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INHIBITION OF HUMAN ERYTHROCYTE Ca^{++}-TRANSPORT ATPase BY PHENOTHIAZINES AND BUTYROPHENONES

Klaus Gietzen, Angela Mansard and Hermann Bader

Abteilung Pharmakologie und Toxikologie, Universität Ulm,
Oberer Eselsberg, D-7900 Ulm, German Federal Republic

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SUMMARY

Antipsychotic agents such as the phenothiazines - trifluoperazine, fluphenazine and chlorpromazine - and the butyrophenones - penfluridol, fluspirilene and pimozide - which were shown to interact with calmodulin in a Ca^{++}-dependent fashion, competitively inhibit Ca^{++}-transport ATPase of human erythrocyte membranes. Activation of the ATPase by calmodulin is half-maximally antagonized by these agents in the concentration range of 2.6-22 μM whereas inhibition of the basal ATPase (in the absence of calmodulin) is achieved at about 10-fold higher concentrations.

INTRODUCTION

According to Schatzmann (1) a (Ca^{++}+ Mg^{++})-dependent ATPase is responsible for the active Ca^{++}extrusion across the erythrocyte membrane. The Ca^{++}-transport ATPase of erythrocytes has been shown to be regulated by a cytoplasmic protein, calmodulin, which combines with the enzyme when the cytoplasmic Ca^{++}concentration rises (2).

Calmodulin, an ubiquitous Ca^{++}-binding protein, has been shown to play a role in the modulation of a wide variety of Ca^{++}-dependent cellular functions. In addition to erythrocyte Ca^{++}-transport ATPase (3), calmodulin mediates control of cyclic nucleotide phosphodiesterase (4), adenylate cyclase (5), myosine light chain kinase (6), phosphorylase kinase (7), cell division via microtubule assembly-disassembly (8), and exocytosis of insulin (9) and neurotransmitters (10).

Levin and Weiss (11) demonstrated that antipsychotic agents selectively bind to calmodulin in a Ca^{++}-dependent fashion and that by this action the activation of nucleotide phosphodiesterase by calmodulin is abolished. Recently it has been shown that amitriptyline and chlorpro-

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mazine also inhibit erythrocyte Ca^{++}-transport ATPase by interfering with calmodulin (12).

The present paper gives evidence that several butyrophenone and phenothiazine derivatives are potent inhibitors of erythrocyte Ca^{++}-transport ATPase activation by calmodulin.

MATERIALS AND METHODS

All reagents were of highest purity available. Trasylol (registered trademark of Bayer A.G.) was obtained from Bayer A.G., Leverkusen, Germany. Penfluridol, fluspirilene and pimozide were kindly provided by Janssen Pharmaceutica, Beerse, Belgium; chlorpromazine by Bayer A.G.; trifluoperazine by Röhm Pharma, Darmstadt, Germany; and fluphenazine by Byk Gulden, Konstanz, Germany.

Penfluridol, pimozide and fluspirilene were added to the assay medium used for determination of the ATPase activity as solutions in ethanol. The final concentration of ethanol in the assay medium was in all cases 0.5% (v/v). To the assay medium of the controls the same amount of ethanol was added. All other pharmacological agents were added as aqueous solutions.

Preparation of calmodulin: Bovine brain calmodulin was prepared as described by Watterson et al.(13). Since human erythrocyte and bovine brain calmodulin are indistinguishable (12,14) we used throughout our experiments bovine brain calmodulin that can be prepared in larger quantities.

Preparation of calmodulin deficient erythrocyte membranes: Membrane-bound human erythrocyte Ca^{++}-transport ATPase was prepared on the basis of the iso-osmotic freeze-haemolysis procedure described by Gietzen et al.(15) modified as follows: Erythrocytes were washed in a solution containing 150 mM KCl and 20 mM 4-morpholinepropanesulphonic acid (MOPS (pH 7.4)). The buffer used for haemolysis contained 150 mM KCl, 10 mM MOPS (pH 7.4), 1 mM EDTA, 10 mM ascorbic acid, 1 000 kallikrein-inhibitor units (KIU) of Trasylol/ml and 1 mM 7-amino-1-chloro-3-L-tosylamidoheptane-2-one (TLCK). The buffer used for the first three washes following haemolysis contained 150 mM KCl, 10 mM MOPS (pH 7.0), 1 mM EDTA, 10 mM ascorbic acid and 100 KIU of Trasylol/ml. For the next three washes EDTA was omitted. After the last centrifugation the resulting pellet of erythrocyte membranes was suspended in an equal volume of 1 M sucrose to give a final protein concentration of about 15 mg/ml.

