The anticalmodulin drugs trifluoperazine and R24571 remove the activation of the purified erythrocyte Ca^{2+}-ATPase by acidic phospholipids and by controlled proteolysis

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1. INTRODUCTION

The Ca^{2+} pumping ATPase purified from the erythrocyte membrane can be stimulated by calmodulin, by a number of acidic phospholipids, by polyunsaturated fatty acids, and by a limited proteolytic treatment [1–3]. Phenothiazine neuroleptics inhibit the Ca^{2+}-pumping ATPase in red cells [4]. This is in agreement with the finding [4,5] that phenothiazines bind to calmodulin and inhibit its interaction with a variety of enzyme targets. A number of so-called anti-calmodulin drugs have since been shown to inhibit the Ca^{2+}-pumping ATPase of red cells, the most notable in terms of potency being the derivative of the antimycotic miconazole R24571 [6].

We have repeatedly observed that phenothiazines and R24571 block the stimulation of the purified Ca^{2+} ATPase, or of the reconstituted purified Ca^{2+}-ATPase by calmodulin. However, these two compounds also remove the activation of ATPase by the alternative treatments mentioned above. This unexpected observation may be very important to the problem of whether calmodulin and the other alternative activating treatments act by the same mechanism or not, and is described in some detail here.

Abbreviations: Hepes, 4-(2-hydroxyethl)-1-piperazine ethanesulfonic acid; EGTA, ethylene glycol bis(aminoethyl ether) *N,N,N',N'*-tetraacetic acid; HEDTA, *N*-(hydroxyethl) ethylenediamine-*N,N',N'*-triacetic acid; DMSO, dimethylsulfoxide; TFP, trifluoperazine

2. MATERIALS AND METHODS

The Ca^{2+}-ATPase was purifed as in [1], and reconstituted in asolectin or phosphatidylcholine, essentially by the cholate dialysis procedure in [2]. The lipid–cholate–ATPase mixture was dialysed overnight at 4°C against 500–2000 vol. 120 mM KCl, 10 mM Hepes (pH 7.2), 50 μM $MgCl_2$ and 1 mM dithiothreitol.

The ATPase activity was monitored spectrophotometrically by the coupled-enzyme assay in [2], in a medium containing, in 1 ml final vol. at 37°C, 120 mM KCl, 60 mM Hepes (pH 7.0), 1 mM $MgCl_2$, 0.5 mM K_2-ATP, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 1 IU of pyruvate kinase, 1 IU of lactate dehydrogenase 500 μM EGTA, 500 μM HEDTA, 0.5 μM A23187, the amounts of calmodulin specified in the figure legends (when added), and 600 μM $CaCl_2$ (total free Ca^{2+}, 10 μM). After 4 min incubation, the reaction was started with the addition of the purified or reconstituted ATPase (2–5 mg protein corresponding to 25–30 μl liposomal suspension). Under these conditions, the rate of ATP hydrolysis was linear for a considerable time. The unstimulated activity is defined here as the activity in the presence of phosphatidylcholine (added phospholipid or liposomes) but in the absence of: (a) calmodulin; or (b) proteolytic treatment. In the case of phosphatidylserine, the stimulation by the latter is defined here as the increment in ATPase activity with respect to a sample incubated and assayed under identical conditions but in the presence of phosphatidylcholine instead of phosphatidyl-

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serine. Appropriate controls showed that the drugs tested inhibited the unstimulated ATPase only negligibly.

The activation of the non-reconstituted Ca^{2+}-ATPase by phosphatidylserine (fig.1) was studied on the enzyme isolated in the presence of 0.05% Triton X-100 and 0.05% phosphatidylcholine. Phosphatidylserine or phosphatidylcholine were microdispersed as indicated in [2] and added at 10 μg/μg enzyme protein.

Proteolysis was performed on 50 μl phosphatidylcholine-reconstituted vesicles (see above) with 2 μg trypsin for 3 min at 37°C. The proteolytic reaction was stopped by the addition of 10 μg soybean trypsin inhibitor. After 2 min on ice, the proteolyzed enzyme was added to the preincubated ATPase assay medium.

Protein was precipitated using deoxycholate and trichloroacetic acid to avoid interference with, e.g., Hepes [8] and determined as in [9].

