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Comparison of the calmodulin antagonists compound 48/80 and calmidazolium

Klaus GIETZEN
Department of Pharmacology and Toxicology, University of Ulm, D-7900 Ulm, Federal Republic of Germany

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The two presumed calmodulin antagonists calmidazolium and compound 48/80 were compared for their effects on several calmodulin-dependent and calmodulin-independent enzyme systems. Compound 48/80 and calmidazolium were found to be about equipotent in antagonizing the calmodulin-dependent fraction of brain phosphodiesterase and erythrocyte Ca^{2+}-transporting ATPase. Compound 48/80 combines high potency with high specificity in that: (1) the basal, calmodulin-independent, activity of calmodulin-regulated enzymes was not suppressed; (2) calmodulin-independent enzyme activities, such as Ca^{2+}-transporting ATPases of sarcoplasmic reticulum, Mg^{2+}-dependent ATPases of different tissues and Na^{+}/K^{+}-transporting ATPase of cardiac sarcolemma, were far less altered, or not altered at all, by compound 48/80 as compared with calmidazolium; and (3) antagonism of proteolysis-induced stimulation as opposed to calmodulin-induced activation of erythrocyte Ca^{2+}-transporting ATPase required a 32 times higher concentration of compound 48/80. In all these aspects compound 48/80 was found to be a superior antagonist to calmidazolium since inhibition of calmodulin-independent events by the other agent occurred at considerably lower concentrations. Therefore compound 48/80 is proposed to be a much more specific and useful tool for studying the participation of calmodulin in biological processes than the presently used agents.

Calmodulin, the ubiquitous Ca^{2+}-dependent regulatory protein, plays a pivotal role in all eukaryotic cells. It confers Ca^{2+}-sensitivity on a multitude of enzyme systems and cell functions. Its properties and functions have been summarized in recent reviews (Cheung, 1980; Klee *et al.*, 1980; Means & Dedman, 1980).

The calmodulin-dependent fraction of these enzymes can be inhibited by a wide range of chemically unrelated substances, such as phenothiazines and butyrophenones (Levin & Weiss, 1976; 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), calmidazolium (Gietzen *et al.*, 1981), formerly referred to as R 24571, and compound 48/80 (Gietzen *et al.*, 1983). Several calmodulin inhibitors were also shown to antagonize the enzymes' activity stimulated by treatments mimicking the action of calmodulin on phosphodiesterase and Ca^{2+}-transporting ATPase (Wolff & Brostrom, 1976; Gietzen *et al.*, 1982*a*). In a recent study a general model has been proposed illustrating the molecular mechanism of activation and inhibition of calmodulin-regulated enzymes simply by the assumption of hydrophobic and ionic interactions (Gietzen *et al.*, 1982*a*). Generally, activators of calmodulin-dependent enzymes (calmodulin, oleic acid or phosphatidylserine) can be considered as anionic amphiphiles, whereas calmodulin antagonists are cationic amphiphiles at physiological pH.

Evidence has been presented that inhibition of a calmodulin-stimulated enzyme may occur according to the following modes: (1) calmodulin is complexed by the cationic amphiphilic antagonist, as a result of their complementary structural features, via ionic and hydrophobic interactions (Weiss *et al.*, 1980); and (2) in addition, several calmodulin antagonists exert their inhibitory effect via direct interaction with the calmodulin effector enzyme (Gietzen *et al.*, 1982*a*,*b*). Therefore, these substances cannot be considered as calmodulin-specific probes. Moreover, almost all described inhibitors are more or less unspecific in that they also inhibit the basal activity of calmodulin-dependent enzymes and even the activity of calmodulin-independent enzymes (Balzer *et al.*, 1968; Levin & Weiss, 1976;

Kobayashi *et al.*, 1979; Gietzen & Bader, 1980; Gietzen *et al.*, 1980, 1981; Luthra, 1982).

From the numerous calmodulin antagonists two were shown to be outstanding. (1) Calmidazolium was reported to be the most potent inhibitor (Gietzen *et al.*, 1981; Van Belle, 1981) and in addition the substance displayed a higher specificity for calmodulin-induced activation of phosphodiesterase and Ca^{2+}-transporting ATPase compared with other modes of stimulation (Gietzen *et al.*, 1982*a*). (2) Compound 48/80 was shown to be the most specific antagonist of calmodulin-dependent Ca^{2+}-transporting ATPase activity as opposed to basal, calmodulin-independent, ATPase activity (Gietzen *et al.*, 1983).

