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Selective inhibition of one of the cyclic AMP phosphodiesterases from rat brain by the neurotropic compound rolipram

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Among the novel neurotropic drugs which have been described in the past few years, the cyclic nucleotide phosphodiesterase inhibitor rolipram (4-[3-cyclopentyloxy-4-methoxyphenyl]-2 pyrrolidone) proves to be of major interest, as it induces in animals a peculiar profile of behavioral modifications [1, 2] and exhibits antidepressant potentialities, in animal trials [3, 4] as well as in preliminary clinical observations [3]. Interestingly, it induces specific increases in cyclic AMP level without affecting cyclic GMP level, in brain cortical slices [5]. Large increases in cyclic AMP level are also observable in several areas of the brain, after *in vivo* treatment of rats by rolipram [6]. *In vitro*, in crude preparation, it proves to be a rather potent inhibitor (in the micromolar range) of cyclic AMP-hydrolyzing, Ca^{2+}-independent phosphodiesterase of rat brain, while it weakly affects cyclic GMP-hydrolyzing phosphodiesterase [5]. In other tissues, such as in beef aorta [7], or in rat heart (our laboratory, unpublished results), it inhibits with a high selectivity the partially purified cyclic AMP-specific isoenzyme. Recently, Davis described the preferential effect of rolipram on the Ca^{2+}-independent fraction of rat brain cortex phosphodiesterase, separated by gel filtration and further cleared up of Ca^{2+}-dependent enzyme by passage on a calmodulin affinity column [8]. Or, gel filtration resolves only two peaks of activity from cerebral tissue [9–11], while other methods point out up to six forms of phosphodiesterase [11–15]. The Ca^{2+}-insensitive brain fraction might thus be constituted of several components with various sensitivities to rolipram. The isolation of an enzyme species particularly sensitive to rolipram inhibition would point it out as a possible candidate as the pharmacological target of the drug.

Materials and methods

Rat brains minus cerebella were homogenized in 9 vol. of 5 mM Tris–HCl pH 7.5, 0.32 M sucrose, in a Teflon–glass potter, and centrifuged for 1 hr at 105,000 *g*. Freshly prepared supernatant was submitted to isoelectric focusing procedure on Sephadex flat gel bed, as in [16]. Alternatively (membrane-bound enzyme studies), the tissue was homogenized in 9 vol. of 20 mM Tris–HCl pH 7.5, 1 mM $MgCl_2$, 0.1 mM dithiothreitol. A first 1000 *g* pellet was discarded. The supernatant was centrifuged for 1 hr at 105,000 *g*. The resulting pellet was washed one time and resuspended in the initial volume of the same buffer containing 1% non-ionic detergent Lubrol PX. The medium was submitted to sonication for 1 min (Branson Sonifier, 80 pulses, power 4), and centrifuged for 1 hr at 105,000 *g*. Nearly 80% of phosphodiesterase activity was found in the supernatant. This solubilized fraction was submitted to isoelectric focusing as in [16].

R_0 20-1724 was a generous gift of Hoffman–La Roche Co. (Switzerland); M & B 22.948 was kindly supplied by May and Baker Co. (U.K.) and Rolipram by Schering Laboratories (FRG). 2′-deoxy cylic AMP, 2′-deoxy cyclic GMP, Lubrol PX, calmodulin (P-0270), were purchased from Sigma Chemical Co. (U.S.A.)

Phosphodiesterase was assayed by the radioisotopic procedure of Thompson and Appleman [9], modified as described in [17]. Assay medium contained 100 mM Tris–HCl pH 8, 5 mM $Mg Cl_2$, 0.5 mg/ml bovine serum albumin, 0.25 μM cyclic nucleotide.

