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Different sensitivities of native and oxidized forms of Na^+/K^+-ATPase to intracellular proteinases

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Abstract

Inactivation of Na^+/K^+-ATPase by partially reduced oxygen metabolites has been implicated in ischemia-reperfusion injury to heart and other organs. Because oxidation of many proteins makes them more susceptible to degradation by intracellular proteinases, we studied the effects of several such proteinases on native and H_2O_2-oxidized preparations of Na^+/K^+-ATPase from canine kidney (containing α_1 isoform of the catalytic subunit) and rat axolemma (containing α_2 and α_3 isoforms). Lysosomal cathepsin D degraded the native and the oxidized preparations at acid pH, but it was significantly more effective against the oxidized forms. m-Calpain had little or no effect on the native Na^+/K^+-ATPase preparations, but it digested the oxidized α-subunits of the axolemma and the kidney enzymes. μ-Calpain's effects were similar to those of m-calpain. Multicatalytic proteinase which is known to degrade a large number of oxidized proteins, did not affect the native or the oxidized forms of Na^+/K^+-ATPase. The findings suggest that (a) during oxidative stress there may be accelerated degradation of the oxidatively damaged Na^+/K^+-ATPase, either through internalization and transport to lysosomes, or by the action of calpains at the membrane; and (b) those isoforms of the enzyme that are more sensitive to oxidants are more susceptible to degradation by the above processes.

Key words: ATPase, Na^+/K^+-; Cathepsin D; Calpain; Multicatalytic proteinase; Proteinase; Lysosome; Oxidant

1. Introduction

Oxidative damage to proteins has been implicated in a variety of pathological conditions that are or seem to be caused by partially reduced oxygen metabolites [1,2]. It has also been shown that upon oxidation, many proteins become more susceptible to proteolytic degradation, suggesting that the enhanced turnover of the damaged proteins may be a defense mechanism against oxidative stress [1–3]. Na^+/K^+-ATPase, the enzyme that catalyzes the active transports of Na^+ and K^+ across the plasma membrane of mammalian cells, is known to be an early target for the oxidant-induced damage to isolated myocytes [4]. There is also evidence to suggest that oxidative inhibition of Na^+/K^+-ATPase contributes significantly to ischemia-reperfusion injury in the intact heart [5,6]. In our initial studies on the inhibition of purified Na^+/K^+-ATPase by partially reduced oxygen metabolites, we noted the increased sensitivity of the oxidized Na^+/K^+-ATPase to trypsin and chymotrypsin [7]. The present studies were undertaken, therefore, to explore the potential role of the intracellular proteolytic systems in the turnover of the oxidant-damaged Na^+/K^+-ATPase. We have examined the effects of multicatalytic proteinase, m-calpain, μ-calpain, and cathepsin D on Na^+/K^+-ATPase isolated from kidney outer medulla and brain stem axolemma. The choice of proteinases was based on the following considerations: the cytosolic multicatalytic proteinase degrades a large number of oxidatively damaged proteins [1,2,8]. Calpains, the ubiquitous Ca^{2+}-dependent intracellular proteinases, are known to interact with membranes, and have been suggested to degrade membrane-bound proteins [9]. The lysosomal cathepsin D was used because a normal pathway for the turnover of Na^+/K^+-ATPase involves internalization of this plasma membrane enzyme, and eventual degradation in lysosomes [10,11]. Cathepsin D and calpains were shown earlier to degrade the oxidized

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form of glutamate synthetase [12]. The two Na^+/K^+-ATPase preparations used were selected because they contain the three well-characterized isoforms of the enzyme's catalytic subunit (α_1, α_2, and α_3), and because of our recent findings indicating different oxidant sensitivities of these isoforms [13].

2. Methods

Na^+/K^+-ATPase from canine kidney medulla (specific activity, 1000 μmol/mg per h) and rat brain stem axolemma (specific activity 120 μmol/mg per h) were prepared by established methods [14,15], and assayed at 37°C through the determination of the initial rate of release of P_i from [γ-^{32}P]ATP [14]. The reaction mixture contained 2 mM ATP, 3 mM $MgCl_2$, 1 mM EGTA, 100 mM NaCl, 25 mM KCl, and 50 mM Tris-HCl (pH 7.4). Activity was calculated as the difference in values obtained in the presence and absence of 1.5 mM ouabain.