In the present paper calmidazolium and compound 48/80 were compared directly under identical experimental conditions. The effects of both antagonists on several Ca^{2+}–calmodulin-dependent, Ca^{2+}-dependent but calmodulin-independent and Ca^{2+}–calmodulin-independent enzymes were investigated. In nearly all respects, compound 48/80 proved to be superior to calmidazolium.

Materials and methods

All reagents were of the highest purity available. Calmidazolium was supplied by Janssen Pharmaceutica (Beerse, Belgium). Compound 48/80 (product no. C4257), oleic acid, 5′-nucleotidase and soya-bean trypsin inhibitor were obtained from Sigma (München, Germany).

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 always 1% (v/v).

Oleic acid microdispersions were prepared by sonication (Branson Sonifier B12; approx. 2 min at setting 2) in a buffer containing 0.1 mM-EGTA and 5 mM-4-morpholinepropanesulphonic acid (Mops, pH 7.0) under a stream of N_2.

Preparation of enzymes and calmodulin

Homogeneous calmodulin was prepared from bovine brain as described by Kakiuchi *et al.* (1981). Calmodulin-sensitive phosphodiesterase was partially purified, based on the method of Wang & Desai (1977) and slightly modified as described by Gietzen *et al.* (1982*a*). Human erythrocyte membranes deficient in calmodulin were prepared by the procedure of Gietzen & Kolandt (1982). Sarcoplasmic-reticulum vesicles were prepared from dog heart by the procedure of Suko & Hasselbach (1976) and from rabbit skeletal muscle by the procedure described by Meissner *et al.* (1973). Vesicles of calf cardiac sarcolemma were purified as reported by Jones *et al.* (1979) and Caroni *et al.* (1980).

Controlled tryptic digestion

Ca^{2+}-transporting ATPase was digested at 37°C by 0.2 mg of trypsin/mg of erythrocyte membrane protein. Proteolysis was performed in the assay medium and was terminated with a 5-fold excess (w/w) of trypsin inhibitor (see Gietzen *et al.*, 1982*a*).

Assay of enzyme activities

ATPase and phosphodiesterase activities were determined at 37°C by measuring the rate of P_i-liberation as reported by Stewart (1974), slightly modified as described by Lanzetta *et al.* (1979).

Briefly, phosphodiesterase activity was assayed by coupling the phosphodiesterase reaction with 5′-nucleotidase reaction (Butcher & Sutherland, 1962; Wang & Desai, 1977) and measuring the P_i produced within 30 min. The assay mixture (final volume, 1 ml) 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-$CaCl_2$.

The reaction of the different ATPases was followed discontinuously over various time periods depending on the specific activity of the respective enzyme. The assay medium for Ca^{2+}-transporting ATPase contained, in a final incubation medium of 1 ml, 25 mM-4-morpholinepropanesulphonic acid (pH 7.0), 100 mM-KCl, 0.25 mM-ouabain, 10 mM-NaN_3, 1 mM-ATP, 2 mM-$MgCl_2$ and a 0.2 mM-Mg^{2+}/0.2 mM-Ca^{2+}/0.4 mM-EDTA buffer to yield a free Ca^{2+} concentration of 36 μM (Wolf, 1973). Ca^{2+}-free controls contained, instead of the Mg^{2+}/Ca^{2+}/EDTA buffer, 0.2 mM-Mg^{2+}/0.4 mM-EGTA. These controls yielded simultaneously the Mg^{2+}-dependent ATPase activity. Ca^{2+}-transporting ATPase activity refers to the difference in activity obtained in the presence and in the absence of Ca^{2+}. The medium for Na^+/K^+-transporting ATPase consisted of 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^+-transporting ATPase activity is defined as the difference in activity obtained in the presence and in the absence of ouabain.

To facilitate comparison, all enzyme assays were performed at the same protein concentration of 30 μg/ml unless otherwise stated. Before the reaction was started with the respective substrate (ATP, cyclic AMP) enzymes were pre-incubated as follows: Mg^{2+}-dependent ATPases, Na^+/K^+-transporting ATPase, sarcoplasmic-reticulum Ca^{2+}-transporting ATPases and tryptically digested erythrocyte Ca^{2+}-transporting ATPase were pre-incubated with the corresponding drug for 10 min at 37°C. Phosphodiesterase and erythrocyte Ca^{2+}-transporting ATPase were first pre-incubated for 10 min with the

drug and additionally for 10 min in the presence or in the absence of an activator.

