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COMPOUND 48/80 IS A SELECTIVE AND POWERFUL INHIBITOR OF CALMODULIN-REGULATED FUNCTIONS

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Compound 48/80, a condensation product of *N*-methyl-*p*-methoxyphenethylamine with formaldehyde, is composed of a family of cationic amphiphiles differing in the degree of polymerization. Compound 48/80 was found to be a potent inhibitor of the calmodulin-activated fraction of brain phosphodiesterase and red blood cell Ca^{2+}-transport ATPase, with IC_{50} values of 0.3 and 0.85 μg/ml, respectively. However, the basal activity of both enzymes is not at all suppressed by the drug at concentrations up to 300 μg/ml. Inhibition of Ca^{2+} transport into inside-out red blood cell vesicles by compound 48/80 follows a similar pattern in that basal, calmodulin-independent, transport is also not affected by the drug. Kinetic analysis revealed that the stimulation of Ca^{2+}-transport ATPase induced by calmodulin is inhibited by compound 48/80 according to a competitive mechanism. It was demonstrated that the inhibitory constituents of compound 48/80 bind to calmodulin in a Ca^{2+}-dependent fashion. Comparison of the specificity of several anti-calmodulin drugs showed that compound 48/80 is the most specific inhibitor of the calmodulin-dependent fraction of red blood cell Ca^{2+}-transport ATPase that has been described hitherto. In addition, compound 48/80 was found to be a rather specific inhibitor of the calmodulin-induced activation of Ca^{2+}-transport ATPase when compared with the stimulation induced by an anionic amphiphile or by limited proteolysis. Half-maximal inhibition of the activity stimulated by oleic acid or mild tryptic digestion required 8- and 32-times higher concentrations of compound 48/80, respectively, compared with the calmodulin-dependent fraction of the ATPase activity. Moreover, calmodulin-independent systems as rabbit skeletal muscle sarcoplasmic reticulum Ca^{2+}-transport ATPase or calf cardiac sarcolemma $(Na^+ + K^+)$-transport ATPase are far less influenced by compound 48/80 as compared with trifluoperazine and calmidazolium. Because of its high specificity compound 48/80 is proposed to be a promising tool for studying calmodulin-dependent processes.

Introduction

Calmodulin is a Ca^{2+}-binding protein of general importance and is a major cytoplasmic receptor for Ca^{2+} [1,2]. Through its dependence on the intracellular Ca^{2+} concentration, calmodulin mediates many of the intracellular effects of Ca^{2+} by the reversible formation of a calmodulin-Ca^{2+} complex which regulates a multitude of important Ca^{2+}-dependent cell functions and enzyme systems [1,2]. Calmodulin not only mediates the effect of an elevated cytoplasmic Ca^{2+} concentration but also terminates the function of the calmodulin-

Abbreviations: Mops, 4-morpholinepropanesulphonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; IC_{50}, concentration producing 50% inhibition.

Ca^{2+} complex by regulating the intracellular free Ca^{2+} concentration by stimulation of Ca^{2+}-transport ATPases [2]. It has been demonstrated for several enzymes that anionic amphiphiles (fatty acids, acidic phospholipids) and limited tryptic digestion mimic the effect of calmodulin [3–5].

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 [6,7], naphthalene sulphonamides [8], Vinca alkaloids [9–11], local anaesthetics [12] and calmidazolium [13,14], formerly referred to as R 24571, which is a derivative of the antimycotic miconazole. Calmidazolium, the most potent inhibitor of calmodulin-mediated functions [13,14], also was found to be a more specific inhibitor of calmodulin-induced activation as compared with the activation induced by anionic amphiphiles or limited proteolysis [15]. In this respect the neuroleptics, trifluoperazine and penfluridol, were found to be far less specific drugs [15].

However, all described calmodulin antagonists are more or less unspecific in that they also inhibit the basal, calmodulin-independent, activity of these enzymes [6–14]. This unspecific effect was observed with soluble enzymes, such as phosphodiesterase [6], but is more prominent with membrane-bound enzymes, like Ca^{2+}-transport ATPase [7,8,13]. The unspecific inhibition may be due to perturbation of the target enzyme itself and/or the lipid environment of these enzymes by the lipophilic inhibitors.

Thus, in searching for a highly specific inhibitor of the calmodulin-dependent fraction of enzyme activities we examined the effects of compound 48/80, commonly used as a histamine releaser, on two calmodulin-regulated enzymes: Ca^{2+}-transport ATPase of human erythrocytes and rat brain cyclic nucleotide phosphodiesterase. This substance fulfils the requirements of a potential inhibitor of calmodulin-mediated functions in being a cationic amphiphile [15,16]. A communication of our investigations has been presented [17].

Materials and Methods

Materials. All reagents were of highest purity available. Compound 48/80, a concensation product of *N*-methyl-*p*-methoxyphenethylamine with formaldehyde, was obtained from Sigma. Compound 48/80 is composed of several constituents differing in the degree of polymerization, which range from the dimer to higher oligomers having structures shown in Ref. 16. The limit of solubility of compound 48/80 is between 700 and 1000 μg/ml at pH 7.0–7.5. Trifluoperazine dihydrochloride was a gift from Röhm Pharma (Darmstadt, F.R.G.) and calmidazolium was supplied by Janssen Pharmaceutica (Beerse, Belgium). Oleic acid, 5′-nucleotidase and soya-bean trypsin inhibitor were obtained from Sigma (Munich, F.R.G.). Trypsin from bovine pancreas was a product of Boehringer (Mannheim, F.R.G.).

