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PURIFICATION OF cAMP-SPECIFIC PHOSPHODIESTERASE FROM RAT HEART BY AFFINITY CHROMATOGRAPHY ON IMMOBILIZED ROLIPRAM

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SUMMARY : Affinity chromatography on a cAMP-specific phosphodiesterase inhibitor related to Rolipram, immobilized to AH Sepharose allowed to perform an efficient purification of the cAMP-specific phosphodiesterase isoenzyme from rat heart cytosol (102-fold purification with a 35 % yield in a single step). This affinity chromatography involved a biospecific interaction since a 2 mM cAMP elution step at 30°C was necessary for releasing the cAMP specific form tightly bound on the affinity gel. The cAMP eluate fraction exhibited a high specificity towards cAMP (cAMP/cGMP hydrolysis ratio 5-10), a marked sensitivity to Rolipram inhibition and could be resolved in two cAMP-specific, highly Rolipram-sensitive peaks of pI 6.7 and 4.8 by IEF on polyacrylamide gel plates. Protein stain of the IEF gel revealed a single band at pI 6.7.

A better understanding of the physiological functions and regulation of the multiple forms of phosphodiesterase implies an accurate separation of the different forms. Affinity chromatography has been already employed in the purification procedure of cN PDE from several tissular and species origins. Among the different ligands linked to a solid matrix for PDE purification one distinguishes : (i) dyes, (1,2) which are not specific of a single cN PDE form, (ii) calcium-binding proteins (3-6) which not only retain Ca-dependent cN PDE but also several other Ca^{2+} dependent proteins, (iii) cN PDE inhibitors. Only few attempts of phosphodiesterase purification by affinity chromatography on columns of immobilized inhibitors were

Abbreviations : cN PDE: cyclic nucleotide phosphodiesterase, IEF : Isoelectric focusing, EDC, HCl : 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide, HCl, DTE : (erythro-2,3-dihydroxy)-1,4-butan dithiol, EGTA: ethylene glycol 2(2-aminoethyl) tetracetic acid.

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reported. Agarose gel substituted by non specific inhibitors such as doxantrazole (7), papaverolin and xanthine derivatives (8) or phenylbutenolide (9) were able to retain PDE with good (7,9) or low (8) activity recovery but with low isoenzyme selectivity. The use, as ligand, of an inhibitor poorly specific toward one of the different cN PDE forms confines the affinity chromatography to a final step of purification when the other cN PDE forms are removed (e.g. the chromatography step on immobilized theophyline used by Marchmont and Houslay) (10). More recently, Umekawa et al. (11) described an affinity chromatography procedure on an OPC-13135-Sepharose column as a final step of purification of human platelet cAMP PDE : only the high-affinity cAMP-PDE was retained on this column, but the yield of this step wa rather low (17%). No endogenous activator such as calmodulin or cGMP can be used for the selective purification of the high affinity cAMP PDE form, in contrast with the other cN PDE forms. Thus, a specific inhibitor of the high affinity cAMP PDE would constitute a potential ligand designed to retain specifically this enzyme form. Rolipram (4-[3-cyclopentyloxy 4-methoxyphenyl] 2-pyrrolidone), described as an antidepressor drug (12) selectively inhibits a rat brain cytosolic cAMP PDE form of pI 5.6 (13) and the partially purified cAMP specific isoenzyme of beef aorta (14). We observed that it also inhibits with a high selectivity the I.E.F. separated cAMP-specific isoenzyme (15) of the rat heart cytosolic fraction (I50 = 4 μM). It is 100 times less potent on cGMP-activatable PDE (I50 = 350 μM) and 150 times less potent on calmodulin-activatable PDE (I50 = 600 μM) (this paper). An analog of Rolipram, AAL 115, with a carboxylic substituent, suitable for linkage on aminoalkyl Sepharose, exhibited a comparable inhibition selectivity and was thus employed as an affinity chromatography ligand, in an attempt to purify the cAMP-specific form of rat heart cytosol.

MATERIALS AND METHODS

Preparation of cytosolic rat heart PDE prior to chromatography. A 105,000 g (1 h) supernatant fraction from Sprague Dawley rat hearts, prepared as described in (9,15) was stored at - 80°C and used as a source of PDE.

