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8-Bromoadenosine 3′:5′-Cyclic Monophosphate as a Promoter of Cell Division in Excised Tobacco Pith Parenchyma Tissue

(auxins/phosphodiesterase/cytokinesins/cytokinins)

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ABSTRACT **8-Bromoadenosine 3′:5′-cyclic monophosphate, when used in association with an auxin, can completely replace the cell-division-promoting activity of either a cytokinesin or a 6-substituted adenylyl cytokinin in excised tobacco pith parenchyma tissue. The 8-bromo derivative of adenosine 3′:5′-cyclic monophosphate was found to be far more resistant to degradation by plant adenosine 3′:5′-cyclic monophosphate phosphodiesterases than was adenosine 3′:5′-cyclic monophosphate. These findings appear to provide further support for the suggestion made earlier that the cytokinesins, which are potent inhibitors of both plant and animal adenosine 3′:5′-cyclic monophosphate phosphodiesterases, exert their cell-division-promoting effects as regulators of adenosine 3′:5′-cyclic monophosphate.**

The processes of cell enlargement and cell division are controlled in higher plant species by the quantitative interaction of two growth-regulating substances, the auxins and the cell-division-promoting factors. The auxins are concerned with cell enlargement (1) and chromosomal DNA synthesis (2-4), while the cell division factors act synergistically with the auxins to regulate cell division. The cell division factors are ineffective in encouraging either cell enlargement or division in the absence of an auxin in tobacco pith parenchyma tissue (5-7). What appears to be an entirely analogous situation has recently been described in the animal literature (8). In that report, an as-yet uncharacterized dialyzable component has been implicated in chromosomal DNA synthesis, while a heat-labile nondialyzable substance(s) has been found to act synergistically with the dialyzable component to promote mitosis and cytokinesis in BHK/21 cells. The nondialyzable component(s), like the cell division factors in plants, is ineffective in encouraging either DNA synthesis or in promoting cytokinesis in the absence of the dialyzable factor.

Our earlier studies had shown that crown gall tumor cells acquire, as a result of their transformation, a capacity to synthesize persistently both auxins and cell-division-promoting factors (9, 10). The cell-division factors, which have been given the trivial name cytokinesins, together with the auxins have been found to play a central role in the development of a capacity for autonomous growth of crown gall tumor cells, since their continued synthesis by such cells establishes and maintains the pattern of synthesis concerned with cell growth and division and, thus, keeps the tumor cells dividing persistently. Normal cells of *Vinca rosea* L. in culture do not commonly synthesize cytokinesins. It was found, however, that if such cells are forced into rapid growth with a 6-substituted adenylyl cytokinin such as kinetin (6-furfurylaminopurine) they synthesize a cytokinesin that is indistinguishable from that produced by the tumor cells in the absence of an exogenous source of kinetin or other 6-substituted adenylyl cytokinin. This finding has led to the suggestion that a compound such as kinetin activates the synthesis of the cytokinesins in normal cell types and that it is those substances, rather than the 6-substituted adenylyl cytokinins, that are directly involved in promoting cytokinesis in cells of higher plant species (10).

Physical and chemical studies have suggested that cytokinesin I is a 3,7-dialkyl-2-alkylthio-6-purinone that contains glucose (11). This compound has been found to be a potent inhibitor of adenosine 3′:5′-cyclic monophosphate (cAMP) phosphodiesterases of both plant and animal (bovine brain) origin (12). The results of that study suggest, then, that the cytokinesins may exert their effects in promoting cell division as protectors of cAMP. The facts that a membrane-bound adenylate cyclase has been found to be present in plant cells (12) and that significant increases in the amount of cAMP have repeatedly been found by us with the use of the radioimmunoassay of Steiner *et al.* (13) to be present in auxin and kinetin-treated pith tissue, appear to strengthen that suggestion. Elevated levels of cAMP were found in those studies to be present during and beyond the "S" phase of the cell cycle, while cAMP levels dropped significantly just prior to the time that the growing tissues had doubled in dry weight. The results of this study will be published elsewhere. If cAMP is, in fact, somehow concerned in the regulation of cell division in higher plant species and if, as postulated, the cytokinesins act specifically as inhibitors of the cAMP phosphodiesterases, then it should be possible to replace the growth-promoting effects of the cytokinesins with either cAMP or with a less readily degradable derivative of that compound. It is with an attempt to test the validity of this concept that the present study is concerned.

