A model for the regulation of the calmodulin-dependent enzymes erythrocyte Ca^{2+} -transport ATPase and brain phosphodiesterase by activators and inhibitors

Klaus GIETZEN, Inge SADORF and Hermann BADER

Department of Pharmacology and Toxicology, University of Ulm, Oberer Eselsberg, D-7900 Ulm, Federal Republic of Germany

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Acidic phospholipids, unsaturated fatty acids and limited proteolysis mimic the activating effect of calmodulin on erythrocyte Ca^{2+} -transport ATPase and on brain cyclic nucleotide phosphodiesterase, as has been reported previously in several studies. Three different antagonists of calmodulin-induced activation of these enzymes were tested for their inhibitory potency on the stimulation produced by the other activators. Trifluoperazine and penfluridol were found to antagonize all the above mentioned types of activation of Ca^{2+} -transport ATPase in the same concentration range. Both inhibitors also can reverse the activation of phosphodiesterase by oleic acid, phosphatidylserine and calmodulin at similar concentrations. However, in contrast with erythrocyte Ca^{2+} -transport ATPase, activation of phosphodiesterase by limited tryptic digestion cannot be antagonized by penfluridol and trifluoperazine. Calmidazolium, formerly referred to as compound R 24571, was found to be a relatively specific inhibitor of calmodulin-induced activation of phosphodiesterase and Ca^{2+} -transport ATPase, since antagonism of the other activators required much higher concentrations of the drug. The results suggest that the investigated drugs exert their inhibitory effect on calmodulin-regulated enzymes not solely via their binding to calmodulin but may also interfere directly with the calmodulin effector enzyme. In addition, a general mechanism of activation and inhibition of calmodulin-dependent enzymes is derived from our results.

Calmodulin is a Ca^{2+} -binding protein that modulates a number of Ca^{2+} -dependent enzymes and cell functions, and is a major cytoplasmic receptor for Ca^{2+} (Cheung, 1980; Means & Dedman, 1980). The calmodulin-dependent fraction of the activity of these enzymes can be inhibited by a wide range of chemically unrelated substances, such as phenothiazines and butyrophenones (Levin & Weiss, 1979; Gietzen *et al.*, 1980), naphthalene sulphonamides (Kobayashi *et al.*, 1979), *Vinca* alkaloids (Watanabe *et al.*, 1979; Gietzen & Bader, 1980), local anaesthetics (Volpi *et al.*, 1981). The most powerful inhibitor for calmodulin-mediated effects that has hitherto been described is calmidazolium, formerly referred to as compound R24571 (Gietzen *et al.*, 1981; Van Belle, 1981), which is a derivative of the antimycotic miconazole.

Abbreviations used: Mops, 4-morpholinepropane-sulphonic acid; IC_{50} , concentration producing 50% inhibition.

It has been demonstrated for several enzymes that unsaturated fatty acids, acidic phospholipids and limited tryptic digestion mimic the effect of calmodulin (Wolff & Brostrom, 1976; Depaoli-Roach *et al.*, 1979; Taverna & Hanahan, 1980; Sarkadi *et al.*, 1980; Niggli *et al.*, 1981). However, phosphatidylserine that was reported to be an effective activator of purified erythrocyte Ca^{2+} -transport ATPase (Niggli *et al.*, 1981) failed to exhibit a pronounced effect on brain phosphodiesterase (Wolff & Brostrom, 1976; Itano *et al.*, 1981).

In a comparative study we investigated the effect of several so-called calmodulin inhibitors on activation of brain phosphodiesterase and erythrocyte Ca^{2+} -transport ATPase induced by calmodulin, oleic acid, phosphatidylserine and limited proteolysis. Three structurally unrelated inhibitors were tested for their specificity: (1) trifluoperazine, a phenothiazine derivative; (2) penfluridol, a butyrophenone analogue; and (3) calmidazolium, a complex imidazolium compound. The present study was designed

to investigate further the mechanism of activation and inhibition of calmodulin-dependent enzymes.