Preparation of a AAL 115-substituted gel. 2 g A-H sepharose 4B (Pharmacia) washed following the manufacturer's instructions, was suspended in 100 ml of deionized water. 250 mg (800 μM) of AAL 115 4-(3-carboxypropyloxy-4-methoxyphenyl)-2-pyrrolidone synthesized as described in (16), were dissolved in 10 ml N.N.-dimethylformamide and added to the AH-Sepharose 4B suspension. EDC-HCl (1.5 g) in 15 ml of deionized water was added dropwise. The pH was brought to 4.5 with dilute hydrochlorid acid and the reaction allowed to proceed for 48-72 h at 30°C with continuous gentle mechanical stirring. pH was frequently readjusted to 4.5. A further overnight incubation in presence of 2 mM acetic acid was performed in order to block any remaining activated group. The resulting substituted gel was washed with a N.N.-dimethylformamide-water (2:1) mixture (700 ml) to remove unreacted ligand, (the disappearance of unbound AAL 115 in the wash was monitored by

determination of absorbance at 280 nm), with 900 ml 1 M K_2HPO_4,1 L of deionized water and finally with storage buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 0.1 mM DTE pH : 7.5). It was suspended in Tris buffer as 2:1 slurry and stored at 4°C untill use. In order to perform several control experiments, acetic acid-substituted gel was alternatively prepared following an identical procedure. In this case the coupling reaction was allowed to proceed overnight. The concentration of AAL 115 attached to the gel was estimated to be 33 µmoles/g dried resin when determined from the ultraviolet spectrum of the hydrolysate (6N HCl hydrolysis at 120°C for 2 h).

Chromatography procedure : Chromatography was performed as indicated in legend of figure 1 and in results.

I.E.F. procedure on polyacrylamide gel plates (LKB produkter) : cAMP eluate from affinity chromatography was concentrated in a Micro-ProDiCon apparatus (PA 10 membranes) which allowed a simultaneous dialysis against a 10 mM pH 7.5 Tris-HCl Buffer. PAG plates pH 3.5-9.5 received two samples of the cAMP eluate, of the cGMP eluate and of the unseparated rat heart cytosolic fraction. Sample droplets (20 µl)were applied directly on the gel surface. The plate temperature was maintained at 4°C. After completion of the focusing, for each sample pair, one lane was submitted to protein silver staining (Bio-Rad Laboratories) the other was sliced in 2.5 mm wide bands. The slices were ground in 1 ml of 160 mM Tris-HCl buffer containing 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mg/ml BSA (pH = 8) with a polytron grinder, and the suspension was assayed for PDE activity. The pH of each slice was measured.

PDE assay : cAMP and cGMP PDE activities were assayed as reported in (17) following a modified method based on the original two-step radioisotopic procedure of Thompson et al. (18) with 0.25 µM cAMP or cGMP as substrates. PDE activity determined in samples from the first KCl elution steps were corrected for underestimation due to inhibition by K^+ ion as reported in (9). Proteins were evaluated by the method of Bradford (19) with bovine serum albumin as standard. In order to measure protein concentration in KCl eluates, KCl was removed by a overnight dialysis.

RESULTS

Among the structural analogs of Rolipram, AAL 115 is a convenient potential ligand since it could be easily linked to ω-aminoalkyl Sepharose via an amide linkage (16). Furthermore some of its amide and ester derivatives exhibited selective inhibition of the cAMP-specific form of heart PDE (not shown). cN PDE from the rat heart cytosolic fraction was entirely bound both to an acetic acid-substituted gel and to the AAL 115-substituted gel (33 µmoles of ligand/g of dried gel). In both cases, the bulk of contaminating proteins (50-60 % of the applied material) without detectable PDE activity was recovered in the first wash with loading buffer (not shown). cN PDE bound to the acetic acid-Sepharose column was eluted by a linear KCl gradient as a single peak of activity at 0.30 M KCl (not shown). Pooling the active fractions of this peak resulted in a 60 % yield in enzyme activity with a 10-15 fold purification. The majority (about 85 %) of the recovered activity was eluted in a narrow range of KCl concentration from 0.25 to 0.54 M. cN PDE was more tightly bound to the inhibitor-coupled Sepharose since only 17 % and 3 % of the applied cytosolic cGMP and cAMP