MATERIALS AND METHODS

Biological Assay. Tobacco pith parenchyma tissue was used to assay cell-division-promoting activity of the several compounds tested. Tobacco pith tissue was isolated aseptically from the middle third of tobacco plants (*Nicotiana tabacum* var. Turkish L.) that were uniform in size and about 1-meter tall. The isolated pith tissue free of internal phloem was cut with sterile precautions into uniform pieces 10-mm long and 3-mm wide and high. These were used in the experiments.

The culture medium used was that described by Linsmaier

and Skoog (14). White's basic culture medium was also used and gave similar results. The media, which contained a final concentration of 0.9% purified Difco powdered agar, were made up in double strength and 5 ml of the appropriate medium was added to 25-ml Erlenmeyer flasks. Where desired, an auxin in the form of naphthalene acetic acid was added to give a final concentration of 0.5 mg/liter. In addition, triple-distilled water was added in amounts required to bring the total volume of the medium in each flask to 9 ml in those instances in which compounds were added aseptically to the medium after sterilization, and to 10 ml where such compounds were not added. The medium was then sterilized in an autoclave at 15 lb/inch^2 pressure for 15 min. The compounds to be tested for cell-division-promoting activity were sterilized by filtration with the use of 0.45-μm Millipore filters and 1 ml of the desired concentration was added to the 9 ml of medium after the sterilized medium had cooled to below 60°. One uniformly cut piece of pith tissue was added to each flask and the flasks were held at a temperature of 23° in diffuse light for 3–4 weeks, after which time the results were recorded.

Source of Compounds Tested. An initial sample of the 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP), as well as certain other 8-substituted derivatives of cAMP, were kindly supplied by Dr. Sidney M. Hess of The Squibb Institute for Medical Research. Later samples were purchased from Plenum Scientific Research, Inc., Hackensack, N.J. The 8-bromo derivative of 5′-adenosine monophosphate (8-Br-5′-AMP) was synthesized by use of the procedure described by Muneyama *et al.* (15). The reaction products were resolved on a Dowex 1 × 8 (formate form, 100–200 mesh) column according to the procedure of Ikehara and Uesugi (16). The [*U*-^{14}C]cAMP (3′:5′) (117 Ci/mol) was purchased from the Amersham/Searle Corp., Arlington Heights, Ill. Approximately 15 μCi of this compound was taken to dryness in a vacuum desiccator and the total reaction mixture used by Muneyama *et al.* (15) for the bromination of cAMP (3′:5′) was scaled down so that the final volume was 1.4 ml. The products of the reaction were resolved on a column containing Dowex 1 × 8 (formate form, 100–200 mesh). The 8-Br-[^{14}C]cAMP fraction was removed and lyophilized. This compound was dissolved in water and diluted so that 2 μl of the solution solubilized in 1 ml of Scintisol-GP and 15 ml of Liquifluor and toluene gave approximately 2000 cpm.

The other compounds tested were obtained commercially.

A stock solution of each of the compounds tested for cell division activity, except 8-Br-cAMP (3′:5′) and 8-Br-5′-AMP, was prepared at a concentration of 1 mM in triple-distilled water just before use. The 8-bromo derivatives were dissolved in a 0.01 N NaOH solution to give a final concentration of 1 mM. The pH of all solutions was adjusted to 6 with HCl. Desired dilutions were made from those stock solutions.

Chromatographic Assay for 5′-AMP Phosphomonoesterases. The 5′-AMP monoesterase was prepared from normal tobacco callus tissue (300 g) grown on the Linsmaier and Skoog medium for 3 weeks. The tissue was harvested and prepared in the same manner as described earlier for the isolation of the phosphodiesterases (12) through the ammonium sulfate precipitation and the dialysis steps. This crude enzyme preparation contained high monoesterase activity.

