EFFECT OF Ca^{2+} ON THE INHIBITION OF CALCIUM-ACTIVATED NEUTRAL PROTEASE BY LEUPEPTIN, ANTIPAIN AND EPOXYSUCCINATE DERIVATIVES

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1. Introduction

Calcium-activated neutral protease (CANP, EC 3.4.22.17) is a typical intracellular thiol protease and its physiological function is now being studied [1]. CANP may be responsible for initiating the turnover of a variety of cellular constituents, e.g., muscle proteins [2], microtubules [3], hormone receptors [4]. In a study of the physiological role of a protease, the effect of protease inhibitors on cultured cells and tissues is often examined. The activity of CANP is inhibited when thiol protease inhibitors such as leupeptin [5], antipain [5], and epoxysuccinate derivatives [6] are added to the assay mixture, i.e., in the presence of Ca^{2+}. Upon addition of Ca^{2+}, the conformation of CANP changes with concomitant appearance of the enzyme activity and the maximum activity is observed at ~1 mM Ca^{2+} [5,7]. This indicates that the inhibition of CANP by thiol protease inhibitors may be affected by Ca^{2+}. In the following studies the effect of Ca^{2+} on the inhibition of CANP was examined and it was revealed that these inhibitors essentially require Ca^{2+} for inhibition of CANP.

2. Materials and methods

CANP was purified from chicken skeletal muscle and the activity was assayed as in [5]. Leupeptin and antipain were obtained from the Protein Research Foundation (Mino-o, Osaka). Epoxysuccinate derivatives, E-64 (*N*-(*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl)agmatin), E-64c (*N*-(*N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl)isoamylamine), and [^{3}H]E-64c (uniformly labeled, 115 mCi/mmol) were obtained from Taisho Pharmaceutical Co. (Tokyo). Iodo[2-^{14}C]acetic acid (54 mCi/mmol) was bought from the Radiochemical Centre (Amersham). Equilibrium dialysis was performed in a Kontron Diapack apparatus model 4000 (Zürich) with 0.2 ml microcells and membranes (Visking tubing 18/32). SDS–Polyacrylamide gel electrophoresis on 7.5% gel was performed as in [8]. Other materials and methods were as in [5,9,10].

3. Results

3.1. *Effect of inhibitors directly added to the assay mixture*

The activity of CANP was measured in the ordinary assay mixture to which various amounts of leupeptin, antipain, E64 and E-64c were added. These inhibitors strongly inhibited CANP activity in the presence of Ca^{2+} and the molar ratios of inhibitor to enzyme causing a 50% decrease of the enzyme activity (ID_{50}) were 5.5, 25, 10 and 15, for leupeptin, antipain, E-64 and E64c, respectively. Since no synthetic substrate for CANP is known, inhibition constants for CANP were measured using casein as a substrate. These inhibitors inhibited CANP noncompetitively and the K_i-values were 0.46, 2.0, 0.90 and 1.8 μM, respectively, for leupeptin, antipain, E-64 and E-64c. The ID_{50} and K_i-values which correspond well to each other, show strong inhibition in the presence of Ca^{2+}. Leupeptin was the most potent inhibitor, while antipain was the least.

3.2. *Effect of preincubation of CANP with leupeptin and antipain*

As a control experiment, CANP was incubated at 30°C in the presence and absence of 1 mM Ca^{2+} with-

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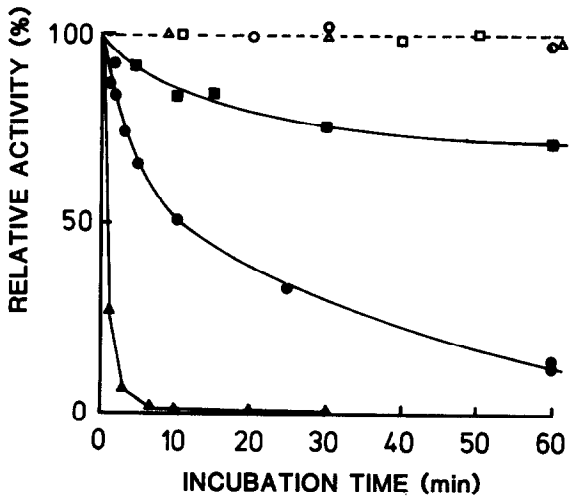

