Inhibition of phosphate uptake by fluphenazine, a calmodulin inhibitor
Analysis of Volvox wild-type and fluphenazine-resistant mutant strains

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1. INTRODUCTION

The marked sensitivity of *Volvox* cells to fluphenazine, a phenothiazine calmodulin inhibitor [1–5], results in cell lysis [6]. The lethal effect, produced within a few hours of incubation, allowed the selection of spontaneously-derived resistant mutant strains. Four such mutant strains were isolated; analyses of partially purified calmodulin preparations derived from the wild-type and 2 mutant strains, demonstrated genetically altered calmodulin activity associated with drug resistance [6]. The mutations were also shown to affect development and morphogenesis [6].

The putative calmodulin mutants provide a system for the study of calmodulin-modulated cellular processes and its involvement in the control of development and differentiation in multicellular eukaryotes.

Here, inhibition of phosphate uptake and possibly ATP synthesis by fluphenazine is shown in *Volvox* wild-type and 3 fluphenazine-resistant strains. One mutant strain, FP^r4, exhibited marked resistance to this inhibitory effect. Inhibition of phosphate uptake and incorporation was evident at low, sublethal, drug concentrations, shown to affect *Volvox* motility and the morphogenic process of embryo inversion [6] and implicate calmodulin modulation of phosphate uptake.

2. MATERIALS AND METHODS

2.1. *Materials*

Fluphenazine–2 HCl was kindly provided by E.R. Squibb and Sons, Inc. Sterile stock solutions (100 μg/ml) were prepared in *Volvox* medium. Sodium [^{32}P]phosphate (100 mCi/mmol) was from Nuclear Research Center (Negev). Silica gel 60 thin-layer aluminum sheets were from Merck (Darmstadt) and polygram PEI-cellulose thin-layer sheets were from Machgerey (Negel). Organic solvents were of analytical grade.

2.2. *Strains and growth conditions*

Volvox carteri f. nagariensis, wild-type strains HK10, was obtained from Dr R.C. Starr. Spontaneous fluphenazine-resistant mutant strains FP^r1, FP^r2, FP^r3 and FP^r4 were derived from strain HK10 as in [6]. Asexual cultures were grown in sterile *Volvox* medium (pH 8.0) [7], with illumination of 3000 Lux scheduled for 16 h and 8 h light–dark periodicity with constant bubbling of sterile air at room temperature. Stock cultures were maintained on agar (1% in *Volvox* medium pH 8.0).

2.3. *Incorporation of* $^{32}PO_4^{2-}$

Asexual spheroids were collected on sterile nylon filters (Nitex, 30 μm) washed with phosphate-free medium (without glycerophosphate) and suspended in 10 ml phosphate free medium, in the presence or absence of fluphenazine. These were incubated for 1 h under dim light. The pretreated cultures were pulsed with $^{32}PO_4^{2-}$ (10 μCi) for 20 min. The spheroids were collected by centrifugation and washed with phosphate-free medium. The washed radiolabeled spheroids were divided into 2 equal portions. One portion was used for trichloroacetic acid (10%) precipitation and the other for lipid extraction. Phospholipid extraction was done as in [8].

Trichloroacetic acid-insoluble material, as well as

the chloroform–methanol-insoluble fraction, was dissolved in 0.5 N NaOH, and protein was determined by Lowry method using bovine serum albumin as a standard.

2.4. *Chromatography*

Nucleotides were resolved by chromatography on PEI-cellulose thin-layer plates using 0.85 M potassium phosphate buffer (pH 3.5) [9]. Phospholipids were resolved by two-dimensional chromatography on silica gel thin-layer plates [8].

2.5. *Polyacrylamide gel electrophoresis*

Trichloroacetic acid (10%) (or chloroform: methanol, 1:2) precipitates were dissolved in sample buffer and electrophoresed on sodium dodecylsulfate (SDS)–polyacrylamide gels (7.5% and 14%) [2]. The gels were stained with Coomassie blue, dried on Whatman 3MM paper and exposed to Kodak XAR-5 X-ray films with intensifier screen, at −70°C.