Materials and methods

All reagents were of the highest purity available. Trifluoperazine dihydrochloride was a gift from Röhm Pharma (Darmstadt, Germany). Penfluridol and calmidazolium were obtained from Janssen Pharmaceutica (Beerse, Belgium). Oleic acid, phosphatidylserine (product no. P7769), 5'-nucleotidase, pepstatin A and soya-bean trypsin inhibitor were supplied by Sigma (München, Germany). Trypsin from bovine pancreas was a product of Boehringer (Mannheim, Germany).

The lipophilic compounds penfluridol and calmidazolium were dissolved in dimethyl sulphoxide and added to the respective assay medium with vigorous mixing. The final concentration of dimethyl sulphoxide in the assay media including the controls was 0.5% (v/v) for Ca^{2+} -transport ATPase and 1% (v/v) for phosphodiesterase.

Oleic acid and phosphatidylserine microdispersions were prepared by sonication (Branson Sonifier B12; approx. 2 min at setting 2) of the substances in a buffer containing 0.1 mM-EGTA and 5 mM-Mops (pH 7.0) under a stream of N_2 . Stock solutions of oleic acid and phosphatidylserine (3 mg/ml) were prepared fresh daily.

Preparation of enzymes and calmodulin

Homogeneous calmodulin was prepared from bovine brain as described by Kakiuchi *et al.* (1981). Membrane-bound human erythrocyte Ca^{2+} -transport ATPase deficient in calmodulin was prepared by the method of Gietzen *et al.* (1980). Calmodulin-sensitive phosphodiesterase was partially purified, based on the method of Wang & Desai (1977). Briefly, phosphodiesterase was obtained from rat brain by homogenization in a buffer containing 10 mM-Tris/HCl (pH 7.5) and 2 mM-EDTA followed by $(\text{NH}_4)_2\text{SO}_4$ (55% saturated) precipitation of the 100 000 g supernatant. The redissolved pellet from the precipitation was applied to a DEAE-cellulose column (Whatman DE52) equilibrated with a buffer consisting of 20 mM-Tris/HCl (pH 7.5), 1 mM-magnesium acetate, 1 mM-imidazole, 10 mM- β -mercaptoethanol, 0.1 mM-EGTA, and phosphodiesterase was eluted with the same buffer by a gradient of 50–350 mM-NaCl. In addition all buffers needed for the preparation of phosphodiesterase contained 0.1 mM-di-isopropyl fluorophosphate and 0.05 μg of pepstatin A/ml.

Tryptic digestion of enzymes

Ca^{2+} -transport ATPase of disrupted erythrocyte membranes was digested at 37°C, usually by 0.2 mg of trypsin/mg of erythrocyte membrane protein. Rat

brain phosphodiesterase was digested at 37°C by 30 μg of trypsin per mg of protein of the phosphodiesterase preparation. Proteolysis of both enzymes was performed in the respective assay medium. Usually, unless otherwise stated, digestion was terminated after 3 min by the addition of a 5-fold excess (w/w) of soya-bean trypsin inhibitor. Control experiments revealed that the presence of trypsin plus soya-bean trypsin inhibitor in the assay medium for all applied concentrations had no significant influence on the ATPase and phosphodiesterase activity.

Assay of ATPase activity

Ca^{2+} -transport ATPase activity was monitored continuously for 8 min at 37°C by the phosphate released from ATP using the automated assay described by Arnold *et al.* (1976). The assay medium contained, in a final incubation medium of 10 ml, approx. 60 μg of erythrocyte membrane protein/ml, 25 mM-Mops (pH 7.0), 100 mM-KCl, 0.2 mM-ouabain, 1 mM-ATP, 2 mM- MgCl_2 and an Mg-Ca-EDTA buffer (0.2/0.2/0.4 mM) to yield a free Ca^{2+} concentration of 36 μM (Wolf, 1973). Ca^{2+} -free controls contained, instead of the Mg-Ca-EDTA buffer, Mg-EDTA (0.2/0.4 mM). Ca^{2+} -transport ATPase activity refers to the difference in activity obtained in the presence and absence of Ca^{2+} .