The lipophilic compound calmidazolium was 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 erythrocyte and sarcoplasmic reticulum Ca^{2+}-transport ATPases and 1% (v/v) for sarcolemma $(Na^+ + K^+)$-transport ATPase.

Oleic acid microdispersions were prepared by sonication (Branson Sonfier B12; approx. 2 min at setting 2) in a buffer containing 0.1 mM EGTA and 5 mM Mops (pH 7.0) under a stream of N_2.

Preparation of enzymes, calmodulin and calmodulin-Sepharose conjugate. Homogeneous calmodulin was prepared from bovine brain as described by Kakiuchi et al [18]. 10 mg of purified calmodulin were coupled per gram of CNBr-activated Sepharose 4B in accordance with the manufacturer's instructions. Membrane-bound human erythrocyte Ca^{2+}-transport ATPase deficient in calmodulin was prepared by a standard procedure [19]. Calmodulin-sensitive phosphodiesterase from rat brain was partially purified based on the method of Wang and Desai [20] with slight modifications as described recently [15]. Sarcoplasmic reticulum Ca^{2+}-transport ATPase was prepared according to the procedure of Meissner et al. [21]. Vesicles of calf cardiac sarcolemma with high $(Na^+ + K^+)$-transport ATPase activity were purified as reported recently [22].

Preparation of calmodulin-deficient inside-out erythrocyte vesicles. Inside-out human erythrocyte vesicles were prepared as described recently [13]. The vesicles were either calmodulin-saturated or

calmodulin-depleted. This could be achieved by inclusion or omission of EGTA (1 mM) in the buffers used for lysis and the subsequent washings. The properties of the inside-out vesicles have been described elsewhere [23].

Ca^{2+} uptake into inside-out erythrocyte vesicles. Ca^{2+}-transport measurements were carried out as described previously [23]. Before starting the transport process with ATP (1.2 mM) the vesicles were first preincubated for 10 min with compound 48/80 and then for 10 min with calmodulin (60 nM) at 37°C in a medium consisting of 125 mM KCl, 25 mM Mops (pH 7.0), 2 mM $MgCl_2$ and Ca^{2+} (plus $^{45}Ca^{2+}$ (0.2 μCi/ml)) as indicated in the legend to Fig. 2. The assay medium contained 150 μg of vesicle protein/ml. To calculate initial transport rates by linear regression, three samples (each 0.1 ml) were taken within the first 3 min after adding ATP. The samples were filtered immediately through Millipore filters with a pore diameter of 0.45 μm, which retained all protein.

Assay of enzyme activities. Sarcoplasmic reticulum Ca^{2+}-transport ATPase, ($Na^+ + K^+$)-transport ATPase and phosphodiesterase activities were determined at 37°C by measuring the rate of P_i-liberation as reported by Stewart [24] with slight modifications as reported recently [25] or for erythrocyte Ca^{2+}-transport ATPase by the automated assay described by Arnold et al. [26].

Briefly, phosphodiesterase assay was performed by coupling the phosphodiesterase reaction with 5'-nucleotidase reaction [20,27] 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 $CaCl_2$.

In the case of erythrocyte Ca^{2+}-transport ATPase the reaction was monitored continuously for 8 min, whereas in the case of sarcoplasmic reticulum Ca^{2+}-transport ATPase and ($Na^+ + K^+$)-transport ATPase the reaction was followed discontinuously over a period of 10 min. The assay medium for Ca^{2+}-transport ATPases contained 30–60 μg of membrane protein/ml, 25 mM Mops (pH 7.0), 100 mM KCl, 0.25 mM ouabain, 10 mM NaN_3, 1 mM ATP, 2 mM $MgCl_2$ and 36 μM Ca^{2+} (as a 0.4 mM Ca^{2+}/Mg^{2+}/EDTA buffer [28]). Ca^{2+}-free controls contained 0.4 mM Mg^{2+}/EGTA instead of the Ca^{2+}/Mg^{2+}/EDTA buffer. Ca^{2+}-transport ATPase activity refers to the difference in activity obtained in the presence and absence of Ca^{2+}. The medium for ($Na^+ + K^+$)-transport ATPase consisted of 30 μg of membrane protein per ml, 100 mM NaCl, 10 mM KCl, 30 mM imidazole · HCl (pH 7.2), 4 mM $MgCl_2$, 10 mM NaN_3, 0.5 mM Tris-EGTA, and 2 mM ATP, with or without 0.25 mM ouabain. ($Na^+ + K^+$)-transport ATPase activity is defined as the difference in activity obtained in the presence and absence of ouabain.

Before the reaction was started with the respective substrate (ATP, cyclic AMP) enzymes were preincubated as follows: ($Na^+ + K^+$)-transport ATPase and sarcoplasmic reticulum Ca^{2+}-transport ATPase were preincubated with the corresponding drug for 10 min at 37°C whereas phosphodiesterase and erythrocyte Ca^{2+}-transport ATPase were first preincubated for 10 min with the drug and additionally for 10 min in the presence and absence of calmodulin.