Oxidized Na^+/K^+-ATPase preparations were obtained by incubating the above enzymes with H_2O_2 as described before [13]. H_2O_2 concentrations and incubation times were chosen based on available data [13] to obtain 60–90% irreversible inhibition. A preparation of the kidney enzyme in which about half of the α-subunits are oxidatively cross-linked in the presence of Cu^{2+} and *o*-phenanthroline was obtained as described before [16].

Native and oxidized preparations of Na^+/K^+-ATPase were exposed to the proteinases as follows: (1) Na^+/K^+-ATPase (0.5 mg/ml) was incubated at 37°C with the indicated concentrations of cathepsin D in 40 mM citrate buffer (pH 3.2–6.2) for 1 h. The reaction was terminated by adjusting the pH of the mixture to 7.0 with Tris, followed either by dilution and assay of Na^+/K^+-ATPase activity or by the addition of SDS-PAGE sample buffer. (2) m-Calpain or μ-calpain (37.5 μg/ml) was mixed with Na^+/K^+-ATPase (0.5 mg/ml)

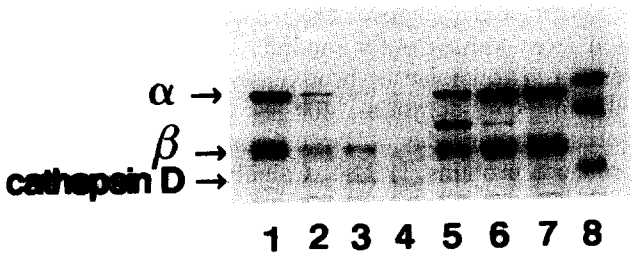

Fig. 1. Digestion of kidney Na^+/K^+-ATPase by cathepsin D at different pH values. The enzyme was exposed to cathepsin D (1 unit/ml) and subjected to electrophoresis as described in Methods. Lane 1, no cathepsin. pH values for samples incubated with cathepsin D were: lane 2, 3.2; lane 3, 4.2; lane 4, 4.8; lane 5, 5.2; lane 6, 5.6; lane 7, 6.2. Lane 8, markers (106 kDa, 80 kDa, 49.5 kDa).

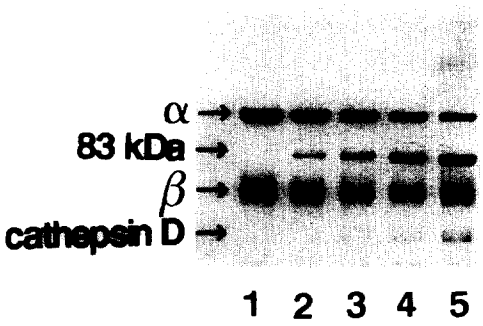

Fig. 2. Digestion of kidney Na^+/K^+-ATPase by varying concentrations of cathepsin D at pH 5.2. Experiments were done as described in Methods. Cathepsin D concentration (units/ml) in each reaction mixture was: lane 1, 0; lane 2, 0.5; lane 3, 1; lane 4, 2; lane 5, 4.

in a solution containing 10 mM KCl, 1 mM dithiothreitol and 50 mM imidazole-HCl (pH 7.4). The reaction was started by the addition of calcium acetate to a final concentration of 2 mM Ca^{2+} for m-calpain, and 0.2

