

## Adenosine 3':5'-Cyclic Monophosphate in Higher Plants

### ISOLATION AND CHARACTERIZATION OF ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE FROM *KALANCHOE* AND *AGAVE*

By ANTHONY R. ASHTON and GIDEON M. POLYA

Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, Australia

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1. 3':5'-Cyclic AMP was extensively purified from *Kalanchoe daigremontiana* and *Agave americana* by neutral alumina and anion- and cation-exchange column chromatography. Inclusion of 3':5'-cyclic [8-<sup>3</sup>H]AMP from the point of tissue extraction permitted calculation of yields. The purification procedure removed contaminating material that was shown to interfere with the 3':5'-cyclic AMP estimation and characterization procedures.
2. The partially purified 3':5'-cyclic AMP was quantified by means of a radiochemical saturation assay using an ox heart 3':5'-cyclic AMP-binding protein and by an assay involving activation of a mammalian protein kinase.
3. The plant 3':5'-cyclic AMP co-migrated with 3':5'-cyclic [8-<sup>3</sup>H]AMP on cellulose chromatography, poly(ethyleneimine)-cellulose chromatography and silica-gel t.l.c. developed with several solvent systems.
4. The plant 3':5'-cyclic AMP was degraded by ox heart 3':5'-cyclic nucleotide phosphodiesterase at the same rates as authentic 3':5'-cyclic AMP. 1-Methyl-3-isobutylxanthine (1 mM), a specific inhibitor of the 3':5'-cyclic nucleotide phosphodiesterase, completely inhibited such degradation.
5. The concentrations of 3':5'-cyclic AMP satisfying the above criteria in *Kalanchoe* and *Agave* were 2–6 and 1 pmol/g fresh wt. respectively. Possible bacterial contribution to these analyses was estimated to be less than 0.002 pmol/g fresh wt. Evidence for the occurrence of 3':5'-cyclic AMP in plants is discussed.

Although cyclic nucleotides have major regulatory roles in animal cells and micro-organisms, the occurrence and significance of cyclic nucleotides in higher plants remains controversial. There have been many claims for the occurrence of cyclic AMP in higher plants, but the adequacy of the purification, quantitative and characterization procedures used have been criticized on many grounds (Lin, 1974; Trewavas, 1976). The major problems variously present in these analyses have been the amount of tissue extracted (and the sensitivity of the detection systems used), bacterial or fungal contamination, the presence of plant compounds that interfere with biochemical procedures used for quantitative analysis and characterization of cyclic AMP and insufficient criteria necessary to establish the identity of plant substances as cyclic AMP. Thus estimates of the cyclic AMP content of plant tissues range from 1 to  $1 \times 10^4$  pmol/g fresh wt. [for discussion, see Bressan *et al.* (1976)]. Recently several groups, using extensively purified plant fractions and careful characterization procedures, have been unable to detect cyclic AMP in higher plants (detection limits 1–25 pmol/g fresh wt.) by using a variety of analytical techniques (Keates, 1973; Amrhein, 1974). Bressan *et al.* (1976) estimated that the maximum concentrations of cyclic AMP in various plant tissues were at the lower limits of

detection and characterization obtainable in their study (2–6 pmol/g fresh wt.). The present paper presents evidence for the occurrence of cyclic AMP in higher plants at minimum concentrations of 1–6 pmol/g fresh wt.

## Experimental

### Materials

1-Methyl-3-isobutylxanthine was obtained from the Aldrich Chemical Co., Milwaukee, WI, U.S.A. All nucleotides, bovine serum albumin and ox heart cyclic nucleotide phosphodiesterase (0.2  $\mu$ mol/min per mg of protein) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 3':5'-Cyclic [8-<sup>3</sup>H]AMP (27.5 Ci/mmol) and [ $\gamma$ -<sup>32</sup>P]ATP (16.6 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Norit GSX was obtained from Norit-Clydesdale Co., Glasgow, U.K., and Norit A from Matheson, Coleman and Bell, Norwood, OH, U.S.A. Before use the Norit A was washed as described by Hardman *et al.* (1969). Casein (vitamin- and fat-free) was obtained from BDH Chemicals (Melbourne, Vic., Australia) and dephosphorylated as described by Reimann *et al.* (1971). Cellulose thin-layer sheets and neutral aluminium oxide were obtained

