HERBICIDES AND FUNGICIDES STIMULATE Ca^{2+} EFFLUX FROM RAT LIVER MITOCHONDRIA

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1. Introduction

Through work on the regulation of microfibril orientation in the algae *Oocystis* [1] and on flagellar regeneration in *Chlamydomonas* [2] it has become clear that the herbicides amiprophosmethyl, oryzalin and trifluralin, all of which show antimitotic properties, affect plant microtubule polymerization and depolymerization. As proposed in [2,3], the action mechanism of these substances in plant cells appears to be through disturbance of the cytoplasmic Ca^{2+} level by interfering with mitochondrial Ca^{2+} accumulation. Furthermore, *Chlamydomonas* flagellae regeneration is inhibited by the two fungicides captan and dichlofluanide (C. Fedtke, personal communication). Both substances are reported to react reversibly with thiol-groups of proteins [4,5]. We have found that captan and dichlofluanide inhibit plant mitochondrial Ca^{2+} accumulation (C. H., D. M., unpublished).

In animal cells, the important regulatory function of free cellular Ca^{2+} is well established [6]. Mitochondria may play an important role in the control of free cytoplasmic $[Ca^{2+}]$ [7]. The widespread agricultural usage of the above herbicides and fungicides might presuppose their ineffectiveness on animal cells. However, as in plant cells, any disturbance of the free cytoplasmic Ca^{2+} level would cause a disturbance of many biochemical and physiological processes in animal cells which depend on well-balanced cellular Ca^{2+} relationships. Therefore, we have investigated the effects of the herbicide amiprophosmethyl, its structural analogues amiprophos, oryzalin and trifluralin and of the fungicides captan and dichlofluanide on rat liver mitochondrial Ca^{2+}-transport.

We show that all of the above pesticides affect rat liver mitochondrial Ca^{2+} accumulation. The two fungicides are even more effective with this animal system than with plants.

2. Materials and methods

2.1. *Chemicals*

Amiprophosmethyl, amiprophos, dichlofluanide and captan were obtained as gifts from Bayer AG, Leverkusen. Trifluralin and oryzalin were obtained as gifts from Lilly Res. Labs., (Indianapolis IN). All substances were dissolved in dimethylsulfoxide (DMSO) and added to the assays to give 1% or 4% final conc.

2.2. *Preparation of mitochondria*

Rat liver mitochondria were prepared as in [8]. Mitochondria from corn (*Zea mays* L.) were prepared as in [3]. Protein was determined as in [8].

2.3. *Ca^{2+}-transport measurements*

Ca^{2+}-uptake into rat liver mitochondria was followed continuously by measuring the spectral changes of arsenazo III as in [9]. Ca^{2+} accumulation into plant mitochondria was determined by the millipore filtration technique [3].

3. Results and discussion

The effects of the herbicides and fungicides on plant mitochondrial Ca^{2+} accumulation are shown in table 1. The data are presented as the amount of pesticide/mg mitochondrial protein which gives 50% inhibition of Ca^{2+} accumulation. From table 1 it is

Table 1
ID_{50} values of various pesticides for the Ca^{2+}-accumulation into corn mitochondria

Addition	ID_{50} (nmol/mg protein)
Captan	800
Oryzalin	400
Amiprophos	160
Amiprophosmethyl	140
Dichlofluanide	140
Trifluralin	70

obvious that the fungicide captan is the least effective compound in plant mitochondria. The data do not allow us to discriminate between effects of the pesticides on uptake or efflux or both. We had shown that amiprophosmethyl and its structural analogue amiprophos inhibited Ca^{2+}-uptake at 5×10^{-5} M [10]; at higher levels Ca^{2+} efflux is also stimulated [3]. All experiments with plant mitochondria were done with P_i present, as, at the experimental conditions employed, no Ca^{2+} accumulation could be observed without phosphate [11].

We have investigated the effects of the pesticides on Ca^{2+} uptake into rat liver mitochondria in the absence of P_i. Phosphate is not necessary for Ca^{2+} accumulation into animal mitochondria; it enhances Ca^{2+} release [9]. At experimental levels, the pesticides have no effect on Ca^{2+} uptake into rat liver mitochondria (not shown). For the herbicides amiprophos and amiprophosmethyl, this conflicted with the data obtained with plant mitochondria where they do inhibit Ca^{2+} uptake [10]. The effect of the other pesticides on plant mitochondrial Ca^{2+} uptake has not been investigated.