Results and discussion

As isoelectric focusing on granular gel plate proves an efficient technique for phosphodiesterase fractionation [16], 105,000 *g* supernatant of rat brain was submitted to this procedure in a 4–8 pH range. A reproducible pattern of phosphodiesterase activity was observed (Fig. 1 A,B). A group of peaks ranging from pI 4.9 to 5.4 hydrolyzed preferentially cyclic GMP. Two individualized cyclic AMP-hydrolyzing peaks focused at pI 5.6 and 6. The cyclic

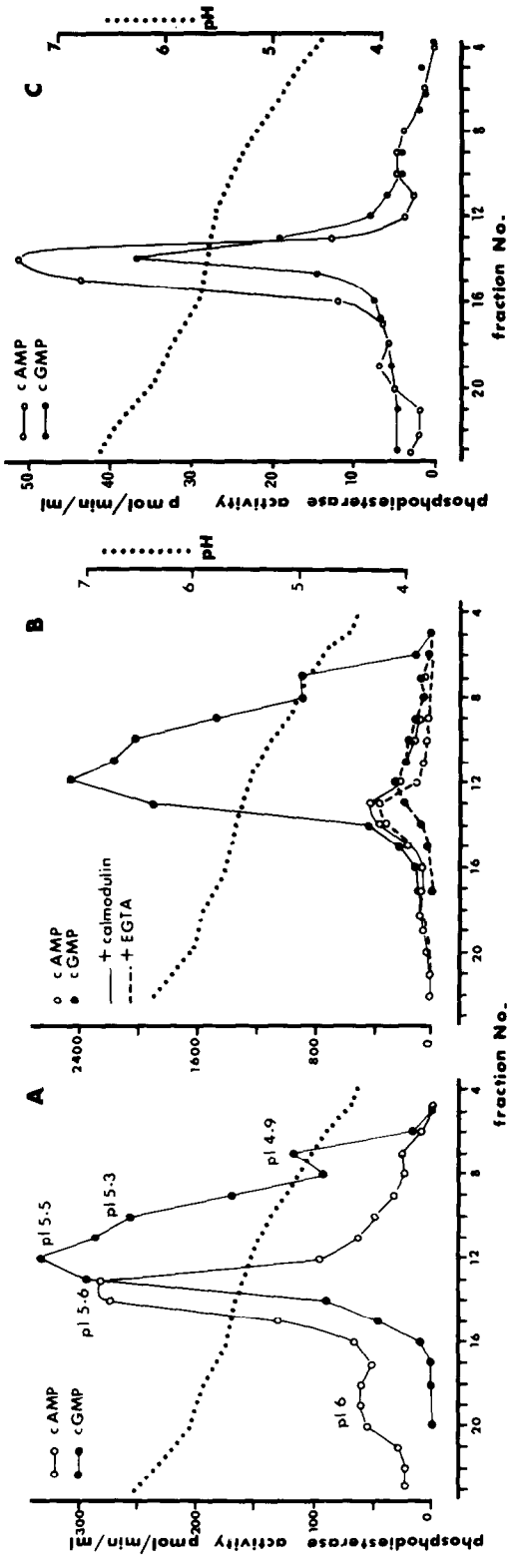

Fig. 1. Isoelectric focusing pattern of brain cytosol: (A) no addition; (B) the same fractions were assayed in the presence of 1 mM EGTA, or in the presence of 0.15 mM $CaCl_2$ and 100 units per assay of calmodulin; (C) fractions No. 13 to 15 of the experiment of Fig. 1A, B were pooled and submitted to a second isoelectric fractionation in the same conditions. Phosphodiesterase was assayed without calmodulin or EGTA addition.

GMP-hydrolyzing peaks proved strongly sensible to Ca^{2+}-calmodulin activation, while the two cyclic AMP hydrolyzing peaks were not (Fig. 1B). The pI 5.6 form was slightly activatable (+20–40%) by micromolar concentrations of cyclic GMP (not shown). As the pI 5.6 form was markedly contaminated by the neighbouring cyclic GMP-specific peaks, it was submitted to a second isoelectric focusing in the same conditions. This gave rise to a peak of the same isoelectric point, hydrolyzing more efficiently cyclic AMP than cyclic GMP (Fig. 1C). Subsequent studies on the pI 5.6 peak were performed with fraction no. 15 as it appears to be only slightly contaminated by the adjacent cyclic GMP hydrolyzing peaks.