Assay of phosphodiesterase activity

Phosphodiesterase activity was determined at 37°C as described by Wang & Desai (1977) based on the method of Butcher & Sutherland (1962). This procedure involved coupling of the phosphodiesterase reaction with 5'-nucleotidase reaction and measuring the P_i produced within 30 min. The assay mixture consisted of 40 mM-Tris/HCl (pH 7.5), 40 mM-imidazole, 3 mM-magnesium acetate, 1.2 mM-cyclic AMP and 0.1 mM- CaCl_2 or 0.1 mM-EGTA.

Results

The effect of various activators on erythrocyte Ca^{2+} -transport ATPase of disrupted membranes, deficient in calmodulin (the calmodulin content was determined to be approx. 20% of the value obtained for calmodulin-saturated membranes) is shown in Fig. 1. Adding back calmodulin to the preparation results in fully activated Ca^{2+} -transport ATPase at a calmodulin concentration ≥ 30 nM. Limited tryptic digestion, using 0.2–0.3 mg of trypsin/mg of erythrocyte membrane protein for 3 min at 37°C, also gives maximum activation of the enzyme. In a narrow concentration range, being optimal at 30–60 μM , oleic acid is a powerful activator of Ca^{2+} -transport ATPase. Higher concentrations of oleic acid lead to a rapid decline in the ATPase activity. Phos-

Fig. 1. Activation of calmodulin-deficient and membrane-bound erythrocyte Ca^{2+} -transport ATPase

Erythrocyte membranes were pre-incubated in the assay medium with different concentrations of the respective activator (calmodulin, oleic acid or phosphatidylserine) for 10 min at 37°C before the enzyme reaction was started with ATP. Activation of Ca^{2+} -transport ATPase by different doses of trypsin (mg/mg of erythrocyte membrane protein) was performed in the assay medium by proteolysis for 3 min at 37°C . Digestion was stopped by the addition of a 5-fold excess (w/w) of soya-bean trypsin inhibitor and the samples were immediately assayed. Note that for activation by tryptic digestion the upper scale is relevant. Ca^{2+} -transport ATPase activity is related to the activity of the maximally stimulated enzyme by calmodulin (100% activity = $50\text{--}70\text{ nmol/min per mg}$ of protein). Abbreviation: PS, phosphatidylserine. Each point represents the mean of at least four independent determinations.

Fig. 2. Activation of calmodulin-deficient brain phosphodiesterase

Brain phosphodiesterase was pre-incubated in the assay medium with different concentrations of the respective activator (calmodulin, oleic acid and phosphatidylserine) for 10 min at 37°C before the enzyme reaction was started by the addition of cyclic AMP. Activation of phosphodiesterase by limited proteolysis was performed in the assay medium at 37°C by digestion for different time intervals with $30\text{ }\mu\text{g}$ of trypsin per mg of protein of the phosphodiesterase preparation. Digestion was stopped after the indicated time periods by the addition of a 5-fold excess (w/w) of trypsin inhibitor. Note that for stimulation by tryptic proteolysis the upper scale is relevant. Activation of phosphodiesterase by tryptic digestion, oleic acid and phosphatidylserine was performed in the assay medium containing 0.1 mM-EGTA , whereas for activation by calmodulin the assay medium contained 0.1 mM-CaCl_2 . Phosphodiesterase activity is related to the activity of the fully stimulated enzyme by calmodulin (100% activity = $0.8\text{--}1\text{ }\mu\text{mol/min per mg}$ of protein). Abbreviation: PS, phosphatidylserine. Each point represents the mean of at least four independent determinations.

phatidylserine was found to be ineffective as an activator of Ca^{2+} -transport ATPase of disrupted erythrocyte membranes in the investigated concentration range. Higher concentrations of phosphatidylserine, as indicated in Fig. 1, could not be used because of interference through turbidity of the ATPase assay.