Fig.1. Time course of inactivation of CANP. CANP (0.5 mg/ml) was incubated at 30°C in 50 mM Tris–HCl, 5 mM 2-mercaptoethanol (pH 7.5) with and without 1 mM $CaCl_2$. Aliquots withdrawn at intervals were assayed and used for the electrophoresis shown in fig.2. The reaction of CANP with iodoacetic acid (100-fold molar excess over CANP) was carried out similarly but at 0°C. The iodoacetic acid taken into the assay mixture together with enzyme had no significant effect on the enzyme activity. (●) 30°C, $+Ca^{2+}$; (○) 30°C, $-Ca^{2+}$; (■) 0°C, $+Ca^{2+}$; (□) 0°C, $-Ca^{2+}$; (▲) 0°C, $+Ca^{2+}$, + iodoacetic acid; (△) 0°C, $-Ca^{2+}$, + iodoacetic acid.

out inhibitors. Though the activity was stable in the absence of Ca^{2+}, a rapid decrease of enzyme activity due to autolysis was observed in the presence of Ca^{2+} [9,10]. The remaining activity after 60 min at 30°C was ~10% (fig.1). During this autolysis the kM_r-value of CANP changes as follows (fig.2A): 82 000 (native CANP) → 79 → 60 → 30–35 → → [10]. Since the 79 000 and 60 000 M_r species are active fragments and almost as equally active as native CANP, the loss of activity during autolysis coincided roughly to the decrease of the total amount of the 82 000, 79 000 and 60 000 M_r bands [10].

Various amounts of leupeptin and antipain were

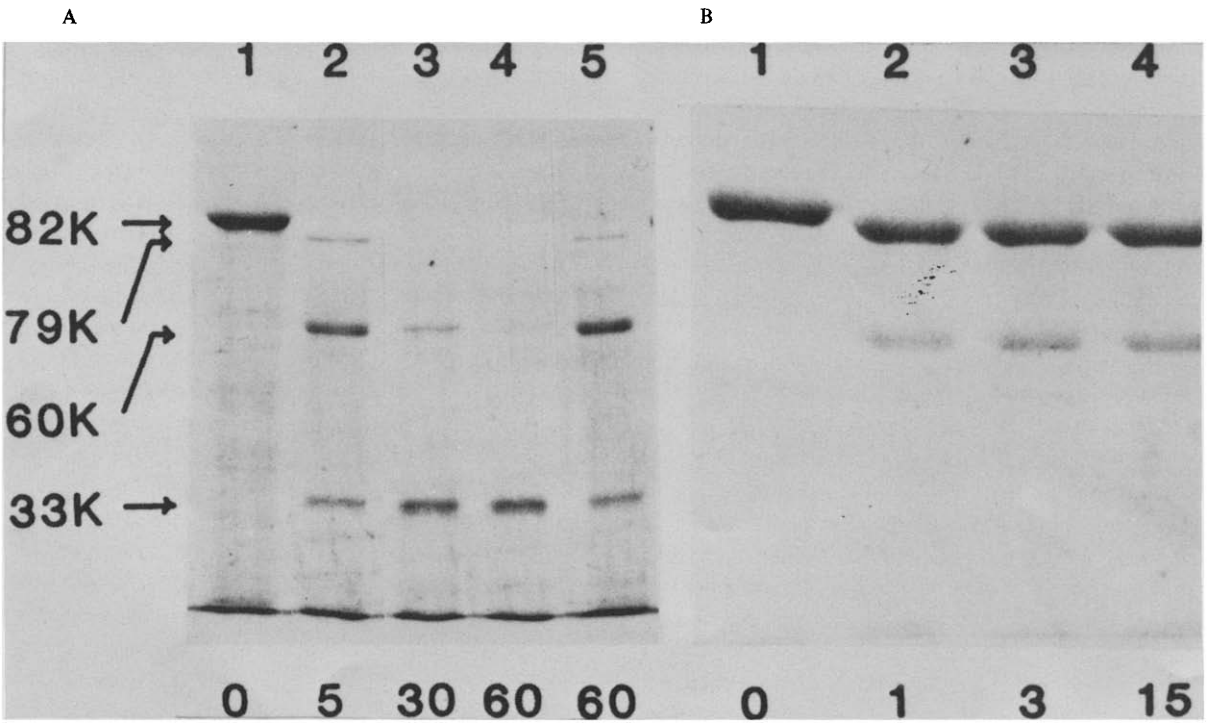

Fig.2. SDS–polyacrylamide gel electrophoresis of CANP: (A) CANP was incubated at 30°C (no. 1–4) and 0°C (no. 5) with 1 mM $CaCl_2$ but without inhibitor; (B) CANP was incubated with iodoacetic acid at 0°C in the presence of 1 mM $CaCl_2$. Figures under the gel indicate incubation times in minutes. For other conditions see fig.1. M_r-Values estimated from standards are shown on the left.

