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# cAMP ANALOGS AND SELECTIVE INHIBITORS USED TO STUDY LOW $K_m$ *Mucor rouxii* cAMP PHOSPHODIESTERASE

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**Abstract**—1. The sensitivity of partially purified low $K_m$ phosphodiesterase (PDE) from *Mucor rouxii* to pharmacological agents and cAMP analogs was studied. The $IC_{50}$ obtained were compared with those reported for PDEs from higher eukaryotes.

2. The best inhibitors of the hydrolysis of 1 $\mu$M cAMP were SQ 65.442 ($IC_{50}$ *c* 10 $\mu$M), dipyridamol and CI 930. cGMP was not an inhibitor ($IC_{50} > 1000$ $\mu$M).

3. The cAMP analogs were tested as inhibitors of the hydrolysis of 0.1 $\mu$M cAMP. 8-Aminohexylamino cAMP was the best inhibitor with an $IC_{50}$ of *c* 1 $\mu$M.

4. A sedimentation profile of *Mucor* PDE was assayed in the presence of several pharmacological inhibitors and cAMP analogs. No isoforms with different sensitivity towards the inhibitors were detected. Forms with slightly different behaviour towards some cAMP analogs were observed.

## INTRODUCTION

Cyclic AMP phosphodiesterases (PDE) play an important role in controlling intracellular cAMP concentration and hence cellular functioning in eukaryotic cells, since they provide the sole means of degrading this key nucleotide. Mammalian cells exhibit multiple forms of PDEs differing on amount and proportions in different cells, subcellular localization, substrate affinities, kinetic characteristic, physico-chemical properties, responsiveness to various effectors or drugs (especially PDE inhibitors) and mechanisms of regulation (Wells and Hardman, 1977; Strada and Thompson, 1978; Vaughan *et al.*, 1981; Beavo *et al.*, 1982; Weishaar, 1987).

Mammalian PDE, which characteristically show a low $K_m$ for cAMP and may be regulated by a variety of hormones (Loten *et al.*, 1978; Francis and Kono, 1982; Houslay, 1986; Manganiello *et al.*, 1987; Macphee *et al.*, 1988; Gettys *et al.*, 1988) belong to the type IV enzymes (Strada and Thompson, 1984). Recent data form several laboratories using a combination of biochemical, immunological and pharmacological approaches have indicated the presence of more than one class of type IV enzyme, differing in their ability to demonstrate allosteric regulation via cGMP inhibition, sensitivity to certain pharmacologic agents and substrate analogs and utilization of cGMP as substrate (Yamamoto *et al.*, 1984; Beebee *et al.*, 1985; Manganiello *et al.*, 1987; Pyne *et al.*, 1987; Reeves *et al.*, 1987; Weishaar *et al.*, 1987; Beebee *et al.*, 1988; Manganiello *et al.*, 1988). The functional relevance of such multiplicity and the mechanism(s) of hormonal activation of these PDEs have not been yet elucidated.

We have demonstrated that in the dimorphic fungus *Mucor rouxii* there is only one form of soluble PDE of the calmodulin-insensitive, low $K_m$ type, highly specific for cAMP, reversibly activatable *in vitro* and *in vivo* by a cAMP-dependent phosphorylation process and irreversibly activatable by limited proteolysis, being both processes not additive but complementary (Galvagno *et al.*, 1979; Cantore *et al.*, 1980; Moreno *et al.*, 1982; Tomes and Moreno, 1990).

In order to attempt a correlation of *Mucor* PDE with a subclass of type IV PDE of mammalian origin, and to search for more than one form within the apparently unique low $K_m$ cAMP PDE, the sensitivity of this enzyme to several pharmacological agents and cAMP analogs was undertaken in this study.

## MATERIALS AND METHODS

### *Cultures*

Spores of *M. rouxii* (NRRL 1894) were produced and stored as described by Haidle and Storck (1966). Aerobic mycelium was grown until mid-exponential phase as previously described (Kerner *et al.*, 1984) except that 1% acid hydrolysed casein replaced peptone in the culture medium. The harvested material was ground in a mortar with liquid $N_2$ and immediately processed.

