THE QUANTITATION OF ADENOSINE 3′:5′-CYCLIC MONOPHOSPHATE IN CULTURED TOBACCO TISSUE BY MASS SPECTROMETRY

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1. Introduction

Although adenosine 3′:5′-cyclic monophosphate (cAMP) is ubiquitous metabolite in mammalian tissues and in microorganisms, its occurrence in higher plants has not been established beyond reasonable doubt [1,2]. Claims that cAMP is present in higher plants have been based almost entirely on the biochemical assay procedures used to measure levels of cAMP in animal tissues. However, plant extracts are now known to contain compounds which interfere markedly in such assays and render them of questionable value for quantitation of cAMP in plants [1–4]. We have developed a specific assay based on combined gas chromatography–mass spectrometry with selected multiple ion detection (GC–MS–MID) which has been used for the quantitation of picomole amounts of cAMP in liver and urine [5]. Based on further studies involving this assay, we now report the detection and quantitation of cAMP in a sterile higher-plant tissue, namely, cultured tobacco-pith callus tissue.

2. Materials and methods

2.1. *Plant tissue and extraction*

Callus tissue, derived from the stem pith of tobacco (*Nicotiana tabacum*, cv. Wisconsin 38) plants, was maintained in culture on a medium (pH 5.9) containing the inorganic nutrients essentially as used in [6] plus the following (mg/l): myo-inositol, 100; 2,4-D, 2.0; kinetin, 0.01; thiamine, 10; nicotinic acid, 1.0; pyridoxine, 1.0; sucrose, 20 000; agar, 10 000. Although the tissue had acquired cytokinin autonomy during repeated subculturing, a low concentration of kinetin was added to the medium. All cultures were grown for 18 days at 27°C in darkness.

Small pieces of tissue were dropped into methanol–chloroform–formic acid–water (12:5:1:2, by vol.; 5 ml/g fresh wt tissue) which had been chilled to 40°C. The following internal standards were added: c[8-^{3}H]AMP (25 Ci/mmol; 0.1 pmol/g tissue); c[2,8-$^{2}H_2$,6-^{15}N]AMP (4 μg 100 g tissue). After 48 h at −20°C to inhibit phosphatase [7], the mixture was homogenized, stirred at 4°C for 24 h and finally filtered at 23°C. The plant residue was stirred with methanol–water–formic acid (20:80:1, by vol.; 5 ml/g tissue) at 23°C for several hours and the mixture was then filtered. The 2 extracts were evaporated to dryness in vacuo, and aqueous suspensions of the residues were combined.

2.2. *Purification of extracts*

Aqueous solutions of the extracts were neutralized with 10 M KOH, centrifuged, concentrated and applied to a column (2 × 10 cm) of neutral alumina (Merck), from which cAMP was eluted with distilled water. The eluate fractions containing c[^{3}H]AMP were pooled, concentrated and applied to a Dowex-50 ion-exchange column (2 × 10 cm; 200–400 mesh; H^+ form). Cyclic AMP was eluted with distilled water and the combined ^{3}H-containing fractions were further purified by TLC on Merck pre-coated silica gel $60F_{254}$ plates using 2-propanol:28% (w/w) aqueous NH_4OH (7:3) as solvent (R_F cAMP, ~0.6). Purified methyl blue chromatographed just below c[^{3}H]AMP and was used to locate cAMP on the TLC plate. A portion of the c[^{3}H]AMP zone (~25%) was discarded to avoid an overlapping fluorescent compound which interfered in the GC–MS–MID assay. The remainder of the TLC zone was eluted with 50% ethanol. Part of the eluate was subjected to GC–MS–MID, while

the remainder was further purified by HPLC as outlined below.

An aqueous solution of the evaporated TLC eluate was centrifuged (10 000 × g, 10 min) and purified isocratically using a DuPont Zorbax C-8 column (10 μm packing; 4.6 mm × 25 cm). The mobile phase consisted of 0.16 M acetic acid containing 4% (v/v) methanol and the flow rate was 1.0 ml/min. In this system the k'-values for cAMP and its isomer adenosine 2′:3′-cyclic monophosphate (2′:3′-cAMP) were 1.1 and 0.40, respectively. A peak which eluted at the retention time of authentic cAMP, and which exhibited a A_{254}/A_{280} ratio similar to that of cAMP, was collected. This peak, which was homogeneous by analytical HPLC, was further examined by GC–MS–MID.