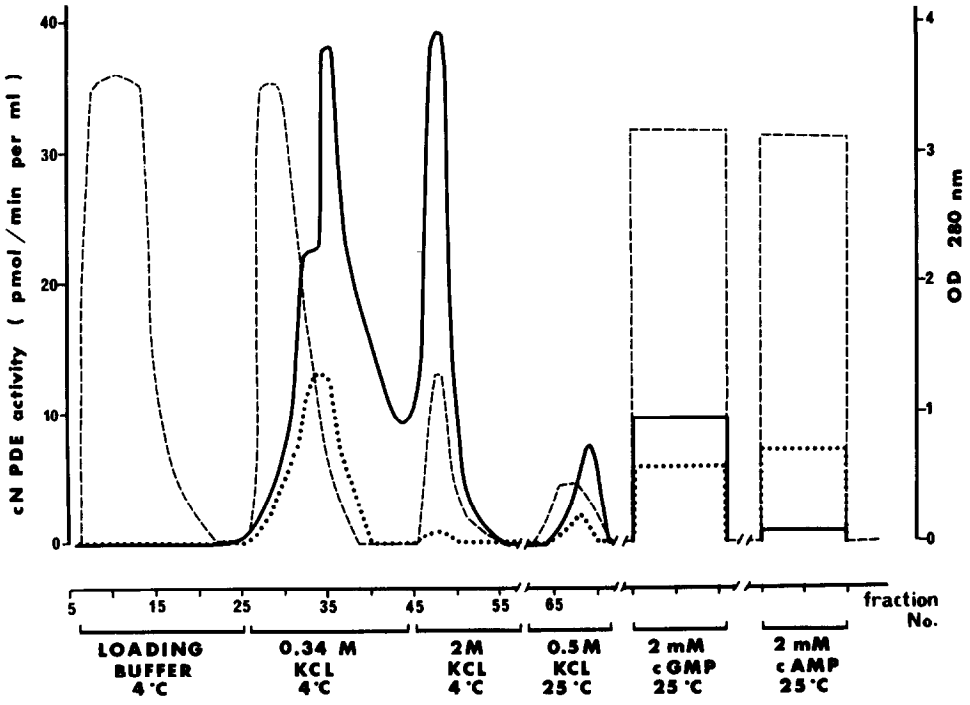

Fig. 1 : Affinity chromatography of rat heart cN PDE on AAL 115-AH Sepharose 4B conjugate (33 µmoles of ligand/g dried gel) : The figure is representative of several experiments leading to similar results. Between two separate experiments the AAL 115 AH-Sepharose 4B gel was washed by 2 M urea. For all elutions, 3 ml fractions were collected and a flow-rate of 30 ml/h was adopted.The gel columm (8 ml) was washed and equilibrated with 50 mM Tris-HCl buffer containing 5mM $MgCl_2$, 0.1 mM DTE, pH : 7.5 at 4°C. The 105 000 g supernatant of the rat heart homogenate (10 ml, about 56 mg proteins) was applied to the column, with a flow-rate of 6 ml/h. The column was washed with 43 ml of Tris-HCl buffer followed by 50 ml 0.34 M KCl in Tris-HCl buffer. When the OD returned to the basal level, the column was washed with 40 ml 2 M KCl in the same Tris-HCl buffer. When the OD returned to the basal level, the column was equilibrated with 50 mM Tris-HCl buffer containing 5 mM $MgCl_2$, 0.1 mM DTE, 0.5 M KCl, pH = 7.5 at room temperature. The column was then washed with 25 ml of the same buffer containing 2 mM cGMP. This elution was followed by an elution with 25 ml of the same buffer containing 2 mM cAMP in the same conditions. For each elution by a substrate, all the eluted fractions with high OD, i.e. the substrate containing fractions, were pooled and applied on a G50 Sephadex column (34 x 1,8 cm) before enzyme assay. cN PDE activities were measured with 0.25 µM cN (····· cAMP, ——— cGMP). All assays were performed in triplicate. Absorbance (-----) was measured at 280 nm. In order to avoid large losses in enzyme activity which occurred upon storage at - 20°C, the purified fraction were stored at + 4°C in presence of bovine serum albumin (1 mg/ml). Before BSA addition, aliquots of each fraction were taken up for protein determination.