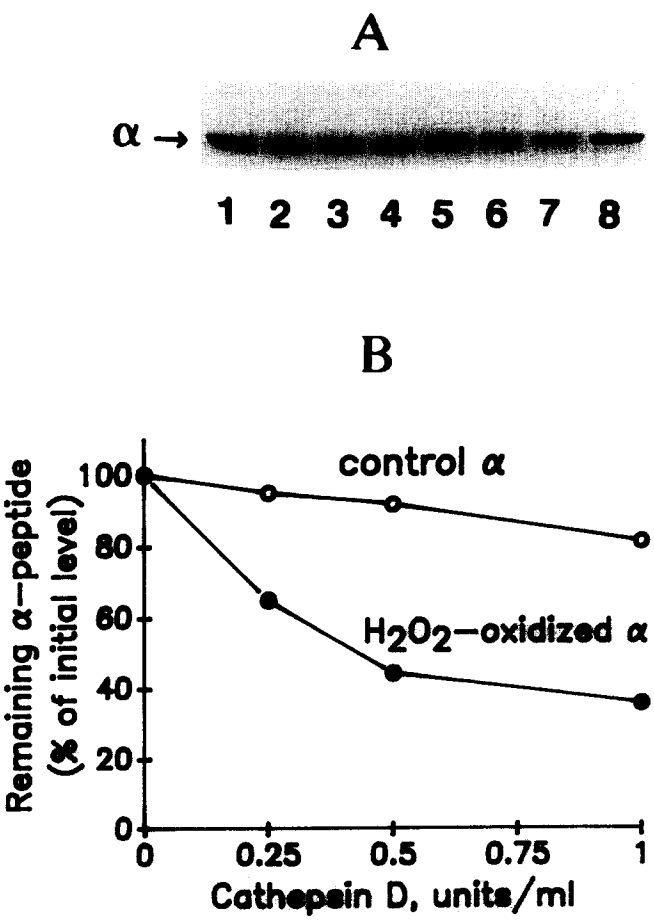

Fig. 3. Effects of varying concentrations of cathepsin D on native and H_2O_2-oxidized kidney Na^+/K^+-ATPase. Experiments were done as described in Methods. (A) Coomassie blue-stained α bands of native (1–4) and oxidized (5–8) Na^+/K^+-ATPase. Cathepsin D (units/ml) per reaction mixture was: lanes 1 and 5, no cathepsin; lanes 2 and 6, 0.25; lanes 3 and 7, 0.5; lanes 4 and 8; 1. (B) Comparison of the densities of the bands shown in A.

mM Ca^{2+} for μ-calpain. After incubation for 45 min at 25°C, a small aliquot of the mixture was removed for electrophoresis, another portion of calpain equal to the original amount was added, and the reaction was continued for 45 min. Calpain additions were repeated as indicated. Reaction was stopped by the addition of EGTA (to a final concentration of 30 mM) and the SDS-PAGE sample buffer. When the assay of Na^+/K^+-ATPase was required, the reaction with calpain was stopped by the addition of EGTA (30 mM), and Na^+/K^+-ATPase was collected by centrifugation at 100000 $\times g$. (3) Multicatalytic proteinase (1 mg/ml) was incubated at 37°C in a solution containing 1 mM dithiothreitol, 50 mM Tris-HCl (pH 8.0) for 5 min. Polylysine was then added to a final concentration of 0.1 mg/ml. After 1–2 min, the activated proteinase (25 μg/ml) was added to a suspension of Na^+/K^+-ATPase (1 mg/ml) in 1 mM dithiothreitol, 50 mM Tris-HCl (pH 8.0) and incubated at 37°C for 2 h. The reaction was stopped by the addition of SDS-PAGE sample buffer.

SDS-solubilized samples containing 5–10 μg of Na^+/K^+-ATPase protein were subjected to electrophoresis according to Laemmli [17]. Gels were stained with Coomassie blue. Alternatively, proteins from unstained gels were transferred to nitrocellulose membranes, probed with antibodies, and detected using second antibody conjugated with alkaline phosphatase [18]. Stained bands on gels and blots were quantified by a soft laser scanning densitometer.

Cathepsin D (bovine pancreas), 'vanadate-free' ATP, H_2O_2, and ouabain were obtained from Sigma (St. Louis, MO). [γ-^{32}P]ATP was bought from DuPont-New England Nuclear (Boston, MA). All reagents for gel electrophoresis were purchased from BioRad (Richmond, CA). Polyclonal antibody against α_1 subunit was kindly provided by Dr. R. Mercer (Washington University, St. Louis, MO). Polyclonal antisera specific for α_2 and α_3 were purchased from Upstate Biotechnology (Lake Placid, NY). Bovine myocardial m-calpain, human erythrocyte μ-calpain, and human erythrocyte multicatalytic proteinase were prepared as previously described [19,20]. All had activities comparable to those of the original preparations.

3. Results

Degradation of Na^+/K^+-ATPase by cathepsin D

In experiments of Fig. 1 the native kidney Na^+/K^+-ATPase, which is known to contain the α_1-isoform of the catalytic subunit, was exposed to cathepsin D at different pH values in the range of 3.2–6.2. Optimal pH seemed to be 4.8 where most of α and β subunits were digested. At pH 5.2 there was little or no effect on β, and the major product of the cleavage of α was a peptide with molecular mass of 83 kDa. At pH 6.2, there was no significant digestion of either subunit. Exposure of the native enzyme to different concentrations of cathepsin D at pH 5.2 showed that with increasing cathepsin D, α decreased and 83 kDa prod-