Results

Effects of compound 48/80 on Ca^{2+}-transport ATPase

Fig. 1. demonstrates the stimulation of erythrocyte Ca^{2+}-transport ATPase by calmodulin

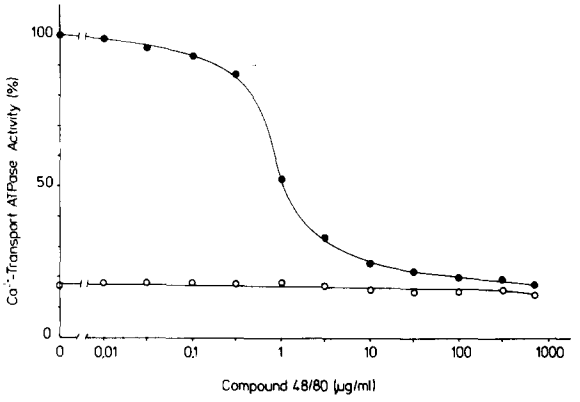

Fig. 1. Effects of compound 48/80 on erythrocyte calmodulin-dependent Ca^{2+}-transport ATPase. Ca^{2+}-transport ATPase activity of disrupted erythrocyte membranes was assayed at 37°C in the absence (○) or presence (●) of 30 nM calmodulin. The data are plotted as a percentage of the activity in the presence of calmodulin and in the absence of the drug (100% activity = 70–85 nmol of P_i/mg membrane protein per min). Each point represents the mean of four to six determinations.

and antagonism of the activation by compound 48/80. In the absence of the agent, as documented by the points on the ordinate, Ca^{2+}-transport ATPase of disrupted erythrocyte membranes could be maximally stimulated by calmodulin 4- to 6-fold above the basal Ca^{2+}-transport ATPase activity ('basal' Ca^{2+}-transport ATPase activity was defined as that activity determined in the absence of added calmodulin). As can be seen from Fig. 1, compound 48/80 specifically antagonized the calmodulin-induced activation of human erythrocyte Ca^{2+}-transport ATPase with an IC_{50} value of 0.85 μg/ml whereas the basal activity was not at all affected at concentrations up to 300 μg/ml and was only slightly inhibited at 700 μg/ml.

Compound 48/80 was also tested for its effects on Ca^{2+} transport into inside-out erythrocyte vesicles in the absence and presence of added calmodulin (Fig. 2A). Ca^{2+} transport of calmodulin-depleted inside-out erythrocyte vesicles was found to be less responsive to added calmodulin (cf. points on the ordinate) as compared with Ca^{2+}-transport ATPase activity of disrupted membranes (see Fig. 1). The calmodulin-dependent fraction of Ca^{2+}-transport activity of inside-out vesicles was inhibited half-maximally by compound 48/80 at a concentration of approx. 7 μg/ml. This value is somewhat higher than that determined for inhibition of Ca^{2+}-transport ATPase activity (see Fig. 1). Note that the calmodulin concentration in the assay medium for Ca^{2+} transport was double (60 nM) of that used for ATPase determinations (30 nM). Ca^{2+}-transport activity of inside-out vesicles without added calmodulin declined slightly to an apparent steady value that was not further suppressed by concentrations up to 700 μg/ml.

The majority of the inside-out vesicles used for the transport studies were tight for Ca^{2+}. This was demonstrated by the experiment shown in Fig. 2B. Only a small fraction of the Ca^{2+} accumulated in the vesicles was released by lowering the Ca^{2+} concentration in the medium to a value under 10^{-8} M, reflecting the population of leaky vesicles. Fig. 2B provides evidence that inhibition of Ca^{2+} transport by compound 48/80 (see Fig. 2A) in-

