For $^{45}Ca^{2+}$ uptake measurements, synaptosomes were resuspended in incubation medium and preincubated at 37° with or without the test agents in a total volume of 250 μl, to allow for the association of the ligands with the receptor. After 30 min, an equal volume of uptake solution was added. After incubation for a defined time (usually 5 sec) $^{45}Ca^{2+}$ uptake was stopped by addition of 5 ml icecold stop buffer and rapid vacuum filtration over prewashed Whatman GF/C filters. The incubation time was measured using a metronome. Filters were washed twice with 5 ml icecold wash buffer, dried and dissolved in 3.5 ml Optiphase MP (LKB, U.K.). The amount of $^{45}Ca^{2+}$ retained on the filters was determined by Liquid Scintillation Counting. Measurements were made in triplicate. When appropriate, significance of differences was tested with the *t*-test.

The binding assay for [^{3}H]DPCPX as described by Bruns *et al.* [13] was modified for use in synaptosomes at 37°. Prior to use the synaptosomes were, unless otherwise indicated, incubated for 30 min with 0.5 IU/ml ADO deaminase (Boehringer, F.R.G.). The binding reaction was initiated by addition of 100 μl preincubated synaptosomal suspension to 300 μl incubation medium, containing *ca.* 0.1 nM [^{3}H]DPCPX (Amersham, U.K., sp. act. 95 Ci/mmol) and other test agents. After incubation for 45 min at 37° the reaction was stopped by addition of 1 ml icecold wash buffer and rapid vacuum filtration over Whatman GF/C filters. Filters were washed twice with 3 ml icecold wash buffer. Specific binding was calculated by subtraction of nonspecific binding, measured in the presence of 10^{-5} M R-phenylisopropyladenosine (R-PIA, Boehringer, F.R.G.) from total binding.

Protein concentrations were determined by the method of Peterson [15].

Results and discussion

Equilibrium [^{3}H]DPCPX binding in the presence of ADO deaminase was reached within 10 min. Treatment with ADO deaminase increased specific [^{3}H]DPCPX binding 3–4-fold (Fig. 1), whereas nonspecific binding and filter binding were not affected. In the presence of ADO deaminase, R-PIA inhibited [^{3}H]DPCPX binding with an IC_{50} of 7 nM (Fig. 2). The displacement curve was shallow, in agreement with the concept of multiple binding states for agonists to the A_1 receptor [13].

From the data from Fig. 1 it can be argued that in the absence of adenosine deaminase a major part of the specific [^{3}H]DPCPX binding is displaced by endogenous ADO. It is difficult to relate this effect of endogenous ADO to a certain concentration as the affinity of ADO for the A_1 receptor is not known. As evident from Fig. 2, however, this concentration is equipotent with *ca.* 70 nM R-PIA. Considering the fact that 20 nM R-PIA is sufficient to depress calcium currents in slices [4], we initially contemplated that endogenous adenosine might exert tonic inhibitory effects on $^{45}Ca^{2+}$ uptake. In analogy to other cases, this effect might be blocked by including ADO deaminase or ADO antagonists in the incubation medium [4, 16, 17].

In the medium, containing 1.0 mM $CaCl_2$, $^{45}Ca^{2+}$ uptake, stimulated by 30 mM KCl was 4.3 ± 0.3 nmol/mg protein/5 sec (N = 8). $^{45}Ca^{2+}$ uptake could be stimulated by veratridine (10 μM), as well, which comprised about 40% of KCl-stimulated uptake.

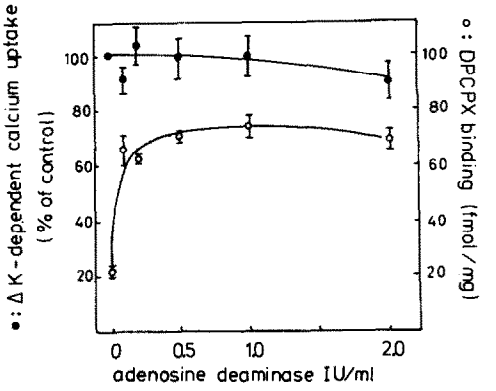

Fig. 1. The effect of ADO deaminase on specific [^{3}H]DPCPX binding (○) and Ca^{2+}-uptake (●) by synaptosomes. 100% corresponds to 4.3 ± 0.3 nmol/mg protein/5 sec KCl-stimulated Ca^{2+}-uptake. [[^{3}H]DPCPX] was 0.11 nM. Represented are means ± SE from 3–4 experiments.