Assay of the Ca^{++}-transport ATPase: ATPase activity was determined at 30°C as described previously (16). The assay medium contained, in a final incubation volume of 10 ml, approximately 700 µg membrane protein, 10 mM Tris-maleate buffer (pH 7.0), 100 mM KCl, 0.1 mM ouabain, 1 mM ATP, 2 mM $MgCl_2$ and 36 µM Ca^{++} (as a Ca^{++}- Mg^{++}-EDTA buffer). For determination of the Mg^{++}-ATPase activity the Ca^{++}- Mg^{++}-EDTA buffer was replaced by 0.4 mM EGTA. Ca^{++}-transport ATPase activity was obtained by subtracting the Mg^{++}-ATPase activity. Before starting the reaction by addition of ATP the enzyme was preincubated with the pharmacological agents for 10 min at 30°C in the presence and absence of calmodulin.

Protein was determined using the method of Lowry et al.(17).

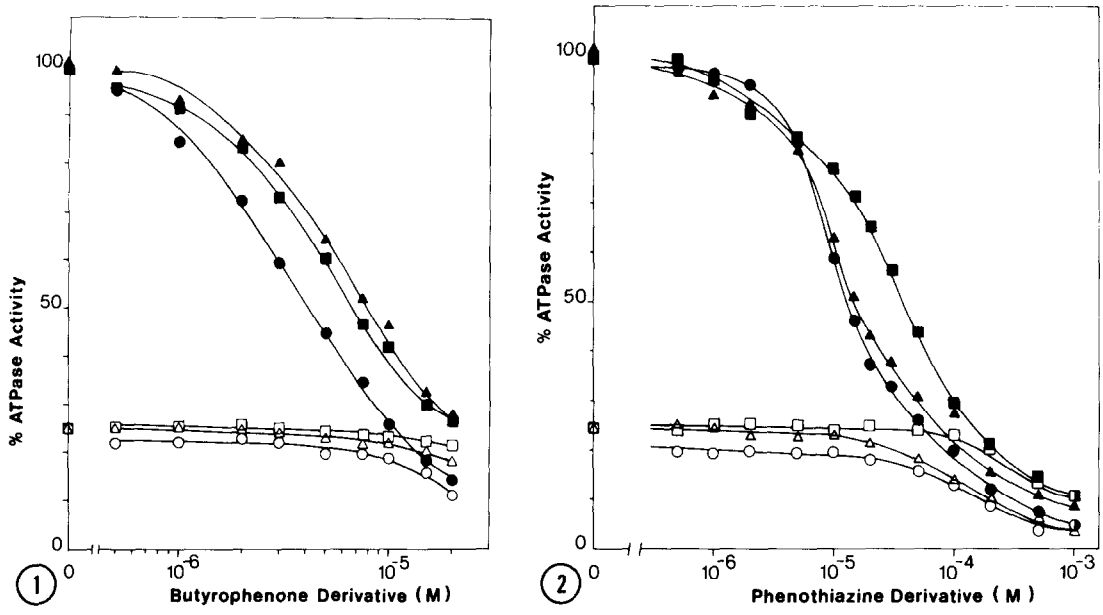

Fig.1. Effects of butyrophenone derivatives (● penfluridol, ■ pimozide, ▲ fluspirilene) on erythrocyte Ca^{++}-transport ATPase in the presence of calmodulin. The assay medium contained 700 μg membrane protein and 4 μg calmodulin (equal to the 10-fold amount needed for half-maximal activation). The open symbols represent the basal Ca^{++}-transport ATPase activity (in the absence of calmodulin). The ATPase activity is related to the calmodulin stimulated enzyme in the absence of the drug. Each point is the mean of at least two experiments.

Fig.2. Effects of phenothiazine derivatives (● trifluoperazine, ■ chlorpromazine, ▲ fluphenazine) on erythrocyte Ca^{++}-transport ATPase in the presence of calmodulin. The assay medium contained 700 μg membrane protein and 4 μg calmodulin. The open symbols represent the basal Ca^{++}-transport ATPase activity. ATPase activity is related to the stimulated enzyme. Each point is the mean of at least two experiments.

