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CETIEDIL INHIBITION OF CALMODULIN-STIMULATED ENZYME ACTIVITY

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Abstract—Cetiedil, an *in vitro* anti-sickling agent, inhibited calmodulin-stimulated cyclic 3′:5′-nucleotide phosphodiesterase (EC 3.1.4.17) and Ca^{2+}-ATPase (ATP phosphohydrolase, EC 3.6.1.3) activities. The drug had no effect on basal enzyme activities in the absence of calmodulin. The inhibition of phosphodiesterase was competitive with respect to the concentrations of both cAMP and calmodulin. Cetiedil did not inhibit calmodulin-stimulated enzyme activities by acting as a calcium chelator, since increasing the concentration of calcium did not reverse the inhibitory effect.

An amino acid substitution at the sixth position of the beta globin chain defines hemoglobin S [1]. With deoxygenation, this abnormal hemoglobin undergoes gelation, changing the cell into its characteristic sickled shape. Such sickled erythrocytes may occlude vessels in the microcirculation and cause ischemia. Typically, this distortion of the sickle erythrocyte membrane is transient, and upon reoxygenation the cells return to their biconcave discoid appearance. Repeated episodes of sickling, however, may permanently damage the cells, producing shape alterations of the cell membranes which are irreversible, even when the hemoglobin is in solution [2]. There is as yet no convincing evidence to suggest that this change results from a coexistent genetic abnormality of the sickle erythrocyte membrane [3].

One hypothesis to explain the alteration of the cell membrane which follows repeated cycles of sickling involves the intracellular accumulation of calcium. Calcium levels as high as those known to exist in the sickle erythrocyte may decrease erythrocyte deformability and alter cell membrane constituents. Because the effect of calcium on many intracellular processes is mediated through an interaction with calmodulin, we postulated that inhibition of calcium–calmodulin activity may be a means of preventing the membrane damage which occurs as a consequence of sickling. To test this hypothesis, we studied the ability of cetiedil, a known anti-sickling agent which does not alter hemoglobin S, to inhibit calmodulin stimulation of various enzyme activities. The findings in this study are compared to those of Bereza *et al.* [4] which were reported during the preparation of this manuscript.

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§ Abbreviations: SDS. sodium dodecyl sulfate; cAMP, adenosine 3′:5′-cyclic monophosphate; PDE, cyclic 3′:5′-nucleotide phosphodiesterase; IC_{50}, inhibitor concentration producing 50% inhibition; K_i, dissociation constant of enzyme–inhibitor complex; and EGTA, ethyleneglycolbis(amino-ethylether)tetra-acetate.

MATERIALS AND METHODS

Purification of calmodulin. Calmodulin was purified to homogeneity from bovine brain by affinity chromatography over a fluphenazine–Sepharose column by the method of Charbonneau and Cormier [5]. The purity of the calmodulin was confirmed by SDS§ polyacrylamide gel electrophoresis. Aliquots were frozen at −70° and retained full activity for at least 1 year.

Preparation of calmodulin-deficient phosphodiesterase. Calmodulin-deficient phosphodiesterase was purified from bovine brain by a modification of the method of Cheung and Lin [6].

Preparation of membranes for ATPase assay. Human erythrocyte membranes for the ATPase assay were prepared by the method of Jarrett and Kyte [7] from recently outdated banked blood.

Phosphodiesterase assay. Phosphodiesterase (PDE) was assayed by the two-step method of Thompson *et al.* [8]. The reaction mixture consisted of 0.015 mg calmodulin-deficient phosphodiesterase, 0.125 mM cAMP (0.1 μCi [^{3}H]cAMP), 0.05 mM $CaCl_2$, 5.0 mM $MgCl_2$, and 40 mM Tris, pH 7.4, in a total volume of 0.4 ml. The reaction was linear with time, and less than 10% of the cAMP was hydrolyzed. Unless otherwise specified, $CaCl_2$ was present at a final concentration of 0.05 mM and calmodulin, when added, was at a final concentration of 10 nM. The effect of various concentrations of cetiedil (0–200 μM) was determined in the presence and absence of 10 nM calmodulin. All samples were assayed in duplicate, and the data reported are the result of at least four independent observations. The effect of various concentrations of calmodulin was determined at a constant concentration of cyclic AMP (0.125 mM) in the presence and absence of

50 μM cetiedil. The effect of variable cyclic AMP concentrations was determined at a fixed concentration of calmodulin (10 nM) in the presence and absence of 50 μM cetiedil.

