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The orphan receptor cDNA RDC7 encodes an A1 adenosine receptor

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The extensive amino acid sequence conservation among G protein-coupled receptors has been exploited to clone new members of this large family by homology screening or by PCR. Out of four such receptor cDNAs we cloned recently, RDC7 corresponds to a relatively abundant transcript in the brain cortex, the thyroid follicular cell and the testis. We have now identified RDC7 as an A1 adenosine receptor. The A1 agonist CPA [N^6-cyclopentyladenosine] decreased by 80% cAMP accumulation in forskolin-stimulated CHO cells stably transfected with RDC7. Specific binding of another A1 adenosine agonist, [^{3}H]CHA [N^6-cyclohexyladenosine], was demonstrated on membranes from Cos cells transfected with a pSVL construct harbouring the RDC7 cDNA insert. The binding characteristics were similar to those of the natural brain A1 receptor. The recombinant and the natural receptors behaved also in the same way in displacement experiments involving a series of A1 adenosine agonists. The binding characteristics of RDC7 were compared to those of RDC8, another orphan receptor recently identified as an A2 adenosine receptor. The two molecular species RDC7 and RDC8 correspond clearly to the A1 and A2 receptor entities defined hitherto on a purely pharmacological basis.

Key words: A1 adenosine receptor/A2 adenosine receptor/G protein-coupled receptors/RDC7/RDC8

Introduction

Signal transduction by hormones, growth factors and neurotransmitters involves specific receptors which can be classified into families according to functional, pharmacological or evolutionary criteria. The G protein-coupled receptors constitute such a family of proteins encoded by genes with a common ancestor (Dixon *et al.*, 1986; Kubo *et al.*, 1986; O'Dowd *et al.*, 1989). They share a monomeric structure with seven putative transmembrane domains and the capacity to modulate the activity of effector enzymes or ion channels via GTP-binding proteins (Gilman, 1987; Spiegel, 1988; O'Dowd *et al.*, 1989). The evolutionary relationship of G protein-coupled receptors has been exploited to clone new members of this gene family by relying on their sequence similarities (e.g. see Fargin *et al.*, 1988; Libert *et al.*, 1989; Parmentier *et al.*, 1989). The result is the accumulation of a series of 'orphan' receptors awaiting the identification of their ligands (Libert *et al.*, 1989; Eva *et al.*, 1990; Hla and Maciag, 1990; Ross *et al.*, 1990).

A cloning strategy using the polymerase chain reaction with a couple of degenerate primers allowed us to clone four such orphan receptors from a thyroid cDNA library (Libert *et al.*, 1989). One of them, RDC8, was shown to encode a transcript present mainly in the central nervous system in the striatum, nucleus accumbens and olfactory tubercle (Schiffmann *et al.*, 1990). A systematic search among the ligands known to bind receptors present in these brain regions led to the identification and preliminary characterization of RDC8 as an A2 adenosine receptor (Maenhaut *et al.*, 1990).

Adenosine receptors are widely distributed in vertebrate tissues (Snyder, 1985; Stiles, 1986a). They have been classified into two main categories: A1 receptors inhibit adenylyl cyclase while A2 receptors stimulate it (Clark *et al.*, 1974; Prémont *et al.*, 1977; Van Calker *et al.*, 1979). Both types of receptors are implicated in regulatory phenomena in the central nervous system as well as the periphery. In particular, A1 receptors constitute a major class of presynaptic inhibitory receptors in the brain (Snyder, 1985; Fredholm and Dunwiddie, 1988). Both the peripheral and central actions of the widely used agents theophyllin and caffeine are thought to result from inhibition of adenosine receptors (Rall, 1990). In the present study, we provide evidence that the orphan receptor cDNA RDC7 (Libert, 1989) encodes an A1 adenosine receptor.