PDE activities respectively were eluted by a linear 0-2 M KCl gradient (not shown). In this case, PDE activity was resolved into two distinct peaks at 0.35 M and 0.6 M KCl. The former peak hydrolysed both substrates, the latter appeared rather cGMP-specific when the activity was measured with low substrate concentration (0.25 µM). When the elution was performed in two KCl steps (0.34 M and 2 M) (fig. 1), the first one eluted 8 % of the

total cGMP and 3 % of the total cAMP PDE activities. This peak hydrolysed 3 times more efficiently cGMP than cAMP and was strongly sensitive to EGTA inhibition (85 %) : it can thus be referred to as the calmodulin-activatable PDE (previously detected at pI 4.9 in IEF experiments) (15). The second one represented 5 % of the total cGMP and 0.07 % of the total cAMP PDE activities. This peak hydrolysed 71 times more efficiently cGMP than cAMP and was less sensitive to EGTA inhibition (33 %) : it might represent a part of the cGMP-activable PDE (previously detected at pI 5.45 in IEF experiments) (15). The KCl elutions were followed by different attempts of biospecific elution at room temperature with PDE substrates (cGMP and cAMP) in 0.5 M KCl containing buffer. During the equilibration of the column at room temperature in 0.5 M KCl a small peak of activity was eluted. This peak represented 0.9 % of the total cGMP and 0.2 % of the total cAMP PDE activities. The column was first washed with 25 ml of the same buffer containing 2 mM cGMP. The eluted activity measured at low concentration of substrate corresponded to 3 % and 1 % of the total cGMP and cAMP PDE activities respectively. The cAMP/cGMP hydrolysis ratio was 0.6. The column was then washed with 25ml of the same buffer containing 2 mM cAMP. The

Table 1 : Purification of rat heart cN PDE using AAL 115-Substituted gel with 16 µmoles of ligand per g dried gel.

Fraction Assayed	Substrate	Total enzyme activity (a) (pmoles/min)	Total proteins (mg)	Specific activity (a)	Yield (%)	Purif. fold
Crude supernatant	cGMP	15 530	87	179	100	1
	cAMP	15 295		176	100	1
Loading buffer elution	cGMP	0	55	-	-	-
	cAMP	0		-	-	-
KCl 2 M 30°C eluate	cGMP	5 746	29	198	37	1.1
	cAMP	743		26	5	0.1
cGMP 2mM 30°C eluate	cGMP	2 754	0.738	3732	18	21
	cAMP	963		1305	6	7.5
cAMP 2mM 30°C eluate	cGMP	1 045	0.294	3074	7	17
	cAMP	5 293		18003	35	102

(a) activity was determined with 0.25 µM of each substrate

eluted activity measured at low concentration of substrate corresponded to 0.4 % and 3 % of the total cGMP and cAMP PDE activities respectively. The cAMP/cGMP hydrolysis ratio was 5. The total yield of PDE activity was rather low : 16.5 % for cGMP activity and 9 % for cAMP activity. A possible explanation might be that PDE activity still remained tightly bound to the immobilized ligand after the various elution procedures. In order to test this hypothesis we used in following experiments a gel presenting a lower degree of substitution by AAL 115 inhibitor. The AAL 115 substituted gel used in the previous experiments was mixed with an equal volume of acetic acid substitued Sepharose to give final concentration of 16 µmoles of ligand bound/g of dried gel.

In a second set of experiments using the less-substituted gel, the column was directly washed by 2 M KCl at 30°C after the first wash with the loading buffer. The eluted activity corresponded to 37 % of the total