from E. Merck, Darmstadt, Germany, Miracloth from Calbiochem, San Diego, CA, U.S.A., and Triton X-114 from Rohm and Haas, Melbourne, Vic., Australia. The plants *Kalanchoe daigremontiana* Hamet et Perr and *Agave americana* var. *marginata* Trel were supplied by Mr. M. Pender of the Botany Department, La Trobe University. AG-50W (X8; 200–400 mesh) and AG-2 (X8; 200–400 mesh) ion-exchange resins were obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A., and washed extensively before use (see Otten *et al.*, 1972). The AG-50W cation-exchange resin was washed successively with 5–10 vol. each of 1M-NaOH, water, 1M-HCl, water, ethanol, 1M-HCl and glass-redistilled water; the AG-2 anion-exchange resin was washed successively with 5–10 vol. each of 1M-NaOH, water, 1M-HCl and glass-redistilled water. Poly(ethyleneimine)-cellulose thin layers were prepared as described previously (Ashton & Polya, 1975). Scintillation fluid A, used for counting the radioactivity of aqueous samples, contained 1 vol. of Triton X-114, 3 vol. of xylene, 0.3% PPO (2,5-diphenyloxazole) and 0.02% dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene]. Scintillation fluid B containing 0.5% PPO and 0.03% dimethyl-POPOP in toluene was used for counting paper discs from protein kinase assays. The counting efficiency was the same for all samples within each radiochemical experiment.

#### *Estimation of bacterial contamination of plant material*

Leaves were wiped with a damp cloth, excised and homogenized in an Ato-Mix blender for 45s with 3 vol. of sterile 100mM-potassium phosphate, pH 6.75. The final pH of the homogenate was approx. 6.3. The bacterial content of the homogenate was estimated by plating serial dilutions on to plates containing 0.75% (w/v) yeast extract and 2.8% (w/v) agar and incubating at 26°C for 36h.

#### *Purification of cyclic AMP from plant tissue*

*Kalanchoe* leaves were wiped with tissue moistened with water before excision from the plants. Leaves (30g or less) were frozen in liquid N<sub>2</sub> and then suspended in 3 vol. of 0.4M-HClO<sub>4</sub> (0°C) containing tracer 3':5'-cyclic [8-<sup>3</sup>H]AMP (0.05 pmol of cyclic AMP/g fresh wt., specific radioactivity 27.5 Ci/mmol) to enable purification of plant cyclic AMP to be monitored. The leaves were blended in an Ato-Mix blender for 1 min at full power, and the homogenate was squeezed through Miracloth and the filtrate centrifuged at 10000g for 15 min at 0°C. The resulting supernatant was neutralized to pH 7.5 by the addition of 10M-KOH and the resulting precipitate removed by centrifugation at 10000g for 10 min at 0°C. The

neutralized supernatant was brought to 50mM with respect to Tris/HCl, pH 7.5. The neutralized extract supernatant was applied to a neutral alumina column which was washed with 1 column vol. of 0.6M-Tris/HCl, pH 7.5. The total eluate from the alumina column was then applied to a column (1.1 cm × 16 cm) of the AG-2 anion-exchange resin. The column was then washed with glass-redistilled water (in all subsequent operations glass-redistilled water was used). The cyclic AMP was eluted with 50mM-HCl. The fractions containing cyclic AMP were pooled, made 0.1M with respect to HCl and applied to a column (1.1 cm × 16 cm) containing the AG-50W cation-exchange resin equilibrated with 0.1M-HCl. The column was washed with 0.1M-HCl and the cyclic AMP subsequently eluted with water. The fraction containing cyclic AMP was concentrated by freeze-drying.

Cyclic AMP was purified from *Agave* leaves by a modification of the above procedure that was introduced to handle the large amounts of plant tissue extracted. The cleaned leaves (approx. 1 kg) were not frozen before being sliced into 50g segments and extracted in 0.4M-HClO<sub>4</sub>. After filtration and centrifugation of the homogenate as described above, the nucleotide was adsorbed on activated charcoal, and the charcoal washed and eluted as described by Hardman *et al.* (1969). Cyclic AMP was subsequently purified by means of chromatography on alumina, AG-2 and AG-50W resins.