### *PDE preparation*

The fine mycelial powder obtained by grinding was brought to 0–4°C and extracted with two volumes of 10 mM Tris–HCl, pH 7.4, 10 mM $\beta$-mercaptoethanol, 4 mM EGTA, 1 mM EDTA, 0.5 mM PMSF, 3 $\mu$g/ml antipain (buffer A) during 30 min at 4°C, aided by two strong mixing periods of 1 min in a Sorvall Omni-mixer at full speed. The extract was centrifuged for 30 min at 10,000 *g* and the supernatant was absorbed to DEAE–Sepharose with gentle agitation at a ratio of 50 mg protein/ml resin for 30 min at 4°C. The resin was collected by filtration through a Büchner funnel and washed with 3 vol of buffer A; PDE activity was eluted with 2 vol of 0.25 M NaCl in buffer A. This fraction, previous dialysis against buffer A, was loaded onto a DEAE–Sepharose column at a ratio of 10–20 mg protein/ml resin. The column was washed with 1 vol of buffer A and eluted with 10 vol of a linear gradient of 0–0.3 M NaCl in buffer A. Fractions of 0.1 vol were

collected. PDE activity was assayed on adequate aliquots under basal conditions and after trypsin treatment. The active fractions were pooled, dialysed against buffer A and concentrated by A DEAE–Sepharose column loaded at 50 mg/ml and eluted with 2 vol of 0.25 M NaCl in buffer A. This eluate was dialysed against buffer A and used as PDE source.

*Sucrose gradient centrifugation*

Linear gradients, 4.5 ml, 5–20% (w/v) sucrose were prepared with seven layers of 0.65 ml each of sucrose in buffer A. The sample was layered in 200 $\mu$l and the gradient spun for 16 hr at 40,000 rpm in a Beckman SW 55 Ti rotor at 4°C. A total of 26 fractions of six drops were syphoned from bottom to top of the tubes. Peroxidase was used as internal marker.

*PDE assay*

PDE activity was assayed by the modified two-step method of Thompson *et al.* (1974) as previously described (Moreno *et al.*, 1982) under basal conditions or after trypsin treatment. The standard incubation mixture contained in a final volume of 0.1 ml: PDE preparation, 50 mM Tris–HCl buffer, pH 7.4, 2 mM $\beta$-mercaptoethanol, 50 $\mu$g bovine serum albumin, 10 mM $MnCl_2$ and 1 $\mu$M or 0.1 $\mu$M [$^3$H]cAMP (150,000 cpm) as indicated, depending on whether the assays were performed with inhibitors or cAMP analogs, respectively. Trypsin treatment was performed by preincubation of the enzymatic preparation of 10 min at 4°C with 10 $\mu$g of trypsin in a final volume of 50 $\mu$l; proteolysis was stopped by the addition of 30 $\mu$g of egg-trypsin inhibitor. The incubation was carried out for 20 min at 30°C. Reaction rates were linear with time and proportional to enzyme–protein concentration. Substrate consumption was not higher than 20%. Data points reported are the average of duplicate determinations performed on representative experiments. All experiments were repeated at least three times.

*Chemicals*

[$^3$H]cAMP (20 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. Bovine pancreatic trypsin (type III), chicken egg-white trypsin inhibitor (type III), snake venom (*Ophiophagus hannah*), Dowex 1X-4, DEAE–Sepharose CL-6B, dipyridamole, IBMX, theophylline, papaverine, 8-azido-cAMP and $N^6$-aminohexylcarbamoylmethyl-cAMP were from Sigma Chemical Co. $N^6$-butyryl-cAMP, 8-Br-cAMP, 8-aminohexylamino-cAMP, 8-thiobenzyl-cAMP and $N^6$-benzoyl-cAMP were generously provided by Dr J. D. Corbin, Vanderbilt University, Nashville, Tenn., U.S.A. SQ 20006, SQ 20009 and SQ 65442 were a gift from E. R. Squibb and Sons, Princeton, N.J., U.S.A. Imazodan (CI 914) and CI 930 were a gift from Warner Lambert Co, Ann Arbor, Mich., U.S.A.; Cilostamide (OPC 3689) and cilostazole (OPC 13013) were generous gifts from Otsuka Pharmaceutical, Co., Osaka, Japan; ICI 63197 and ICI 118233 were kindly given by ICI Pharmaceuticals, Cheshire, U.K.: Rolipram (ZK 62711) was a gift from Schering, Berlin, West Germany. RO 20-1724 was kindly given by Hoffman-La Roche, Nutley, N.J., U.S.A.; Amrinone and Milrinone were generously provided by Sterling Winthrop Research Centre, Northumberland, U.K. A 20 mM sample of stock solution of each compound were prepared as follows: IBMX, theophylline, papaverine, SQ 20006, SQ 20009, CI 930 and CI 914 in water; cilostamide was dissolved in methyl alcohol; ICI 63197, RO 20-1724 and Rolipram were dissolved in ethyl alcohol; SQ 65442, ICI 118233, cilostazole, dipyridamole, amrinone and milrinone were dissolved in dimethyl sulphoxide. Stock solutions of cAMP analogs were made in water.

