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METABOLISM OF HALOGENATED BISPHOSPHONATES BY THE CELLULAR SLIME MOULD *DICTYOSTELIUM DISCOIDEUM*

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Methylenebisphosphonate and its monofluoro-, difluoro- and dichloro- derivatives inhibited growth of amoebae of *Dictyostelium discoideum*. Dichloromethylenebisphosphonate was the most potent inhibitor of amoebal growth whereas difluoromethylenebisphosphonate was the least potent inhibitor. Each of the bisphosphonates was taken up by the amoebae and incorporated into the corresponding ß,γ–methylene analogue of adenosine triphosphate. Two of the bisphosphonates were also incorporated into the corresponding analogues of diadenosyl tetraphosphate. No correlation was found between the ability of the bisphosphonates to inhibit amoebal growth and the extent to which they were metabolised.

Abbreviations:
Ap_4A 5',5'''-diadenosyl P^1,P^4-tetraphosphate; $AppCCl_2p$ adenosine 5'-(ß,γ-dichloromethylenetriphosphate); $AppCF_2p$ adenosine 5'-(ß,γ-difluoromethylenetriphosphate); AppCFp adenosine 5'-(ß,γ-fluoromethylenetriphosphate); AppCp adenosine 5'-(ß,γ-methylenetriphosphate); AppCppA diadenosine 5'5'''-P^1,P^4-(P^2,P^3-methylene tetraphosphate); CDTA trans-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; Cl_2MBP dichloromethylenebisphosphonate; F_2MBP difluoromethylenebisphosphonate; FMBP monofluoromethylenebisphosphonate; FPLC fast protein liquid chromatography; MBP methylenebisphosphonate; MES 2(N-morpholino) ethanesulphonic acid.

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Methylenebisphosphonate (MBP) has a structure similar to that of pyrophosphate but with the phosphoanhydride oxygen bridge of the latter replaced by a methylene group. It is therefore an analogue of pyrophosphate that cannot be hydrolysed in reactions catalysed by enzymes such as pyrophosphatase. However, the ionisation of MBP is different from that of pyrophosphate so that, in the physiological pH range, MBP tends to be less negatively charged than pyrophosphate. Because of this, monofluoro- and difluoro-methylenebisphosphonates (FMBP, F_2MBP) have been synthesised in the expectation that they would be improvements on MBP as non-hydrolysable analogues of pyrophosphate owing to their more acidic ionisation constants (1,2).

Amoebae of the cellular slime mould *Dictyostelium discoideum* are able to take up MBP and incorporate it into adenosine 5'-(ß,γ-methylenetriphosphate) (AppCp) and 5',5'''-P^1,P^4-(P^2,P^3- methylene-tetraphosphate) (AppCppA) in reactions probably catalysed by the amoebal aminoacyl-tRNA synthetase enzymes (3). It has been proposed that intracellular accumulation of these metabolites then causes the inhibition of *Dictyostelium* growth that occurs when MBP is included in the growth medium. Amoebae of *Dictyostelium discoideum* therefore appeared to be an ideal system for comparing *in vivo* the effectiveness of the fluorinated methylenebisphosphonates and MBP as analogues of pyrophosphate.

Dichloromethylenebisphosphonate (Cl_2MBP) has been used clinically for treatment of bone diseases, such as Paget's disease and hypercalcaemia of malignancy (4-7), in which there is excessive bone resorption owing to over-activity of the osteoclast cells. The drug was developed as a result of early investigations of bone metabolism that suggested that pyrophosphate is involved in the control of bone formation and destruction by osteoblast and osteoclast cells, and that non-hydrolysable analogues of pyrophosphate might therefore be helpful in treating diseases in which the activities of these bone cells become uncoordinated (8-12). Nevertheless, the mechanism of action of Cl_2MBP and other bisphosphonate drugs is unknown (13,14). It therefore seemed appropriate to include Cl_2MBP in investigations of the metabolism of halogenated bisphosphonates by amoebae of *D. discoideum* since clues about its cellular actions might be revealed.