All reagents were of the highest purity available. The phospholipids were grade I, from Lipids Products (South Nuffield). Calmodulin was isolated from bovine brain [10]. Trypsin and soybean trypsin inhibitor were obtained from Calbiochem-Behring (La Jolla CA). R 24571 was a kind gift of Dr Van Belle, Janssen Pharmaceuticals (Beerse) and was dissolved in DMSO. Trifluoperazine was a kind gift of Smith, Kline and French (Philadelphia PA), and was dissolved in 5 mM HCl.

3. RESULTS AND DISCUSSION

Fig.1 shows that the potent anticalmodulin drug R24571 blocks the activation of the purified ATPase by calmodulin: half-maximal inhibition was observed at 3 μM R24571, and complete inhibition at 8 μM. In a series of 4 experiments of this type, the variability range for the $K_{½}$ was 3–6 μM, and for 100% inhibition 8–10 μM. In fig.1, 1 μg calmodulin was used. Under identical experimental conditions, the inhibition curve was shifted somewhat to the right if 1.5 μg calmodulin were used. R24571 removed the stimulation of the ATPase induced by phosphatidylserine in the absence of calmodulin. The degree of activation of the ATPase was the same in the case of calmodulin and phosphatidylserine, but the amount of R24571 required for 50% and 100% inhibition of the activation was greater in the latter case. This, however, may reflect the possible preferential binding of R24571 to phosphatidylserine.

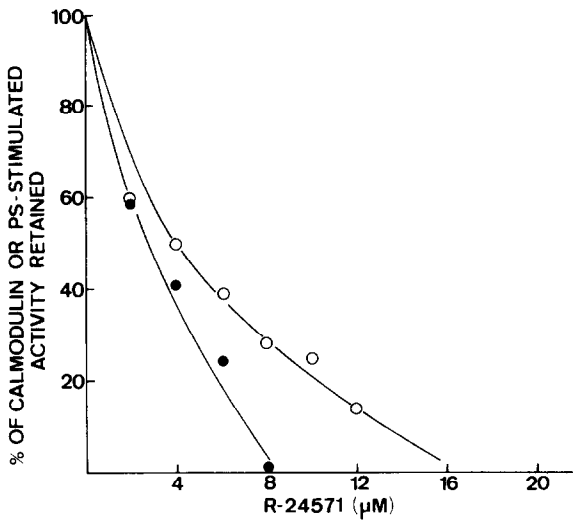

Fig.1. Effect of R24571 on the activation of the purified erythrocyte Ca^{2+}-ATPase by calmodulin or by phosphatidylserine. Details are described in section 2: 1 μg calmodulin present; (○) phosphatidylserine; (●) calmodulin (phosphatidylcholine).

Fig.2–4 show the effect of trifluoperazine on the activation of the reconstituted Ca^{2+}-ATPase by either calmodulin (phosphatidylcholine liposomes) acidic phospholipids (asolectin liposomes), or controlled proteolysis (phosphatidylcholine liposomes). The acidic phospholipid in this case was cardiolipin, which represents ~ 15% of the phospholipids of asolectin. It is evident from fig.1–4 that trifluoperazine counteracted very efficiently not only the activation produced by calmodulin, but also that produced by acidic phospholipids and by limited proteolysis. Varying amounts of liposomal phospholipids were added to the reaction media. Since trifluoperazine partitions into organic phases, it was important to compare its effects on the activations by calmodulin, asolectin, and limited proteolysis at equivalent amounts of phospholipids added to the medium. Fig.2 (●), fig.3 (△) and fig.4 (○) compare the effects obtained by adding 25–30 μl phospholipids. Under these conditions (i.e., equivalent amounts of phospholipids) the inhibition curves in the case of calmodulin and asolectin appeared similar and reached 100% over 150–100 μM, whereas the kinetics of the inhibition in the