Calcium-ATPase assay. As an initial step, Mg^{2+}-ATPase activity was assayed by incubating human erythrocyte membranes (0.09 mg/ml) in 100 mM NaCl, 20 mM KCl, 0.5 mM EGTA, 3 mM $MgCl_2$, 3 mM ATP, 0.1 mM ouabain, and 40 mM imidazole–HCl, pH 7.0, for 15 min at 37°. Ouabain was added to inhibit $(Na^+–K^+)$ATPase activity. Ca^{2+}-ATPase activity was determined by subtracting Mg^{2+}-ATPase activity from that of membranes assayed exactly as above, except for the addition of 0.8 mM $CaCl_2$ [9]. To determine the effect of cetiedil, the membranes were preincubated on ice for 15 min with various concentrations of cetiedil, with or without added calmodulin (6.6 nM). The reaction was stopped by the addition of 0.7 ml of 0.5 M H_2SO_4, 0.5% ammonium molybdate, and 2% sodium dodecyl sulfate followed by 0.02 ml of 1.2% sodium metabisulfite, 1.2% sodium sulfite, and 0.2% 1-amino-2-naphthol-4-sulfonic acid. The color was developed for 30 min at 25°, and inorganic phosphate was determined by absorbance at 650 nm on a Gilford spectrophotometer. The reaction was linear with time, and less than 5% of the ATP was hydrolyzed. All samples were run in duplicate, and the data reported are the result of at least four independent experiments.

Protein concentrations were determined by the method of Lowry *et al.* [10] using bovine serum albumin as the standard.

The data are reported as the mean value of the specific activity ± the standard error of the mean (S.E.M.). Each value is the mean of at least four independent observations. Statistical analysis was by Student's unpaired *t*-test.

RESULTS

The effects of various concentrations of cetiedil on the specific activity of basal and calmodulin-stimulated phosphodiesterase activity are demonstrated in Fig. 1. In the absence of calmodulin, basal activity was 7.40 ± 0.45 nmoles·(mg protein)$^{-1}$·min^{-1} and was not affected by cetiedil. Calmodulin (10 nM) stimulated the basal activity 5-fold to 40.00 ± 0.55 nmoles·(mg protein)$^{-1}$·min^{-1}. Under these conditions, cetiedil produced a dose-dependent inhibition of calmodulin-stimulated enzyme activity. Significant inhibition was observed at all concentrations of cetiedil ≥25 μM ($P < 0.001$), and the half-maximal inhibitory concentration (IC_{50}) was 30 μM.

To investigate further the mechanism of inhibition, phosphodiesterase activity was assayed in the presence and absence of 50 μM cetiedil with variable concentrations of calmodulin. Figure 2 is a double-reciprocal plot which illustrates that the inhibition by cetiedil was competitive with respect to the calmodulin concentration (K_i = 16.5 μM). Similarly, Fig. 3 demonstrates that the inhibition by cetiedil was competitive with respect to the cyclic AMP concentration (K_i = 20.6 μM). In each instance, the addition of 50 μM cetiedil resulted in a decrease in the binding affinity but no change in the maximal enzymatic velocity.

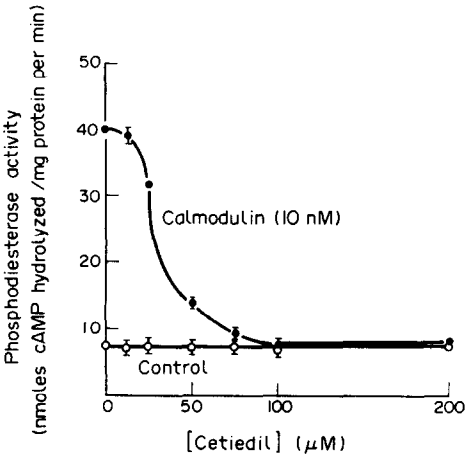

Fig. 1. Effects of cetiedil on basal and calmodulin-stimulated phosphodiesterase activity. Phosphodiesterase activity was determined, as described in Materials and Methods, in the absence (○) or presence (●) of 10 nM calmodulin. Vertical bars indicate the S.E.M.