2.3. *Quantitation by mass spectrometry*

The equipment and experimental details of the GC–MS–MID step in the assay for cAMP have been published [5] and were used with only minor modification. Here, improved sensitivity was obtained by using the GC-column (1% OV-17 on Gas Chrom Q) bleed peak at m/z 281 as the MID lock-channel reference mass rather than the bleed peak at m/z 207 as in [5]. Quantitation using the GC–MS–MID assay is based on peak-height responses at m/z 530 and 533 (for identity of ions, see below).

3. Results and discussion

For the determination of cAMP in liver extracts and in urine by the GC–MS–MID assay, the only purification step required was chromatography on Dowex-50 [5]. However, because of the considerably lower cAMP level in plant tissue and the interference from other endogenous compounds, several additional purification steps were necessary for cAMP quantitation in tobacco callus tissue. After inactivation of phosphatase at −20°C, the tissue was extracted with solvent containing c[8-^{3}H]AMP and c[2,8-$^2H_2$,6-^{15}N]-AMP. The former enabled cAMP-containing fractions to be detected by radiotracer monitoring during extract purification and also provided a basis for calculation of cAMP recoveries; the latter served as the internal standard for GC–MS–MID quantitation [5]. The tobacco tissue extract was purified by sequential chromatography on columns of alumina and Dowex-50 and then by TLC on silica gel. The overall recovery of c[8-^{3}H]AMP was ~20%.

The eluate of the c[8-^{3}H]AMP-containing zone was trimethylsilylated and the trimethylsilyl (TMS) derivatives were analysed by GC–MS–MID. The monitored fragment ions derived from tri-TMS cAMP and their identity are as follows:

m/z 533 (M^+-CH_3 of the $^2H_2$,^{15}N-labelled standard);
m/z 530 (M^+-CH_3 of endogenous cAMP); and
m/z 310 (a robose phosphate fragment ion, common to both endogenous and isotopically-labelled cAMP).

In the GC system used, the retention times of c[2,8-$^2H_2$,6-^{15}N]AMP and unlabelled cAMP are 5.8 and 5.9 min, respectively, i.e., there is a slight isotopic fractionation. The ion-current for m/z 533, 530 and 310 for the TLC-purified extract exhibited peaks at the exact retention times of authentic labelled and unlabelled cAMP, but in addition, there were several other prominent peaks at differing retention times. Hence, the purification procedure was not sufficient to remove from the plant extract all compounds which were capable of giving rise to ions at the same m/z values as those used to monitor cAMP and its labelled analogue. Although these compounds did not appear to interfere in the quantitation of cAMP, the TLC eluate was further purified by HPLC on a Zorbax C-8 column which effectively separates cAMP from its 2′:3′-isomer. The fraction containing c[^{3}H]AMP was collected and subjected to GC–MS–MID. The ion-current traces for m/z 533 and m/z 530 each showed only one prominent peak corresponding to the retention time of labelled and unlabelled cAMP, respectively. The ratio of the heights of the 533, 530 and 310 ion-current peaks at the retention time of cAMP was the same as that measured before HPLC purification.

Hence, we have detected in tobacco callus extracts a compound which co-chromatographs with c[^{3}H]AMP during initial alumina and Dowex-50 column chromatography, and during subsequent TLC and HPLC purification procedures. The TMS derivative of the compound exhibited the same GC retention time as tri-TMS–cAMP and fragmented to yield high-mass ions characteristic of the TMS derivative of cAMP during electron-impact mass spectrometry. In view of this substantial evidence, we conclude that the compound in the extracts is cAMP. Using the GC–MS–MID assay with c[2,8-$^2H_2$,6-^{15}N]AMP as an internal standard, the level of cAMP in the tobacco tissue, cultured as defined in section 2, was calculated to be 93 ± 9 pmol/g fresh wt. In another sample of tissue,

cultured in the absence of exogenous cytokinin, the cAMP level was found to be 137 ± 12 pmol/g fresh wt.

For tobacco callus tissue, reported levels of cAMP based on biochemical assays range from <0.5 pmol/g fresh wt [8] to 900 pmol/g dry wt [9]. This wide variation probably reflects the lack of specificity of these assays for cAMP when applied to plant extracts. Extraction of plant tissue before inactivation of phosphatase may be another relevant consideration.

HPLC had been used for purification of nucleotides, including cAMP [10], from tissue extracts. Usually these separations have involved the use of anion exchangers and buffered salt solutions for elution. However, the reverse-phase HPLC system we describe, gives excellent resolution, separates cAMP from its 2′:3′-isomer, and avoids the use of buffers which introduce electrolyte into purified fractions to interfere in trimethylsilylation and GC–MS–MID.

The experiments detailed herein constitute reliable evidence for the occurrence of cAMP in a higher plant tissue. The GC–MS–MID method for quantitating cAMP should provide a chemical basis on which to carry out physiological studies of this nucleotide in plants.

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