MATERIALS AND METHODS

Chemicals

Methylenebisphosphonic acid was obtained from Aldrich, and antifoam A and CDTA were from Sigma. Disodium

dichloromethylenebisphosphonate was a gift from Instituto Gentili S.p.A., Pisa, Italy. FMBP and F_2MBP were synthesised as described in (2). Synthesis of ATP analogues was as described in (15).

Growth of Dictyostelium discoideum

Amoebae of the strain Ax-2 were grown axenically as previously described (16). Cultures were inoculated at an initial density of 10^4 cells.ml^{-1} and growth of the cultures was monitored by counting samples in a model ZM Coulter Counter.

Preparation of cell extracts

700ml cultures were harvested at a density of 8 x10^6 cells.ml^{-1} and the amoebae were washed twice with 20mM MES buffer, pH 6.3 at 0°C. The amoebae were resuspended to a final volume of 20ml in the MES buffer at 22°C or in the MES buffer containing 5mM bisphosphonate at 22°C. Approximately 10µl antifoam A were added to each suspension through which O_2 was then bubbled slowly. The suspensions were maintained at 22°C for 2h and were kept stirring by magnetic followers. After 2h, the amoebae were harvested, washed three times with the MES buffer at 0°C and resuspended in 20ml distilled water at 0°C. 2.5ml 70% $HClO_4$ at 0°C were then added and the extracts were centrifuged. The supernatants were then neutralised with a saturated solution of $KHCO_3$, left at 0°C for several hours and then centrifuged. Supernatants that were intended for NMR analysis were made 7mM with CDTA. The resulting supernatants were lyophilised and the residues redissolved in 1.5ml distilled water (for FPLC) or 0.5ml D_2O (for NMR).

NMR and FPLC analysis of cell extracts

^{31}P NMR spectra were collected at 20°C on a Bruker AMX-500 spectrometer operating at 202.46 MHz, using a 90° pulse, a spectral width of 18.5 kHz and an acquisition time of 0.44s. A total relaxation delay of 2s was allowed between pulses. Broad-band proton-decoupling was applied during data acquisition using the WALTZ16 pulse sequence, with a field strength of 2.8 kHz. ^{19}F NMR spectra were collected at 470.50 MHz, using a 90° pulse, a spectral width of 11.9 kHz and an acquisition time of 0.7s. A total relaxation delay of 1.7s was allowed between pulses. Signals in the NMR spectra were identified by comparison with the spectra of authentic compounds.

50µl samples of *Dictyostelium* cell extracts were also analysed by FPLC on a 1.0ml MonoQ anion-exchange column (Pharmacia), by elution in a gradient of NH_4HCO_3 (120mM - 360mM NH_4HCO_3 over 17 minutes, followed by 360mM - 120mM NH_4HCO_3 over 3 minutes, 1.5 ml.min^{-1}). Eluted nucleotides were detected by their absorption at 254nm, and were identified by comparison with the retention times of standards, and by spiking extracts with authentic compounds.

RESULTS

All of the halogenated methylenebisphosphonates inhibited growth of *Dictyostelium* amoebae (Fig. 1.). Cl_2MBP was the most potent