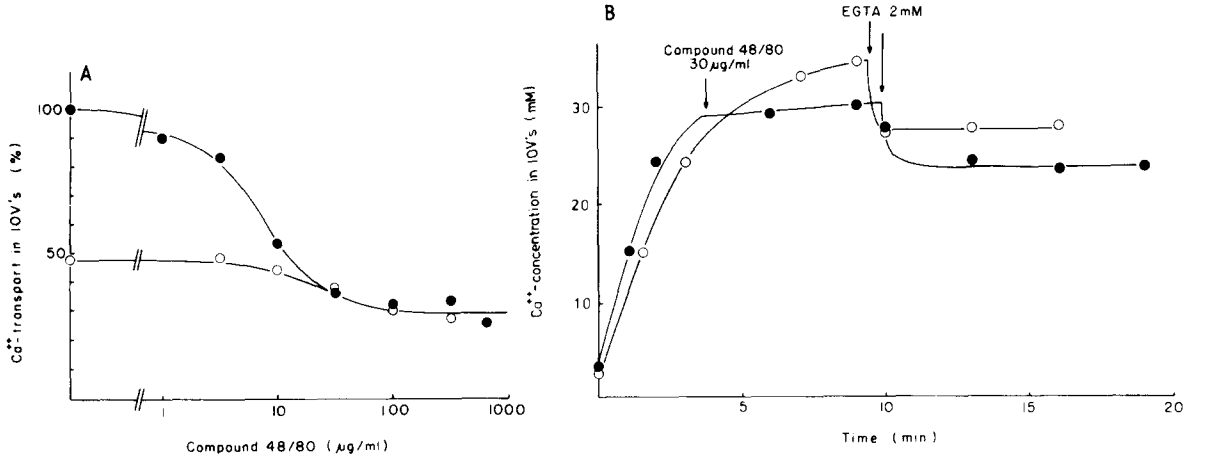

Fig. 2. Inhibition of calmodulin-dependent Ca^{2+} transport into inside-out vesicles (IOV's) by compound 48/80. (A) Inhibition of Ca^{2+} transport into calmodulin-depleted inside-out erythrocyte vesicles by compound 48/80 in the absence (○) and in the presence (●) of added calmodulin (60 nM). Ordinate: relative initial rate (0–3 min after adding ATP) of $^{45}Ca^{2+}$ uptake into vesicles (100% activity = 9.01 ± 0.70 (S.E.) nmol of Ca^{2+}/mg vesicle protein per min). The medium contained 0.2 mM EGTA and 0.2 mM $CaCl_2$ leading to a concentration of 5.3 μM free Ca^{2+} at the prevailing ATP and Mg^{2+} concentrations. The points represent the mean of two or three determinations with three different preparations. For experimental details see the methods section. (B) Ca^{2+} uptake and retention in inside-out vesicles saturated with calmodulin in the presence and absence of compound 48/80. (○) Time-course of $^{45}Ca^{2+}$ uptake. At the time indicated by an arrow Ca^{2+} concentration in the medium (5.3 μM) was lowered to $<10^{-8}$ by adding EGTA. (●) In this paralled experiment at 3.5 min calmodulin-dependent Ca^{2+} pumping was blocked by compound 48/80 and 6.5 min later EGTA was added as in the control. At 10 min internal Ca^{2+} concentration had reached about 30 mM. Notice that compound 48/80 does not cause a leak exceeding that seen in the control. Single experiment. Intravesicular Ca^{2+} concentration was estimated from the accumulated radioactivity ($^{45}Ca^{2+}$) and the vesicle volume determined by the method reported in Ref. 35.

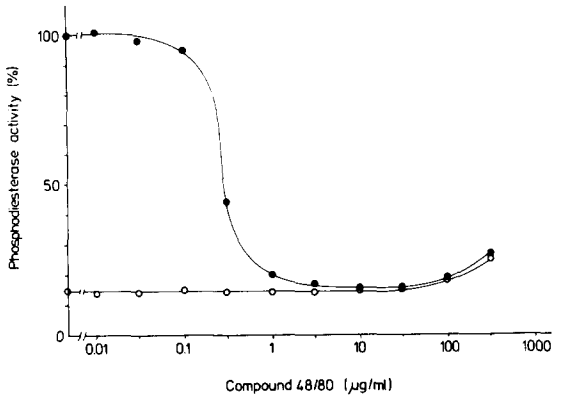

Fig. 3. Effects of compound 48/80 on rat brain phosphodiesterase. Phosphodiesterase activity was determined at 37°C in the absence (○) or presence (●) of 30 nM added calmodulin. The activity is related to the calmodulin-stimulated enzyme in the absence of the drug (100% activity = 0.8–1 μmol/mg protein per min). Each point is the mean of six determinations.

deed was caused by blocking the calmodulin-dependent Ca^{2+} transport into inside-out vesicles, and not simulated by leaky vesicles as a consequence of the action of compound 48/80 on the membrane permeability. Compound 48/80 at a dose of 30 μg/ml did not release Ca^{2+} that had been accumulated in the vesicles.

Effects of compound 48/80 on phosphodiesterase

Compound 48/80 was examined for its inhibitory potency on phosphodiesterase, another enzyme modulated by calmodulin. Calmodulin-induced activation of rat brain phosphodiesterase was antagonized half-maximally by compound 48/80 at a concentration of 0.3 μg/ml (Fig. 3). As was also shown for Ca^{2+}-transport ATPase, the basal activity of phosphodiesterase could not be suppressed by compound 48/80. This agent had the opposite effect on basal phosphodiesterase activity in that it slightly stimulated the enzyme activity above its basal level in the concentration range of 100–300 μg/ml (Fig. 3).

Mechanism of action of compound 48/80 on the function of calmodulin

To obtain stronger evidence for the involvement of calmodulin in the inhibition of erythrocyte Ca^{2+}-transport ATPase by compound 48/80, the antagonistic effect of compound 48/80 was studied as a function of calmodulin concentration. In the absence of the agent approx. 7 nM calmodulin was required for half-maximal activation of the calmodulin-dependent fraction of ATPase activity (Fig. 4A). In the presence of 0.3 and 1 μg/ml compound 48/80 the dose-effect curves of