Fig. 2 shows the effect of calmodulin, oleic acid, phosphatidylserine and proteolysis on calmodulin-depleted brain phosphodiesterase. The enzyme is maximally activated in the presence of $\geq 30\text{ nM}$ -calmodulin and $\geq 30\text{ }\mu\text{M}$ -oleic acid. In contrast with erythrocyte Ca^{2+} -transport ATPase, brain phosphodiesterase can only be stimulated by limited tryptic digestion up to 70% of the activity of the maximally stimulated enzyme by calmodulin. Initially, limited proteolysis has an inhibitory effect on phosphodiesterase. Within 30 s, digestion by trypsin leads to a 30% loss of activity related to the maximally stimulated enzyme, as was revealed by assaying phosphodiesterase activity after proteolysis in the presence of calmodulin (results not shown). With $130\text{--}400\text{ }\mu\text{M}$ -phosphatidylserine, brain

phosphodiesterase was stimulated to about 82% of the activity induced by saturating calmodulin concentrations. Activation of phosphodiesterase by tryptic digestion, oleic acid or phosphatidylserine did not require the presence of Ca^{2+} , as was the case for calmodulin-induced activation (Fig. 2). However, similar dose-response curves for the Ca^{2+} -independent activators were also obtained in the presence of Ca^{2+} (results not shown). Activation of both phosphodiesterase and Ca^{2+} -transport ATPase by oleic acid, phosphatidylserine or tryptic digestion was accompanied by a simultaneous elimination of calmodulin stimulation to the same extent as activation by the other activator processes (results not shown).

The effect of several so-called calmodulin inhibitors on erythrocyte Ca^{2+} -transport ATPase and brain phosphodiesterase, activated by optimal

Fig. 3. Inhibition of activated Ca^{2+} -transport ATPase by trifluoperazine (a), penfluridol (b) and calmidazolium (c) Ca^{2+} -transport ATPase was activated either by calmodulin (O, 30 nM), oleic acid (Δ , 50 μM) or by limited tryptic digestion (●, for 3 min at 37°C with 0.2 mg of trypsin per mg of erythrocyte membrane protein). Trypsin digestion was terminated with a 5-fold excess (w/w) of trypsin inhibitor. In the case of stimulation of erythrocyte Ca^{2+} -transport ATPase by trypsin, after digestion the samples were pre-incubated for 10 min at 37°C with the inhibitor. In the case of activation by calmodulin or oleic acid

concentrations of different activators, was then investigated. The optimum concentrations of the activators were obtained from the activation experiments shown in Figs. 1 and 2. Fig. 3(a) shows that stimulation of Ca^{2+} -transport ATPase induced by oleic acid or tryptic digestion is inhibited by trifluoperazine in the same concentration range as the activation of the enzyme by calmodulin. The activity of Ca^{2+} -transport ATPase dependent on oleic acid is inhibited in a biphasic fashion. The concentration of trifluoperazine producing 50% inhibition (IC_{50}) of oleic acid- or proteolysis-induced activation of Ca^{2+} -transport ATPase is only approximately a factor of 2 lower or higher respectively, compared with the IC_{50} value obtained for the calmodulin-dependent ATPase activity. The butyrophenone derivative penfluridol is equally effective in antagonizing all types of activators (calmodulin, oleic acid or tryptic digestion), as shown in Fig. 3(b). In contrast with penfluridol and trifluoperazine, calmidazolium was found to be a more specific inhibitor of the calmodulin-induced activation of Ca^{2+} -transport ATPase (Fig. 3c). Half-maximal inhibition of the activating effects of limited proteolysis or oleic acid on Ca^{2+} -transport ATPase requires 7 and 25 times higher concentrations of the drug respectively, compared with the calmodulin-dependent fraction of the ATPase activity.

