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Different sensitivities of the Na^+/K^+-ATPase isoforms to oxidants

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Abstract

Inhibition of Na^+/K^+-ATPase by partially reduced oxygen metabolites has been suggested to be involved in ischemia-reperfusion injury to heart and other organs. Since various isoforms of the enzyme have different sensitivities to ouabain and several other inhibitors, we studied the effects of H_2O_2 and the hydroxyl radical on enzyme activity and phosphoenzyme formation in Na^+/K^+-ATPase preparations with known α-subunit isoform composition in order to assess the oxidant sensitivities of the isoforms. Rat axolemma enzyme (α_2 and α_3) which has higher sensitivity than the rat kidney enzyme (α_1) to ouabain also showed higher oxidant sensitivity than the kidney enzyme. No significant difference between the oxidant sensitivities of the α_2 and α_3 of the axolemma was noted. In the ferret heart enzyme (α_1 and α_3), we confirmed that α_3 has higher ouabain sensitivity than α_1, and we established that α_3 also has higher oxidant sensitivity than α_1. The rat kidney enzyme (α_1) and the canine kidney enzyme (a variant of α_1 with much higher ouabain sensitivity than the rat kidney enzyme) exhibited similar oxidant sensitivities. The findings suggest that (a) oxidant sensitivity is related to structural features that distinguish α_1 from α_2 and α_3, rather than to features that control ouabain sensitivity; and (b) different isoform compositions of the various tissues may contribute to their relative susceptibilities to oxidant stress.

Key words: ATPase, Na^+/K^+-; Isoform; Hydrogen peroxide; Ouabain; Oxidant; Oxygen radical

1. Introduction

Na^+/K^+-ATPase is the membrane-bound enzyme responsible for the active transport of Na^+ and K^+ across the plasma membrane of mammalian cells. The enzyme consists of α and β subunits, both of which are essential for function [1]. While the α-subunit contains the catalytic site, the β-subunit is thought to assure proper localization and assembly of the holoenzyme in the plasma membrane [1–3]. Both subunits have multiple isoforms. The three well characterized isoforms of α exhibit different sensitivities to proteases and several inhibitors including ouabain and related cardiac glycosides [1–3].

Partially reduced oxygen metabolites (hydrogen peroxide, hydroxyl radical, and superoxide radical) have been implicated as causes of ischemia-reperfusion injury to the heart and several other organs [4,5]. Our studies on the isolated adult rat heart myocytes that are exposed to these oxidants showed that partial inhibition of Na^+/K^+-ATPase is an early event in oxidant-induced damage to these cells, and that a small fraction of the Na^+/K^+-ATPase of these cells that is more sensitive to oxidants also seems to have higher sensitivity than the oxidant-resistant fraction to ouabain [6]. Because the adult rat heart contains different isoforms of the enzyme's α subunit and since these rodent isoforms are known to have different ouabain sensitivities [2,3], we suggested that isoforms with higher sensitivity to ouabain may also be more sensitive to oxidants [6]. The studies reported here were done to test this hypothesis. Using enzyme preparations with known isoform compositions, we show that both ouabain-sensitive and ouabain-insensitive variants of α_1 isoform are more resistant than α_2 and α_3 isoforms to the inhibitory effects of the oxidants.

2. Methods

Ouabain, 'vanadate-free' ATP, and H_2O_2 were obtained from Sigma (St. Louis, MO). [γ-^{32}P]ATP and [^{32}P]P_i were bought from DuPont-New England Nu-

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clear (Boston, MA). [^{32}P]P_i was purified before use [7]. Ferret hearts were purchased from Marshall Farms (North Rose, NY). Peptide directed rabbit antisera specific for N terminal sequences of rat α_3 isoform (GDKKDDKSSPKKS) and rat α_1 isoform (DKYEPAAVSEHGD) were kindly provided by Dr. R. Mercer (Washington University, St. Louis). Rabbit polyclonal antiserum against rat α_2 fusion protein [8] was bought from Upstate Biotechnology (Lake Placid, NY). All reagents for gel electrophoresis, and prestained protein markers, were obtained from Bio-Rad (Richmond, CA).