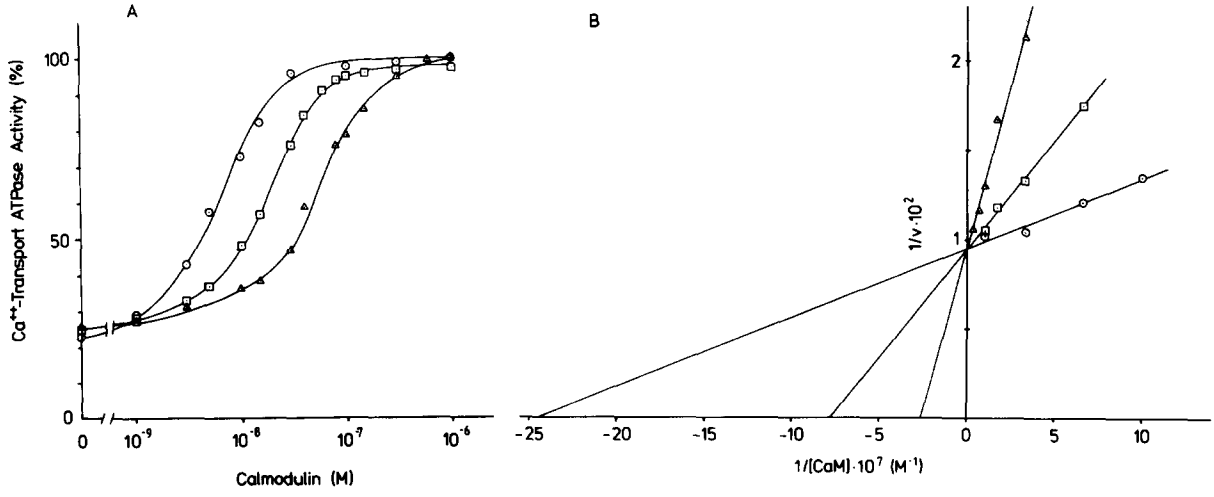

Fig. 4. Antagonism of calmodulin-induced activation of Ca^{2+}-transport ATPase by compound 48/80. (A) Effects of calmodulin on erythrocyte Ca^{2+}-transport ATPase in the absence of compound 48/80 (⊙) and in the presence of 0.3 μg/ml (□) and 1 μg/ml (△) compound 48/80. The ATPase activity is related to the activity in the presence of 60 nM calmodulin and in the absence of the drug. Points on the ordinate represent the basal Ca^{2+}-transport ATPase activity, i.e., the activity obtained in the absence of added calmodulin. Each point represents the mean of four independent determinations. (B) Double-reciprocal plot of dependence of rate (v) of Ca^{2+}-transport ATPase reaction on calmodulin (CaM). v represents % activity as given in Fig. 2A. The lines of best fit were drawn by linear regression analysis.

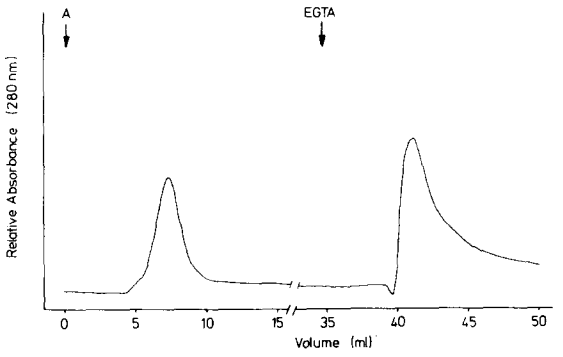

Fig. 5. Ca^{2+}-dependent binding of compound 48/80 to calmodulin-Sepharose conjugate. A calmodulin-Sepharose column (0.9×7 cm) was preequilibrated with Ca^{2+} containing buffer similar to that used for the assay of Ca^{2+}-transport ATPase: 25 mM Mops (pH 7.0), 100 mM KCl, 2 mM $MgCl_2$, and 36 μM free Ca^{2+} (as a 0.4 mM Ca^{2+}/Mg^{2+}/EDTA buffer). Arrow A marks the application of 300 μg compound 48/80, dissolved in 1 ml of the above mentioned buffer, to the column. Elution was first performed with the Ca^{2+} containing buffer equivalent to five bed volumes before an EGTA containing buffer (25 mM Mops (pH 7.0), 100 mM KCl, 2 mM $MgCl_2$ and 2 mM EGTA) was applied. Elution of the column was carried out at a flow rate of 0.5 ml/min. The elution pattern was obtained by recording the absorbance at 280 nm.

calmodulin were shifted to the right. The lines in the double-reciprocal plot (Fig. 4B) represent computer-fitted linear regressions of the data and yielded estimates of half-maximal stimulation by 4.1, 13 and 38 nM calmodulin in the absence and in the presence of the two indicated concentrations of compound 48/80, respectively. Increasing concentrations of compound 48/80 obviously did not change the extrapolated maximal activity of the enzyme. The data are compatible with the interpretation that the stimulation of erythrocyte Ca^{2+}-transport ATPase induced by calmodulin is antagonized by compound 48/80 according to a competitive mechanism.

Fig. 5 provides evidence that the function of calmodulin may be abolished by interaction of compound 48/80 with calmodulin. Passing compound 48/80 in the presence of Ca^{2+} over a calmodulin-Sepharose column revealed that part of the constituents bind Ca^{2+}-dependently to calmodulin since they were exclusively eluted from the column after an EGTA-containing buffer had been applied to the column. Another fraction of compound 48/80 did not bind in the presence of Ca^{2+} to the calmodulin-Sepharose gel and was immediately eluted after the sample had been ap-

TABLE I

SPECIFICITY OF DIFFERENT DRUGS FOR ANTAGONISM OF CALMODULIN-INDUCED ACTIVATION OF Ca^{2+}-TRANSPORT ATPase

The specificity is expressed as the ratio of the IC_{50} value of the basal erythrocyte Ca^{2+}-transport ATPase activity to the IC_{50} value of the calmodulin-dependent fraction of the ATPase activity. Part A of the table compares IC_{50} values of calmodulin antagonists determined in our laboratory under the same experimental conditions with a calmodulin concentration of 30 nM. In order to compare compound 48/80 with W-7 and W-9 its IC_{50} values were also determined under the conditions applied in Ref. 8, namely 180 nM calmodulin and 110 μg erythrocyte membrane protein/ml. These results are shown in Part B of the Table.