Results

Tissue distribution of RDC7 transcripts

Both RDC7 and RDC8 have been cloned from a dog thyroid cDNA library (Libert *et al.*, 1989). From the comparison of their primary structure, it was suggested that the two receptors constitute a subfamily with some characteristics in common: 51% identity in amino acid sequence in transmembrane regions, very short N-terminal domains devoid of N-glycosylation acceptor sites, and absence of the aspartate residue in the third transmembrane domain which is constantly present in receptors to charged amines (Strader *et al.*, 1988; Libert *et al.*, 1989). *In situ* hybridization experiments had demonstrated RDC7 mRNA in the brain, with preferential localization in cortical structures (Schiffmann *et al.*, 1990). The tissue distribution of RDC7 transcripts was further studied by Northern blotting of RNA from 10 tissues. Apart from the brain, the thyroid gland and, in lower amounts, the kidney and heart (Libert *et al.*, 1989; Schiffmann *et al.*, 1990), RDC7 transcripts were found in the testis (Figure 1). Within the thyroid, RDC7 mRNA

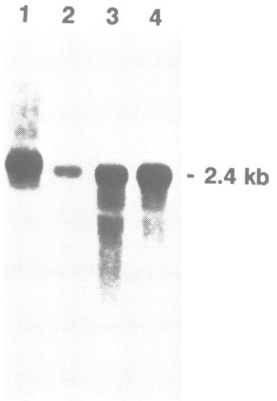

Fig. 1. Tissue distribution of transcripts corresponding to the A1 adenosine receptor cDNA (clone RDC7). A Northern blot of poly(A)$^+$ RNA (10 μg per lane) prepared from dog tissues was hybridized with ^{32}P-labelled RDC7 clone. The size of the transcript was deduced from ^{32}P-labelled lambda DNA marker digested with *Eco*RI. Tissues examined were brain (lane 1), testis (lane 2), thyroid (lane 3) and dog thyrocytes (lane 4) that had been cultured for 3 days in the presence of 10^{-5} M forskolin (Roger *et al.*, 1985). Exposure of the radioautograph was for 20 h with intensifying screen at −70°C.

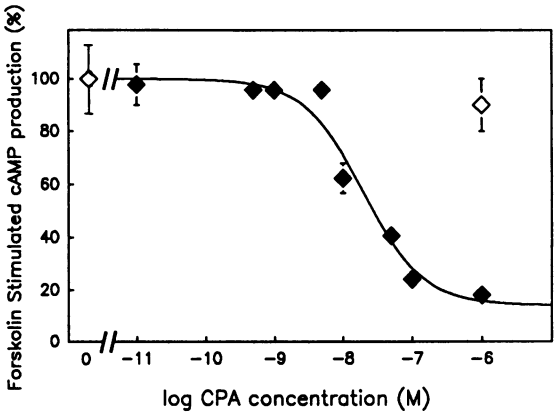

Fig. 2. Inhibition by CPA of forskolin-stimulated cAMP accumulation in a CHO cell line expressing stably the RDC7 receptor. Results are expressed as percentage of maximal stimulation of cAMP accumulation observed with 1 μM forskolin alone: control cells (◇); cells expressing RDC7 (◆). The basal levels of cAMP in Ro 20-1724 and adenosine deaminase treated cells (see Materials and methods) were 0.86 ± 0.1 pmol and 0.52 ± 0.12 pmol per tube for the selected clone RDC7/CHO and CHO cells stably transfected with pSV2NEO vector alone (control cells) respectively. Forskolin increased levels of cAMP to 62 ± 0.7 pmol and 74.2 ± 11 pmol per tube for RDC7/CHO and control cells respectively.

resides in the follicular cells as demonstrated by Northern blotting of RNA from thyrocytes in primary culture (Figure 1).

CPA inhibits cAMP accumulation in CHO cells expressing RDC7

Its similarity to RDC8, recently identified as an A2 adenosine receptor (Maenhaut *et al.*, 1990), together with its tissue distribution was compatible (Bruns *et al.*, 1986; Bergman *et al.*, 1986) with RDC7 being an A1 adenosine receptor. This hypothesis was tested in CHO cells stably transfected with RDC7 cDNA. As shown in Figure 2, the A1 adenosine agonist CPA [N^6-cyclopentyladenosine] inhibited by 80% forskolin-induced accumulation of cAMP in CHO cells expressing RDC7, as expected for an A1 adenosine receptor (Van Calker *et al.*, 1979). No significant inhibition of cAMP accumulation was observed in forskolin treated CHO cells transfected with the pSV2NEO vector alone.