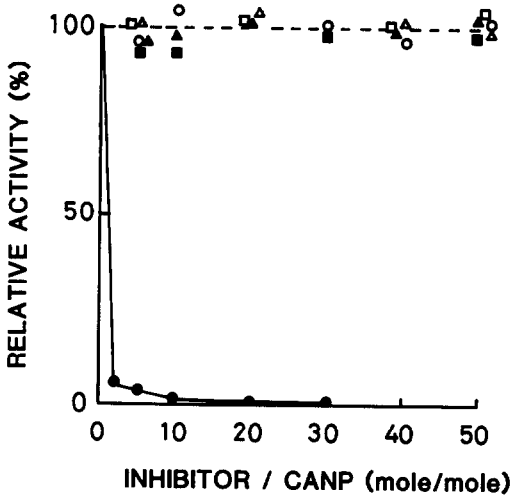

Fig.3. Effect of preincubation of CANP with inhibitors. CANP (0.5 mg/ml) was incubated for 60 min at 30°C with various amounts of inhibitors in 50 mM Tris–HCl, 5 mM 2-mercaptoethanol (pH 7.5) with and without 1 mM $CaCl_2$ and was used for activity measurement and the electrophoresis in fig.4. (○,●) E-64c; (□,■) leupeptin; (△,▲) antipain. Open and closed symbols show the absence and presence of Ca^{2+}, respectively.

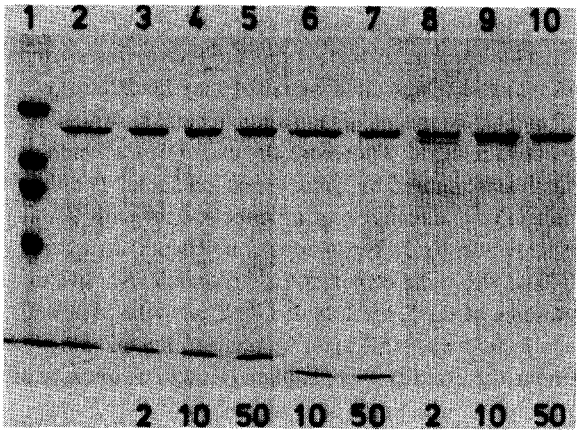

Fig.4. SDS–Polyacrylamide gel electrophoresis of CANP incubated for 60 min at 30°C with various amounts of leupeptin (no. 3–5), antipain (no. 6,7) and E-64c (no. 8–10) with 1 mM $CaCl_2$. Figures under the gel indicate the molar ratio of inhibitor to CANP. (1) Marker proteins: from the top, phosphorylase *a* (94 kM_r); bovine serum albumin (68 kM_r); pyruvate kinase (57 kM_r); ovalbumin (43 kM_r). (2) CANP as control. For other conditions see fig.3.

added to this incubation mixture and after incubation for 60 min at 30°C, each mixture was dialyzed to remove unreacted inhibitors and Ca^{2+}. The dialyzed solution was subjected to enzyme activity measurement and SDS–polyacrylamide gel electrophoresis. Full enzyme activity was recovered at all concentrations of inhibitors even in the presence of Ca^{2+} (fig.3). This indicates that in the presence of Ca^{2+}, leupeptin and antipain completely inhibited the autolysis of CANP as can be seen in the electrophoretogram (fig.4). However, this inhibition was reversible and the full activity was regained after dialysis. Note that the concentration of leupeptin and antipain was $\geqslant$20-times higher than K_i-values even at the lowest concentration used. Reversible inhibition by leupeptin and antipain is conceivable, because when aldehyde inhibitors react with thiol protease, a non-covalent enzyme–inhibitor complex or thiohemiacetal adduct is assumed to be formed [11]. The full recovery of CANP activity in the absence of Ca^{2+} can be explained by assuming that the same reaction as in the presence of Ca^{2+} occurred, or that the inhibitors had no effect on CANP. No changes of the electrophoretic pattern of CANP were observed after incubation with inhibitors in the absence of Ca^{2+} (not shown).