3. RESULTS AND DISCUSSION

The effect of fluphenazine, a phenothiazine inhibitor of various calmodulin-modulated cellular processes [1–5], on phosphate uptake and incorporation into various classes of phosphorylated molecules, has been investigated using intact *Volvox* wild-type and fluphenazine-resistant mutant strains. The time course of in vivo protein phosphorylation in *Volvox* cells has been studied by in-

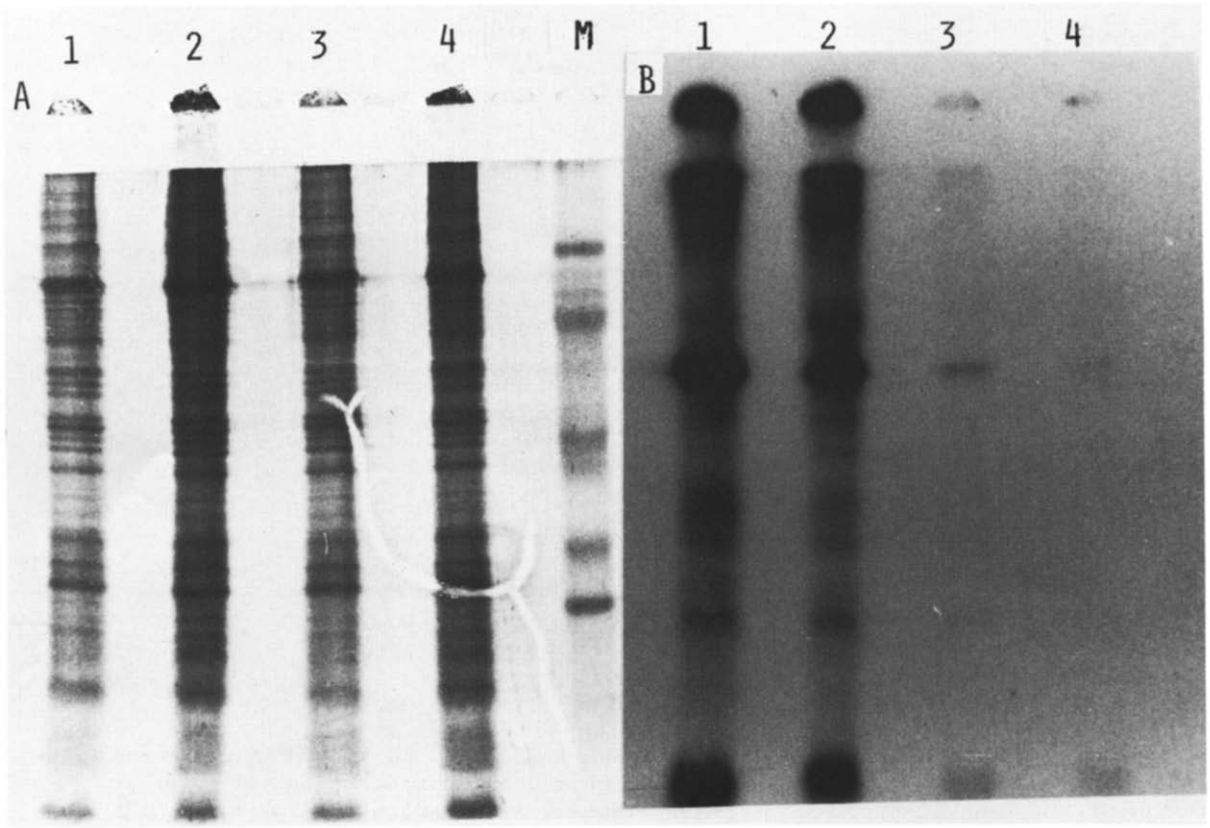

Fig.1. SDS–polyacrylamide gel (14%) electrophorogram of *Volvox* proteins: (A) Coomassie blue staining; (B) Autoradiograhy of in vivo phosphorylated proteins. Lanes 1–4 are aliquots taken from trichloroacetic acid precipitates of intact spheroids incubated with 0, 0.2, 0.4 and 0.6 μg fluphenazine/ml in phosphate free medium prior to 20 min incubation with $^{32}PO_4^{2-}$. Lane H, protein standards M_r: lysozyme (14 300); α-lactoglobulin (18 400); trypsinogen (24 000); pepsin (34 700); egg albumin (45 000); and bovine albumin (66 000) (from Sigma).