Results

The stimulation of erythrocyte Ca^{2+}-transporting ATPase and brain phosphodiesterase by calmodulin and antagonism of the activation by compound 48/80 and calmidazolium is demonstrated in Figs. 1(*a*) and 1(*b*). In the absence of the agents, as documented by the points on the ordinate (Fig. 1*a*), Ca^{2+}-transporting ATPase of disrupted erythrocyte membranes could be maximally stimulated by calmodulin 5–6-fold above the basal enzyme's activity ('basal' Ca^{2+}-transporting ATPase activity was defined as that activity determined in the absence of added calmodulin). Phosphodiesterase displayed a somewhat higher sensitivity towards calmodulin in that calmodulin stimulated the enzyme in the absence of drugs 6.5–8-fold above its basal activity (Fig. 1*b*).

As can be seen from Fig. 1(*a*) calmidazolium antagonized the calmodulin-induced activation of erythrocyte Ca^{2+}-transporting ATPase with an IC_{50} value (concentration producing 50% inhibition) of 0.35 μM. However, also the calmodulin-independent activity of the enzyme was inhibited by this drug at higher concentrations (IC_{50} = 9 μM). On the other hand compound 48/80 specifically antagonized the calmodulin-dependent fraction of erythrocyte Ca^{2+}-transporting ATPase activity with an IC_{50} value of 0.85 μg/ml, whereas the basal activity was not at all affected at concentrations ⩽300 μg/ml (Fig. 1*a*).

Both calmidazolium and compound 48/80 inhibited the calmodulin-stimulated fraction of rat brain phosphodiesterase with high potency and IC_{50} values of 0.15 μM and 0.3 μg/ml respectively (Fig. 1*b*). Again calmidazolium also antagonized the basal activity of this enzyme (IC_{50} = 20 μM). As was shown for Ca^{2+}-transporting 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. 1*a*).

The two drugs were also tested for their effects on two Ca^{2+}-transporting ATPases of sarcoplasmic reticulum of different tissues, being not calmodulin-dependent, at least not directly. Ca^{2+}-transporting ATPase of dog cardiac sarcoplasmic reticulum was inhibited half-maximally by calmidazolium at 2.1 μM and by compound 48/80 at 16 μg/ml (Table 1). Half-maximal inhibition of skeletal muscle sarcoplasmic-reticulum Ca^{2+}-transporting ATPase by calmidazolium occurred at 2.9 μM, whereas a rather high concentration (80 μg/ml) of compound 48/80 was required to give the same effect (Table 1).

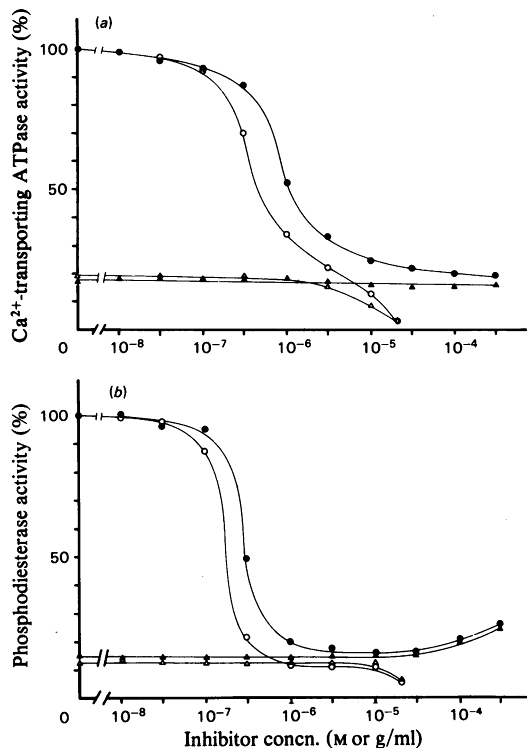

Fig. 1. *Effects of compound 48/80 and calmidazolium on erythrocyte Ca^{2+}-transporting ATPase* (*a*) *and rat brain phosphodiesterase* (*b*)

Basal, calmodulin-independent (▲ and △), and calmodulin (30 nM)-activated (● and ○) enzyme activities were determined in the absence and in the presence of various concentrations of compound 48/80 (● and ▲) and calmidazolium (○ and △). Note that the concentrations of compound 48/80 are given as g per ml and those of calmidazolium as M. Ca^{2+}-transporting ATPase (100% activity = 70–85 nmol/min per mg of protein) and phosphodiesterase (100% activity = 0.9–1 μmol/min per mg of protein) activities are related to the calmodulin-stimulated enzymes in the absence of drug. Each point represents the mean of four to six determinations.