All other chemicals were of analytical grade.

## RESULTS AND DISCUSSION

*Sensitivity of Mucor PDE towards several PDE inhibitors*

Further characterization of *Mucor* PDE was attempted by studying its sensitivity towards various structurally distinct PDE inhibitors.

Table 1 shows the effect of increasing concentrations of the different inhibitors on the hydrolysis of

Table 1. Effect of inhibitor on cAMP hydrolysis by PDE from *M. rouxii*

| Drug | Concentration ($\mu$M) | PDE activity (% of control) |
|---|---|---|
| None | — | 100 |
| cGMP | 1 | 100 |
| | 10 | 100 |
| | 100 | 86 |
| | 1000 | 80 (100) |
| IBMX | 1 | 100 |
| | 10 | 70 |
| | 100 | 40 (60) |
| | 1000 | 10 |
| Theophylline | 1000 | 85 |
| | 5000 | 65 |
| | 10000 | 50 (100) |
| | 20000 | 33 |
| Papaverine | 10 | 85 (100) |
| | 100 | 25 (50) |
| | 1000 | 10 |
| SQ 20006 | 10 | 90 |
| | 100 | 55 |
| | 1000 | 45 (100) |
| SQ 20009 | 10 | 100 |
| | 100 | 80 |
| | 1000 | 50 (80) |
| SQ 65442 | 5 | 90 |
| | 10 | 50 |
| | 100 | 20 (65) |
| | 1000 | 10 |
| ICI 63197 | 10 | 100 |
| | 100 | 80 |
| | 1000 | 35 (65) |
| ICI 118233 | 200 | 100 |
| | 500 | 70 |
| | 1000 | 40 (100) |
| Rolipram | 10 | 100 |
| | 100 | 70 |
| | 1000 | 40 (55) |
| RO 20–1724 | 10 | 100 |
| | 100 | 80 |
| | 1000 | 50 (100) |
| Dipyridamole | 10 | 95 |
| | 100 | 50 (80) |
| | 1000 | 10 |
| CI 914 | 10 | 100 |
| | 100 | 87 |
| | 1000 | 50 (93) |
| CI 930 | 10 | 100 |
| | 100 | 65 |
| | 1000 | 23 (50) |
| Cilostamide | 10 | 100 |
| | 100 | 70 |
| | 1000 | 30 (83) |
| Cilostazole | 10 | 100 |
| | 100 | 73 |
| | 1000 | 50 (50) |
| Milrinone | 10 | 90 |
| | 100 | 50 |
| | 1000 | 33 (70) |
| Amrinone | 10 | 100 |
| | 100 | 50 |
| | 1000 | 38 (60) |

PDE activity was assayed in the standard reaction mixture at 1 $\mu$M cAMP in the presence of the indicated inhibitor. Values between parentheses represent percentage of control activity at 10 $\mu$M cAMP. Drug responses were controlled for solvent and snake venom effects.

1 $\mu$M cAMP by PDE. Methylxanthines and papaverine are classically used in the literature as inhibitors of cyclic nucleotide PDEs, without selectivity for the different isoforms. Weishaar (1987), in reviewing results from several authors reports that the three PDE isozymes isolated from different systems are not selectively inhibited by theophylline, IBMX nor papaverine, being the $IC_{50}$ for any of the isozymes *c* 300 $\mu$M for theophylline and 10 $\mu$M for IBMX and papaverine. A previous report on *Mucor* PDE (Cantore *et al.*, 1980) indicated that it was insensitive to classical methylxanthines such as theophylline, theobromine and caffeine. In Table 1 it can be observed that the results with theophylline were corroborated but that both the xanthine derivative IBMX as well as the non-specific inhibitor papaverine, were quite potent, with $IC_{50}$ in the range of 10–100 $\mu$M.