RDC7 binds A1-specific adenosine agonists

The identification of RDC7 as a putative A1 adenosine receptor was explored further by assaying binding of the A1-specific agonist [^{3}H]CHA (N^6-cyclohexyladenosine) (Bruns *et al.*, 1980) to membranes from Cos-7 cells transfected with RDC7 cDNA (Figure 3A). Binding sites with the characteristics expected for an A1 receptor were demonstrated (see Table I; Bruns *et al.*, 1990; Ferkany *et al.*, 1986). No specific binding was detected in Cos-7 cells that have not been transfected with the pSVL/RDC7 construct or that have been transfected with the pSVL vector alone (see Materials and methods). In order to provide direct comparison with *bona fide* A1 adenosine receptors, binding experiments were performed in parallel with membranes from canine cortex (Figure 3C). The receptors in both preparations clearly presented similar characteristics (Table I). The A1 nature of these receptors and their identity were further demonstrated by displacement experiments with a series of agonists (Figure 3B,D; Table I) (Bruns *et al.*, 1980; Ferkany *et al.*, 1986). When compared with A1 receptors from different species for the order of potency of these ligands, RDC7 and the natural A1 receptor from dog brain are close to the guinea-pig A1 receptor (see Table I). Together with the effects on cAMP accumulation, these binding studies qualify RDC7 as an A1 adenosine receptor.

RDC8 encodes a pharmacologically distinct A2 receptor

To compare the RDC7 and RDC8 receptors and to define better the pharmacology of the latter, the binding characteristics of RDC8 expressed in Cos-7 cells were studied in parallel with those of the striatal A2 adenosine receptor (Bruns *et al.*, 1986). As shown in Figure 4, both membrane preparations displayed similar binding sites for the A2-specific ligand [^{3}H]CGS-21680 (Jarvis *et al.*, 1989) (Figure 4A,C and Table I). No specific binding was observed in untransfected Cos-7 cells or Cos-7 cells transfected with the pSVL vector alone (see Materials and methods). Displacement of the tracer was tested with the same series of adenosine agonists as used to characterize the RDC7−A1 receptor. The recombinant and natural receptors again behaved in a similar fashion (Figure 4B,D) and in agreement with the expectations for a high affinity A2 receptor (Bruns *et al.*, 1986; Jarvis *et al.*, 1989) (Table I).

Discussion

Among the available strategies leading to 'orphan' receptor identification, mere sequence comparison may provide a first useful hint. Out of four such receptors we cloned recently, RDC7 and RDC8 resemble more each other than any other member of the G protein-coupled receptor family. On this basis, they were suggested to constitute a new subfamily of receptors (Libert *et al.*, 1989). Among other characteristics they displayed a very short N-terminal (extracellular) extension lacking N-glycosylation sites. This misled us