Drug	Ref.	IC_{50} of basal ATPase (μM, * = μg/ml)	IC_{50} of calmodulin-dependent ATPase (μM, * = μg/ml)	Specificity (IC_{50} basal ATPase/IC_{50} calmodulin-dependent ATPase)
A				
Penfluridol	7	20	2.6	8
Trifluoperazine	7	160	9	18
Fluphenazine	7	200	10	20
Chlorpromazine	7	500	22	23
Calmidazolium	13	10	0.35	29
Compound 48/80		> 700 *	0.85 *	> 824
B				
W-7	8	2000	100	20
W-9	8	1200	23	52
Compound 48/80		> 700 *	3.3 *	> 212

plied to the column. The fraction not retained by the column in the presence of Ca^{2+} had only negligible inhibitory efficacy (results not shown).

Specificity of compound 48/80

Table I provides a list of IC_{50} values of several calmodulin inhibitors for the basal and calmodulin-dependent fraction of the activity of erythrocyte Ca^{2+}-transport ATPase. The ratio of IC_{50} value of basal ATPase to IC_{50} value of calmodulin-dependent ATPase activity is a measure for the specificity of the drug to inhibit the calmodulin-induced stimulation. The butyrophenone derivative, penfluridol, exhibits an extremely low specificity whereas trifluoperazine, fluphenazine, chlorpromazine and calmidazolium are somewhat more specific as indicated by a ratio of 18, 20, 23 and 29, respectively. Compound 48/80, however, is by far the most specific inhibitor of calmodulin-induced activation of erythrocyte Ca^{2+}-transport ATPase (specificity ratio: > 824) as compared with any of the inhibitors shown in Table I part A. Data in parts A and B of Table I differ in that they were obtained at a 6-fold higher calmodulin concentration in B than in A. In addition, the data shown in Table I part B reveal a greater specificity of compound 48/80 over W-7 and W-9 in inhibiting calmodulin-stimulated as opposed to basal Ca^{2+}-transport ATPase.

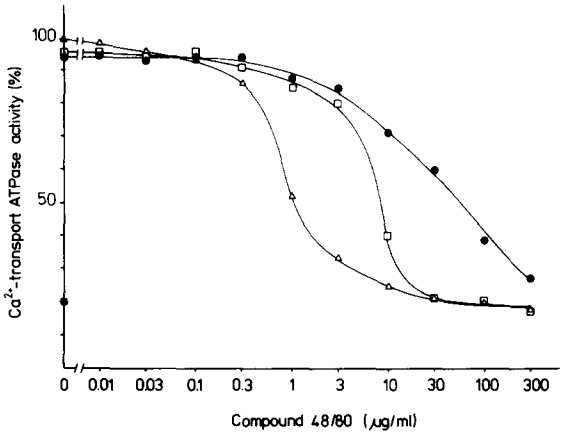

Fig. 6. Inhibition of Ca^{2+}-transport ATPase stimulated by calmodulin, oleic acid or limited proteolysis. Ca^{2+}-transport ATPase was activated either by calmodulin (△, 30 nM), oleic acid (□, 50 µM) or by limited tryptic digestion (●, for 3 min at 37°C with 0.2 mg of trypsin/mg erythrocyte membrane protein). Trypsin digestion was carried out in the assay medium and was terminated with a 5-fold excess (w/w) of trypsin inhibitor [15]. In the case of stimulation of erythrocyte Ca^{2+}-transport ATPase by trypsin, after digestion the samples were preincubated for 10 min at 37°C with the inhibitor. In the case of activation by calmodulin or oleic acid Ca^{2+}-transport ATPase was first preincubated 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+}-transpoert ATPase activity (without added calmodulin) in the absence of the drug. Each point represents the mean of four to six determinations.

TABLE II

EFFECTS OF CALMODULIN ANTAGONISTS ON CALMODULIN-DEPENDENT AND CALMODULIN-INDEPENDENT ATPases

Three calmodulin antagonists were tested for their potency to inhibit a Ca^{2+}/calmodulin dependent, a Ca^{2+}-dependent but calmodulin-independent, and a Ca^{2+}/calmodulin-independent enzyme. The investigated enzymes were human erythrocyte Ca^{2+}-transport ATPase, rabbit skeletal muscle sarcoplasmic reticulum Ca^{2+}-transport ATPase and calf cardiac sarcolemma $(Na^+ + K^+)$-transport ATPase. For comparison's sake the inhibition studies were perfored for each ATPase with the same protein concentration (30 µg/ml). The specificity for inhibition of calmodulin effects is expressed as the ratio of the IC_{50} value of the calmodulin-independent enzyme's activity to the IC_{50} value of the calmodulin-dependent fraction of erythrocyte Ca^{2+}-transport ATPase activity. The value in parentheses indicates the percentage of inhibition at the highest drug concentration used (300 µg/ml).