Kinetic properties for cyclic AMP hydrolysis were investigated in peaks of pI 6 and 5.6. Both Lineweaver–Burk plots proved of the concave-downward type, with apparent extrapolated K_m values of 24 and 0.7 μM for the pI 6 form, and 43 and 1.7 μM for the pI 5.6 form. Hill representation of kinetic data allowed the calculation of $K_m = 6$ μM, N = 0.6 and $K_m = 14$ μM, N = 0.7 for the two forms respectively (not shown). No striking difference was thus observable between the two forms at this stage of the study.

The 105,000 *g* pellet from brain homogenate was also submitted to isoelectric focusing after solubilization of the major part of phosphodiesterase activity by non-ionic detergent treatment. The resulting profile is shown in Fig. 2. Briefly, two cyclic AMP-hydrolyzing peaks were focused at pI 5.9 and 5.6. An enzymatic form hydrolyzing both substrates was found at pI 5.5. A peak hydrolyzing preferentially cyclic GMP, and slightly sensible to Ca^{2+}-calmodulin activation was found at pI 5.3.

Inhibition studies were then undertaken in order to further characterize the cytosolic and particulate enzymatic forms (Table 1).

R_0 20-1724, which inhibits preferentially the cyclic AMP-specific isoenzyme in aorta [7], heart [18], lung [19], and the Ca^{2+}-independent fraction in brain [8], is found to be a potent inhibitor of pI 5.6 cytosolic peak. Compound M & B 22.948, a preferential inhibitor of cyclic GMP hydrolyzing phosphodiesterases in other tissues [7, 18, 19], shows here no marked specificity. Deoxy cyclic AMP, a specific inhibitor of Ca^{+}-independent fraction in brain [8] is active here both on the pI 6 and the pI 5.6 cytosolic forms. Deoxy cyclic GMP, which inhibits specifically Ca^{2+}-dependent form in

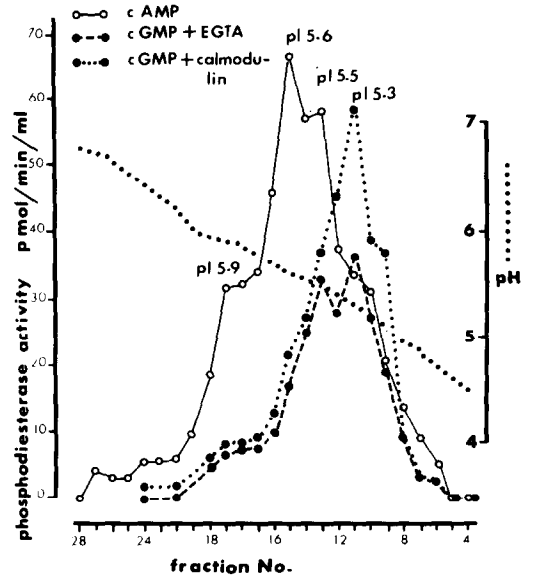

Fig. 2. Isoelectric focusing pattern of solubilized brain membrane fraction. Assay conditions were the same as in Fig. 1.

Table 1. Inhibition of various brain phosphodiesterase preparations by reference compounds and rolipram (I_{50} μM)*

	Enzyme preparations				
	Crude cytosol		Isoelectrically-fractionated cytosol		
Compounds tested	without EGTA	with EGTA	pI 6	pI 5.6	pI 5.3
Rolipram†	800	32	44	0.3	1000
Ro 20-1724			79	2	1000
M & B 22,948			36	47	29
2'-deoxy cAMP			6	13	316
2'-deoxy cGMP			660	1000	120
	Crude membranes		Isoelectrically-fractionated membranes		
	without EGTA	pI 5.9	pI 5.6	pI 5.5	pI 5.3
Rolipram†	63	110	23	91	91

* All the I_{50} values were determined with cyclic AMP as substrate. Isoelectrically-fractionated forms were assayed without calmodulin or EGTA addition.