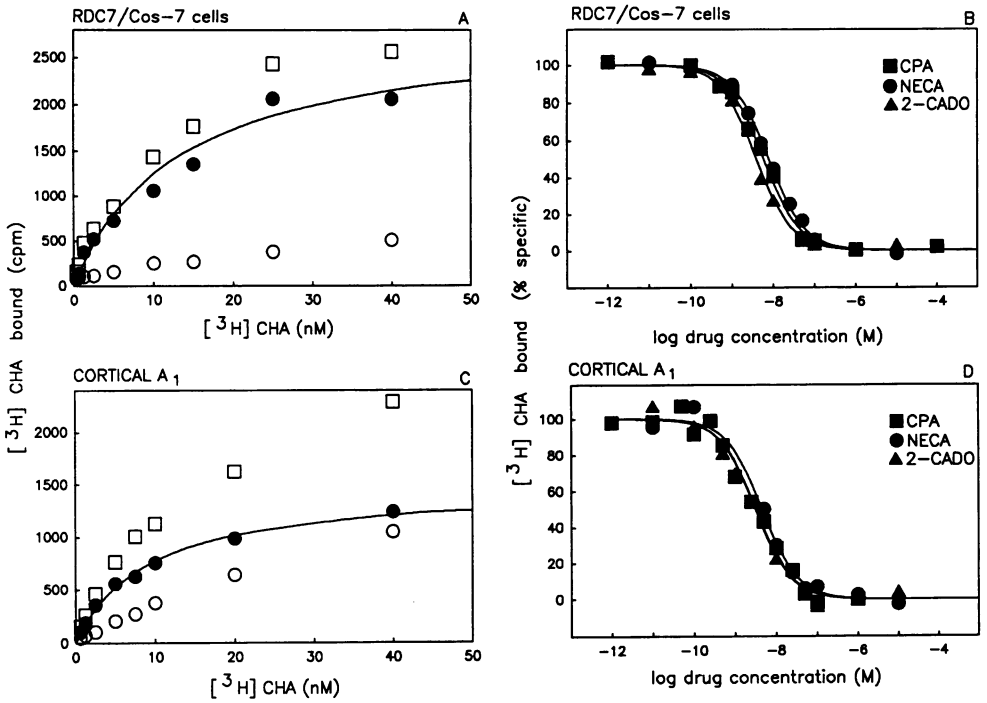

Fig. 3. Comparison of binding of [^{3}H]N^6-cyclohexyladenosine (CHA) to membranes prepared from Cos-7 cells transfected with RDC7 cDNA clone (**A**,**B**) or from dog cerebral cortex (**C**,**D**). Data are mean of duplicate determinations and curves are representative of at least two independent experiments. Both saturation and competition binding data were analysed by non-linear least-square curve fitting using a one-site model. Panels **A**,**C**: saturation of isotherms of the total (□), specific (●) and non-specific (○) binding (see Table I for K_d values). Panels **B**,**D**: competitive inhibition of [^{3}H]CHA specific binding by different adenosine agonists. The 50% inhibitory concentration values (IC_{50}) were computed and converted to K_i values (Cheng and Prussof, 1973) (see Table I).

Table I. Equilibrium binding constants and pharmacological profiles of the recombinant A1 and A2 canine adenosine receptors; comparison with the natural receptors of dog, rat and guinea-pig

	K_d (nM) [^{3}H]CHA	K_i (nM) [^{3}H]CHA		
		CPA	NECA	2-CADO
RDC7/Cos-7 cells	10.1 ± 2.6	3.54 ± 0.33	4.55 ± 0.67	2.34 ± 0.40
A1 (dog cortex)	6.9 ± 1.3	2.96 ± 0.75	4.17 ± 1.56	1.74 ± 0.01
A1 (rat cortex)[1]	1.49 ± 0.21	0.59 ± 0.02	6.26 ± 0.52	9.33 ± 0.58
A1 (guinea-pig)[2]	6.59 ± 1.67	–	6.83 ± 0.29	2.46 ± 0.41
	K_d (nM) [^{3}H]CGS-21680	K_i (nM) [^{3}H]CGS-21680		
		CPA	NECA	2-CADO
RDC8/Cos-7 cells	26.4 ± 3.4	1351 ± 91	26.4 ± 4.4	332 ± 66
A2 (dog striatum)	10.1 ± 2.5	613 ± 18	10.5 ± 2.6	66 ± 3.6
A2 (rat striatum)[3]	15.5 ± 2	890	12	120

Data are mean of duplicate determinations. The K_d and IC_{50} values were determined by non-linear least-square curve fitting using a one-site model, and K_i values were derived using the Cheng–Prussof relationship (Cheng and Prussof, 1973). Saturation and competition binding data for A1 [^{3}H]CHA binding assays in rat brain and guinea-pig membranes were from [1]Bruns *et al.* (1986) and [2]Ferkany *et al.* (1986) respectively. The data for [^{3}H]CGS-26180 binding assay in rat brain membranes were from [3]Jarvis *et al.* (1989).

to the conclusion that RDC7 and RDC8 would encode non-glycosylated receptors. We know now that, as adenosine receptors, they must be glycosylated (Stiles, 1986b; Barrington *et al.*, 1990; Nakata, 1990), which implies that the N-glycosylation acceptor sites in their second extracellular loop (Libert *et al.*, 1989) must be functional.

A second hint to receptor identification may be given by the tissue distribution of their transcripts. RDC7 and RDC8 were both abundant in the brain (Libert *et al.*, 1989; Schiffmann *et al.*, 1990). The striking restriction of RDC8 distribution to striatal medium-sized neurons (Schiffmann *et al.*, 1990) together with the finding that it promoted activation of adenylyl cyclase when expressed in various cell types (Maenhaut *et al.*, 1990), led to its eventual identification as an A2 adenosine receptor (Maenhaut *et al.*, 1990). Apart from the brain cortex (Schiffmann *et al.*, 1990), RDC7 transcripts are relatively abundant in the thyrocytes and in the testis (Libert *et al.*, 1989; Figure 1). While no saturable binding site for A1 adenosine ligands could be detected in the thyroid (Murphy and Snyder, 1981), A1 receptors have been demonstrated in thyrocytes by inhibition of cAMP production (Bergman *et al.*, 1986). The testis