The reaction mixture consisted of 32 mM Tris·HCl (pH 7.0), 0.64 mM dithiothreitol, 0.64 mM $MgCl_2$, 1.29 mM 5′-AMP or 1.29 mM 8-Br-5′-AMP, and 25 μl of crude enzyme preparation. The total volume of the reaction mixture was 155 μl. The reaction was run at 30° for 5, 15, and 30 min, and was terminated by immersing the tubes in boiling water for 3 min. The cooled reaction mixture was centrifuged at 5900 × *g* for 10 min and 50 μl of the supernatant was chromatographed as described (12). Controls consisting of 5′-AMP or 8-Br-5′-AMP were carried through the procedure with 25 μl of distilled water in place of the enzyme solution. Absorbing spots on the paper, which were revealed with a UV lamp, were cut out in such a way that the total area of the paper removed was the same in all instances. Paper controls of the same size were cut from the chromatogram in areas free of absorbance but having R_F values comparable to the absorbing samples, and the eluants were used as the blanks for spectrophotometric analysis. Paper containing the absorbing spots, as well as the paper controls, were eluted with water according to the procedure described by Heppel (17). The eluants were in all instances adjusted to a total volume of 2 ml and their absorbance was measured with a Cary model 15 recording spectrophotometer.

Phosphodiesterase Assay. The cAMP (3′:5′) phosphodiesterases used in this study were isolated from 300 g of freshly isolated Turkish tobacco pith tissue according to methods previously described (12), except that the enzyme preparation was used after the dialysis step. The reaction mixture consisted of 40 mM Tris·HCl (pH 7), 0.8 mM dithiothreitol, 0.4 mM $MgCl_2$, 0.48 mM cAMP (3′:5′) or 0.4 mM 8-Br-cAMP (3′:5′), 5 μl of [^{14}C]cAMP (3′:5′) or 20 μl of 8-Br-[^{14}C]cAMP (3′:5′) prepared as indicated above, and 100 μl of the enzyme preparation. The total volume of the reaction mixture was 250 μl. The reaction was terminated after 60 min by immersing the tubes in boiling water for 3 min. To the cooled tubes, 50 μl of 2 mM 5′-AMP or 8-Br-5′-AMP and 50 μl of adenosine were added, and the tubes were centrifuged at 5900 × *g* for 10 min. 50-μl Aliquots of the supernatant liquid were removed for chromatography. The cAMP (3′:5′) assay was chromatographed (12). The 8-Br-cAMP (3′:5′) and 8-Br-5′-AMP were chromatographed on precoated cellulose 20 × 20 cm thin-layer chromatography plates with the use of *n* butyl alcohol–glacial acetic acid–water 4:1:1 as the solvent. In this system the 8-Br-cAMP had an R_F of 0.12, while the 8-Br-5′-AMP had an R_F of 0.64. In all instances the UV-absorbing spots were carefully scraped off the plates, placed in a Liquifluor–toluene solution that contained 13% ethanol to more readily solubilize the compounds to be tested, and counted as described (12).

RESULTS

Since it was the purpose of the present study to test the suggestion made earlier that the cytokinesins may exert their cell-division-promoting effects as regulators of cAMP, an attempt was made to learn whether cAMP or one of its derivatives, when applied in a culture medium, was capable of replacing the cell-division-promoting effects of the cytokinesins in excised tobacco pith parenchyma tissue. Representative results of that study, together with appropriate controls, are pictured in Fig. 1.

cAMP, when applied in the culture medium at final concentrations of 100 μM, 10 μM, or 1 μM, was essentially

ineffective in promoting cell division in the tobacco pith assay, either with or without 1% dimethylsulfoxide in the culture medium. The tissues in all instances showed typical auxin responses in which growth was largely, if not entirely, due to cell enlargement (Fig. 1*G*). The 8-bromo derivative of cAMP was, on the other hand, highly effective in promoting cell division in the pith parenchyma cells when incorporated into the culture medium at a final concentration of 100 μM (Fig. 1*E*). Both the rate of growth and type of growth obtained were in every way comparable to that observed under similar conditions of culture when either kinetin or cytokinesin I were used in place of the 8-bromo derivative of cAMP (compare Fig. 1*C* and *D* with *E*). The 8-bromo derivative of cAMP was less effective in promoting cell division when applied at a final concentration of 10 μM.