The rest of the inhibitors shown in Table 1 are currently used in the literature in order to attempt a unique classification for the different isoforms of PDE from mammalian origin. The results obtained with *Mucor* PDE show that complete inhibition was attained only with SQ 65442, dipyridamole and CI 930. SQ 65442 was the most potent inhibitor with an $IC_{50}$ *c* 10 $\mu$M. The sensitivity to the other inhibitors was very low and even at a concentration of 1 mM complete inhibition was not attained. Higher concentrations could not be assayed due to the inhibitory effect of the organic solvent used to prepare the inhibitor stock solutions. The mechanism of inhibition of each compound was not thoroughly investigated, but from the results shown in parentheses in Table 1, reporting the degree of inhibition at 10 $\mu$M cAMP, a competitive type of inhibition is suggested for all the compounds except for cilostazole.

According to the literature (Weishaar, 1987; Manganiello *et al.*, 1987; Weishaar *et al.*, 1987; Pyne *et al.*, 1987; Kariya and Dage, 1988; Epstein *et al.*, 1982) the high affinity cAMP PDE sensitive to inhibition by cGMP (cGMP-inhibitable type IV) is very sensitive to cilostamide, cilostazol, CI 914, CI 930, amrinone, milrinone and OPC 3911, whereas the high affinity cAMP PDE insensitive to inhibition by cGMP [cGMP non-inhibitable type IV, or type V as suggested by Kariya and Dage (1988)] is sensitive to Rolipram, RO-20-1724, dipyridamole, ICI 63197 and SQ 65442. The role of compounds such as SQ 65442 as selective inhibitors of cGMP non-inhibitable type IV PDEs is not very clear since there is a report in the literature (Pyne *et al.*, 1987) describing that SQ 20009 (SQ 65442 is a 4-thiomethyl analog of SQ 20009) is a very good inhibitor ($IC_{50}$: 4.6 $\mu$M) of the "dense-vesicle" PDE from rat liver (which can be homologed to the cGMP-inhibitable type IV PDE) and a very poor inhibitor ($IC_{50} \sim 1000$ $\mu$M) of the peripheral-plasma membrane enzyme from the same tissue (which can be compared to cGMP non-inhibitable type IV PDE).

Although the $IC_{50}$ value is not a kinetic parameter and is absolutely dependent on the concentration of the substrate used in the assay, it permits to estimate that the apparent $K_i$ of SQ 65442 for *Mucor* PDE is *c* 5 $\mu$M since $K_i = IC_{50}/1 + s/K_m$, assuming competitive inhibition. This value is quite higher than the apparent $K_i$ of 0.06 $\mu$M described for the inhibition of type IV PDE from dog kidney by SQ 65442 (Epstein *et al.*, 1982).

In order to decide if the apparent high $K_i$ observed was the result of the coexistence of more than one form of *Mucor* PDE with different degrees of sensitivity towards the inhibitor, a sample of PDE was centrifuged through a sucrose density gradient and aliquots of each fraction were assayed for PDE activity under basal conditions or after addition of 500 $\mu$M ICI 63197, 200 $\mu$M Rolipram, 10 $\mu$M SQ 65442, 300 $\mu$M CI 930, 1 mM cilostazole, 100 $\mu$M milrinone and 100 $\mu$M amrinone. The profile of PDE activity obtained in the presence of the inhibitors exactly reproduced the shape of the one obtained under basal conditions, with 50% lower activity (data not shown). This results suggest that no isoforms with different sensitivity towards the inhibitors coexisted in the PDE preparation.