As shown in Fig. 4(a) trifluoperazine antagonizes activation of brain phosphodiesterase induced by calmodulin, oleic acid or phosphatidylserine with almost equal potency. However, in contrast with erythrocyte Ca^{2+} -transport ATPase (Fig. 3a), activation of phosphodiesterase by limited proteolysis is not inhibited by trifluoperazine in the investigated concentration range ($\leq 300 \mu\text{M}$). The same pattern of inhibition of activated phosphodiesterase is obtained with the butyrophenone derivative penfluridol (Fig. 4b) as with the phenothiazine compound trifluoperazine (Fig. 4a). Evidently also penfluridol is unable to antagonize the activation of phosphodiesterase induced by mild tryptic digestion (Fig. 4b). Calmidazolium, the most potent inhibitor of calmodulin-mediated effects, is rather specific in antagonizing calmodulin-induced activation of phosphodiesterase (Fig. 4c). Antagonism of the other activators of phosphodiesterase requires consider-

Ca^{2+} -transport ATPase was first pre-incubated for 10 min at 37°C with the inhibitor followed by another 10 min with the activator before the enzyme reaction was started. Ca^{2+} -transport ATPase activity is related to the activity of the maximally stimulated enzyme by calmodulin (30 nM). ■, Basal Ca^{2+} -transport ATPase activity (without added calmodulin) in the absence of the drug. Each point represents the mean of four determinations.

Fig. 4. Inhibition of activated phosphodiesterase by trifluoperazine (a), penfluridol (b) and calmidazolium (c). Phosphodiesterase was activated by calmodulin (O, 30 nM), oleic acid (Δ , 50 μM), phosphatidylserine ($\square$, 130 μM) or limited tryptic digestion ($\bullet$, for 3 min at 37°C with 30 μg of trypsin per mg of protein of the phosphodiesterase preparation). Activation of phosphodiesterase by tryptic digestion, oleic acid and phosphatidylserine was performed in the assay

ably higher concentrations of calmidazolium. In contrast with trifluoperazine and penfluridol, calmidazolium also antagonizes the activation of phosphodiesterase by tryptic digestion. Half-maximal inhibition of the stimulation produced by oleic acid, mild proteolysis or phosphatidylserine demands at least a 40-fold higher concentration of calmidazolium in comparison with the calmodulin-initiated activation of phosphodiesterase (Fig. 4c). It is noteworthy that all inhibitors exhibit, at lower concentrations, as where they produce their inhibitory effects, some ability to amplify slightly the effect of some activators of phosphodiesterase (Figs. 4a–4c).

Discussion

Several acidic and hydrophobic compounds (Wolff & Brostrom, 1976; Al-Jobore & Roufogalis, 1981; Niggli *et al.*, 1981) and limited proteolysis (Cheung, 1971; Depaoli-Roach *et al.*, 1979; Taverna & Hanahan, 1980; Sarkadi *et al.*, 1980; Klee, 1980; Niggli *et al.*, 1981) were reported to mimic the regulatory effect of calmodulin on enzymes.

Activation of erythrocyte Ca^{2+} -transport ATPase by oleic acid and mild tryptic digestion as reported by Niggli *et al.* (1981) for the purified enzyme and by Sarkadi *et al.* (1980) for erythrocyte inside-out vesicles was confirmed by our work on the ATPase of disrupted membranes (Fig. 1). In contrast with previous reports (Al-Jobore & Roufogalis, 1981; Niggli *et al.*, 1981), phosphatidylserine was ineffective as an activator of Ca^{2+} -transport ATPase (Fig. 1). This discrepancy might be due to different preparations and conditions applied in these studies. Purified ATPase (Niggli *et al.*, 1981) and crude ATPase (Al-Jobore & Roufogalis, 1981) were