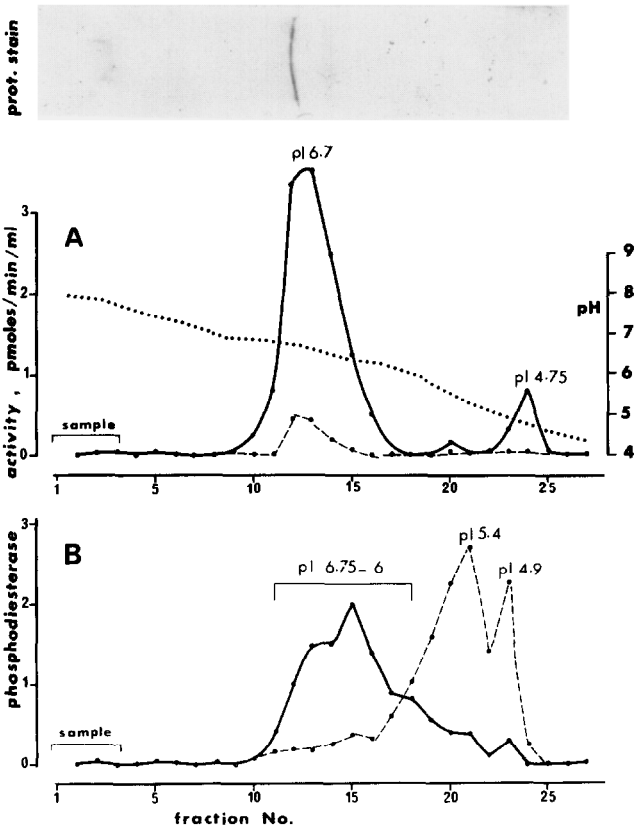

Fig. 2 : IEF patterns of cAMP eluate from affinity chromatography (A) and of total rat heart supernatant (B) on polyacrylamide plate. 2.5 µg of proteins of cAMP eluate were applicated for PDE pattern determination (A), and 62.5 ng for protein staining. 120 µg of proteins of total rat heart supernatant were applicated (B). PDE activity was measured with 0.25 µM cN (—●— cAMP, -●-●- cGMP). pH : .....

cGMP and 5 % of the total cAMP PDE activities. The cAMP/cGMP hydrolysis ratio was 0.13. The column was then washed at 30°C by 35 ml of 2 mM cGMP in 0.5 M KCl containing buffer : the recoveries in cGMP PDE and cAMP PDE activities were about 18 % and 6 % respectively. The cAMP/cGMP hydrolysis ratio was 0.35. The I 50 value for Rolipram, determined on this eluate with 0.25 µM cAMP as substrate was 1778 µM. The column was then eluted by 78 ml of 2 mM cAMP at 30°C in the same buffer : the recovery in cGMP PDE activity was about 7 % ; that in cAMP PDE activity about 35 % with a 102-fold purification. The cAMP/cGMP hydrolysis ratio was 10. The I50 value for Rolipram determined with 0.25 µM cAMP as substrate was 2 µM. The purification schedule is presented in table 1. The enzyme from the substrate eluate steps and the crude unseparated cytosolic fraction were submitted to IEF on polyacrylamide plate (fig. 2). The unseparated cytosolic fraction gave the classical pattern of PDE activity that we routinely find for heart enzyme (20). The cGMP eluate gave rise to a major peak of PDE activity at pI 5.4, hydrolysing preferentially cGMP (cAMP/cGMP hydrolysis ratio : 0.24) ; an intensely stained group of protein bands focused between pI 5 and 5.5 (not shown). The cAMP eluate gave rise to two well-separated peaks of activity : a major peak of pI 6.7 hydrolysed preferentially cAMP, a minor peak of pI 4.75 hydrolysed only cAMP. The protein silver staining revealed one major band at pI = 6.7 and a light band at pI = 6.4 but no detectable protein at pI = 4.75. The I50 value for Rolipram, determined with 0.25 µM

Table 2 : Comparison of I50 values for Rolipram measured on different fractions of heart cytosol

Tested fraction		ROLIPRAM I50 (µM)
Crude Supernatant (a)		890
IEF separated supernatant		
cAMP PDE	pI 6.3-6.75	4
cGMP PDE	pI 5.4	350 (c)
Calm.PDE	pI 4.9	600 (c)
Affinity chromatography separated supernatant		
cAMP eluate		2
cGMP eluate		1 178
IEF separated cAMP eluate (b)		
	pI 4.75 peak	1
	pI 6.7 peak	0.9

(a) 105.000 g supernatant fraction of rat heart homogenate. (b) cAMP eluate from AAL 115 substituted gel (16 µmoles of ligand/g dried gel) submitted to IEF on polyacrylamide gel plate gave rise to two well separated cAMP peaks at pI 4.75-6.7. I50 of Rolipram was determined with 0.25 µM cAMP or with 0.25 µM cGMP (c) in the presence of 1 % of DMSO.

cAMP as substrate was 1 μM for the pI 4.75 peak and 0.9 μM for the pI 6.7 peak as compared with 4 μM for cAMP-specific form and 350 μM for cAMP/cGMP form of the IEF separated rat heart cytosolic fraction. Both cAMP PDE peaks were unresponsive to 1 mM EGTA, to 1 μM cGMP and to calmodulin (100 U/assay). The properties of the two cAMP PDE obtained after the IEF step are summarized and compared to properties of other rat heart enzyme preparations in table 2.