The properties of the apparently unique form of *Mucor* PDE can thus be summarized as follows: the enzyme shows an apparent $K_m$ for cAMP of 1–2 $\mu$M (Cantore *et al.*, 1980; Moreno *et al.*, 1982); cGMP is a poor substrate (M. A. Galvagno, unpublished results) and inhibits cAMP hydrolysis only at very high concentrations ($K_i > 1000$ $\mu$m); SQ 65442 is the best inhibitor ($K_i \sim 5$ $\mu$M); the apparent molecular weight is *c* 60–70 kdaltons (Tomes *et al.*, 1989). From the comparison of these properties with those of PDEs from higher eukaryotic tissues, it can be concluded that *Mucor* PDE can be included in the type IV, cGMP non-inhibitable PDE, such as the low $K_m$ PDEs from dog, pig and rat kidney (Epstein *et al.*, 1982; Thompson *et al.*, 1988).

*Effect of cAMP analogs on hydrolysis of cAMP by Mucor PDE*

Cyclic nucleotide analog specificity for *Mucor* PDE was assayed in order to establish a comparison with PDEs from higher eukaryotes, as well as to characterize one of the determinants of analog potency in intact cells. The analogs were tested by inhibition of 0.1 $\mu$M [$^3$H]cAMP hydrolysis. Comparisons were made using approximate $IC_{50}$ values. Results are shown in Table 2.

The lowest $IC_{50}$ was attained with 8-aminohexylamino-cAMP (*c* 1 $\mu$M). The $IC_{50}$ for the other three 8-substituted analogs was *c* 10 $\mu$M; for $N^6$-monobutyryl-cAMP was 50 $\mu$M and for $N^6$-benzoyl-cAMP ranged between 10–100 $\mu$M. There are some important differences with data reported by Beebee *et al.* (1985) for low $K_m$ cAMP PDEs from rat adipocytes and hepatocytes. $IC_{50}$ for 8-aminohexylamino-cAMP could not be measured, since it was > 1 mM, while for *Mucor* PDE it was an excellent inhibitor; $IC_{50}$ for $N^6$-benzyl-cAMP, $N^6$-monobutyryl-cAMP and $N^6$-aminohexylcarbamoylmethyl-cAMP were between 10–40 $\mu$M, *c* 700 $\mu$M and > 1 mM respectively; the three $N^6$ substituted analogs were better inhibitors for *Mucor* PDE. The data reported for 8-Br-cAMP and 8-thiobenzyl-cAMP were *c* 20 and 40 $\mu$M respectively, quite similar to those obtained for *Mucor* PDE.

Although 50% inhibition of *Mucor* PDE activity was attained with relatively low concentrations of the analogs, complete inhibition was attained with concentrations two or more orders higher. This

phenomenon was specially observed with $N^6$-aminohexylcarbamoylmethyl-cAMP, 8-azido-cAMP, 8-Br-cAMP and 8-thiobenzyl-cAMP. These results suggested the possible coexistence of isoforms with different degrees of sensitivity towards the analogs. This possibility was analysed assaying the degree of inhibition of cAMP hydrolysis by the individual fractions obtained from the sedimentation of a sample of the PDE preparation through a sucrose density gradient. The concentrations of cAMP analogs used produced an inhibition *c* 50% when assayed on the whole sample of PDE. Results are shown in Fig. 1 (A, B and C). Figure 1A shows the profile of PDE activity assayed at 0.1 $\mu$M cAMP and at 1 $\mu$M cAMP without or with prior trypsin treatment. PDE activity at 1 $\mu$M yields a quite symmetrical peak, being all the fractions activatable by limited proteolysis. The shape of the peak changes when assayed at 0.1 $\mu$M cAMP suggesting the coexistence of species with slightly different $K_m$ for cAMP being those with higher sedimentation velocity the ones which show the lower $K_m$ values. Figures 1B and C show the effect of two $N^6$ and two C8-cAMP analogs respectively on the hydrolysis of 0.1 $\mu$M [$^3$H]cAMP by PDE. The effect of $N^6$-monobutyryl-cAMP is similar throughout the whole peak of PDE activity while the effect of the other three analogs is slightly greater on the fractions with lower sedimentation coefficient.

We suppose that this microheterogeneity of PDE forms, detected through different and opposed sensitivity towards the inhibition of hydrolysis of 0.1 $\mu$M [$^3$H]cAMP by 1 $\mu$M cAMP or by the three cAMP analogs, is the result of the difference in behaviour of the different forms derived from a unique PDE by endogenous proteolysis (Kerner *et al.*, 1984; Tomes *et al.*, 1985). These different forms could not be detected by assay with the PDE inhibitors (not shown).