#### *Enzymic assays for cyclic AMP*

Cyclic AMP-dependent protein kinase was purified from fresh ox hearts as described by Kuo & Greengard (1972) up to and including chromatography on DEAE-cellulose. This preparation was used in both protein kinase activation and radiochemical saturation assays for cyclic AMP. Cyclic AMP concentration was measured by a radiochemical saturation assay essentially as described by Tovey *et al.* (1974). Cyclic AMP concentration was also estimated by the protein kinase activation assay procedure of Wastila *et al.* (1971) with the cyclic AMP-dependent protein kinase obtained from ox heart. The reaction mixture was incubated for 90 min at 30°C and then stopped and processed as described by Mayer *et al.* (1975).

#### *Enzymic degradation of cyclic AMP*

Cyclic AMP (approx. 5 pmol/50 μl of reaction medium) was degraded at 30°C in a reaction medium containing 50mM-Tris/HCl, pH 7.5, and 1mM-MgCl<sub>2</sub> in the presence and absence of 1mM-1-methyl-3-isobutylxanthine. The reaction was initiated by the addition of ox heart cyclic nucleotide phosphodiesterase (1.5 μg/50 μl of reaction medium)

and terminated at various times by the addition of EDTA (to 4mM) and then by heating samples in a boiling-water bath for 5 min. The cyclic AMP content of the samples was determined by saturation assay as described above.

The degradation of cyclic [8-<sup>3</sup>H]AMP was examined in a reaction mixture identical with the medium described above for degradation of cyclic AMP but which also contained cyclic [8-<sup>3</sup>H]AMP (1.7 μCi/ml). The reaction was initiated by the addition of ox heart cyclic nucleotide phosphodiesterase and terminated by spotting a 5 μl sample on to a poly(ethyleneimine)-cellulose thin-layer sheet. The poly(ethyleneimine)-cellulose thin-layer sheets were developed with solvent A as described by Ashton & Polya (1975). The AMP spot was detected under u.v. light, cut out and placed in a scintillation vial containing 0.75 ml of 0.7M-MgCl<sub>2</sub> in 2mM-Tris/HCl, pH7.5, and the nucleotide was eluted at room temperature for 1 h. Scintillation fluid A (10ml) was added to the vial and the sample counted for radioactivity at 30% efficiency in a Packard model 3003 scintillation spectrometer. When the AMP was not eluted before counting the efficiency was only 6%.

## Results and Discussion

### Purification of cyclic AMP from *Kalanchoe* and *Agave*

The purification procedure was designed to minimize steps involving extensive concentration of the plant extracts and to ensure reproducibility. After acid extraction and neutralization the extract was passed through a column of neutral alumina, which does not retain cyclic AMP, but which binds many other nucleotides (White & Zenser, 1971). When extracts were not passed through neutral alumina before chromatography on AG-2 resin, these anion-exchange columns were overloaded and cyclic AMP was not retained. Chromatography on AG-2 resin effected removal of a large amount of material that inhibits the binding of cyclic AMP to the protein kinase in the saturation assay. The remainder of this contaminant was removed by chromatography of the fraction containing cyclic AMP on an AG-50W cation-exchange column. The inhibitory material was not retained by the column and was well resolved from cyclic AMP. This contaminating material inhibited binding of cyclic AMP to the protein kinase in the saturation assay, but did not behave like authentic cyclic AMP when diluted in the assay (Fig. 1). Unlike this inhibitory material, cyclic AMP purified from *Kalanchoe* did behave like authentic cyclic AMP on dilution in the saturation assay. The inhibitory fraction resolved from cyclic AMP by chromatography on AG-50W cation-exchange resin also contained an inhibitor of ox heart cyclic nucleotide phosphodiesterase. A concentration of this fraction

that inhibited cyclic AMP-binding by 38% in the standard saturation assay completely inhibited the ox heart cyclic nucleotide phosphodiesterase (assayed as described in the Experimental section with 1 μM-cyclic AMP as substrate). The resolution of cyclic AMP from this inhibitory material was clearly necessary for our subsequent analysis. It should be noted that elution of cyclic AMP from AG-50W resin with water (Krishna *et al.*, 1968) was not satisfactory.