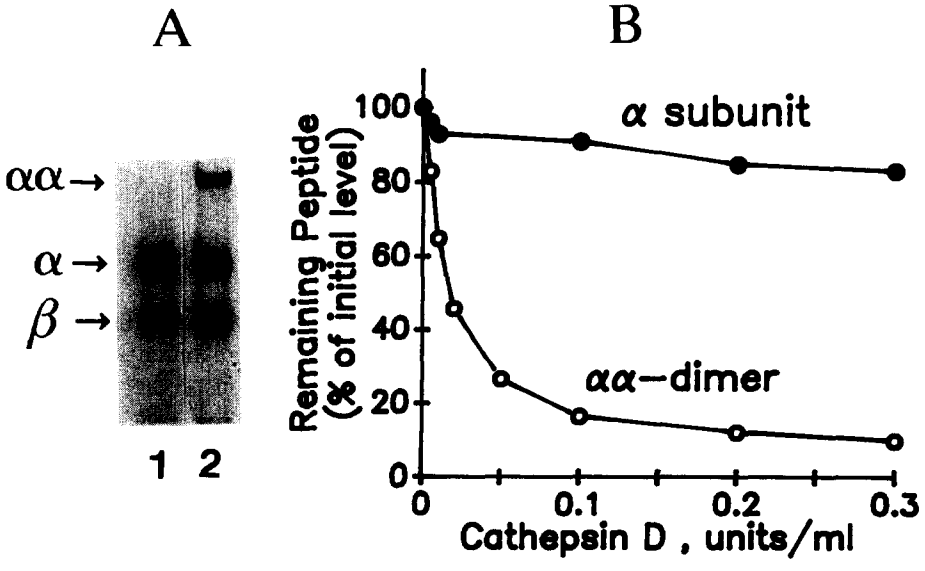

Fig. 4. Effects of varying concentrations of cathepsin D on the α-subunit and the oxidatively cross-linked α,α-dimer of the kidney Na^+/K^+-ATPase. (A) Stained gels of the control and the partially cross-linked kidney enzyme. (B) Densitometer scans of the Coomassie blue-stained α-monomer and α,α-dimer of the cross-linked enzyme exposed to the indicated amounts of cathepsin D under conditions described in Methods.

uct increased proportionately (Fig. 2). In the same experiments described in Fig. 2 a portion of Na^+/K^+-ATPase sample that was exposed to 4 units/ml of cathepsin D was also assayed for activity (Methods) prior to electrophoresis. The 61% decrease in activity corresponded to 65% decrease in the amount of stained α.

Experiments of Fig. 3 showed that when the kidney enzyme was irreversibly inhibited by H_2O_2, its α-subunit became more sensitive to degradation by cathepsin D. Because protein oxidation often leads to the formation of cross-linked oligomers [2], a cross-linked dimer of α-subunit was formed through oxidation of sulfhydryl groups in the presence of Cu^{2+}-phenanthroline, and the sensitivity of the dimer to cathepsin D was compared with that of the α-monomer (Fig. 4). The dimer was considerably more sensitive than the monomer. It was evident from the examination of the gels (not shown) that cathepsin D converted the α,α-dimer to fragments much smaller than the α-monomer.