medium containing 0.1 mM-EGTA, whereas for activation by calmodulin the assay medium contained 0.1 mM- CaCl_2 . Trypsin digestion was stopped with a 5-fold excess (w/w) of trypsin inhibitor. In the case of stimulation of phosphodiesterase by trypsin after proteolysis the samples were pre-incubated for 10 min at 37°C with the inhibitor. In the case of activation by calmodulin, oleic acid or phosphatidylserine, phosphodiesterase was first pre-incubated for 10 min at 37°C with the inhibitor followed by another 10 min with the activator before assaying phosphodiesterase activity. After termination of the phosphodiesterase assay samples containing phosphatidylserine were clarified by the addition of 10 μl of 10% (w/v) sodium dodecyl sulphate before measuring the absorbance. Phosphodiesterase activity is related to the activity of the maximally stimulated enzyme by calmodulin (30 nM). ■, Basal phosphodiesterase activity (without added calmodulin) in the absence of drug. Each point represents the mean of four determinations.

both in the solubilized state and thus the incubation media contained a considerable amount of detergent, which might account for the activating effect of phosphatidylserine in their preparations.

Stimulation of brain phosphodiesterase by mild proteolysis and oleic acid (Fig. 2) confirms the results from several laboratories (Cheung, 1971; Wolff & Brostrom, 1976; Klee, 1980). A new finding is that phosphatidylserine at concentrations between 130 and 400 μM (100–300 $\mu\text{g/ml}$) also stimulates phosphodiesterase up to 82% of the activity of the maximally stimulated enzyme by calmodulin (Fig. 2). In contrast, Wolff & Brostrom (1976) obtained, even with 2 mg of phosphatidylserine/ml, only a 25% increase over the basal phosphodiesterase activity and Itano *et al.* (1981) found no activating effect of phosphatidylserine at concentrations $\leq 300 \mu\text{g/ml}$. The preparation of phosphatidylserine vesicles is of crucial importance (Gietzen *et al.*, 1982b). Only vesicles of the smallest possible size are capable of activating calmodulin-deficient phosphodiesterase. This is in agreement with the finding of Itano *et al.* (1981), who showed a stimulation of phosphodiesterase by phosphatidylserine when adding small amounts of Triton X-100, which results in the formation of smaller particles of phosphatidylserine.

'Calmodulin inhibitors', such as trifluoperazine and penfluridol, can antagonize the effects of all types of activators (calmodulin, oleic acid, limited proteolysis) on erythrocyte Ca^{2+} -transport ATPase in a similar fashion and concentration range (Figs. 3a and 3b). In addition, both drugs exhibit an equal potency to inhibit activation of brain phosphodiesterase by oleic acid, phosphatidylserine or calmodulin (Figs. 4a and 4b). However, in contrast with Ca^{2+} -transport ATPase, trifluoperazine and penfluridol failed to inhibit activation of phosphodiesterase by limited tryptic digestion (Figs. 4a and 4b). From these results the following conclusions can be drawn. (1) Penfluridol and trifluoperazine bind, in addition to calmodulin, also to erythrocyte Ca^{2+} -transport ATPase, since activation by both tryptic digestion and calmodulin can be completely antagonized in a similar concentration range. Inhibition of trypsin-activated Ca^{2+} -transport ATPase due to perturbation of the lipid environment of the enzyme is improbable, since in a previous report (Gietzen *et al.*, 1980) it was shown that inhibition of the basal (in the absence of calmodulin) Ca^{2+} -transport ATPase activity requires at least 10 times higher concentrations of the drugs compared with the calmodulin- or trypsin-stimulated fraction of the ATPase activity. However, our experiments do not allow us to ascertain whether the binding site on the ATPase is made accessible by trypsin digestion or alternatively whether it is already accessible in the native enzyme. In a recent

report we demonstrated that vinblastine, another inhibitor of calmodulin-mediated effects, has a binding site on native Ca^{2+} -transport ATPase (Gietzen *et al.*, 1982a). So it may be that the binding site for penfluridol and trifluoperazine on Ca^{2+} -transport ATPase is already accessible in the native enzyme. (2) Brain phosphodiesterase obviously has no binding site for these drugs, since it was reported that native phosphodiesterase does not bind antipsychotic drugs (Weiss & Sellinger-Barnette, 1981) and our results indicate that trypsin-treated phosphodiesterase cannot be inhibited by penfluridol and trifluoperazine. (3) Antagonism by these drugs of the activators (calmodulin, oleic acid and phosphatidylserine) of Ca^{2+} -transport ATPase and phosphodiesterase, occurring in the same concentration range, implies a general mode of activation and inhibition of these enzymes.