RESULTS

The iso-osmotic freeze-haemolysis procedure (see Materials and Methods) for preparing erythrocyte membranes yields a Ca^{++}-transport ATPase that can be maximally stimulated by calmodulin on an average 4 to 5-fold above the basal activity. The specific activity of the basal Ca^{++}-transport ATPase is 7 - 9 nmoles·mg protein^{-1}·min^{-1} and of the maximally activated enzyme 30 - 40 nmoles·mg protein^{-1}·min^{-1}. Ca^{++}-independent Mg^{++}-ATPase activity is less than 5% of the total activity of the preparation.

The effects of two types of antipsychotic agents - butyrophenone and phenothiazine derivatives - on human erythrocyte Ca^{++}-transport ATPase are shown in Fig.1 and Fig.2. Butyrophenones as well as phenothiazines are potent inhibitors of the calmodulin activated fraction of the Ca^{++}-transport ATPase whereas inhibition of the basal Ca^{++}-transport ATPase activity (in the absence of calmodulin) requires about 10-fold higher concentrations of the drugs. The Ca^{++}-independent Mg^{++}-ATPase activity is not influenced by the concentrations of antipsychotic agents used. Butyrophenones exert their inhibitory effect on ATPase activation by calmodulin at lower concentrations as compared to the phenothiazines. Penfluridol is the most potent agent of the investigated substances followed by pimozide, fluspirilene, trifluoperazine, fluphenazine and chlorpromazine. The concentrations of these agents producing 50% inhibition (I_{50}) of the Ca^{++}-transport ATPase activation by calmodulin and the basal Ca^{++}-transport ATPase are summarized in Table 1.

By increasing the amount of calmodulin at a constant membrane protein concentration the I_{50} of penfluridol and trifluoperazine subsequently shifted in a competitive fashion to higher concentrations (Fig.3, 4 and Table 1).

DISCUSSION

The present results demonstrate that butyrophenones and phenothiazines can antagonize the activation of erythrocyte Ca^{++}-transport ATPase by calmodulin. It is known that these agents bind to calmodulin in a Ca^{++}-dependent fashion (11). The question whether the binding of these antipsychotic agents to calmodulin inhibits the formation of the ATPase-calmodulin complex or alters calmodulin that it can still bind to but not activate the ATPase, is unsolved and presently under investigation. Results, shown in Fig.3 and 4, indicate that free calmodulin competes for the ATPase with the calmodulin-drug complex. It is

Table 1: Inhibitory effect of several antipsychotic agents on erythrocyte Ca^{++}-transport ATPase activation by calmodulin

Inhibitor	Concentration of inhibitor for half-maximal inhibition (I_{50}, μM)[a]			
	Calmodulin			
	0 μg (basal ATPase)	4 μg	8 μg	16 μg
Penfluridol	20	2.6	3.4	4.5
Pimozide	n.d.[b]	4.5		
Fluspirilene	n.d.[b]	5.5		
Trifluoperazine	160	9	13	18
Fluphenazine	200	10		
Chlorpromazine	500	22		

ATPase activity of human erythrocyte membranes was determined as described in the Materials and Methods sections.

a. The concentration of a compound which produced 50% inhibition of Ca^{++}-transport ATPase activation by calmodulin is defined as I_{50} of that substance. I_{50} of the basal Ca^{++}-transport ATPase is defined as the concentration of a drug which produced 50% inhibition of the ATPase in the absence of calmodulin. The I_{50}-values were obtained graphically from data of Fig.1, 2, 3 and 4.

b. n.d. = not detectable, since these compounds are not soluble in an aquous medium in concentrations higher than 20 uM.