This purification procedure was very reproducible and resulted in yields of tracer cyclic [8-<sup>3</sup>H]AMP of 30–50%. The amounts of cyclic AMP found in *Kalanchoe* leaves (appropriately corrected for losses of tracer cyclic [8-<sup>3</sup>H]AMP) ranged from 2 to 6 pmol/g fresh wt. as determined by the saturation assay. The amount of cyclic AMP contributed to the assay by elution of the three 'blank' chromatographic columns (i.e. in the absence of plant extract) was less than 1% of the observed cyclic AMP.

A modification of the procedure applied to 20–30g of *Kalanchoe* leaves was developed to enable large-scale purification of cyclic AMP from *Agave* leaves (approx. 1 kg). The final purification step resulted in elution from a column of AG-50W resin of one peak of 'cyclic AMP' (as detected by the saturation assay) that coincided exactly with a peak of tracer cyclic [8-<sup>3</sup>H]AMP. No other peaks of substances interfering with the saturation assay were observed. The overall yield of tracer cyclic [8-<sup>3</sup>H]AMP in this large-scale procedure was 25%. The amount of cyclic AMP in *Agave* leaves (appropriately corrected for

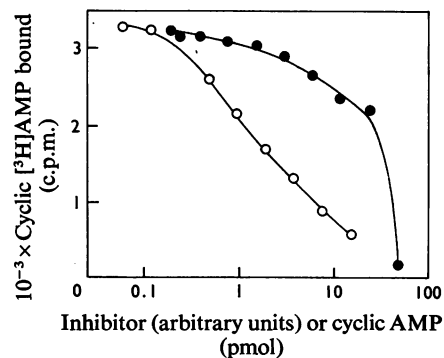


Fig. 1. Interaction of inhibitory material from *Kalanchoe* with cyclic AMP saturation assay

Inhibitory fractions eluted from a column of AG-2 anion-exchange resin and resolved from cyclic [8-<sup>3</sup>H]-AMP were pooled, freeze-dried and redissolved in assay buffer. The material was assayed at various dilutions in the saturation assay and the results were superimposed on an authentic cyclic AMP standard curve. O, Authentic cyclic AMP; ●, inhibitory material from *Kalanchoe*.

cyclic [ $8\text{-}^3\text{H}$ ]AMP losses) was 1 pmol/g fresh wt. as determined by the saturation assay.

#### *Activation of protein kinase by cyclic AMP from plants*

The amounts of cyclic AMP in extensively purified fractions from *Kalanchoe* and *Agave* were determined by an assay involving activation of an ox heart cyclic AMP-activated protein kinase as well as by the saturation assay. Quite different results have been given by these two assay procedures when applied to insufficiently purified cyclic AMP fractions from plants (Bressan *et al.*, 1976). The cyclic AMP concentrations determined by the protein kinase activation assay were approx. 90% of values determined by the saturation assay. The cyclic AMP from both plant sources behaved like authentic cyclic AMP on dilution in both assay systems. Ascending silica-gel t.l.c. of purified material from *Agave* [developed with butan-1-ol/methanol/ethyl acetate/aq.  $\text{NH}_3$  (sp.gr. 0.880) (7:3:4:4, by vol.)] revealed one peak of cyclic AMP-like material (measured by both the protein kinase activation assay and the saturation assay) that exactly co-migrated with authentic cyclic AMP.

#### *T.l.c. of cyclic AMP from plants*

The putative cyclic AMP from *Kalanchoe* exactly co-migrated with authentic cyclic [ $8\text{-}^3\text{H}$ ]AMP on cellulose t.l.c. developed with propan-2-ol/aq.  $\text{NH}_3$  (sp.gr. 0.880)/water (14:3:3, by vol.) (see Goldberg & O'Toole, 1971). After purification as detailed above, the cyclic AMP-containing fraction from *Agave* was analysed by poly(ethyleneimine)-cellulose t.l.c. (developed separately with the following solvents: 0.5M-ammonium acetate/5% (w/v) boric acid, pH 7.5; 0.5M-ammonium acetate, pH 7.5; 0.5M-LiCl; 0.5M-sodium formate, pH 4.2). These chromatographic systems were chosen to resolve cyclic AMP ( $R_f$  values 0.4–0.6) by a variety of criteria (see Randerath & Randerath, 1964; Khym, 1967). Analysis of the chromatograms revealed, in all cases, only one peak of material that interacts with the cyclic AMP-binding protein, and this material exactly co-migrated in all systems with authentic cyclic [ $8\text{-}^3\text{H}$ ]AMP. Note that the putative cyclic AMP from both *Agave* and *Kalanchoe* also exactly co-chromatographed with cyclic [ $8\text{-}^3\text{H}$ ]AMP on elution from the AG-50W cation-exchange columns.