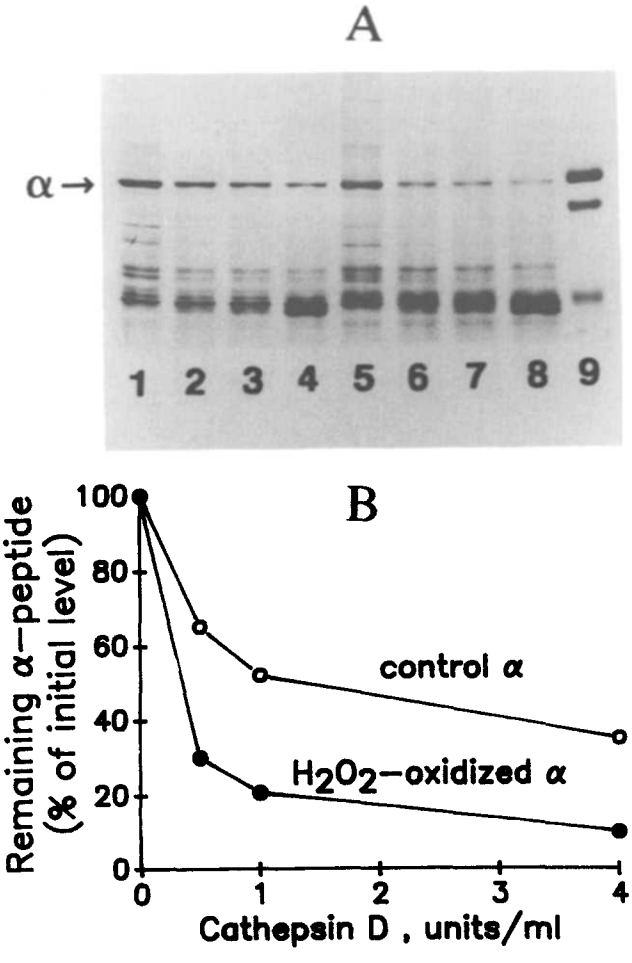

Fig. 5. Effects of varying concentrations of cathepsin D on native and H_2O_2-oxidized axolemma Na^+/K^+-ATPase. Experiments were done as described in Methods. (A) Coomassie blue-stained gels of native (1–4) and oxidized (5–8) Na^+/K^+-ATPase preparations. Lane 9, markers (106 kDa, 80 kDa, 49.5 kDa). (B) Comparison of the densities of the α bands shown in A.

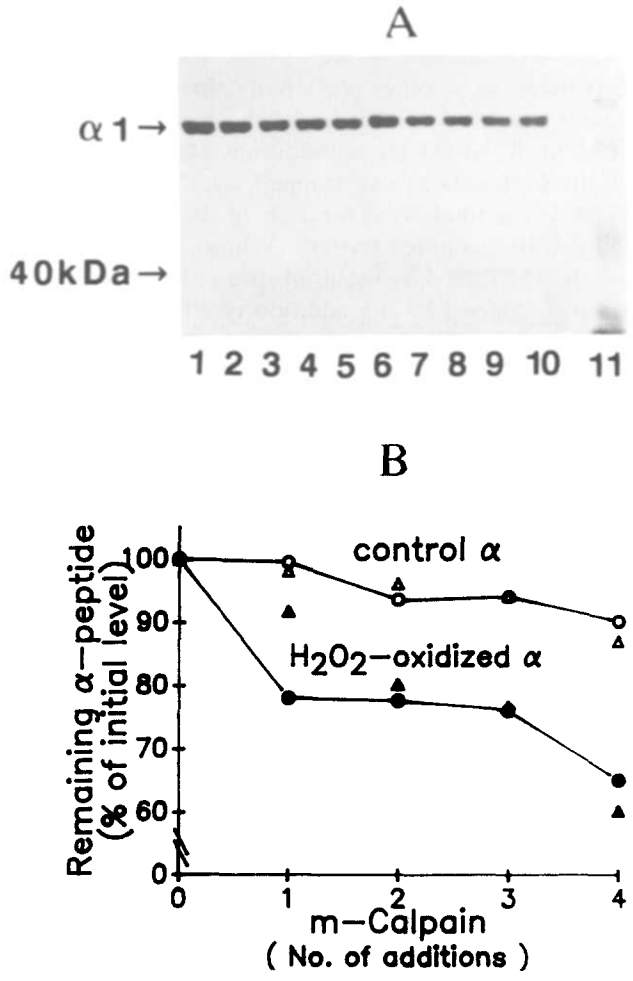

Fig. 6. Effects of m-calpain on the α-subunits of the native and the oxidized kidney Na^+/K^+-ATPase. The two enzyme samples were exposed to m-calpain repeatedly (four times) as described in Methods. At the end of each exposure period, samples were removed, subjected to electrophoresis, and immunostained with an anti-α antibody as described in Methods. (A) Western blots. Lanes 1–5, native enzyme. Lanes 6–10, oxidized enzyme. No calpain (1 and 6); one calpain addition (2 and 7); two calpain additions (3 and 8); three calpain additions (4 and 9); four calpain additions (5 and 10). Lane 11, prestained markers (106 kDa, 80 kDa, 49.5 kDa, 32.5 kDa). (B) Densitometer scans of the Western blots shown in A (○, ●), and Coomassie blue-stained α bands (not shown) from the same experiments (△, ▲).

H_2O_2-oxidation of the axolemma Na^+/K^+-ATPase also led to increased susceptibility of the α-subunit of this preparation to cathepsin D (Fig. 5). Since the α-subunit band of this preparation on gels such as those of Fig. 5 is known to consist of a mixture of two isoforms (α_2 and α_3), duplicate gels were immunostained with two antibodies that are specific for α_2 and α_3. Sensitivities of α_2 and α_3 to cathepsin D did not differ significantly from that of the mixture shown in Fig. 5 (data not shown).