In preliminary experiments, ADO deaminase (2 IU/ml) seemed to inhibit $^{45}Ca^{2+}$ uptake to a great extent. This, however, was due to the presence of $(NH_4)_2SO_4$ in the initially used product. When another commercially available ADO-deaminase preparation, dissolved in 50% glycerol, was used, up to 2 IU/ml ADO deaminase did not influence $^{45}Ca^{2+}$ uptake (Fig. 1). Moreover, the ADO antagonists 8-phenyltheophylline (10^{-5}, 10^{-6} M) and CGS 15943 (10^{-5} M) [18] and the ADO uptake inhibitors dipyridamole and nitrobenzylthioinosine (10^{-5} M) were without effect on $^{45}Ca^{2+}$ uptake.

Subsequently, the ADO agonist R-PIA was tested. From Fig. 2 it was concluded, that 10^{-7} M R-PIA occupies most A_1 receptors. Under standard conditions (30 min preincubation with drug, 5 sec incubation with $^{45}Ca^{2+}$) this concentration R-PIA did not have any effect on basal or KCl-stimulated $^{45}Ca^{2+}$ uptake. Higher concentrations R-PIA (10^{-6}, 10^{-5} M) lacked also effect, as did 10^{-5} M 5′-N-ethyl-carboxamide-adenosine (NECA). It should be realized that A_2 mediated effects are unlikely to occur, as the A_2 receptor is present only in the striatum [19], where it comprises *ca.* 50% of the ADO receptor population. From the relative paucity of striatal tissue it can be estimated that only 3% of the ADO receptors in brain tissue are of the A_2 subtype.

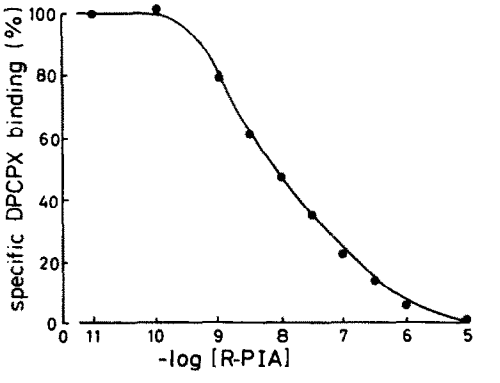

Fig. 2. Displacement of [^{3}H]DPCPX binding by R-PIA. Data are from a representative experiment.

Surmising that the preincubation or incubation conditions might not be optimal, we tried to evoke an effect by R-PIA under several experimental conditions.

(1) At a lower (0.1 mM) Ca^{2+} concentration: it is known that effects of ADO and other modulators of presynaptic release on neuronal activity can be antagonized by high calcium concentrations [20, 21].

(2) Different preincubation times (0, 1, 15 min).

(3) Different incubation times (1 sec up to 10 min). As reported by Nachshen [22] two phases of calcium entry can be distinguished: the fast phase, which is voltage dependent, lasts about 1 sec and is followed by a slow phase, in which calcium exchange mechanisms play an important role.

(4) In the absence of ADO deaminase and using a crude synaptosomal (P_2) fraction, as done by Wu *et al.* [6]. In this preparation dipyridamole and 8-phenyltheophylline lacked effect as well.

(5) With veratridine (10^{-5} M) as stimulator instead of KCl, to evoke depolarization by Na^+-influx.

(6) In the presence of GTP (10^{-4} M), in case activation of a G-protein would be essential.

Under all of these circumstances R-PIA lacked effect on basal or stimulated $^{45}Ca^{2+}$ uptake.

A relatively simple explanation for the lack of effect could be that ADO receptors controlling Ca^{2+} uptake are present only in a subset of the synaptosomes. This, however, does not explain the discrepancies between the results of Barr *et al.* [8] or our group vs Wu *et al.* [6] and Ribeiro *et al.* [7]. The question remains, whether the receptor–effector coupling is impaired in synaptosomes or whether ADO and $^{45}Ca^{2+}$ uptake in nerve endings are not related at all. Our results may support Silinsky's [23] point of view, that in the neuronal cell, ADO analogues influence the sensitivity for calcium rather than the calcium concentration.

Summarizing, we state that although adenosine A_1 receptors are present in synaptosomes, it is unlikely that they are linked to functionally intact voltage sensitive calcium channels.