† Rolipram was assayed in 1% dimethyl sulfoxide. Inhibition exerted by this latter compound on the various preparations was around 10%.

the brain [8] is only active on cytosolic pI 5.3 peak. Rolipram inhibits with a great potency the cytosolic pI 5.6 form (I_{50} = 0.3 μM). Its selectivity is shown by the fact that its potency is higher than those of R_0 20-1724 or deoxy cyclic AMP in pI 5.6 form and lower or equal on the other forms. The main difference between the two cytosolic cyclic AMP-hydrolyzing peaks is thus a greater sensitivity of the pI 5.6 form towards R_0 20-1724 and rolipram inhibition in our experimental conditions. This pI 5.6 cytosolic form is by far the most sensible to rolipram inhibition in the conditions employed, the I_{50} of the other isoelectric forms, from the cytosol as well as from the membrane fraction, being 100- to 1000-fold higher than the I_{50} of pI 5.6 form. The study of crude cytosolic preparations confirms the insensitivity of Ca^{2+}-dependent forms (assay without EGTA). There is also a large discrepancy between inhibition indexes measured with crude supernatant in the presence of EGTA and with the pI 5.6 peak. Indeed, the dose–response curve of inhibition of crude supernatant appears biphasic and reveals that the rolipram-sensible component of the activity is only of minor importance (not shown). In contrast, the rolipram-sensible peak is predominant on isoelectric pattern of cyclic AMP-hydrolyzing activity (Fig. 1A). One can thus suppose that this enzyme form is particularly stable during isoelectrofocusing procedure, while the others would be artefactually underestimated.

It must be noted that the dissociation constants observed for the binding of [3H]-rolipram to brain protein sites are in the 10^{-9} M range [20], i.e. two orders of magnitude lower than the inhibition index measured on partially purified pI 5.6 cytosolic form. Nevertheless, the strong activity of rolipram on one of the cerebral phosphodiesterase forms is consistent with the hypothesis implicating the selective inhibition of a cyclic AMP-specific phosphodiesterase in the mechanism of the neurotropic actions of this drug [1].

The isoelectric fractionation of cerebral phosphodiesterase allows the detection of numerous enzyme forms, only one of which, the cytosolic pI 5.6 form, being strongly inhibited by rolipram. This partially purified enzyme form hydrolyzes preferentially cyclic AMP, is insensitive to calmodulin effect, exhibits non-Michaelian negatively cooperative kinetics and could represent the pharmacological target of the neurotropic compound rolipram.

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Evidence that (+)[^{3}H]amphetamine binds to acceptor sites which are not MAO-A

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The existence in rat hypothalamus of two binding sites for (+)amphetamine, with high [1–3] and low [2, 3] affinity has been observed. More precisely only those of lower affinity were discussed and characterized in regard of amphetamine pharmacological profile: a role in anorexia was postulated [2]. In a previous paper [3] we have noticed that several monoamine oxidase type-A (MAO-A) inhibitors and especially harmaline displace (+)[^{3}H]amphetamine ([^{3}H]AMPH) from its binding sites. Since [^{3}H]harmaline was used to label the MAO-A site [4] and that this compound exhibited a very good affinity for the high-affinity site [3] (which was very close to the one reported by Nelson *et al.* [4] for the [^{3}H]harmaline binding), it was suggested that MAO-A could represent the [^{3}H]AMPH high-affinity site [3, 5]. This study was conducted in order to verify this hypothesis.

We have also examined the possibility that (+)amphetamine could bind to a monoamine carrier since this drug inhibits the uptake of dopamine and noradrenaline *in vitro* [6]. Finally regional and subcellular properties of rat brain [^{3}H]AMPH-binding sites, in various incubation mediums, were studied.

Materials and methods

(+)[^{3}H]Amphetamine sulfate (sp. act. 15.1 or 18.6 Ci/mmole), was purchased from New England Nuclear, Boston, MA. (+)Amphetamine sulfate was from Coopération Pharmaceutique Française. Chloroquine and harmaline were obtained from Serva (Heidelberg, F.R.G.) and Sigma Chemical Co. (St. Louis, MO), respectively. Desipramine, nomifensine and clorgyline were synthesized by the Department of Organic Chemistry, Centre de Recherche Delalande. 1-Deprenyl was a gift from Prof. J. Knoll, Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary. Other chemicals were of analytical grade and purchased from Merck (Darmstadt, F.R.G.).