#### *Degradation of cyclic AMP from plants by a phosphodiesterase*

In some studies, plant material interacting in the saturation assay and co-purifying with cyclic AMP was not degraded by mammalian cyclic nucleotide phosphodiesterase (Niles & Mount, 1974; Bressan

*et al.*, 1976). Degradation of plant 'cyclic AMP' by specific cyclic nucleotide phosphodiesterase has been reported (Brewin & Northcote, 1973; Kessler & Levinstein, 1974; Elliott & Murray, 1975), but, unless appropriate controls are applied, this result can be misinterpreted. Thus, if the saturation assay is measuring only non-specific protein denaturants, e.g. polyphenols or quinones (Loomis & Battaile, 1966), then the added phosphodiesterase may non-specifically adsorb such material and decrease the amount available for interaction with the binding protein. Albano *et al.* (1974) have shown that the presence of inactivated phosphodiesterase can alter the shape of the standard curve. Phosphodiesterase preparations may contain traces of other hydrolytic enzymes (e.g. see Goldberg *et al.*, 1969).

Such ambiguities were avoided in the present work by studying the time courses of degradation catalysed by a specific ox heart cyclic nucleotide phosphodiesterase in the presence and absence of 1mM-1-methyl-3-isobutylxanthine, a specific inhibitor of the enzyme (Beavo *et al.*, 1970) (Fig. 2). The cyclic AMP from *Kalanchoe* was degraded at approx. 85% of the rate obtained with authentic cyclic AMP in the same conditions. This small difference in rates of degradation may derive from an inhibitory effect of residual contaminants in the plant cyclic AMP preparation. The *Kalanchoe* cyclic AMP preparation used in the degradation shown in Fig. 2 was purified as described in the Experimental section, but in addition was purified by cellulose t.l.c. (developed with propan-2-ol/aq.  $\text{NH}_3$ /water, 14:3:3, by vol.). When this additional purification step was omitted, rates of degradation were only 10% of the rates obtained with authentic cyclic AMP. In addition, when cyclic [ $8\text{-}^3\text{H}$ ]AMP with a high specific radioactivity was included with *Kalanchoe* cyclic AMP in a degradation experiment, the plots of percentage cyclic [ $8\text{-}^3\text{H}$ ]AMP remaining and percentage cyclic AMP remaining (determined by saturation assay) versus time were identical within experimental error (Fig. 2). Some 95% of the *Kalanchoe* cyclic AMP was degraded by the mammalian cyclic nucleotide phosphodiesterase (Fig. 2). In a similar experiment cyclic AMP from *Agave* was degraded at the same rate as authentic cyclic [ $8\text{-}^3\text{H}$ ]AMP under the same conditions. Some 95% of 'cyclic AMP' purified from *Agave* was degraded by mammalian cyclic nucleotide phosphodiesterase. Inclusion of 1mM-1-methyl-3-isobutylxanthine completely prevented disappearance of cyclic AMP from *Agave* and from *Kalanchoe* (Fig. 2).

#### *Estimates of cyclic AMP concentrations not of plant origin*

The cyclic [ $8\text{-}^3\text{H}$ ]AMP used was estimated (by means of the saturation assay) to have a specific radioactivity of 29.2 Ci/mmol, which agrees well with

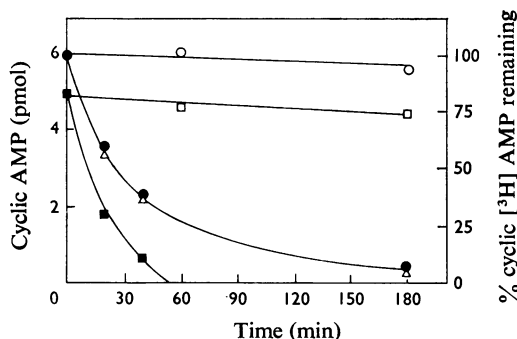


Fig. 2. Degradation of cyclic AMP from *Kalanchoe* by cyclic nucleotide phosphodiesterase