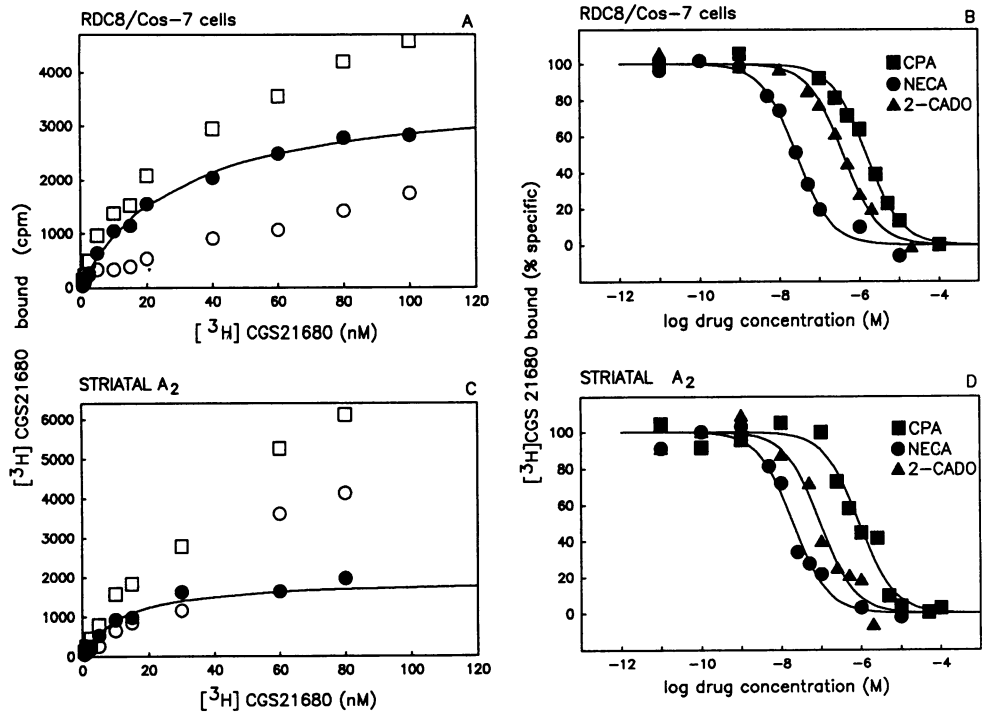

Fig. 4. Comparison of binding of [^{3}H]CGS-21680 to membranes prepared from Cos-7 cells transfected with RDC8 cDNA clone (**A**,**B**) or from dog striatum (**C**,**D**). Experimental points are mean of duplicate determinations and curves are representative of at least two independent experiments. The data were analysed as described in the legend to Figure 3. Panels **A**,**C**: saturation isotherms of the total (□), specific (●) and non-specific (○) binding (see Table I for K_d values). Panels **B**,**D**: competitive inhibition of [^{3}H]CGS-21680 specific binding by different adenosine agonists. The IC_{50} were computed and converted to K_i values (Cheng and Prussof, 1973) (see Table I).

is reportedly the second tissue after the brain for the concentration of A1 agonist binding sites (Bruns *et al.*, 1986). RDC7 was thus a good candidate for an A1 adenosine receptor. The lower amounts of its transcripts found in various tissues including the kidney and the heart (Libert *et al.*, 1989) were compatible with the ubiquity of A1 adenosine receptors as defined by their pharmacological effects (Rall, 1990).

Adenosine A1 and A2 receptors have been defined originally by their effects on adenylyl cyclase rather than by binding studies (Van Calker *et al.*, 1979). However, a clear distinction between two types of receptors has been provided by binding characteristics of the A1-specific agonist CHA (Bruns *et al.*, 1980) and, more recently, by the use of the A2-specific agonist CGS-21680 (Jarvis *et al.*, 1989). This led Hamprecht and Van Calker to propose a redefinition of the adenosine receptor subtypes (Hamprecht and Van Calker, 1985).