Drug	IC_{50} of calmodulin-dependent erythrocyte Ca^{2+}-ATPase (µM, * = µg/ml)	IC_{50} of sarcoplasmic reticulum Ca^{2+}-ATPase (µM, * = µg/ml)	Specificity (IC_{50} sarcoplasmic reticulum Ca^{2+}-ATPase/IC_{50} erythrocyte Ca^{2+}-ATPase)	IC_{50} of $(Na^+ + K^+)$-ATPase (µM, * = µg/ml)	Specificity (IC_{50} $(Na^+ + K^+)$-ATPase/IC_{50} erythrocyte Ca^{2+}-ATPase)
Trifluoperazine	9	60	6.7	450	50
Calmidazolium	0.35	2.9	8.3	15	43
Compound 48/80	0.85 *	80 *	94	> 300 * (25)	> 350

Table II summarizes the effects of trifluoperazine, calmidazolium and compound 48/80 on following ion transporting ATPases: (1) calmodulin-dependent erythrocyte Ca^{2+}-transport ATPase; (2) calmodulin-independent sarcoplasmic reticulum Ca^{2+}-transport ATPase; and (3) Ca^{2+}/calmodulin-independent sarcolemma ($Na^+ + K^+$)-transport ATPase. The ratio of IC_{50} value of the calmodulin-independent enzyme's activity to IC_{50} value of calmodulin-dependent ATPase activity is used as a measure to monitor the specificity of the drug to inhibit the calmodulin-dependent system. Trifluoperazine and calmidazolium had only a 7- to 8-times higher specificity for calmodulin-dependent erythrocyte ATPase than for sarcoplasmic reticulum ATPase whereas the specificity of compound 48/80 for the calmodulin-dependent system is more than 10-times higher (ratio: 94). Moreover, the specificity of compound 48/80 for the calmodulin-dependent enzyme as compared with the Ca^{2+}/calmodulin-independent ($Na^+ + K^+$)-transport ATPase is at least by a factor of 7 higher than for the two other drugs. This is reflected by the specificity ratios of 50, 43 and > 350 for trifluoperazine, calmidazolium and compound 48/80, respectively.

Investigation of the potency of compound 48/80 to antagonize the stimulation of erythrocyte Ca^{2+}-transport ATPase by different activators revealed that this agent is a rather specific inhibitor of the calmodulin-induced activation of the enzyme (Fig. 6). Half-maximal inhibition of the activating effects of calmodulin, oleic acid or limited proteolysis occurred at concentrations of 0.85, 7 and 27 μg of compound 48/80 per ml.

Discussion

In the present work compound 48/80 has been identified as a potent and highly specific antagonist of calmodulin-mediated effects. Antagonism of calmodulin had been expected since compound 48/80 fulfils the general requirements of calmodulin inhibitors in having cationic amphiphilic properties [15,16]. The substance is composed of a family of homologous hydrophobic polycations [16].

Compound 48/80 displayed a high potency in antagonizing the calmodulin-induced activation of brain phosphodiesterase and erythrocyte Ca^{2+}-transport ATPase with observed IC_{50} values of 0.3 and 0.85 μg/ml, respectively. The constituents of compound 48/80 displaying full efficacy for inhibition of calmodulin-mediated effects presumably range from the tetramer to the hexamer with an average molecular weight of 1000 (Adamczyk-Engelmann, P. and Gietzen, K., unpublished data). Thus the potency of this calmodulin antagonist, expressed in terms of molarity, can be estimated to be in the range of 0.1 to 0.3 μM for phosphodiesterase and Ca^{2+}-transport ATPase when taking into account that the active constituents make up just a fraction of compound 48/80. This would mean that the active constituents of compound 48/80 exhibit a potency to antagonize calmodulin effects comparable with that of calmidazolium tested under identical conditions in our laboratory [13,15].

The paralled inhibition of Ca^{2+} transport and Ca^{2+}-ATPase by compound 48/80 is not surprising since recently identity of Ca^{2+}-ATPase and Ca^{2+}-pump protein clearly was demonstrated [29]. The concentration of compound 48/80 producing half-maximal inhibition of the calmodulin-dependent fraction of Ca^{2+} transport into inside-out erythrocyte vesicles was found to be somewhat higher ($IC_{50} = 7$ μg/ml) as compared with the corresponding ATPase activity ($IC_{50} = 0.85$ μg/ml). This discrepancy may, at least in part, be due to the fact the calmodulin concentration in the assay medium for Ca^{2+} transport was higher by a factor of two as compared with that for ATPase. The decline of Ca^{2+} transport into inside-out vesicles (without added calmodulin) to a seemingly steady value with increasing inhibitor concentrations may reflect some residual calmodulin in the preparation. This view is supported by the facts that the decline occurs in the same concentration range as found for Ca^{2+} transport in the presence of added calmodulin (Fig. 2A) and that basal Ca^{2+}-transport ATPase activity of disrupted membranes was not at all affected by compound 48/80 (see Fig. 1).

The predominant feature of compound 48/80 is the observed specificity in antagonizing exclusively the calmodulin-induced stimulation of phosphodiesterase and Ca^{2+}-transport ATPase without suppression of the basal activity of these enzymes.