Reaction mixtures containing cyclic AMP from *Kalanchoe* or authentic cyclic AMP were degraded by ox heart phosphodiesterase in the presence or absence of 1mM-1-methyl-3-isobutylxanthine as described in the Experimental section. The cyclic AMP was measured by the saturation assay. Cyclic [8-<sup>3</sup>H]AMP (1.7  $\mu$ Ci/ml) was degraded in the presence of the *Kalanchoe* extract as described in the Experimental section.  $\square$ ,  $\blacksquare$ , Authentic cyclic AMP (pmol) in the presence and absence of 1mM-1-methyl-3-isobutylxanthine respectively;  $\circ$ ,  $\bullet$ , cyclic AMP (pmol) from *Kalanchoe* in the presence and absence of 1mM-1-methyl-3-isobutylxanthine respectively.  $\Delta$ , % of original cyclic [8-<sup>3</sup>H]AMP remaining in reaction mixture containing *Kalanchoe* extract.

the manufacturer's stated specific radioactivity of 27.5Ci/mmol. Cyclic [8-<sup>3</sup>H]AMP was added at the point of tissue extraction at only 0.05pmol/g fresh wt.

Bacterial contamination of *Kalanchoe daigremontiana* was estimated to be less than 60000 bacteria/g fresh wt. by the method described in the Experimental section. If the volume of a single bacterium is taken as  $10^{-12}$ cm<sup>3</sup> and the intracellular cyclic AMP concentration assumed to be 40 $\mu$ M (Peterkofsky & Gazdar, 1974), then it can be calculated that bacterial contamination could account for less than 0.002 pmol of cyclic AMP/g fresh wt. This is approximately 3 orders of magnitude less than the concentrations of cyclic AMP found in *Kalanchoe*. Similar determinations conducted with *Agave* leaves indicated that possible bacterial contribution to the observed cyclic AMP was less than 0.5fmol/g fresh wt. Leaves of these succulent plants were in fact chosen for this study because of their low degree of microbiological contamination.

#### Cyclic AMP concentrations in higher plants

The saturation assay using a high-affinity cyclic AMP-binding protein has been the most widely used method for estimating cyclic AMP in plants (Lin,

1974; Sachar *et al.*, 1975; Bressan *et al.*, 1976). However, the presence in the assay of any substance that interacts with or denatures proteins may produce erroneously high values for cyclic AMP concentrations (Albano *et al.*, 1974; Wright & Price, 1975). In the present study the concentrations of such substances in *Kalanchoe* exceeded the concentrations of cyclic AMP by a factor of  $10^4$  (as measured in terms of the saturation assay). Such substances, eliminated in our procedure, have clearly contributed to high estimates of plant cyclic AMP concentrations from saturation assays (see Bressan *et al.*, 1976). Although the plant cyclic AMP concentrations found in the present study are similar to estimates by Amrhein & Filner (1973), Amrhein (1974) and Bressan *et al.* (1976), they are 2-3 orders of magnitude lower than those reported by some other workers (Raymond *et al.*, 1973; Brown & Newton, 1973). It is possible that these differences arise from degradation of plant cyclic AMP before equilibration with tracer cyclic [3H]AMP during tissue extraction. However, cyclic AMP concentrations found for *Kalanchoe* and *Agave* leaves were similar, although only the former were frozen in liquid N<sub>2</sub> before extraction.

The present results provide evidence for the occurrence of cyclic AMP in leaves of two higher-plant species at minimum concentrations of 1-6pmol/g fresh weight. Identification of plant material as cyclic AMP in this study involved satisfying a more exacting set of criteria than in previous work. If cyclic AMP is localized cytoplasmically in the highly vacuolated *Agave* and *Kalanchoe* cells, the concentrations found would correspond to cytoplasmic concentrations of approx. 0.1 $\mu$ M, similar in magnitude to the steady-state cyclic AMP concentrations in some animal cells (Wastila *et al.*, 1971).

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