By both the old and the new criteria, RDC7 and RDC8 behave as typical A1 and A2 adenosine receptors, respectively. RDC7 expressed in Cos-7 cells displays a K_d for [^{3}H]CHA and K_is for CPA, NECA and 2-CADO in the same range as the natural canine receptor (Table I). When it is compared to the A1 receptor of the rat and guinea-pig for these parameters, it is definitely closer to the latter. In particular, its shares with the guinea-pig the characteristic that 2-CADO is more potent than NECA in displacing [^{3}H]CHA (Table I). This differentiates it from the A1 receptors in many species including man and the rat (Table I; Ferkany *et al.*, 1986). The K_d for [^{3}H]CGS-21680 of RDC8 expressed in Cos-7 cells is comparable to that of the canine and rat striatal A2 receptors (Table I). The rank order of potency for the tested agonists is typical of A2 receptors: NECA > 2-CADO > CPA (see Table I for comparison with the rat receptor, Bruns *et al.*, 1986). The K_ds of both RDC7 and RDC8 for their specific ligands are somewhat higher than that of their natural counterparts. This could be due to a different lipid environment in Cos-7 cells and brain membranes, or to differences in post-translational modifications (glycosylation?) of the receptors in the two systems.

RDC7 and RDC8 display the expected biological activities, in agreement with the earlier definition. RDC8 stimulates adenylyl cyclase when it is expressed in various cell types (Maenhaut *et al.*, 1990). The stimulation occurs in the absence of any added adenosine agonist, which implies activation of the receptor by ambient, endogenous adenosine (Maenhaut *et al.*, 1990). Further studies are required to appreciate the role of this 'constitutive' activation of the A2 receptor when it is expressed in its normal site. As expected for an A1 receptor, RDC7 expression in CHO cells leads to inhibition of forskolin-stimulated accumulation of cAMP following addition of CPA (Figure 2). It may be significant that A1 adenosine receptors or their transcripts (Figure 1) are relatively abundant in the testis and the thyroid. Both tissues are under the control of the evolutionarily related pituitary hormones (gonadotrophins and thyrotrophin, respectively) which act by increasing cAMP generation.

From all the above, it is concluded that RDC7 and RDC8 cDNA encode the main A1 and A2 adenosine receptor types which have been defined hitherto on a pharmacological basis. In addition, the mol. wts of the encoded polypeptides agree with those reported for the most purified receptor preparations [36 387 versus 34 000–38 000 and 44 971

versus 45 000 for the recombinant and natural A1 and A2 receptors, respectively (Barrington *et al.*, 1989; Nakata, 1989)].

The identification of A1 and A2 adenosine receptor cDNAs opens the way to a new pharmacology of their ligands and to the cloning of further receptor subtypes (Ribeiro and Sebastiao, 1986; Ali *et al.*, 1990). Considering the known implication of adenosine receptors in behavioural, metabolic, respiratory and cardiovascular problems (Snyder, 1985; Stiles, 1986a; Fredholm and Dunwiddie, 1988; Rall, 1990), the corresponding gene loci constitute interesting candidates for a series of genetic diseases. The genes for RDC7 and RDC8 have been localized. RDC7−A1 is on chromosome 22q11.2 (Libert *et al.*, 1991); two loci have been identified for the RDC8−A2 receptor: chromosome 11q12-13.1 and 10q26 (Libert *et al.*, 1991). Finally and more prosaically, one may expect new insight on the central and peripheral effects of the excitatory agents present in tea, coffee, cola and cocoa.

Materials and methods

Northern blot analysis

The total RNA from tissues of adult dogs and dog thyrocytes cultured for 3 days in the presence of 10^{-5} M forskolin (Roger *et al.*, 1985) were purified by the thiocyanate guanidinium method (Chirgwin *et al.*, 1979). Poly(A)$^+$ RNA was extracted by oligo(dT) cellulose chromatography as described (Maniatis *et al.*, 1990). Poly(A)$^+$ RNA samples (10 μg) were treated with glyoxal, fractionated on 1% agarose gel (McMaster and Carmichael, 1977) and transferred to Nylon membrane (Pall-Biodyne). Hybridization was performed with an RDC7 probe labelled with [α-^{32}P]dATP by the random priming procedure (Feinberg and Vogelstein, 1983) to a specific radioactivity of ~ 10^8 c.p.m./μg. The blot was incubated in the presence of 3 × 10^6 c.p.m./ml and 10% dextran sulphate, and washed under standard high stringency conditions (four washes in SSC × 0.1 SDS 0.1% at 60°C for 30 min).