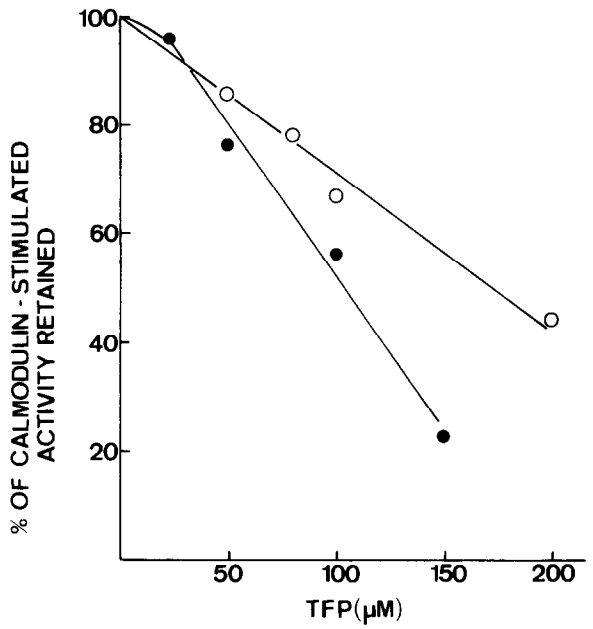

Fig.2. Effect of trifluoperazine on the activation of the reconstituted Ca^{2+}-ATPase by calmodulin. Details are described in section 2: μg calmodulin was present; (•) 25 μl phosphatidylcholine liposomes; (○) 50 μl phosphatidylcholine liposomes.

case of limited proteolysis differed significantly. The activation decreased more rapidly than in the case of calmodulin and asolectin at low trifluoperazine concentrations, but more slowly at higher concentrations. The end-values never reached 100% in the range of trifluoperazine concentrations tested (up to 250 μM). At equivalent phospholipids concentrations, the amount of trifluoperazine required for 50% and 100% inhibition (asolectin and calmodulin) were ~10-times higher than those of R24571, and increased progressively as the amount of liposomes in the medium increased (fig.2,3). This was observed also in the case of limited proteolysis (fig.4). Evidently, the drug became non-specifically bound to the phospholipids of the liposomes.

Two anticalmodulin drugs interfere with the activation of the purified or reconstituted Ca^{2+}-ATPase of erythrocytes by alternative, calmodulin-unrelated treatments. The finding has obvious relevance to the problem of the mechanism of stimulation of the Ca^{2+}-ATPase by calmodulin. More

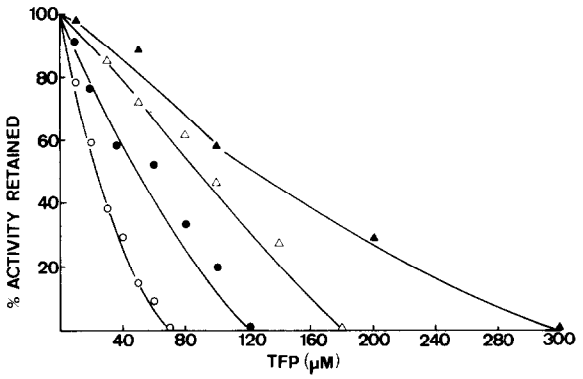

Fig.3. Effect of trifluoperazine on the activation of the reconstituted Ca^{2+}-ATPase by asolectin. Details are described in section 2: 1 μg calmodulin was present; (▲) 50 μl asolectin liposomes; (△) 30 μl asolectin liposomes; (•) 30 μl asolectin liposomes; (○) 10 μl asolectin liposomes.

specifically, the finding is relevant to the question of whether calmodulin and the other alternative activating treatments act by the same mechanism. Taking the problem one step further, one can ask the question of whether anticalmodulin compounds act on calmodulin only, or interact with the ATPase molecule also. While it is now accepted that phenothiazines interact directly with calmodulin (see [11], our evidence now indicates that phenothiazines bind to the Ca^{2+}-ATPase as well.

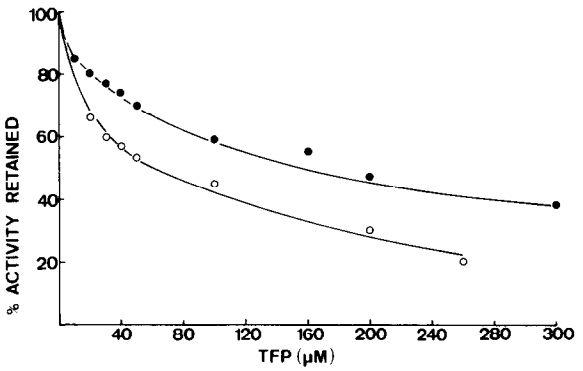

Fig.4. Effect of trifluoperazine on the activation of the reconstituted Ca^{2+}-ATPase by limited proteolysis. Details are described in section 2: 1 μg calmodulin was present; (•) 50 μl phosphatidylcholine liposomes; (○) 25 μl phosphatidylcholine liposomes.

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