In all previous experiments, the concentration of $CaCl_2$ was 0.05 mM. To test the hypothesis that cetiedil was acting as a calcium-binding agent, the effect of the drug on calmodulin-stimulated phosphodiesterase activity was determined in the presence of 0.05 or 3.00 mM $CaCl_2$ (Table 1). It was observed that the inhibitory effect of cetiedil was the same even if the amount of calcium in the medium was increased to a concentration 60-fold in excess of the cetiedil concentration.

The effect of variable concentrations of cetiedil on the specific activity of Ca^{2+}-ATPase of human erythrocyte membranes is demonstrated in Fig. 4. Basal activity was 17.00 ± 0.99 nmoles·(mg protein)$^{-1}$·min^{-1}, while the addition of 6.6 nM calmodulin increased activity to 61.23 ± 1.38 nmoles·(mg protein)$^{-1}$·min^{-1}. As with phosphodiesterase, cetiedil had no inhibitory effect

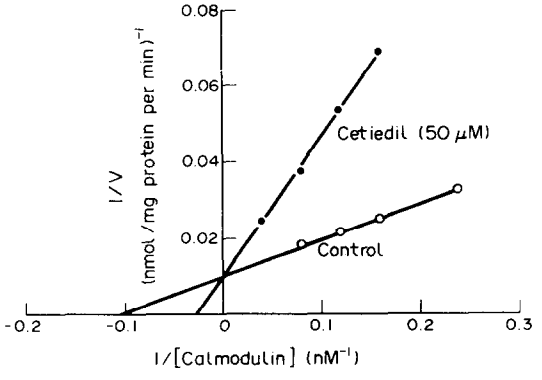

Fig. 2. Effect of calmodulin concentration on cetiedil-induced inhibition of calmodulin-stimulated phosphodiesterase activity. Phosphodiesterase activity was assayed as described in Materials and Methods with the exception that the concentration of calmodulin was varied from 4 to 25 nM. Samples were assayed in the presence (●) and absence (○) of 50 μM cetiedil.

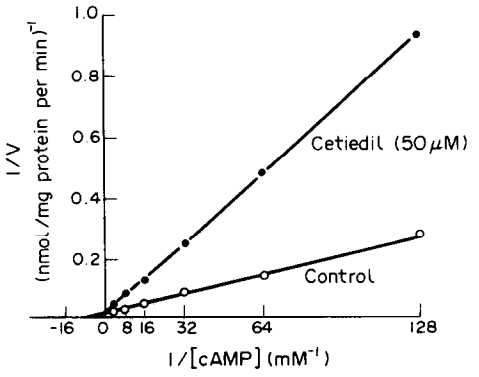

Fig. 3. Effect of cAMP concentrations on cetiedil-induced inhibition of calmodulin-stimulated phosphodiesterase activity. Phosphodiesterase activity was assayed as described in Materials and Methods with the exception that the concentration of cAMP was varied from 0.0075 to 0.25 mM. All samples had calmodulin added to a final concentration of 10 nM. Samples were assayed in the presence (●) and absence (○) of 50 μM cetiedil.

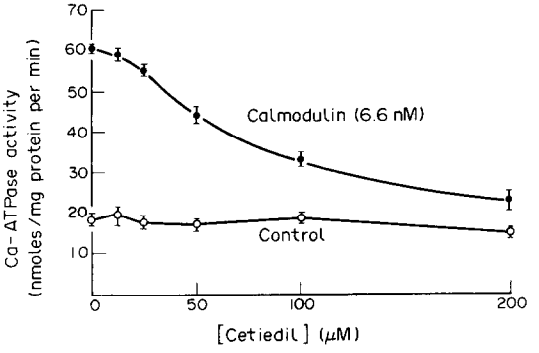

Fig. 4. Effects of cetiedil on basal and calmodulin-stimulated Ca^{2+}-ATPase activity. Ca^{2+}-ATPase activity of human erythrocyte membranes was assayed as described in Materials and Methods. In the presence of 6.6 nM calmodulin (●) all samples with cetiedil ≥25 μM were significantly different ($P < 0.01$) from the activity in the absence of drug. Open circles (○) indicate the absence of calmodulin. Vertical bars indicate the S.E.M.

on Ca^{2+}-ATPase in the absence of calmodulin. In the presence of calmodulin, cetiedil produced a dose-dependent inhibition of enzyme activity with an IC_{50} of 60 μM.