As controls in these experiments, adenosine, 5′-AMP, and 8-Br-5′-AMP were incorporated individually into an auxin-containing culture medium at a concentration of 100 μM. The tissues grown on medium containing adenosine or 5′-AMP showed typical auxin responses in which growth was largely, if not entirely, due to cell enlargement (Fig. 1*H* and *I*). The 8-Br-5′-AMP, like 8-Br-cAMP, encouraged active cell division in the tobacco pith system. The question that arose, therefore, was why the 8-bromo derivatives of cAMP and of 5′-AMP were biologically active in this assay system while the unsubstituted compounds were not. It has been reported that certain other 8-substituted cAMPs are significantly more resistant to degradation by animal cAMP phosphodiesterases than is the unsubstituted compound (15). An attempt was made, therefore, to learn whether the 8-bromo derivatives of cAMP and of 5′-AMP were more resistant than were the unsubstituted compounds to degradation by plant cAMP phosphodiesterases and phosphomonoesterases, respectively. Typical results of those studies demonstrated that while 94% of cAMP was hydrolyzed in a 60-min period by a crude phosphodiesterase preparation isolated from tobacco pith tissue, only 2% of the 8-Br-cAMP was degraded in that period of time. Similarly, a crude monoesterase preparation obtained from tobacco callus tissue completely hydrolyzed 5′-AMP in 15 min, while 84% of the 8-Br-5′-AMP remained unhydrolyzed during that period. It is clear from those results that the 8-bromo derivatives of both compounds are far more resistant than are the unsubstituted compounds to degradation by their respective enzymes. This finding provides a possible explanation as to why the 8-bromo derivatives possess biological activity in this assay system while the unsubstituted compounds do not if it is assumed that 8-Br-5′-AMP can be converted into a stable 8-Br-cAMP through 8-Br ATP by the pith cells. This pathway for synthesis of cAMP is, of course, well established in other systems for the natural compounds. It will, nevertheless, be necessary to follow the fate of 8-Br-5′-AMP in the pith cells before this point is established.

It was found, moreover, that a stock culture of normal tobacco callus tissue responded with profuse growth accompanied by cell division to the 8-bromo derivative of cAMP when that substance was used in the medium at a final concentration of 100 μM. This tissue normally requires an exogenous source of either a 6-substituted purine cytokinin or a cytokinesin if cell division is to occur. Soybean callus tissue, on the other hand, did not respond with growth to the 8-Br-cAMP. The reason for this is not clear.