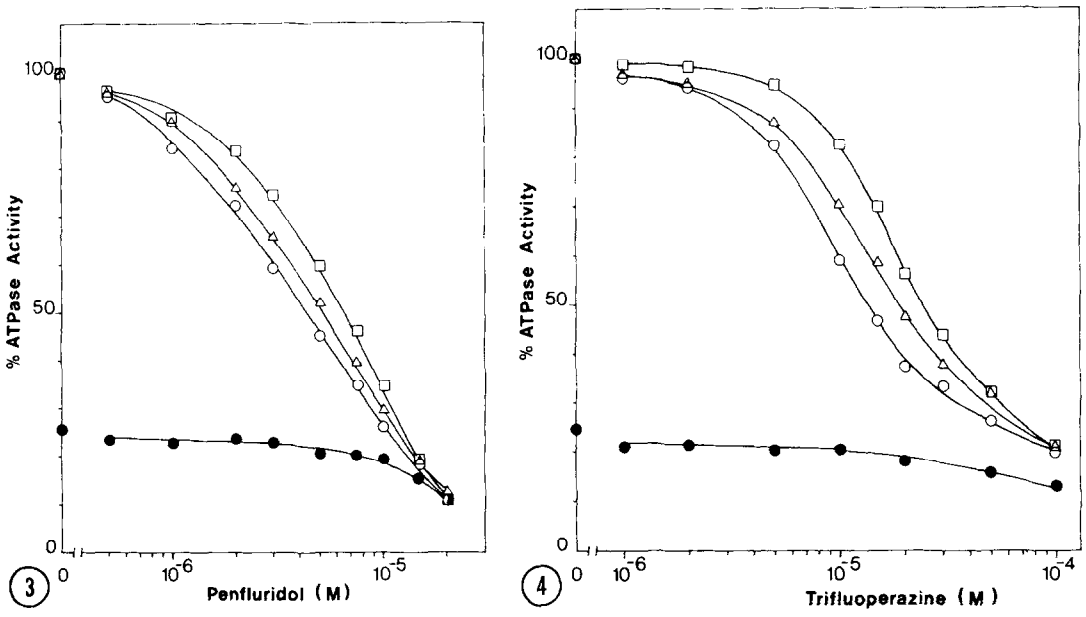

Fig.3. Influence of different amounts of calmodulin (○ 4 μg, △ 8 μg, □ 16 μg) on the inhibitory effect of penfluridol on Ca^{++}-transport ATPase activity (700 μg membrane protein). ● : basal Ca^{++}-transport ATPase activity. ATPase activity is related to the stimulated enzyme. Each point is the mean of two experiments.

Fig.4. Influence of different amounts of calmodulin (○ 4 μg, △ 8 μg, □ 16 μg) on the inhibitory effect of trifluoperazine on Ca^{++}-transport ATPase activity (700 μg membrane protein). ● : basal Ca^{++}-transport ATPase activity. ATPase activity is related to the stimulated enzyme. Each point is the mean of two experiments.

interesting that the I_{50} of the antipsychotic agents obtained in this study for Ca^{++}-transport ATPase are very similar to these for phosphodiesterase (11). The inhibition of the basal Ca^{++}-transport ATPase by the investigated agents occurs at significant higher concentrations and is presumably due to a different mechanism.

The order of potency of the different agents to inhibit phosphodiesterase (11) and Ca^{++}-transport ATPase correlates with that of their clinical antipsychotic activity. Amitriptyline, an antidepressant with similar side effects as the antipsychotic agents, was also shown to be an inhibitor of calmodulin-dependent Ca^{++}-transport ATPase activation (12) but it excerted a significant lower potency than the antipsychotic agents described here. This is in agreement with the binding studies of Levin

and Weiss (11) which showed that antianxiety and antidepressant compounds display considerable less Ca^{++}-specific binding to calmodulin and are less effective to inhibit calmodulin-dependent phosphodiesterase activation than the antipsychotic agents.

The results obtained for erythrocyte Ca^{++}-transport ATPase presumably can be applied to the corresponding ATPase from excitable cells (18) since recently an erythrocyte-type Ca^{++}-transport ATPase was found to be responsible, at least in part, for the low intracellular Ca^{++}concentration in nerve cell (19,20). In addition, it has been shown that several cellular functions in excitable cells are modulated by calmodulin (4,5,10). All calmodulin-controlled processes seem to be targets for the action of the phenothiazines and the butyrophenones (9,11,12,21,22). In view of the widespread distribution of calmodulin, it is tempting to speculate that the interference of the antipsychotic agents with calmodulin-controlled processes may account for some of the side effects and (or) the antipsychotic effect of these compounds. In addition, these agents may prove to be useful tools to elucidate Ca^{++}- and calmodulin-dependent cell functions.

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