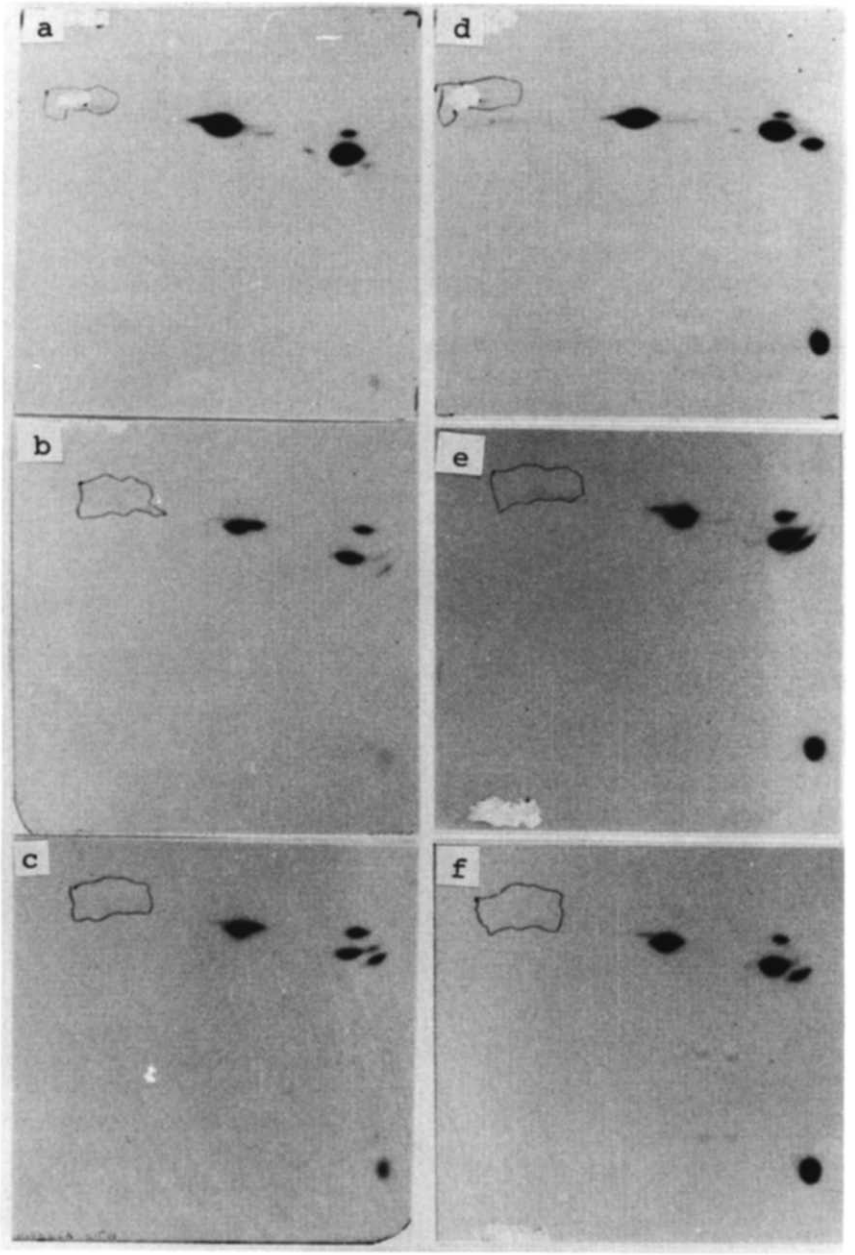

Fig.2. Autoradiograms of two-dimensional chromatography of ^{32}P-labeled phospholipids: (1–3) phospholipids extracted from wild-type HK10 strain incubated with 0, 0.3 and 0.4 μg fluphenazine/ml prior to incubation with $^{32}PO_4^{2-}$, respectively; (4–6) phospholipids extracted from fluphenazine-resistant mutant strain FP^{r}4 incubated with 0, 3.4 and 0.4 μg fluphenazine/ml prior to incubation with $^{32}PO_4^{2-}$. Each chromatogram was loaded with aliquots containing equal cpm.

cubation of intact wild-type spheroids with $^{32}PO_4^{2-}$ in phosphate-free medium, for 15, 30 and 60 min, and detection of radiolabeled phosphorylated proteins by autoradiography following resolution by electrophoresis on SDS–polyacrylamide slab gels (7.5%, not shown). Optimal ^{32}P-incorporation into

proteins was observed in samples incubated with radioactive precursor for 30 min.

The marked inhibition of total $^{32}PO_4^{2-}$ uptake and incorporation into nucleotides, phospholipids and trichloroacetic acid-insoluble macromolecules was observed at low, sublethal fluphenazine concentrations which correspond to effective concentrations for inhibition of *Volvox* motility and the morphogenic process of embryo inversion [6]. The patterns of ^{32}P incorporation into proteins, phospholipids and nucleotides were analysed by autoradiography following resolution by electrophoresis on SDS polyacrylamide gel (14%), two dimensional thin-layer chromatography on silica gels, and chromatography on PEI-cellulose thin-layer, as shown in fig.1–3, respectively. The inhibition of ^{32}P incorporation into the various classes of phosphorylated molecules does not appear to result from changes in pattern of incorporation, but rather reflect the marked inhibition of phosphate uptake. This was apparent from equally reduced incorporation into the resolved phospholipids and nucleotides (fig.2,3). Only one fluphenazine-resistant mutant strain, FP^r4, exhibited lower sensitivity to fluphenazine inhibition of phosphate uptake and incorporation into the various classes of phosphorylated molecules (table 1). The other 3 spontaneously-derived fluphenazine-resistant strains (FP^r1, FP^r2, FP^r3) were as sensitive as the wild-type. Autoradiographs of nucleotides from FP^r4 spheroids radiolabeled in the presence of fluphenazine revealed a possible inhibition of ^{32}P incorporation into ADP and ATP (fig.3).