Construction of RDC7 and RDC8 expression vectors

*Fnu*DII−*Xho*I and *Xba*I−*Nci*I restriction fragments corresponding respectively to the dog RDC7 and RDC8 cDNA (Libert *et al.*, 1989) were inserted in the pSVL vector (Pharmacia). These constructs contained 10 and 35 bases of 5′, and 281 and 288 bases of 3′ non-coding regions from the original RDC7 and RDC8 cDNAs, respectively.

Transfection of Cos-7 cells and binding studies

Cos-7 cells were transfected with RDC7- and RDC8-pSVL cDNA constructs using the DEAE−dextran method (Maniatis *et al.*, 1990). Seventy-two hours after transfection, cells were scraped and a 40 000 *g* particulate fraction was prepared (Bruns *et al.*, 1986). Membranes from dog brain were similarly prepared. Before storage, membranes were incubated for 30 min at 37°C in the presence of adenosine deaminase (Boehringer; 2.5 U/ml).

In saturation experiments performed with membrane preparations of RDC7/Cos-7 cells and cortical brain tissue, particulate fractions (100 μg protein) were incubated in the presence of 0.2 U/ml adenosine deaminase with increasing concentrations (0.625−40 nM) of [^{3}H]CHA (34.1 Ci/mmol, NEN) for 120 min at 25°C in a total volume of 500 μl. Non-specific binding was defined in the presence of 100 μM CPA. For competition binding experiments, membranes were incubated with 5 nM [^{3}H]CHA and various concentrations of adenosine agonists, N^6-cyclopentyladenosine (CPA), 5′-*N*-ethylcarboxamidoadenosine (NECA) and 2-chloroadenosine (2-CADO). Non-specific binding at 5 nM [^{3}H]CHA typically represented 10−15% of total bound radioactivity (Cos-7 cells: 850 c.p.m., cortex 1100 c.p.m.). For comparison, total binding to membranes from untransfected Cos-7 cells amounted to 125 c.p.m. Assays were terminated by rapid vacuum filtration followed by three washes with ice-cold Tris−Cl buffer (Bruns *et al.*, 1986).

Specific binding of [^{3}H]CGS-21680 to membrane preparations from Cos-7 cells transfected with RDC8 (80 μg protein) and dog striatum (150 μg protein) was measured using increasing concentrations (0.625−100 nM) of [^{3}H]CGS-21680 (48.1 Ci/mmol, NEN) for 90 min at 23°C in a total volume of 500 μl. For competition binding experiments, membranes were incubated with 5 nM [^{3}H]CGS-21680 and various concentrations of adenosine agonists (CPA, NECA, 2-CADO). Non-specific binding at 5 nM [^{3}H]CGS-21680 typically represented 10−15% on RDC8/Cos-7 cells membranes and 20−30% on striatal membranes. Total bound radioactivity: RDC8/Cos-7 cells 1800 c.p.m., striatum 900 c.p.m. For comparison, total binding to membranes from untransfected Cos-7 cells amounted to 250 c.p.m.

CHO cell transfection and cloning

CHO cells were co-transfected with a pSVL construct containing the coding region of the dog RDC7 cDNA and the plasmid pSV2NEO using a modified calcium phosphate precipitate method (Velu *et al.*, 1989). Neomycin resistant cell lines were selected in the presence of 0.4 mg/ml G418 and screened for RDC7 receptor expression by measuring specific binding of 5 nM [^{3}H]CHA to a membrane fraction, as described above.

Bioassay performed with CHO/RDC7

About 10^5 cells of a clone selected for its high expression level were seeded in glass tubes and further incubated for 48 h under standard culture conditions (Perret *et al.*, 1990). Control CHO cells were treated similarly. The cells were then exposed to 1 μM forskolin in the presence of 20 U/ml adenosine deaminase, 1 mM of the phosphodiesterase inhibitors Ro 20-1724 (gift from Hoffman-La Roche, Nutley, NJ, USA) and increasing concentrations of CPA for an incubation period of 20 min. The incubation was stopped by pouring off the medium and adding boiling water; cAMP levels were quantified by radioimmunoassay as described previously (Brooker *et al.*, 1979; Van Sande and Dumont, 1973).

Acknowledgements

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