Calmidazolium, the most potent inhibitor of calmodulin-regulated functions (Gietzen *et al.*, 1981; Van Belle, 1981), was found to be a more specific inhibitor of calmodulin-induced activation of both enzymes since antagonism of the other activators required much higher concentrations of the drug. In addition, calmidazolium inhibits proteolysis-induced activation of phosphodiesterase, which cannot be reversed by penfluridol and trifluoperazine. The specificity of calmidazolium antagonism of calmodulin stimulation is more pronounced in the phosphodiesterase system, compared with the Ca^{2+} -transport ATPase. The discrepancy could be due to the distribution of the lipophilic compound in the lipid bilayer of the erythrocyte membranes, so that the concentration, at the target in this system is unknown and less than expected. So it cannot be excluded that the same differences in affinity, as determined for the phosphodiesterase system, may exist also in the Ca^{2+} -transport ATPase system. Inhibition even of the proteolysis-induced activation of phosphodiesterase by calmidazolium may be due to its high potency.

On the basis of the results from several laboratories, including ours, we have developed a hypothetical model (Fig. 5) by which all types of activation and inhibition phenomena of calmodulin-regulated enzymes can be described simply by the assumption of hydrophobic and ionic interactions. (1) Calmodulin has two important structural features: acidity (Cheung, 1980) and hydrophobicity, of which the latter was shown to be induced by binding of Ca^{2+} (LaPorte *et al.*, 1980). Generally calmodulin can be considered to be an anionic amphiphile. Most substances that can mimic the activity of calmodulin, such as phosphatidylserine or oleic acid (Wolff & Brostrom, 1976; Niggli *et al.*, 1981; Al-Jobore & Roufogalis, 1981), share with calmodulin both characteristics. (2) All inhibitors of calmodulin-mediated effects are positively charged

Fig. 5. Mechanism of activation and inhibition of calmodulin-regulated enzymes

The scheme describes our hypothesis for the mechanism by which calmodulin-dependent enzymes may be activated and by which the induced activation may be antagonized. The phenomena simply are described on the basis of hydrophobic and ionic interactions. Activators (calmodulin, oleic acid or phosphatidylserine) are depicted as anionic amphiphiles, whereas so-called calmodulin inhibitors are depicted as cationic amphiphiles. The hatched areas symbolize hydrophobic regions. An inhibitory peptide sequence of the calmodulin-regulated enzymes, being positively charged and having a hydrophobic site, is assumed. The inhibitory effect of this polypeptide is suppressed by interaction with anionic activators leading to its displacement or by cleavage with trypsin. The activated enzyme may be inhibited by complexation of the activator by the inhibitor and for some enzymes in addition by direct interaction of the inhibitor with the calmodulin-dependent enzyme. Abbreviation used: AC, active centre. Only a section of the calmodulin-dependent enzyme is shown.

and possess in addition a hydrophobic region (Vincenzi, 1981). They can be considered as cationic amphiphiles. Most 'calmodulin inhibitors' were shown to bind to calmodulin in a Ca^{2+} -dependent manner (Levin & Weiss, 1979). Therefore binding probably occurs because of the above noted complementary structural features via ionic and hydrophobic interactions. Weiss *et al.* (1980) have shown the importance of the opposite charges in calmodulin and the inhibitors. A complex was only formed when the pH was above the isoelectric point of calmodulin and below the pK_a of the inhibitor. (3) Limited proteolysis of calmodulin-regulated enzymes mimics the activation induced by calmodulin and by this action simultaneously calmodulin sensitivity is eliminated. It was suggested that these enzymes possess a peptide sequence whose inhibitory action on the enzymes is suppressed by binding of calmodulin as well as by its cleavage through trypsin (Klee, 1980; Sarkadi *et al.*, 1980). The calmodulin receptor of these enzymes is thought to be located on the inhibitory peptide sequence. When phosphodiesterase was treated with trypsin in the presence of calmodulin it was shown that cleavage of the inhibitory protein proceeds faster, which is compatible with the explanation that the enzyme unfolds on binding of calmodulin (Klee, 1980).