This makes compound 48/80 superior to the calmodulin antagonists presently known since all of them affect also the basal activity of calmodulin-dependent enzymes (see Table I). Inhibition of basal activity by the other calmodulin antagonists might be a direct effect on the target enzyme but was also interpreted to be a consequence of perturbation of the lipid environment in the case of membrane-bound enzymes [30,31]. The high specificity of compound 48/80 may be determined by the fence-like structure of the homologous polycations [16] of which compound 48/80 is composed. This structure possibly hinders incorporation of the agent into the lipid bilayer of biological membranes and thus no perturbation of the lipid environment of the enzyme may occur. If this interpretation is right compound 48/80 also should not be able to pass the cell membrane. Therefore it is tempting to speculate that compound 48/80-induced histamine release may not be related to calmodulin antagonism in mast cells. This view is supported by the fact that, in contrary, membrane-permeable calmodulin inhibitors, like pimozide, trifluoperazine or W-7, inhibit mast cell secretion elicited by any of several secretagogues [32].

In an other set of experiments the high specificity of compound 48/80 over other calmodulin antagonists in antagonizing a calmodulin-stimulated system as opposed to calmodulin-independent enzymes has been established (Table II). In contrast to trifluoperazine and calmidazolium, compound 48/80 obviously has a much higher specificity for calmodulin-dependent erythrocyte Ca^{2+}-transport ATPase as compared to calmodulin-independent sarcoplasmic reticulum Ca^{2+}-transport ATPase and Ca^{2+}/calmodulin-independent $(Na^+ + K^+)$-transport ATPase.

Another aspect of the specificity of compound 48/80 concerns the antagonism of activating treatments mimicking the calmodulin-induced stimulation of enzymes. Compound 48/80 was found to be a rather specific inhibitor of the calmodulin-dependent activation of erythrocyte Ca^{2+}-transport ATPase, since half-maximal inhibition of the activating effects of oleic acid or limited proteolysis on the enzyme required 8- and 32-times higher concentrations of the substance, respectively, compared with the calmodulin-dependent fraction of the ATPase activity. Inhibition of the trypsin-activated Ca^{2+}-transport ATPase by calmodulin inhibitors was interpreted as direct effect on the enzyme whereas inhibition of anionic amphiphile-induced stimulation of a enzyme was proposed to occur via complexation of the activator by the cationic amphiphilic calmodulin antagonist [15]. From the results presented here it is obvious that compound 48/80 in addition to its effect on calmodulin directly influences Ca^{2+}-transport ATPase. However, this property was far less pronounced as was found for the antipsychotics, trifluoperazine and penfluridol. These substances inhibited the trypsin-activated Ca^{2+}-transport ATPase at similar concentrations as they inhibited the calmodulin-stimulated enzyme [15]. Another inhibitor, calmidazolium, shares with compound 48/80 the property to antagonize preferentially the calmodulin-induced stimulation of Ca^{2+}-transport ATPase as compared with the activation induced by other treatments [15]. However, the order of IC_{50} values obtained for the oleic acid treated and trypsinized enzyme is inverse for the two calmodulin antagonists. Since compound 48/80 possesses multiple positive charges per molecule at physiological pH as opposed to one charge for calmidazolium a higher potency to complex the negatively charged oleic acid micelles can be expected for compound 48/80. In a previous study [33] inhibition of two Ca^{2+}-dependent ATPases, one from erythrocytes and the other from bovine muscle myosin, has been reported to occur at 50-times higher concentrations of compound 48/80 ($IC_{50} = 45\mu g/ml$) as compared with inhibition of calmodulin-dependent Ca^{2+}-transport ATPase reported here. A possible explanation of this discrepancy may be that this investigation [33] was performed with a proteolytically degraded enzyme since this IC_{50} value is in good agreement with that found for erythrocyte Ca^{2+}-transport ATPase stimulated by limited proteolysis.

The mechanism of action by which the calmodulin-induced stimulation of erythrocyte Ca^{2+}-transport ATPase is antagonized by compound 48/80 was elicited. It was shown that the inhibitory constituents of compound 48/80 bind to calmodulin in a Ca^{2+}-dependent fashion and by this action the formation of a calmodulin-enzyme complex is probably prevented. It is thought that

calmodulin antagonists bind to a specific hydrophobic site on calmodulin whose exposure is caused by the binding of Ca^{2+} to calmodulin [34]. The concentration of calmodulin required for half-maximal activation of Ca^{2+}-transport ATPase was increased by compound 48/80 and the inhibition of the enzyme was completely overcome by excess calmodulin. Kinetic analysis of the data revealed that the activation of Ca^{2+}-transport ATPase induced by calmodulin is inhibited by compound 48/80 according to a competitive mechanism.

The experiments dealing with the binding of compound 48/80 to calmodulin-Sepharose gel revealed that compound 48/80 contains constituents that do not interact, or only weakly interact with calmodulin. Since compound 48/80 is composed of several hydrophobic polycations, each of them being a potential calmodulin inhibitor, investigations are under way to determine the active constituents and their respective potency and efficacy in order to obtain a properly defined and characterized calmodulin antagonist.

In conclusion, compound 48/80 proved to be an outstanding calmodulin antagonist in that: (1) it exhibits high potency; and (2) most important, it is highly specific in antagonizing selectively the calmodulin-dependent fraction of enzyme activities. Therefore compound 48/80 is proposed to be a promising tool for studying calmodulin-dependent processes.

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