The determinations of Ca^{2+}-ATPase described above were carried out using membranes derived from normal erythrocytes. Additional experiments (data not shown) were performed with erythrocyte membranes obtained from patients with sickle cell disease. Cetiedil resulted in a similar degree of inhibition of calmodulin-stimulated Ca^{2+}-ATPase activity independent of the source of the erythrocyte membranes.

The cetiedil utilized in the previous assays was provided as cetiedil citrate. Appropriate control experiments demonstrated that the inhibition was, in fact, due to cetiedil rather than the complexing anion since sodium citrate had no inhibitory effect on calmodulin-stimulated phosphodiesterase or Ca^{2+}-ATPase activities.

DISCUSSION

The data reported in this paper demonstrate that cetiedil, an *in vitro* anti-sickling agent, is a competitive inhibitor of calcium–calmodulin-stimulated enzyme activity. While no inhibitory effect on basal enzyme activity was observed, cetiedil did inhibit both Ca^{2+}-ATPase and cyclic nucleotide PDE in the presence of calmodulin. During the preparation of this manuscript, a calmodulin-inhibitory effect of cetiedil was also reported by Bereza *et al.* [4]. The IC_{50} values for cetiedil which we have reported (30 μM for PDE and 60 μM for Ca^{2+}-ATPase) differ considerably from those found by Bereza (800 μM for both PDE and Ca^{2+}-ATPase). The reason for this disparity is unclear. It should be noted that our values correlate closely with the drug levels found to inhibit both deoxygenation-induced sickling and the calcium-mediated potassium leak pathway (the Gardos phenomenon) [11]. In addition, cetiedil concentrations of 500 μM or more have been found to produce rapid red cell lysis and are thus unlikely to be of physiologic importance [12].

The calcium–calmodulin inhibitory properties of cetiedil may be important in protecting the sickle erythrocyte from calcium-induced membrane damage since: (a) the calcium content of the sickle erythrocyte is elevated and rises dramatically upon deoxygenation [13]; and (b) many independent observations have shown that high levels of intracellular calcium may have adverse consequences for the erythrocyte membrane [14, 15]. Red cell deformability is known to be decreased by elevated levels of intracellular calcium [16]. Additionally, because of the external orientation of phosphatidyl serine which occurs upon deoxygenation of the sickle erythrocyte [17], calcium can precipitate cell–cell fusion and may lead to the reported adherence of sickle red cells to vascular endothelium [18, 19]. Calcium may also, by causing potassium loss and cell dehydration, accelerate the gelation of hemoglobin S [20]. While the precise role of calmodulin in the calcium-mediated events resulting in membrane damage of the sickle red cell is at present unknown, it is not unreasonable to speculate that it may play such a role since calmodulin is involved in many other calcium-mediated cellular phenomena [21]. The

Table 1. Effect of various concentrations of $CaCl_2$ on the ability of cetiedil to inhibit calmodulin-stimulated phosphodiesterase activity

Cetiedil	$CaCl_2$	Calmodulin-stimulated phosphodiesterase activity [nmoles·(mg protein)$^{-1}$·min^{-1}]
0	0.05 mM	32.34 ± 0.63
0	3.00 mM	33.35 ± 0.42
50 μM	0.05 mM	10.05 ± 0.14
50 μM	3.00 mM	9.95 ± 0.25

observation that the anti-sickling agent cetiedil is a potent inhibitor of calcium–calmodulin enzyme activity should serve as an impetus to investigate further the role of calmodulin in the sickling process and the resultant membrane pathology. This suggestion is supported by data demonstrating that other calmodulin antagonists, such as phenothiazines, also possess *in vitro* anti-sickling properties [22]. While there is circumstantial evidence that phenothiazines may reduce the morbidity of patients with sickle cell disease, controlled trials to document the efficacy of such therapy have not yet been performed [23]. If calmodulin is found to be important in the development of these membrane changes, calmodulin inhibition may prove to be a useful target for anti-sickling therapy.

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