Binding assay. Male Sprague–Dawley rats (150–200 g) were used. Brain regions were rapidly dissected on ice according to Glowinski and Iversen [7] and crude membranes were prepared as previously described [3]. Binding assay was conducted at 0°. Unless otherwise stated, 75 nM [^{3}H]AMPH was used. Specific binding was defined as the difference between the binding observed in the presence and in the absence of 10^{-2} M non radioactive (+)amphetamine sulfate. After 15 min incubation the ligand-receptor complex was separated by ultracentrifugation and the bound ligand was extracted according to Lesage *et al.* [3]. Incubation buffers were 50 mM Tris HCl, pH 7.4/500 mM NaCl/5 mM KCl or 50 mM Tris HCl, pH 7.4/5 mM EDTA or 10 mM Tris HCl, pH 7.4 supplemented with various sucrose concentrations. All assays were conducted generally in duplicate or triplicate (for pH and sucrose experiments). Unless otherwise stated, results are the mean of three independent determinations.

Subcellular fractionation. Intra- and extrasynaptosomal mitochondria and synaptic membranes were prepared from whole brain homogenates as described by Urwyler and Von Wartburg [8] using discontinuous density gradient of sucrose. Each fraction was resuspended in the Na^+/K^+ incubation medium (Ultraturrax, setting $\frac{3}{4}$ for 15 sec) and used immediately.

Data analysis. Data from competition experiments and saturation isotherms were analysed with a non-linear fitting program as essentially described by Munson and Rodbard [9]. In each case, models up to 3 binding sites were tested and the calculations were conducted on a 4052 Tektronix computer.

Protein concentrations were measured by the method of Lowry *et al.* [10] using bovine serum albumin as a standard.

Results and discussion

Specific [^{3}H]AMPH binding increased linearly with tissue concentration over the range of 0.1–1 mg of membrane protein. Assays were routinely conducted at 0.4 mg (final incubation volume 200 μl).

Results from [^{3}H]AMPH/harmaline displacement curves obtained with the three subcellular fractions are summarized in Table 1. Harmaline was chosen as displacer agent in regard of its high selectivity for MAO-A [4]. These experiments showed clearly that [^{3}H]AMPH-binding sites represent a complex mixture of sites since triphasic patterns were observed in intra- and extrasynaptosomal mitochondria (Fig. 1). Monoamine oxidase is a mitochondrial enzyme which the type-A, in contrast to the type-B form, is preferentially localized in nerve terminal mitochondria [8]. Thus MAO-A cannot be the acceptor site labelled by [^{3}H]AMPH since an enrichment of the high-affinity site in the intrasynaptosomal mitochondria fraction was not obtained. Moreover, the high-affinity site was still present in synaptic membranes. This was corroborated by the persistence of the amphetamine-binding sites after irreversible inhibition of MAO-A and -B due to a preincubation [11], in the presence of both clorgyline and 1-deprenyl in large excess, of the crude membrane preparation (data not shown).

The regional distribution of [^{3}H]AMPH-binding sites was also rather intriguing (Table 1): in the presence of a high- or a low-ionic strength medium the distribution of binding sites was almost completely reversed. The most striking differences were observed for cerebellum and striatum. Saturation isotherms were determined from rat hypothalamic membranes in two different incubation buffers. In each case a single class of binding site was apparent (Hill number close to unity) in the concentration range of [^{3}H]AMPH used (5–500 nM). The K_D values obtained in the presence (1.4 ± 0.4 μM) or absence (1.20 ± 0.12 μM) of Na^+/K^+ ions were very close, but the binding capacity (B_{max}) was greatly enhanced in the absence of ions (16.9 ± 0.4 vs 46.4 ± 3.4 pmoles/mg protein). It is impor-