DISCUSSION

The linkage of AAL 115 inhibitor, selective for the cAMP-specific PDE form, to the Sepharose matrix considerably modifies the retention of the enzyme on the gel. Indeed, cN PDE bound to the acetic acid-substituted Sepharose column is entirely eluted with a KCl gradient, while it largely remains bound in the same conditions on a AAL 115-substituted Sepharose column. The affinity chromatography on AAL 115-Sepharose reported here seems to actually involve a biospecific interaction, since elution steps by warm (30°C) substrates are necessary to elute substantial amounts of phosphodiesterase activity. The retention of the enzyme on the column cannot be attributed to hydrophobic interactions with the hexyl-substituted Sepharose matrix as the adoption of elution conditions designed to lower hydrophobic interactions (1 % nonionic detergent lubrol WX in 20 mM Tris HCl buffer, pH = 8,5 (21,22) does not remove any detectable activity (data not shown). The fraction eluted by the substrate cGMP is markedly different from that eluted by the substrate cAMP. When submitted toan IEF fractionation, the cGMP eluate gives rise to a major peak of PDE activity at pI 5.4 which is likely to be referred to as the "cGMP-activatable" isoenzyme, since it shows a higher affinity for cGMP than for cAMP, and focuses at the same pI (fig. 2). When submitted to IEF, the cAMP-eluate gives rise to a major peak of activity at pI 6.7, corresponding to the major protein band and a minor peak of activity at pI 4.75. A light protein band at pI = 6.4 is devoid of PDE activity. Both PDE peaks hydrolyze preferentially cAMP. The pI 4.75 peak does not obviously correspond to any IEF-separated peak from crude supernatant, and could thus artefactually derive from the other cAMP-specific peak(s) of the supernatant focusing in the pI 5.75-6.75 region. Onali et al.(23) described two high affinity cAMP PDE forms in human acute myelogenous leukemic cells : one form similar to the known type IV PDE,the other highly cAMP-specific but without immunological cross reactivity to the first one. The purified dog kidney cAMP PDE of pI 4.8 (24) exhibited two species with the same immunoreactivity but with different molecular weights and specific activities. The authors postulated that one form could produce the other

one by an unknown modification process (25). So, it seems reasonable to hypothesize in our case either that one form can give rise to the other or, that a native form of pI 6-6.75 similar to those observed in crude supernatant IEF-pattern is separated during the affinity chromatography step in two different active subunits. Further studies like determination of the molecular weights will perhaps answer this question. The purified pI 6.7 and 4.75 peaks exhibit the same high sensitivity to Rolipram inhibition (I50 values around 1 µM). These values were significatively lower than that observed with the cAMP-specific peak isoelectrically separated from crude supernatant. It denotes a more thorough separation from the other Rolipram insensitive forms which exhibited I50 in the mmolar range. Thompson et al. have recently reported that the cGMP/cAMP hydrolysis ratio is not very adequate as a criterium of purification because the purified dog kidney cAMP enzyme exhibits a cGMP PDE activity (24,25). Our results lead us to consider the Rolipram inhibition index as a more valuable criterium of purity for the cAMP-specific form. The affinity chromatography procedure reported in the present paper seems to be a convenient primary step for the purification of cAMP PDE : it allows a 102-fold purification of cAMP PDE with a reasonable yield (35 %). As the other isoenzymatic forms present in the crude supernatant are not devoid of cAMP-hydrolyzing activity, the apparent loss of activity is partially due to the elimination of these other forms and is not only attributable to enzyme inactivation. Furthermore, these experiments bring out some information on the mechanism of PDE inhibition by Rolipram. The presence of substrate cAMP being a prerequisite for elution of enzyme bound to the inhibitor, one may infer that Rolipram acts at the catalytic site of the enzyme.

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