One possible scheme that illustrates our hypo-

thesis for the mechanism by which calmodulin-dependent enzymes may be activated and by which the induced activation may be inhibited is shown in Fig. 5 and may be explained as follows. A section of an enzyme, being in its low-activity state, is shown at the lower-left of the Figure. The inhibitory peptide sequence of the enzyme, being positively charged and having a hydrophobic site, somehow hinders the active centre. These features of the inhibitory peptide sequence are assumed because of the anionic amphiphilic character of the activators. Cleavage of the inhibitory polypeptide makes the active centre easily accessible and by this transforms the enzyme to a high-activity state. Binding of calmodulin to the enzyme results in a conformational change, which again opens the active centre and results in an enzyme being in the high-activity state. The other activators, which are anionic amphiphiles like calmodulin, are thought to transform the enzyme in the same mode as calmodulin does. This view is supported by the finding of Niggli *et al.* (1981) that these activators not only transform Ca^{2+} -transport ATPase to a high-activity state but also increase the affinity of the system for Ca^{2+} . These transformations of the enzyme are identical with those induced by calmodulin. Since calmodulin is a rather large molecule compared with phosphatidylserine or oleic acid, we were interested in the question whether

these activators perform their activating effect via their monomeric or via their micellar (vesicular) form. Measurements with the fluorescent probe pyrenedecanoic acid, also mimicking calmodulin activation, revealed that the micelles of this activator are the activating species for phosphodiesterase (Gietzen *et al.*, 1982b). Antagonism of all anionic amphiphilic activators by the cationic amphiphilic inhibitors is believed to occur by complex-formation, as a result of their complementary structural features, via ionic and hydrophobic interactions. The enzyme then flips back into its low activity state. That indeed such complexes are formed at the relevant concentrations could be demonstrated by fluorescence measurements with the fluorescent inhibitor flupenthixol (Y.-H. Xü, H.-J. Galla & K. Gietzen, unpublished work). From our experiments dealing with the inhibition of trypsin-activated enzymes the conclusion can be drawn that 'calmodulin inhibitors' do not exclusively exert their inhibitory effect on calmodulin-dependent enzymes via interaction with calmodulin but also act, at least in the case of Ca^{2+} -transport ATPase, via direct interaction with the enzyme. Attachment of an inhibitor to the enzyme may influence the active centre by an allosteric effect. The similar patterns of inhibition obtained with trifluoperazine and penfluridol imply that the binding site on Ca^{2+} -transport ATPase may have similar structural features to calmodulin (i.e. anionic amphiphilic). An attractive idea is the hypothesis that Ca^{2+} -transport ATPase might contain calmodulin as an integral EGTA-undissociable subunit in addition to that calmodulin which is Ca^{2+} -dependently bound. Such a dual role of calmodulin was shown to be relevant for phosphorylase kinase (Cohen *et al.*, 1978).

The fact that calmidazolium displays a higher specificity for calmodulin-induced activation compared with the other types of stimulation may be due to its bulky structure. Complexation of this compound by oleic acid or phosphatidylserine demands its incorporation into the micellar or vesicular structure, which might be more difficult than simple attachment of the drug to calmodulin. Also the low affinity of calmidazolium for the trypsin-treated ATPase may be due to a steric problem.

We are aware that part of our proposed mechanism describing the activation of calmodulin-regulated enzymes and the inhibition of the induced activation is speculative, but it may serve as a useful working hypothesis for further experimental work.

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