3.3. *Effect of preincubation of CANP with E-64c*

Similar experiments to above were carried out with E-64c. E-64c inhibited CANP irreversibly in the presence of Ca^{2+} (fig.3). The loss of activity can not be ascribed to autolysis as judged by SDS–polyacrylamide gel electrophoresis (see fig.4). The electrophoretogram indicates that partial autolysis occurred, especially at lower concentrations of E-64c, in contrast to the cases of leupeptin and antipain where autolysis was stopped completely. For the reaction of E-64c with CANP, though it is very rapid, it may take some time before the autolysis is stopped completely.

Without Ca^{2+}, E-64c did not inhibit CANP. Even after incubation of CANP with a 200-fold molar excess of E-64c for 24 h at 30°C, no loss of activity was observed. To analyze further the results obtained in the absence of Ca^{2+}, equilibrium dialysis of CANP with [^{3}H]E-64c was performed. E-64c was examined over 1×10^{-7}–5×10^{-5} M. CANP did not show affinity to E-64c even at the highest concentration examined, which is $\geqslant$25-times higher than the K_i-value in the presence of Ca^{2+}. Thus it was concluded that E-64c did not react with CANP in the absence of Ca^{2+}. Further, this fact also suggests that in the absence of Ca^{2+}, leupeptin and antipain do not react with CANP, either. The results obtained with E-64 were essentially the same as those obtained with E-64c.

3.4. *Reaction of iodoacetic acid with CANP*

As leupeptin, antipain, and E-64c are, in a sense, substrate analogs, their behavior may be different from that of inhibitors with no structural similarity to the substrate. Therefore the effect of Ca^{2+} was further examined by using iodoacetic acid. The time course of the reaction of iodoacetic acid with CANP is shown in fig.1. The incubation was performed at 0°C to show the effect of Ca^{2+} more clearly. In the absence of Ca^{2+}, the loss of CANP activity with iodoacetic acid was <5% after incubation for 60 min under the conditions examined. However, upon addition of Ca^{2+}, the rate of inactivation of CANP was accelerated remarkably and complete loss of activity was observed within 10 min. SDS gel electrophoresis of this carboxymethylated sample (fig.2B) showed that this inactivation was ascribed to carboxymethylation, although the 79 000 M_r species together with a small amount of the 60 000 M_r species was produced from CANP by autolysis. The incorporation of carboxymethyl groups into CANP was 0.8–0.9 mol/mol enzyme after 30 min. The apparent first-order rate constants of inactivation of CANP were 0.0008 min^{-1} and 1.0 min^{-1}, respectively, in the absence and presence of Ca^{2+}. Hence Ca^{2+} enhanced the apparent inactivation rate of CANP by iodoacetic acid >1000-times.

4. Discussion

These results clearly indicate that the inhibiton of CANP by various inhibitors strongly depends on the presence of Ca^{2+}. This effect of Ca^{2+} is specific to CANP and is not seen with other thiol proteases like papain [12] and cathepsin B [13].

In the presence of Ca^{2+} a conformation change of CANP occurs and the active-site SH-group becomes exposed to the solvent [5]. Although all the inhibitors used are usually considered to react with SH-groups, their site of reaction with CANP is not clear. However, preliminary results indicate that the CANP treated with E-64c loses the SH-group exposed by Ca^{2+}. Thus it is highly possible that the inhibitors react with the SH-group of the enzyme active site. The acceleration of the inactivation rate of CANP is mainly ascribed to the conformation change of CANP which exposes the active-site SH-group. To explain the very fast inactivation rate of CANP with inhibitors in the presence of Ca^{2+}, exposure of the active-site SH-group is not sufficient and a decrease of pK-value of the reactive site, presumably the SH-group, should occur concomitantly. It is not clear whether Ca^{2+} is directly involved in this decrease of pK-value of the SH-group.

In the absence of Ca^{2+}, since the active-site SH-group is buried, and the pK-value is higher, the inhibitor can not react with the SH-group to inactivate CANP. The fact that Ca^{2+} is essential for the inhibition of CANP by various inhibitors is quite important in assessing the effect of these inhibitors in vivo.

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