Na^+/K^+-ATPase from canine kidney medulla (spec. act. 1000 μmol/mg per h) and rat kidney medulla (spec. act. 520 μmol/mg per h) were prepared as described before [7,9]. Rat axolemma Na^+/K^+-ATPase (spec. act. 80 μmol/mg per h) was isolated from brain stem by the procedure of Sweadner [10]. Partially purified ferret heart enzyme (spec. act. in the range of 8–20 μmol/mg per h) was prepared according to Ng and Akera [11].

Na^+/K^+-ATPase activity was assayed at 37°C by the determination of the initial rate of release of P_i from [γ-^{32}P]ATP [7]. 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 observed in the presence and absence of 1.5 mM ouabain. Unless indicated otherwise, each value shown in the graphs is the mean of triplicate determinations with the range not exceeding the size of the symbol.

Phosphorylation of the enzyme by P_i or ATP was done as described before [7,12] in one of two ways: (1) The enzyme was incubated with the indicated concentrations of [^{32}P]P_i in the presence of 2 mM $MgCl_2$, either with or without 1.5 mM ouabain, at 37°C for 20 min. (2) The enzyme was reacted with 10 μM [γ-^{32}P]ATP in the presence of 2 mM $MgCl_2$, and either 100 mM NaCl or 20 mM KCl, for 30 s on ice. The reactions were stopped by the addition of SDS-containing sample preparation buffers of the appropriate gel system. Solubilized samples containing 20–100 μg of protein were subjected to electrophoresis on either 7.5% acid gels [13], or 7.5% Laemmli gels [14], or 5% Laemmli gels [3,11]. Only the latter gel system is capable of separating α_2 and α_3 from α_1. The gels were stained and autoradiographed by conventional procedures. The bands were quantified by a soft laser scanning densitometer. Multiple exposures of the radioactive bands were analyzed to assure that the signals were within the linear range of the film.

Western blot analysis was done as described by Blake et al. [15]. After electrophoresis, the separated peptides were transferred to a nitrocellulose sheet, blots were probed with isoform specific antisera, and the complexes were detected using second antibody conjugated with alkaline phosphatase.

3. Results

We showed before that several partially reduced oxygen metabolites cause irreversible inhibition of Na^+/K^+-ATPase either without gross structural changes or concomitant with fragmentation or aggregation of the enzyme subunits [16]. Since H_2O_2 and the hydroxyl radical generating system of $H_2O_2 + Fe^{2+}$ + ascorbate are the oxidants used in the studies to be presented here, it is appropriate that we summarize the structural consequences of their inhibitory effects: H_2O_2 causes time-dependent irreversible inhibition of the enzyme without inducing significant gross structural changes. This is exemplified by the results of the experiments of Fig. 1 in which the purified kidney enzyme was incubated with 50 mM H_2O_2 (one of the highest concentrations used in the present studies) and after different incubation periods enzyme samples were assayed for activity and subjected to SDS-gel electrophoresis. As evident from the data, time-dependent inhibition occurred with no significant changes in the amounts of the stained subunits on the gels; and only after prolonged incubation (2 h) there was evidence of small quantities of high molecular weight cross-linked aggregates of enzyme subunits (Fig. 1). On the other hand, rapid irreversible inhibition caused by $H_2O_2 + Fe^{2+}$ + ascorbate is accompanied by partial fragmentation of the α-subunit to small peptides. This phenomenon was shown before [16], and will also become evident from experiments presented below.