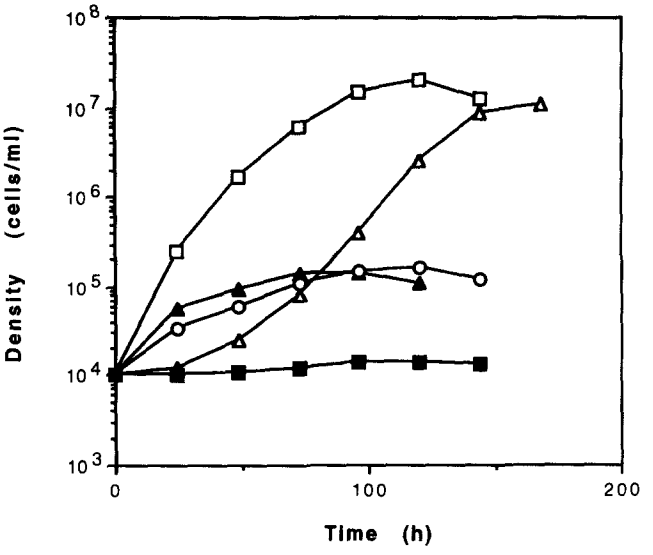

Fig.1. Inhibition of growth of *D. discoideum* amoebae by four bisphosphonates (□ control; △ 2mM F_2MBP; ▲ 2mM FMBP; ○ 2mM MBP; ■ 1mM Cl_2MBP).

growth inhibitor and, when it was included in the growth medium at 500μM, there was only half the number of cell divisions that occurred in its absence. Similar inhibition by the other bisphosphonates occurred only at higher concentrations (2mM for MBP and FMBP; 3mM for F_2MBP).

^{31}P NMR spectroscopy was used to detect metabolites of the bisphosphonates in extracts of amoebae that had been incubated with 5mM bisphosphonate for 2h. Analogues of ATP (ie AppCp) and Ap_4A (ie AppCppA) were found in extracts of amoebae that had been incubated with MBP, confirming previous observations (3). It was also found that FMBP was incorporated into an ATP analogue (ie AppCFp) but no Ap_4A analogue was detectable. Metabolites of F_2MBP and Cl_2MBP could not be detected by ^{31}P NMR but studies of the spectra of authentic samples of $AppCF_2p$ and $AppCCl_2p$ suggested that most of the NMR signals from these compounds would have been obscured by signals from other phosphorus-containing compounds in the cell extracts. However, the AppCFp metabolite of FMBP and the $AppCF_2p$ metabolite of F_2MBP were found in cell extracts by using ^{19}F NMR (Fig. 2.). AppCFp and $AppCF_2p$ were identified by spiking the extracts with the authentic compounds. The $AppCF_2ppA$ metabolite of F_2MBP was also observed at a concentration less than 10% of that of $AppCF_2p$ (data not shown).

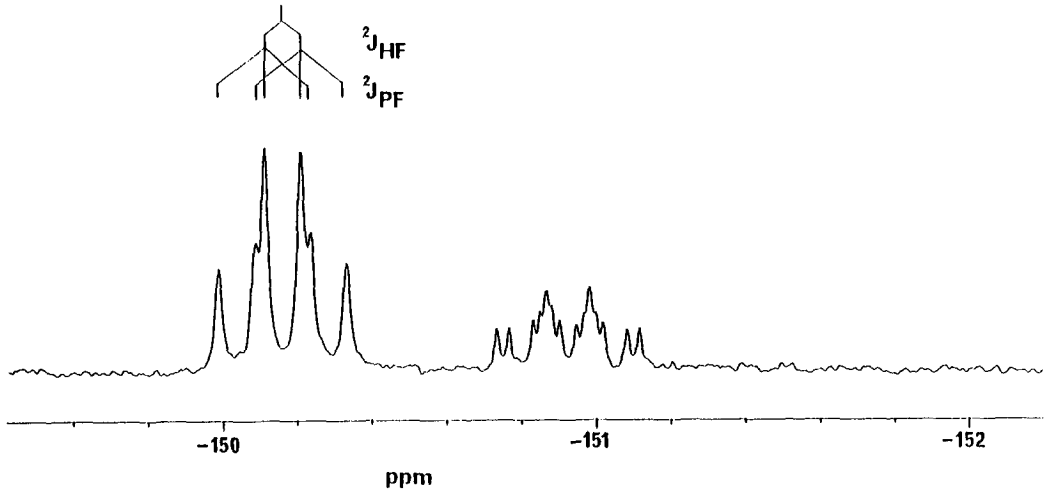

Fig. 2. ^{19}F NMR spectrum of a cell extract prepared from amoebae incubated in 5mM FMBP for 2h. Splittings arising from two-bond *J*-coupling are indicated above the cluster of peaks for PCFP. The coupling pattern for AppCFp is similar, but each peak is doubled because the compound is present as a diastereomeric mixture.