In addition, the effects of both calmodulin antagonists on several Ca^{2+}–calmodulin-independent enzymes were investigated. Table 1 gives evidence that calmidazolium half-maximally antagonized calf cardiac sarcolemma Na^{+}/K^{+}-transporting ATPase at 15 μM. However, compound 48/80 at the highest concentration used (300 μg/ml) inhibited this enzyme only by 25%. Calmidazolium proved also to possess inhibitory potency against Mg^{2+}-dependent ATPases of rabbit skeletal muscle sarcoplasmic reticulum and human erythrocytes with observed IC_{50} values of 3.3 and 20 μM

Table 1. *Effects of calmidazolium and compound 48/80 on different enzyme activities*
Enzyme activities were determined as described in the Materials and methods section at a concentration of 30 μg of protein/ml, except for the cases marked *, in which the protein concentration was 60 μg/ml. The IC_{50} values were obtained graphically from dose–effect curves. The coefficients of specificity represent the factors by which the inhibitors are more specific in antagonizing the calmodulin-dependent fraction of erythrocyte Ca^{2+}-transporting ATPase activity as compared with the listed enzyme activities, or, vice versa, the numbers give the factors by which the inhibitor concentrations were found to be higher in order to achieve half-maximal inhibition of the respective enzyme activities as opposed to the calmodulin-dependent activity. The coefficients were calculated by dividing the IC_{50} values of the listed enzyme activities by the IC_{50} value (determined in the presence of 30 or 60 μg of protein/ml respectively) of the calmodulin-dependent erythrocyte Ca^{2+}-transporting ATPase activity. Abbreviations: SR, sarcoplasmic reticulum; SL, sarcolemma.

	IC_{50} values		Coefficient of specificity	
Enzyme	Calmidazolium (μM)	Compound 48/80 (μg/ml)	Calmidazolium	Compound 48/80
Cardiac SR Ca^{2+}-transporting ATPase	2.1	16	6	19
Skeletal-muscle SR Ca^{2+}-transporting ATPase	2.9	80	8.3	94
Cardiac SL Na^+/K^+-transporting ATPase	15	>300	43	>353
Skeletal-muscle SR Mg^{2+}-dependent ATPase	3.3	⩾300	9.5	⩾353
Erythrocyte Mg^{2+}-dependent ATPase	20	⩾300	57	⩾353
Erythrocyte Ca^{2+}-transporting ATPase				
Basal	9	⩾300	26	⩾353
Oleic acid-activated*	10	7	25	8.2
Proteolysis-activated*	4	27	7	32

respectively (Table 1). In contrast, compound 48/80 did not significantly inhibit the activity of both Mg^{2+}-dependent ATPases in the investigated concentration range (Table 1).

The results of investigations to determine the potency of calmidazolium and compound 48/80 in antagonizing the stimulation of erythrocyte Ca^{2+}-transporting ATPase induced by different activating treatments are shown in Table 1. Half-maximal inhibition of the activity stimulated by oleic acid or mild tryptic digestion required 25 and 7 times higher concentrations of calmidazolium respectively, compared with the calmodulin-dependent fraction of the ATPase activity when assayed under identical conditions. Compound 48/80 shares with calmidazolium the property of antagonizing preferentially the calmodulin-induced stimulation of Ca^{2+}-transporting ATPase. 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/ml respectively.

Discussion

In various studies evidence has been presented that putative calmodulin antagonists not only bind to calmodulin but may have more targets, as pointed out in the introduction. Because of the unspecific effects of so-called calmodulin inhibitors many studies are questionable when inhibition of some process by a presumed calmodulin antagonist is taken as evidence for a regulatory role of calmodulin in that process. Therefore it is desirable to have a more specific tool elucidating the possible involvement of calmodulin in biological processes.

In the present study the two powerful calmodulin antagonists calmidazolium and compound 48/80, were compared with respect to their specificity in antagonizing effects mediated by calmodulin. The results presented here demonstrate the greater specificity of compound 48/80 over calmidazolium in inhibiting calmodulin-dependent, as opposed to calmodulin-independent, enzyme activities. This is summarized in Table 1, which provides a list of specificity coefficients of the two calmodulin antagonists for several enzyme activities. The coefficients represent the factors by which the IC_{50} values obtained for the mentioned enzyme activities were found to be higher as compared with the IC_{50} value for the calmodulin-dependent erythrocyte Ca^{2+}-transporting ATPase activity.