3.1. Oxidant and ouabain sensitivities of the rat kidney and the rat brain stem axolemma enzymes

It is well documented that rat kidney contains only the α_1 isoform and that the rat brain stem axolemma

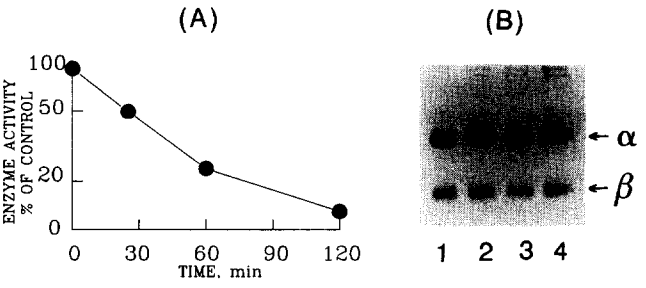

Fig. 1. Time-dependent effects of H_2O_2 on the purified kidney Na^+/K^+-ATPase. The canine kidney enzyme (0.2 mg/ml) was incubated with 50 mM H_2O_2 at 37°C in a solution of 50 mM Tris-HCl (pH 7.4). A control was similarly incubated but without H_2O_2. Aliquots were removed at indicated times, centrifuged at 100000× g, washed with 0.25 mM sucrose, 30 mM histidine, 1 mM EDTA (pH 6.8), and assayed for activity (A) as indicated in Methods. Samples containing the same amount of protein were also subjected to electrophoresis on Laemmli gels and stained (B). Lane 1, enzyme not exposed to H_2O_2. Lanes 2, 3, and 4, samples exposed to H_2O_2 for 25 min, 60 min, and 120 min, respectively.

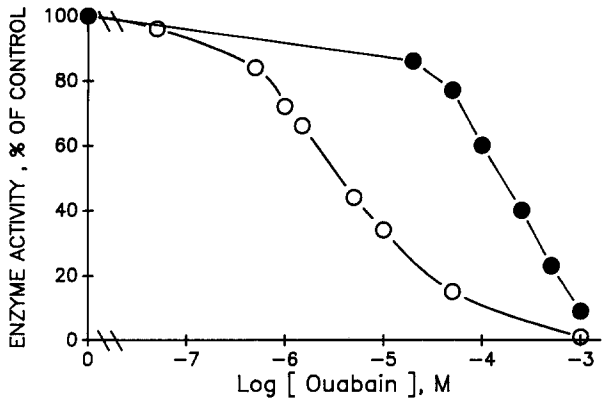

Fig. 2. Effects of various concentrations of ouabain on the activities of rat axolemma (○) and rat kidney (●) Na^+/K^+-ATPase. The enzymes were incubated in the assay media lacking ATP with the indicated concentrations of ouabain for 20 min. ATP was added and assays were done as indicated in Methods.

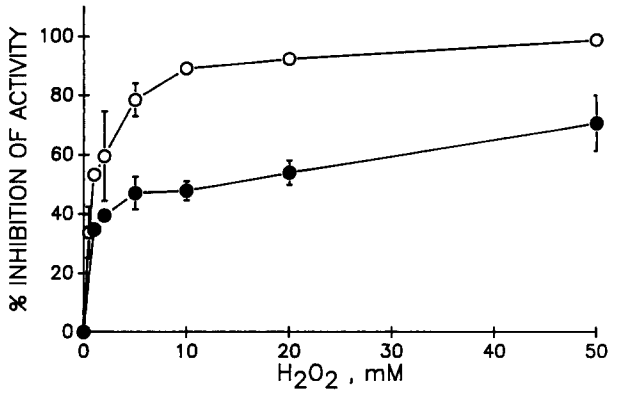

Fig. 3. Irreversible inhibitions of rat axolemma (○) and rat kidney (●) Na^+/K^+-ATPase by various H_2O_2 concentrations. Each enzyme (0.2 mg/ml) was incubated for 25 min at 37°C in a solution containing the indicated concentration of H_2O_2 and 50 mM Tris-HCl (pH 7.4). Control was similarly treated but without H_2O_2. Preparations were then collected by centrifugation at 100000× g, washed with 0.25 M sucrose, 30 mM histidine, and 1 mM EDTA (pH 6.8); and assayed as described in Methods. Values are means ± S.E. from four separate experiments.