Ion-exchange chromatography was also used to separate the adenine nucleotides in the cell extracts. MBP was incorporated into both AppCp and AppCppA (Fig. 3C.) whereas F_2MBP and FMBP appeared to be metabolised only into the ATP analogues (ie $AppCF_2p$ and AppCFp) (Fig. 3A. and 3B.), although small amounts of the Ap_4A analogues may have been obscured by other peaks in the spectra. Both AppCFp and $AppCF_2p$ eluted from the column close to ATP whereas AppCp eluted earlier. This was consistent with the ionisation characteristics of the ATP analogues (15).

The amount of Ap_4A found in amoebae incubated in the absence of bisphosphonate varied from one experiment to another. Addition of bisphosphonates to the amoebae had no obvious effects on intracellular Ap_4A accumulation.

No metabolites of Cl_2MBP could be detected in extracts prepared from amoebae that had been incubated with this bisphosphonate for 2h. However, $AppCCl_2p$ was found in extracts prepared from amoebae that had been grown for 95h in a sub-lethal concentration (300μM) of Cl_2MBP and harvested at a cell density of 4 x 10^6 cells.ml^{-1}. Again, no analogue of Ap_4A was detected (Fig. 4.). In amoebae grown for 95h in 500μM MBP, there was accumulation of both the ATP analogue (AppCp) and the Ap_4A analogue (AppCppA).

