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INHIBITION OF CALPAINS BY CALMIDAZOLIUM AND CALPASTATIN

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INTRODUCTION

The calpains (EC 3.4.22.17) are a group of calcium-activated cysteine proteinases which are found in a wide variety of eucaryotic cells.[1] Although the precise functions of these enzymes remain unclear, they may be involved in intracellular turnover of membrane and cytoskeletal proteins,[2–4] and the activation of phospholipase C[5] and protein kinase C.[6] In addition to control of the activity of calpains by calcium ion concentration, the activity of these enzymes *in vivo* may also be modulated by their interaction with a specific endogenous protein inhibitor, known as a calpastatin,[7,8] which is present at different ratios to calpain in different cell types.

Analysis of the primary structure of calpains[9–11] has revealed that the dimeric protein contains two "copies" of the calcium-binding calmodulin sequence, with one copy in each of the large catalytic and the small non-catalytic subunits. As proteins of the calmodulin family can bind phenothiazine analogues such as trifluoperazine and calmidazolium (CDZ; 1-[bis(4-chlorophenyl)-methyl]-3-[2-(2,4-dichlorophenyl)-methoxyl]-ethyl]-1H-imidazolium chloride)[12,13] with changes in their calcium-binding properties and their effects on target enzymes,[14] we have investigated the effects of CDZ on the proteolytic activity of calpain and the calpain-calpastatin interaction.

MATERIALS AND METHODS

CDZ (Compound R 24571) was purchased from Sigma Chemical Co., and Hammersten grade casein was obtained from the United States Biochemical Corporation.

Azocasein was prepared by the procedure of Barrett and Kirschke.[15] Assays of calpain activity and inhibition of calpain by calpastatin were performed using azocasein as the substrate,[16] and in assays involving CDZ, a 10 mM stock solution of CDZ in ethanol was prepared immediately before use. In order to determine whether CDZ

ABBREVIATIONS: CDZ, calmidazolium (1-[bis(4-chlorophenyl)-methyl]-3-[2-(2,4-dichlorophenyl)-methoxyl]-ethyl]-1H-imidazolium chloride.

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had inhibitory or activating effects in the systems studied, the calcium ion concentration in the calpain assays was set to give half maximal activity (approx. 1.0 mM Ca^{2+}) and in the calpain-calpastatin experiments, an amount of calpastatin was used to give half maximal inhibition of the calpain at 2.5 mM Ca^{2+}.

Hamster skeletal muscle and chicken gizzard calpain II were prepared as described previously.[16,17] Crude chicken gizzard calpastatin which inhibited both calpain and desminase was prepared as described by Johnson *et al.*[18] In order to remove contaminating proteins and to inactivate traces of proteinase activity in the pooled DEAE-Sepharose fractions, the calpastatin pool was heated to 100°C for 5 min, after which the clarified supernatant was used in the calpain inhibition assays.[16]

RESULTS AND DISCUSSION

In the absence of calpastatin, CDZ inhibited the activities of both chicken gizzard (Figure 1) and hamster skeletal muscle (Figure 2) calpain II enzymes with the inhibitory effect being more pronounced on the hamster enzyme (approx. 60% inhibition at 50 μM CDZ) than on the chicken gizzard enzyme. These results show that the calpains, like other members of the calmodulin family are inhibited by micromolar levels of CDZ, although CDZ does not appear to be as effective an inhibitor of these enzymes as it is for calmodulin-dependent enzymes such as sarcoplasmic reticulum Ca^{2+}-ATPase, erythrocyte Ca^{2+}-ATPase[14] and myosin light chain kinase.[19]

A small but statistically significant activation at 1 μM CDZ was observed for the