The inhibitory effect of fluphenazine on phosphate uptake, at low drug concentration, implicates calmodulin modulation of this activity. Activation

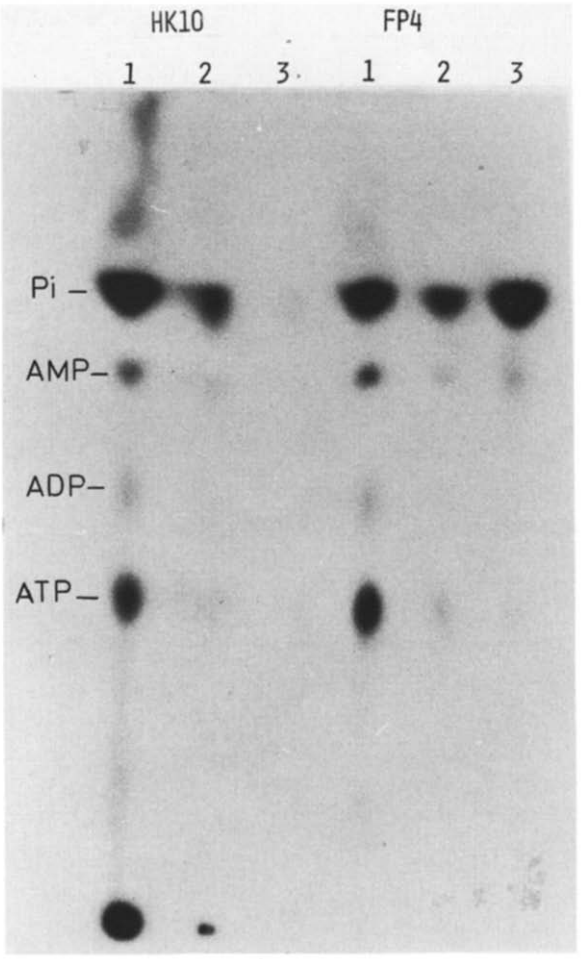

Fig.3. Autoradiography of PEI-cellulose chromatography of ^{32}P-labeled nucleotides extracted from wild-type HK10 and fluphenazine-resistant FP^r4 strains: (1–3) samples taken from cultures incubated with 0, 0.3 and 0.4 μg fluphenazine/ml, respectively, prior to incubation with $^{32}PO_4^{2-}$ in phosphate-free medium.

Table 1
Inhibition of ^{32}P incorporation (% of control) by fluphenazine (0.3 μg/ml)

Strain	HK 10	FP^r1	FP^r2	FP^r3	FP^r4
Trichloroacetic acid (10%)					
-soluble	3	5	5	4	14
-insoluble	4	10	6	2	11
Phospholipids	5	7	5	4	15
Aqueous phase of lipid extraction	5	10	5	4	19

of phosphate uptake was shown to be among the earliest membrane-dependent changes in serum – or mitogens-induced stimulation of quiescent animal cells [10–13] and dependent on, or accompanied by, activation of various cation pumps. These membrane-dependent properties may be calmodulin modulated, either directly or through modulation of other cellular processes, such as cytoplasmic pH. Indeed intracellular calcium may have an important role in the regulation of intracellular pH [14,15].

Fluphenazine resistance is expected to result from altered calmodulin activity in the spontaneously-derived mutant strains. Aberrant calmodulin activity in two strains, FP^r1 and FP^r3, have been shown by analysis of the activation of erythrocyte Ca^{2+}, MG^{2+}-ATPase by preparation of partially purified calmodulin [6]. Whereas partially purified wild-type calmodulin was found to activate Ca^{2+}-ATPase and the activation was sensitive to inhibition by fluphenazine, no activation was detected by a partially purified preparation from mutant strain FP^r1 and activation by the preparation derived from mutant strain FP^r3 was not sensitive to fluphenazine. These results, together with the evidence presented for reduced sensitivity of FP^r4 cells to inhibition of phosphate uptake by fluphenazine, may indicate that the drug resistance in these spontaneously-derived strains results from mutations which selectively affect the interactions of the mutant calmodulin with specific cellular components, the activity of which is calmodulin-modulated.

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