The pesticides do affect the ruthenium red-insensitive Ca^{2+} efflux from rat liver mitochondria. When the organelles are loaded for 15 s with Ca^{2+} in the absence of pesticides and uptake blocked by ruthenium red (1 nmol/mg protein) Ca^{2+} is released at a slow rate only (fig.1, ○,□). If the herbicide amiprophosmethyl and the fungicide captan are added before mitochondria are loaded with Ca^{2+}, in the presence of ruthenium red, they cause an increase of the Ca^{2+} efflux rate (fig.1, ● for amiprophosmethyl and ■ for captan). Similar to these two the other pesticides also enhance the ruthenium red-insensitive Ca^{2+} efflux (table 2).

The two fungicides dichlofluanide and captan exert the greatest effect on Ca^{2+} release from rat liver mitochondria, whereas, most obviously for captan,

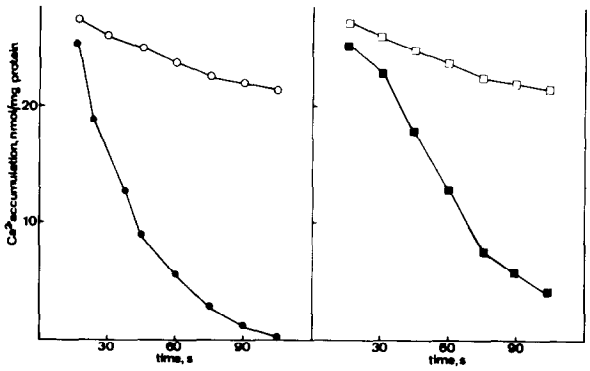

Fig.1. Effects of the herbicide amiprophosmethyl and the fungicide captan on the ruthenium red insensitive Ca^{2+} efflux from rat liver mitochondria. Amiprophosmethyl (200 nmol/mg protein, ●) and captan (1 nmol/mg protein, ■) were added before Ca^{2+}-uptake. The Ca^{2+} uptake was started by the addition of mitochondria to the assay medium (2 mg/ml). After 15 s the uptake was almost completed. At this time ruthenium red (1 nmol/mg protein) was added to the controls (○,□) or to the pesticide-treated samples (●,■).

they are much less effective in plant mitochondria (table 1). The herbicides seem to be almost equally effective in plant and rat liver mitochondria.

The increase of the Ca^{2+}-efflux by these herbicides and fungicides is observed in the presence of ruthenium red, a specific inhibitor of mitochondrial Ca^{2+}-uptake [12]. Since it has been shown that ruthenium red inhibits the Ca^{2+}-uniporter also in de-energized mitochondria [13], this rules out the possibility that the effect is caused by a reversal of the electrophoretic Ca^{2+}-uniporter; e.g., by a collapse of the transmembrane electrical potential. The pesticides may activate an independent, yet unknown, Ca^{2+}-release pathway

Table 2
Amounts of pesticides at which the ruthenium red-insensitive rate of Ca^{2+} efflux from rat liver mitochondria is stimulated 3-fold

Addition	Pesticide (nmol/mg protein)
Oryzalin	200
Amiprophos	125
Amiprophosmethyl	100
Trifluralin	50
Dichlofluanide	40
Captan	10

The pesticides were added before Ca^{2+} uptake; ruthenium red (1 nmol/mg protein) was added 15 s after Ca^{2+} uptake

[13]. An unspecific effect of the compounds on membrane permeability is unlikely as they do not affect the ATP-dependent Ca^{2+}-accumulation into a plant microsomal fraction ([3], C. H., D. M., unpublished).

If the herbicides and fungicides are resorbed and are not immediately metabolized in animal cells, they are able to provoke changes of the free cytoplasmic Ca^{2+} level by releasing Ca^{2+} from the mitochondrial stores and thus to interfere with Ca^{2+}-dependent biochemical and physiological processes in animals as they do in plants.

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