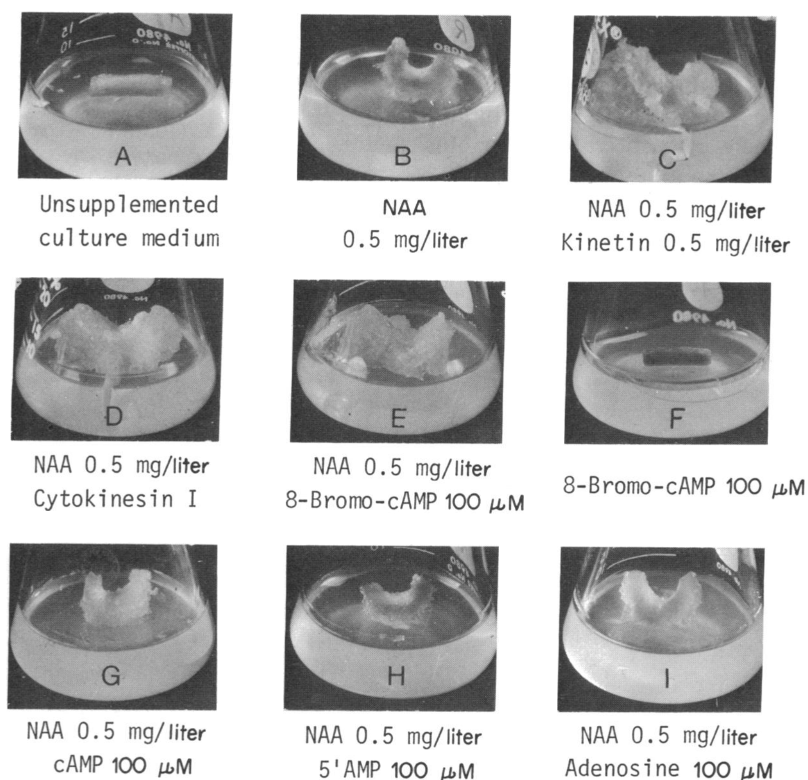

FIG. 1. Response of excised Turkish tobacco pith tissues grown on the Linsmaier and Skoog medium supplemented as indicated. Note tissues in flasks *C, D,* and *E* show profuse growth accompanied by cell division. Tissues in flasks *B, G, H,* and *I* show typical auxin responses in which growth is due largely, if not entirely, to cell enlargement. Tissue in flask *F* has not grown, indicating that 8-Br-cAMP cannot replace auxin in this system. Pictures were taken 3 weeks after the experiment began. NAA: naphthalene acetic acid.

DISCUSSION AND CONCLUSIONS

The results reported here demonstrate that an exogenously supplied source of 8-Br-cAMP can completely replace either a 6-substituted adenylyl cytokinin, such as kinetin, or a cytokinesin as a cell-division-promoting factor in excised tobacco pith parenchyma tissue. cAMP is, on the other hand, ineffective in that system, suggesting either that the unsubstituted compound is not taken up by the pith cells or that it is more readily degraded than is the 8-bromo derivative by the cAMP phosphodiesterases present in those cells. There is, as yet, no information available on the relative rates of uptake of the two compounds by the pith cells, or whether, in fact, these substances penetrate at all but, rather, remain localized at the surface of the cells. The results reported here nevertheless indicate that the 8-bromo derivative of cAMP is far more resistant to degradation by plant cAMP phosphodiesterases than is cAMP. This finding could account for the observed differences in the biological activity shown by the two compounds. That 8-Br-cAMP is at least as effective as is cAMP and can completely replace cAMP in the cAMP-dependent protein kinase reaction has been demonstrated by others (15). Certain other 8-substituted cAMPs were found, moreover, to be significantly more resistant to degradation by animal phosphodiesterases than was cAMP (15).

The 8-bromo derivative of 5′-AMP, but not 5′-AMP, was also shown to be effective in promoting cell division in tobacco pith tissue. The 8-bromo derivative of 5′-AMP was found in these studies to be far more resistant to degradation by plant phosphomonoesterases than was the unsubstituted compound. This property, together with the well known conversion of 5′-AMP to cAMP through ATP, suggests that the synthesis of 8-Br-cAMP from 8-Br-5′-AMP may occur in the pith cells, leading to a stable biologically active form of cAMP.

There are at least two other explanations that could account for the observed results. The first of these is that the 8-bromo derivative of cAMP, because it is a stable structural analogue of cAMP, could act as a competitive inhibitor of cAMP for binding sites on the phosphodiesterases and thus serve to protect cAMP synthesized by the pith cells. It seems unlikely that 8-Br-5′-AMP would act in that way to promote cell division. The 8-bromo derivative of 5′-AMP might, on the other hand, act by way of substrate inhibition and thus force the equilibrium of the phosphodiesterase reaction in such a way as to maintain an elevated concentration of cAMP in the pith cells during critical periods in the cell cycle. An attempt is now being made to resolve these several possibilities.

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