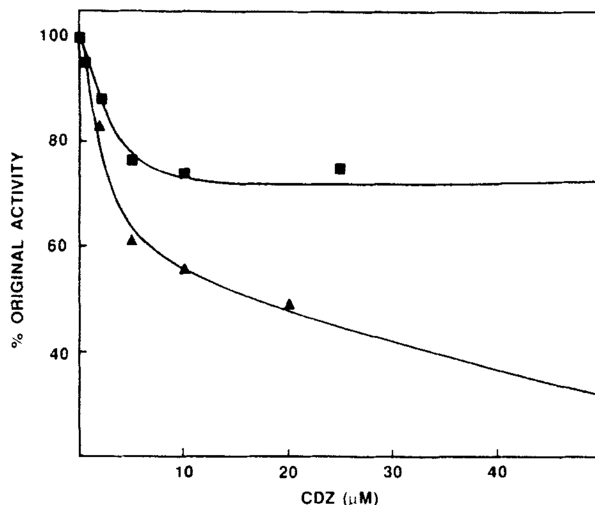

FIGURE 1 Effect of CDZ on chicken gizzard calpain II activity in the presence and absence of calpastatin. The enzyme was preincubated with CDZ for 5 min in the presence (▲) and absence (■) of calpastatin before addition of substrate. Activities were determined by measurement of absorbance of reaction supernatants at 366 nm after deproteinization, and the results are expressed as a percentage of the activity of control (no CDZ) samples.

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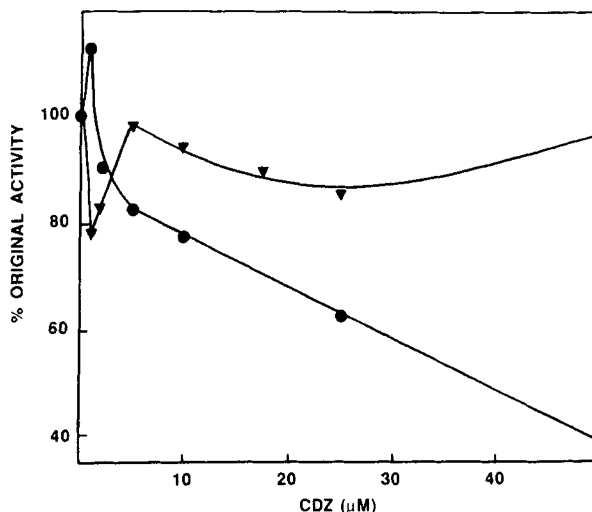

FIGURE 2 Effect of CDZ on hamster calpain II activity in the presence and absence of calpastatin. The enzyme was preincubated with varying concentrations of CDZ for 5 min in the presence (▼) and absence (●) of calpastatin before addition of substrate. Activities were determined by measurement of absorbance of reaction supernatants at 366 nm after deproteinization, and the results are expressed as a percentage of the activity of control (no CDZ) samples.

hamster but not for the chicken gizzard enzyme. A significant difference between the two enzymes is that the chicken gizzard preparation contains both catalytic and regulatory subunits,[17] whereas the hamster enzyme contains only the large catalytic polypeptide.[16] It is possible that the unique activation by 1 μM CDZ of the hamster enzyme is related to this difference in view of the possibility that additional CDZ may be bound by the calmodulin-like domain in the small regulatory subunit of the chicken enzyme.

When the chicken gizzard calpain-calpastatin complex was preincubated with CDZ in the presence of calcium, additional inhibition of calpain activity was observed in comparison to inhibition levels in control experiments of calpain-CDZ (Figure 1). In contrast to these results, similar experiments (Figure 2) with the hamster calpain-chicken gizzard calpastatin complex showed that the presence of calpastatin prevents the inhibition of calpain by CDZ above 2 μM CDZ. The results on the dimeric chicken gizzard calpain suggest that the binding sites for CDZ on calpain are separate from the calpastatin binding site and that the binding of CDZ to calpain is enhanced by the interaction between calpain and calpastatin. In the case of the monomeric hamster enzyme, the results suggest that the prior binding of calpastatin to calpain may either prevent CDZ binding to the hamster enzyme or prevent bound CDZ from having an inhibitory effect. A possible explanation for the different results obtained in the calpastatin experiments is that in the case of the hamster enzyme, CDZ binding is only possible on the catalytic subunit, whereas with the chicken enzyme, CDZ may bind to both the catalytic and regulatory subunits with the additional binding of CDZ on the regulatory subunit being responsible for the observed synergistic inhibition in the presence of CDZ and calpastatin. Further studies are therefore in progress which will provide more detail about the binding of CDZ and other trifluoperazines to the calpains.

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