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REFERENCES

1. Dunwiddie TV, Worth TS and Olsson RA, Adenosine analogs mediating depressant effects on synaptic transmission in rat hippocampus: structure activity relationships for the N^6 subregion. *Naunyn-Schmiedeberg's Arch Pharmacol* **334**: 77–85, 1986.
2. Fredholm BB and Lindgren E, Effects of N-ethylmaleimide and forskolin on noradrenaline release from hippocampal slices. Evidence that prejunctional adenosine and α_2 receptors are linked to N-proteins but not to adenylate cyclase. *Acta Physiol Scand* **130**: 95–105, 1987.
3. Scott RH and Dolphin AC, Inhibition of calcium currents by an adenosine analogue 2-chloroadenosine. In: *Topics and Perspectives in Adenosine Research* (Eds. Gerlach E and Becker BF), pp 449–558. Springer Verlag, Berlin, 1984.
4. Schubert P, Heinemann U and Kolb R, Differential effect of adenosine on pre- and postsynaptic calcium fluxes. *Brain Res* **376**: 382–386, 1986.
5. Ribeiro JA and Sebastiao AM, Adenosine receptors and calcium: basis for proposing a third (A_3) adenosine receptor. *Prog Neuropharmacol* **26**: 179–209, 1986.
6. Wu PH, Phillis JW and Thierry DL, Adenosine receptor agonists inhibit K^+-evoked Ca^{2+} uptake by rat brain cortical synaptosomes. *J Neurochem* **39**: 700–708, 1982.
7. Ribeiro JA, Sa-Almeida AM and Namorado JM, Adenosine and adenosine triphosphate decrease $^{45}Ca^{2+}$ uptake by synaptosomes stimulated by potassium. *Biochem Pharmacol* **28**: 1297–1300, 1979.
8. Barr E, Daniell LC and Leslie SW, Synaptosomal calcium uptake unaltered by adenosine and 2-chloroadenosine. *Biochem Pharmacol* **34**: 713–715, 1985.
9. Bradford HF, Respiration in vitro of synaptosomes from mammalian cerebral cortex. *J Neurochem* **16**: 675–684, 1969.
10. Williams M and Braunwalder A, Effects of purines on the binding of [^{3}H]cyclopentyladenosine to adenosine A-1 receptors in rat brain membranes. *J Neurochem* **47**: 88–97, 1986.
11. Goodman RR, Cooper MJ, Gavish M and Snyder SH, Guanine nucleotide and cation regulation of binding of [^{3}H]cyclohexyladenosine and [^{3}H]diethylphenylxanthine to adenosine A_1 receptors in brain membranes. *Mol Pharmacol* **21**: 329–335, 1982.
12. Ukena D, Poeschla E and Schwabe U, Guanine nucleotide and cation regulation of radioligand binding to R_i adenosine receptors of rat fat cells. *Naunyn-Schmiedeberg's Arch Pharmacol* **326**: 241–247, 1984.
13. Bruns RF, Fergus JH, Badger EW, Bristol JA, Santay LA, Hartman JD, Hays SJ and Huang CC, Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn-Schmiedeberg's Arch Pharmacol* **335**: 59–63, 1987.
14. Hajos F, An improved method for the preparation of synaptosomal fractions in high purity. *Brain Res* **93**: 485–489, 1975.
15. Peterson GL, A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal Biochem* **83**: 346–356, 1977
16. Prestwich SA, Forda SR and Dolphin AC, Adenosine antagonists increase spontaneous and evoked transmitter release from neuronal cells in culture. *Brain Res* **405**: 130–139, 1987.
17. Fredholm BB, Jonzon B and Lindstrom K, Effect of adenosine receptor agonists and other compounds on cyclic AMP accumulation in forskolin-treated hippocampal slices. *Naunyn-Schmiedeberg's Arch Pharmacol* **332**: 173–178, 1986.
18. Williams M, Francis J, Ghai G, Braunwalder A, Psychoyos S, Stone GA and Cash WD, Biochemical characterization of the triazoloquinazoline CGS 15943, a novel, non-xanthine adenosine antagonist. *J Pharmacol Exp Ther* **241**: 415–420, 1987.
19. Bruns RF, Lu GH and Pugsley TA, Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. *Mol Pharmacol* **29**: 331–346, 1986.
20. Wessler I, Eschenbruch V, Halim S and Kilbinger H, Presynaptic effects of scopolamine, oxotremorine, noradrenaline and morphine on acetylcholine release from the mesenteric plexus at different stimulation frequencies and calcium concentrations. *Naunyn-Schmiedeberg's Arch Pharmacol* **335**: 597–604, 1987.
21. Dunwiddie TV, Interactions between the effects of adenosine and calcium on synaptic responses in rat hippocampus in vitro. *J Physiol* **350**: 545–559, 1984.
22. Nachshen DA and Blaustein MP, Some properties of potassium-stimulated calcium influx in presynaptic nerve endings. *J Gen Physiol* **76**: 709–728, 1980.
23. Silinsky EM, Inhibition of transmitter release by adenosine: are Ca^{2+} currents depressed or are the intracellular effects or Ca^{2+} impaired. *Trends Pharmacol Sci* **7**: 180–185, 1986.

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