Effects of calpains

In experiments of Fig. 6 effects of m-calpain on native and H_2O_2-oxidized preparations of the kidney Na^+/K^+-ATPase were compared. Because of the known autolytic inactivation of m-calpain, in these experiments multiple samples of m-calpain were added to the reaction mixtures periodically (Methods). As evident from the data of Fig. 6B, m-calpain had little or no effect on the native α-subunits, but after prolonged incubation about 35 percent of the oxidized α was digested. A peptide of about 40 kDa was a product of the digestion of the oxidized α (Fig. 6A). Comparison of the Coomassie blue-stained gels of the experiments of Fig. 6 (not shown) indicated that the β-subunit of Na^+/K^+-ATPase was not digested by m-calpain.

The native and the H_2O_2-oxidized preparations used in experiments of Fig. 6 were assayed for Na^+/K^+-ATPase activity before and after four exposures to m-calpain. Prior to exposure to m-calpain the specific activity of the oxidized preparation was 30% of that of the native enzyme preparation because of H_2O_2-induced inactivation. Neither the activity of the native preparation nor that of the oxidized preparation was altered significantly after exposure to m-calpain. The simplest explanation for this finding is that the active native enzyme molecules are not degraded by m-calpain, and that the degradations noted in Fig. 6 are due to proteolysis of inactivated and oxidized enzyme molecules.

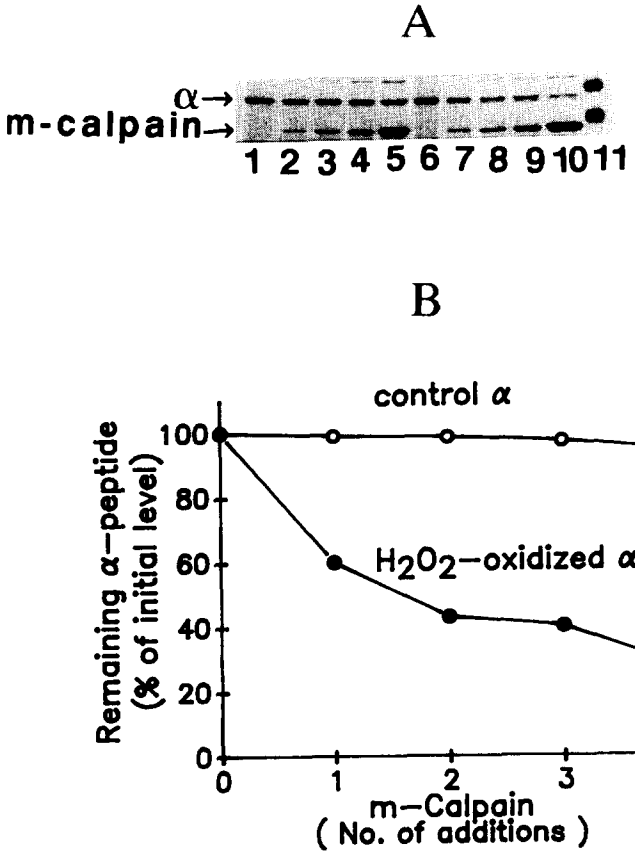

Fig. 7. Effects of m-calpain on native and H_2O_2-oxidized axolemma Na^+/K^+-ATPase. The two enzyme samples were exposed to m-calpain repeatedly (four times) as described in Methods and in legend to Fig. 6. (A) Coomassie blue-stained α bands ($\alpha_2 + \alpha_3$). Lane descriptions the same as indicated in legend to Fig. 6. (B) Densitometer scans of the α bands in A.