Compound 48/80 was found to be superior to calmidazolium in the following ways. (1) This substance exclusively antagonized the calmodulin-induced stimulation of phosphodiesterase and erythrocyte Ca^{2+}-transporting ATPase without suppression of the basal activity of these enzymes. Calmidazolium and other calmodulin antagonists also affect the basal activity of calmodulin-dependent enzymes (Levin & Weiss, 1976; Kobayashi

et al., 1979; Gietzen *et al.*, 1980, 1981, 1983). (2) Compound 48/80 altered calmodulin-independent Ca^{2+}-transporting ATPases far less than calmidazolium (see Table 1) or phenothiazines (Balzer *et al.*, 1968; K. Gietzen, P. Adamczyk-Engelmann, A. Wüthrich, A. Konstantinova & H. Bader, unpublished work) did. (3) Ca^{2+}–calmodulin-independent enzymes, like Mg^{2+}-dependent ATPases from different tissues and Na^{+}/K^{+}-transporting ATPase of cardiac sarcolemma were not, or only slightly, affected by compound 48/80 (Table 1). Calmidazolium (Table 1) and phenothiazines (Luthra, 1982; K. Gietzen, P. Adamczyk-Engelmann, A. Wüthrich, A. Konstantinova & H. Bader, unpublished work) also inhibited these enzymes, although at higher concentrations than those needed for calmodulin antagonism. (4) Antagonism of proteolysis-induced erythrocyte Ca^{2+}-transporting ATPase activity required only a seven times higher concentration of calmidazolium but a 32-fold higher concentration of compound 48/80 as compared with inhibition of the calmodulin-stimulated ATPase activity by the respective drug (Table 1). In contrast, trifluoperazine and penfluridol were shown to be equipotent in antagonizing both the calmodulin- and proteolysis-stimulated activity of erythrocyte Ca^{2+}-transporting ATPase (Gietzen *et al.*, 1982*a*).

Inhibition of basal activity of calmodulin-regulated enzymes and antagonism of the activity of calmodulin-independent enzymes by calmidazolium and other putative calmodulin antagonists, as mentioned in points (1)–(3) of the preceding paragraph, might be a direct effect on the target enzyme and/or a consequence of perturbation of the lipid environment in the case of membrane-integral enzymes (Raess & Vincenzi, 1980; Au, 1981; Luthra, 1982). The high specificity of compound 48/80 may be determined by the polymeric structure of compound 48/80, as was suggested by Gietzen *et al.* (1983). It was proposed that this structure may hinder incorporation of the agent into biological membranes and thus perturbation of the lipid environment of membrane-bound enzymes may be less favourable.

In addition to its high specificity compound 48/80 displayed a high potency to antagonize the calmodulin-dependent fraction of phosphodiesterase and erythrocyte Ca^{2+}-transporting ATPase activity. Experiments aimed at the identification of the active constituents of compound 48/80 indicate an average M_r of approx. 1000 for these species (P. Adamczyk-Engelmann & K. Gietzen, unpublished work). Thus IC_{50} values of this calmodulin antagonist for phosphodiesterase and Ca^{2+}-transporting ATPase, expressed in terms of molarity, are comparable with those of calmidazolium.

In summary, it may be concluded that compound 48/80 is an outstanding calmodulin inhibitor in that this substance combines high potency and high specificity in antagonizing preferentially calmodulin-mediated enzyme activities. Therefore compound 48/80 is proposed to be a much more specific tool for studying the involvement of calmodulin in biological functions than the existing substances.

For the gift of skeletal-muscle sarcoplasmic-reticulum Ca^{2+}-transporting ATPase, cardiac sarcolemma Na^{+}/K^{+}-transporting ATPase and cardiac sarcoplasmic-reticulum Ca^{2+}-transporting ATPase I thank Dr. S. Fleischer, Vanderbilt University, Nashville, TN, U.S.A., Dr. P. Rosenbeiger, University of München, München, Germany, and Dr. Dagmar Hartweg, University of Ulm, respectively. I also thank Professor H. Bader for his encouragement to perform this study. I am grateful to Ms. Angela Mansard-Glogger and Ms. Anka Konstantinova for their excellent technical assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

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