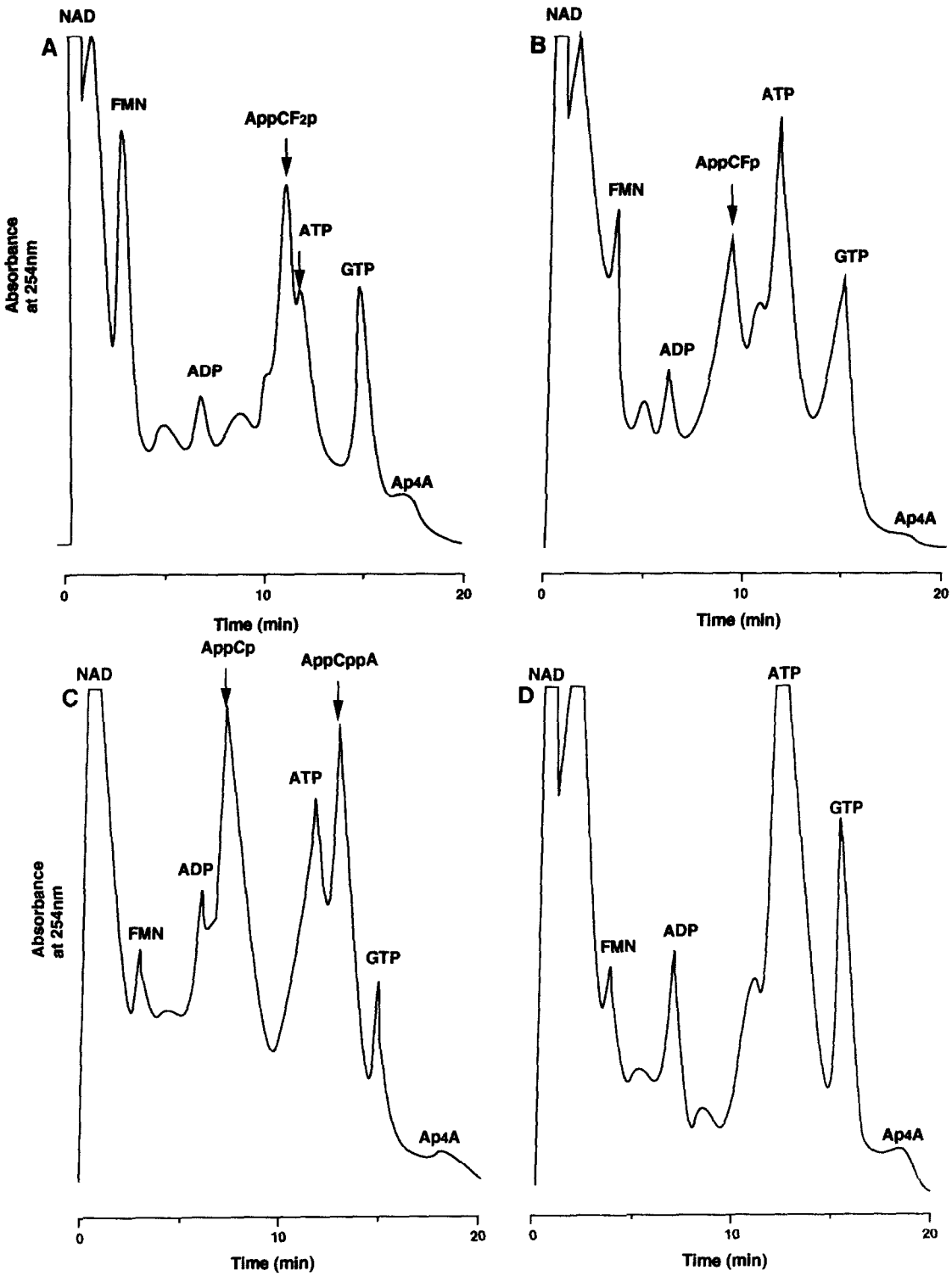

Fig. 3. FPLC elution profiles of extracts from *Dictyostelium* amoebae that had been incubated with bisphosphonates. All the extracts were prepared identically from the same number of amoebae and 50µl samples were analysed by FPLC. Amoebae were incubated for 2h with 5mM F_2MBP (A), 5mM FMBP (B), 5mM MBP (C), or in the absence of bisphosphonate (D).

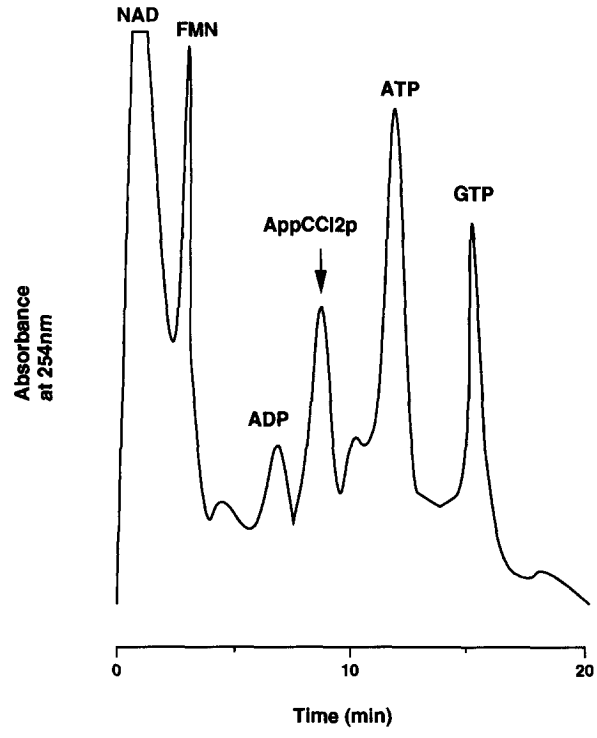

Fig. 4. FPLC elution profile of a *Dictyostelium* cell extract prepared from amoebae grown for 95h in 300μM Cl_2MBP.