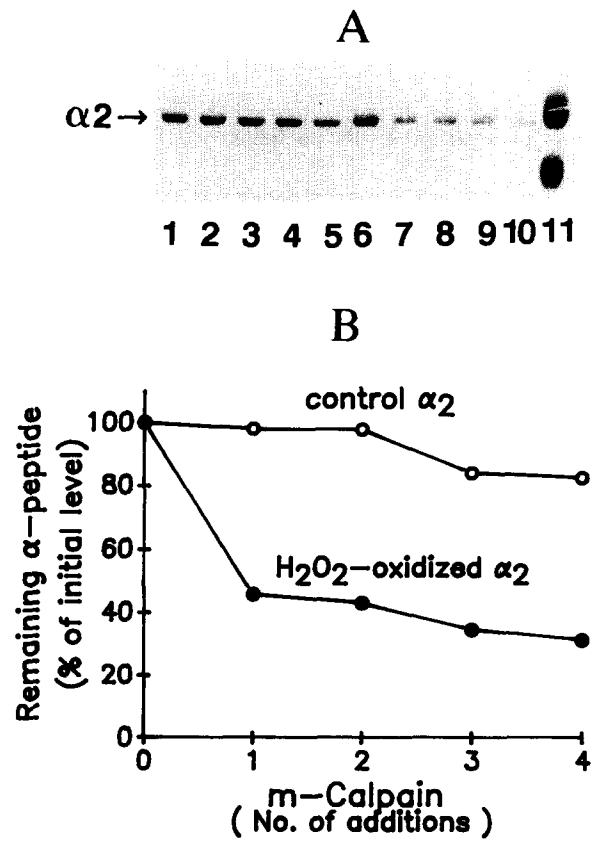

Fig. 8. Effects of m-calpain on native and H_2O_2-oxidized α_2-isoform of the axolemma Na^+/K^+-ATPase. Experiments were done as in Fig. 7. The gels were immunostained with an antibody specific for α_2-isoform (A), and scanned with a densitometer (B).

Native and H_2O_2-oxidized samples of the axolemma Na^+/K^+-ATPase were exposed to m-calpain in experiments similar to those described for the kidney enzyme. After electrophoresis, comparison of the intensities of the Coomassie blue-stained α-subunit bands showed considerable digestion of the oxidized α, but no digestion of the native α (Fig. 7). To compare the sensitivities of the α_2- and α_3-isoforms of this preparation, after experiments similar to those of Fig. 7, samples were immunostained with isoform-specific antibodies. The results showed that the oxidized forms of both isoforms were considerably more susceptible to m-calpain than the native isoforms (Figs. 8 and 9).

Experiments of Fig. 10 showed that oxidation of axolemma Na^+/K^+-ATPase also increased the susceptibility of the α-subunit to μ-calpain.

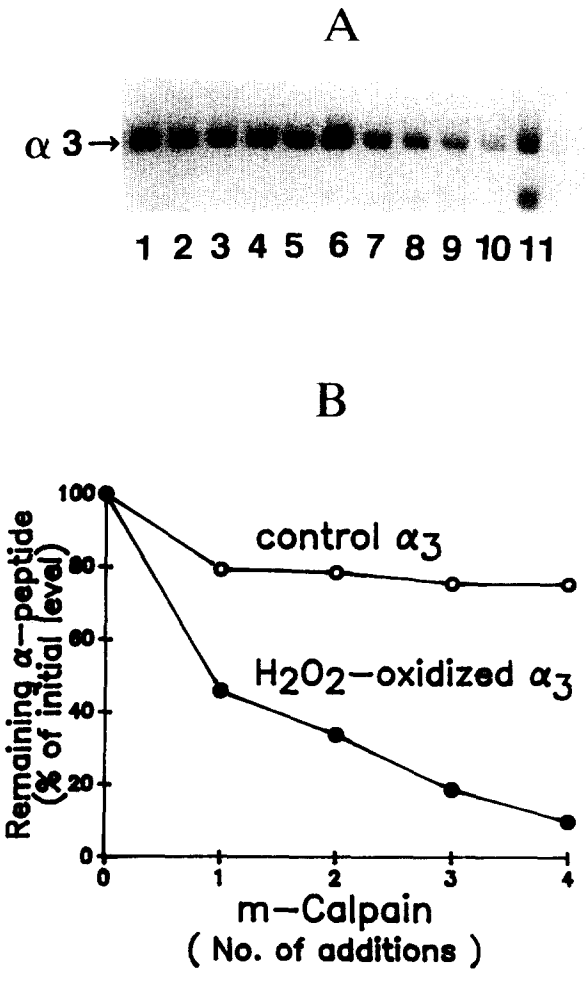

Fig. 9. Effects of m-calpain on native and H_2O_2-oxidized α_3-isoform of the axolemma Na^+/K^+-ATPase. Experiments were done as in Fig. 7. The gels were immunostained with an antibody specific for α_3-isoform (A), and scanned with a densitometer (B).