Table 2. Effect of cAMP analogs on cAMP hydrolysis by PDE from *M. rouxii*

| cAMP analog | Concentration ($\mu$M) | PDE activity (% of control) |
|---|---|---|
| None | — | 100 |
| cAMP | 1 | 70 |
| | 10 | 25 |
| cGMP | 10 | 95 |
| | 100 | 90 |
| 8-Br-cAMP | 0.1 | 75 |
| | 1 | 60 |
| | 10 | 50 |
| | 100 | 25 |
| | 1000 | 8 |
| 8-thio-benzyl-cAMP | 1 | 66 |
| | 10 | 55 |
| | 100 | 27 |
| 8-azido-cAMP | 1 | 63 |
| | 10 | 54 |
| | 100 | 42 |
| | 1000 | 12 |
| 8-aminohexylamino-cAMP | 1 | 55 |
| | 10 | 30 |
| | 50 | 29 |
| $N^6$-aminohexylcarbamoylmethyl-cAMP | 1 | 60 |
| | 10 | 60 |
| | 100 | 48 |
| | 1000 | 36 |
| $N^6$-benzoyl-cAMP | 10 | 71 |
| | 100 | 13 |
| | 500 | 0 |
| $N^6$-monobutyryl-cAMP | 10 | 85 |
| | 50 | 50 |
| | 100 | 30 |
| | 1000 | 0 |

PDE activity was assayed in the standard reaction mixture at 0.1 $\mu$M cAMP in the presence of the indicated cAMP analog.

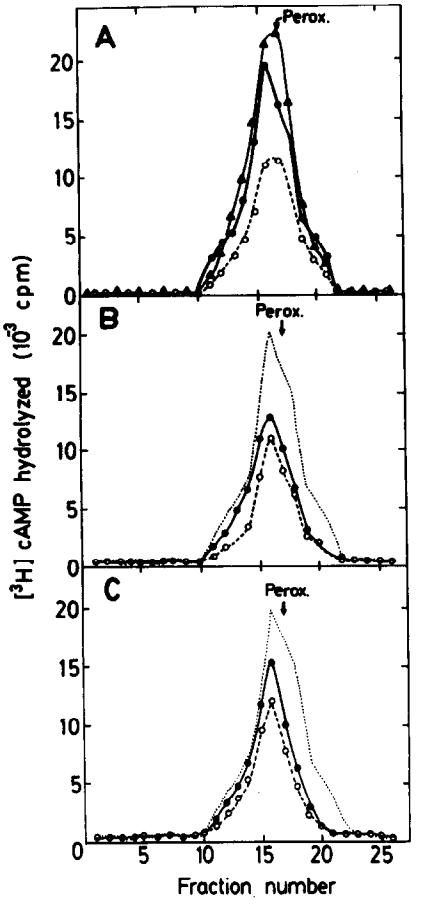


Fig. 1. Sucrose density gradient centrifugation of *Mucor* PDE. A sample from the PDE preparation was loaded onto 5–20% sucrose gradient and centrifuged as described under Materials and Methods (fractions were numbered from bottom to top). Aliquots of each fraction were assayed for PDE activity at 0.1 $\mu$M [$^3$H]cAMP with the following additions: (A) none (▲), 1 $\mu$M cAMP under basal conditions (○) and after trypsin treatment (●); (B) none (. . . .), 100 $\mu$M $N^6$-aminohexylcarbamoylmethyl-cAMP (●), 50 $\mu$M $N^6$-monobutyryl-cAMP (○); (C) none (. . . .), 5 $\mu$M 8-azido-cAMP (●), 5 $\mu$M 8-Br-cAMP (○).

The $IC_{50}$ values reported reflect the interaction between *Mucor* PDE and cyclic nucleotide analogs and give a preliminary information concerning their hydrolysis. Conclusive data requires the study of the mechanism of inhibition of cAMP hydrolysis.

These results complement a previous study on the order of effectiveness of cAMP analogs on the activation of *Mucor* cAMP-dependent protein kinase (Paveto *et al.*, 1989). These two parameters, together with the partitioning characteristics of the analogs (Beebee *et al.*, 1985) are the determinants on which the analogs potency in intact cells is based and the pharmacological significance of each can be analysed.

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