DISCUSSION

Zamecnik (17) showed that MBP is a substrate for purified aminoacyl-tRNA synthetase enzymes and acts as an analogue of pyrophosphate in the back reaction (reaction ii). The AppCp so formed can then take part in a further reaction (reaction iii) giving an analogue of Ap_4A. It therefore seemed reasonable to conclude that formation of AppCp and AppCppA in *Dictyostelium* amoebae incubated with MBP was also catalysed by the amoebal aminoacyl-tRNA synthetases (3). Furthermore, by incubating the amoebae with halogenated methylenebisphosphonates, it would also be possible to determine the effectiveness of these bisphosphonates as substrates for the amoebal aminoacyl-tRNA synthetase enzymes *in vivo*.

(i) ATP + amino acid ⟶ aminoacyl-AMP + pyrophosphate

(ii) aminoacyl-AMP + MBP ⟶ AppCp + amino acid

(iii) AppCp + aminoacyl-AMP ⟶ AppCppA + amino acid

FMBP, F_2MBP and Cl_2MBP were all incorporated into ATP analogues but further metabolism into analogues of Ap_4A was almost undetectable. AppCp thus appeared to be a better analogue of ATP for reaction (iii) than were $AppCF_2p$ or AppCFp, even though the latter have ionisation characteristics more similar to those of ATP (15). However, an alternative explanation for these observations could be that both FMBP and F_2MBP are readily catabolised to give analogues of Ap_4A in *Dictyostelium* but, unlike AppCppA, these products are hydrolysed back to the ATP analogues almost as rapidly as they are formed.

The extent of bisphosphonate metabolism was clearly no greater in amoebae incubated with FMBP or F_2MBP than in amoebae incubated with MBP. Thus, although FMBP and F_2MBP have ionisation constants that are closer to those of pyrophosphate than are the ionisation constants of MBP (Table 1), FMBP and F_2MBP are apparently not better than MBP as analogues of pyrophosphate in reaction (ii) catalysed by the aminoacyl-tRNA synthetase enzymes *in vivo*. At least for these enzymes, it would seem that steric properties are more important than ionisation characteristics in determining how effectively bisphosphonates can act as analogues of pyrophosphate.

Metabolism of Cl_2MBP by the amoebae was poor and could not be detected during short incubations. This indicated that Cl_2MBP was the least effective of the bisphosphonates studied as an analogue of pyrophosphate in reaction (ii). Nevertheless, Cl_2MBP was considerably more potent than the other bisphosphonates as an inhibitor of amoebal growth.

Differences in the extent to which each bisphosphonate was metabolised did not appear to be due to differences in the extent to which the compounds were taken up by the amoebae, since comparable results were obtained in preliminary experiments using cell extracts of *Dictyostelium* amoebae instead of intact cells.

Table 1. Comparison of the pK_a3 and pK_a4 values of four bisphosphonates (PXP) with pyrophosphate (POP)

X	pK_a3	pK_a4
O	5.77	8.22
CF_2	5.80	8.00
CFH	6.15	9.35
CCl_2	6.11	9.78
CH_2	7.45	10.96

There was, therefore, no obvious correlation between inhibition of amoebal growth and the extent of bisphosphonate metabolism. This would cast doubt on the proposal that *Dictyostelium* growth is inhibited by bisphosphonates owing to the accumulation of ATP analogues (3).

Cl_2MBP is used clinically particularly in the treatment of hypercalcaemia of malignancy and bone metastases since it inhibits osteoclast-mediated bone resorption (18). While our studies of Cl_2MBP metabolism in *D. discoideum* amoebae have as yet given no clear indication of how Cl_2MBP disrupts cellular function, they have shown that, contrary to the widely-held view, this drug can be metabolised, the product being a non-hydrolysable analogue of ATP. We expect that future work will give some insight into the mechanism by which Cl_2MBP inhibits the growth of *Dictyostelium* and what relationship this has to the inhibition of osteoclastic bone resorption by bisphosphonates.

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