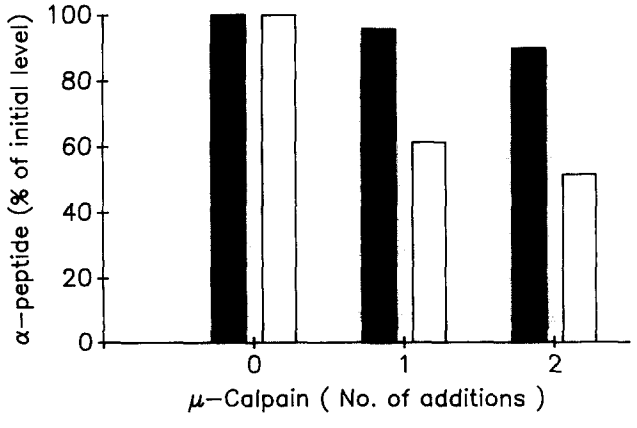

Fig. 10. Effects of μ-calpain on native and H_2O_2-oxidized axolemma Na^+/K^+-ATPase. The two enzyme samples were exposed to μ-calpain repeatedly (twice) as described in Methods. Samples were removed for electrophoresis prior to calpain addition and after each calpain addition. Coomassie blue-stained α bands were scanned by a densitometer. Solid bars, native enzyme; open bars, oxidized enzyme.

Lack of effect of multicatalytic proteinase

In several experiments, native and oxidized preparations of Na^+/K^+-ATPase from kidney and axolemma were exposed to multicatalytic proteinase under conditions where this enzyme is fully active against casein (Methods). No cleavage of any oxidized or native subunit of Na^+/K^+-ATPase was detected.

4. Discussion

The findings presented here show clearly that cathepsin D, m-calpain, and μ-calpain degrade the oxidized catalytic subunits of Na^+/K^+-ATPase more effectively than the unoxidized forms, and that multicatalytic proteinase does not digest either the native or the oxidized Na^+/K^+-ATPase. The latter observation is in contrast to the previously observed ability of multicatalytic proteinase to degrade a large number of oxidized proteins in an ATP-independent manner [1,2,8]. The multicatalytic proteinase, however, is also a component of the ATP, ubiquitin-dependent proteolytic system [20]. The possibility remains, therefore, that Na^+/K^+-ATPase may be a substrate for this system under conditions different from those used in our experiments.

Although the studies of Cook et al. [10] suggested that degradation of Na^+/K^+-ATPase in lysosomes occurred during the normal turnover of the enzyme, this suggestion was tempered by the inability of the same investigators to show the existence of endosomes containing Na^+/K^+-ATPase [10]. Subsequent demonstration of a role of Na^+/K^+-ATPase in the regulation of internal pH of endosomes [21,22], however, confirms that internalization of Na^+/K^+-ATPase by endocytosis, and fusion of the resulting endosomes with lysosomes, are indeed involved in the degradation of this plasma membrane enzyme. Therefore, our data showing the degradation of all isoforms of the catalytic subunit of Na^+/K^+-ATPase by the lysosomal cathepsin D, and the greatly increased susceptibility of the oxidized isoforms to cathepsin D, support the notion that the enhanced turnover of the oxidatively damaged Na^+/K^+-ATPase is indeed a secondary defense mechanism under pathological conditions where this important plasma membrane enzyme is inactivated by reactive oxygen metabolites.

Interaction of native Na^+/K^+-ATPase with calpain was first reported briefly by Cova and Sweadner [23]. In agreement with their findings, our experiments show little or no effects of calpains on the native preparations of Na^+/K^+-ATPase. We show, however, that oxidation of Na^+/K^+-ATPase increases the susceptibilities of the various α-subunits to calpains. While there is no evidence to indicate the involvement of

calpains in the turnover of oxidized Na^+/K^+-ATPase of intact cells, this possibility should be considered seriously because oxidative stress to the heart causes not only inactivation of Na^+/K^+-ATPase, but also an increase in intracellular Ca^{2+} [6]. It is plausible, therefore, that the resulting activation of calpains under these conditions may provide another pathway for degradation and removal of damaged Na^+/K^+-ATPase from the cell membrane.

Our previous studies [13] demonstrated that α_2- and α_3-isoforms of Na^+/K^+-ATPase from different tissues have higher oxidant sensitivities than several variants of α_1-isoform, and suggested strongly that these differences were related to structural features that distinguish α_1 from α_2- and α_3-isoforms. The present experiments show that the oxidized forms of the various isoforms are more susceptible than the native forms to cathepsin D and calpains. It seems, therefore, that degradation by intracellular proteinases may be more pertinent to the enhanced turnover of those isoforms of Na^+/K^+-ATPase that are more prone to damage under oxidative stress.

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