contains a mixture of α_2 and α_3 isoforms with only traces of the α_1 isoform [3,10]. Experiments of Fig. 2 confirmed previous observations [3,9] showing that the sensitivity of the axolemma enzyme to ouabain is about two orders of magnitude greater than the ouabain sensitivity of the kidney enzyme. Rodent α_2 and α_3 isoforms both have higher sensitivities than rodent α_1 to ouabain [3].

In experiments of Fig. 3 inhibitory effects of varying concentrations of H_2O_2 after a fixed preincubation period (25 min) were determined for the rat kidney and the axolemma enzymes. The data show clearly that the kidney enzyme is more resistant to H_2O_2 *. To explore this difference between the preparations further, in experiments of Fig. 4 the two enzymes were preincubated with the same concentration of H_2O_2 and then exposed to [γ-^{32}P]ATP to measure the level of the Na^+-dependent and K^+-sensitive phosphointermediate of the enzyme. The data show that H_2O_2-induced depression of the phosphoenzyme is more pronounced in the axolemma enzyme than it is in the kidney enzyme.

Although the combined data of Figs. 3 and 4 demonstrate that α_2 and α_3 isoforms are more sensitive than α_1 isoform to H_2O_2, they do not provide information about the relative oxidant sensitivities of α_2 and α_3. Because hydroxyl radicals generated by $H_2O_2 + Fe^{2+}$ + ascorbate cause fragmentation of the α-subunit to smaller peptides [16], we exposed the axolemma enzyme to the hydroxyl radical generating system for a fixed period of time, and measured the

* Although further studies on the kinetics of oxidant-induced inhibition of the enzyme are to be presented in subsequent reports, it is appropriate to point out that inactivation time-courses of all enzymes used here, including the purified preparations consisting of one isoform, are biphasic and similar to the limited data shown in Fig. 1. As discussed by Fritzsch [17], such kinetics which have been noted with several other irreversible inhibitors of this enzyme, may be explained not only by multiple enzyme species, but also by multiple inhibitor binding sites with different affinities, or multiple states of the same enzyme with different inhibitor affinities.

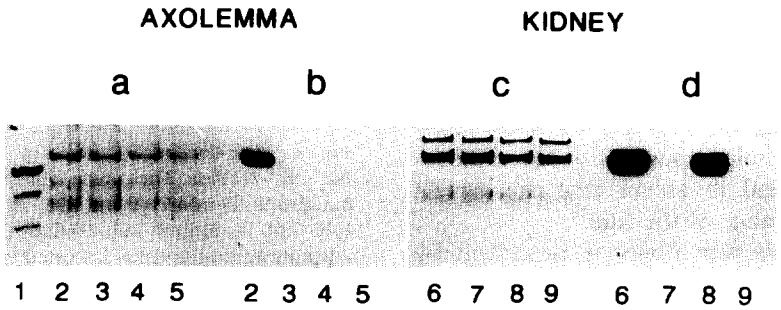

Fig. 4. Effects of H_2O_2 on Na^+-dependent phosphorylations of the rat axolemma and rat kidney Na^+/K^+-ATPase. Enzymes were pretreated with 20 mM H_2O_2 as described in Fig. 3, and then phosphorylated and placed on acid gels as indicated in Methods. The gels were stained (a and c) and autoradiographed (b and d). Lane 1, marker proteins; Lanes 2, 3, 6, and 7, control enzymes; Lanes 4, 5, 8, and 9, H_2O_2-treated enzymes; Lanes 2, 4, 6, and 8, phosphorylated in the presence of Na^+; Lanes 3, 5, 7, and 9, phosphorylated in the presence of K^+.

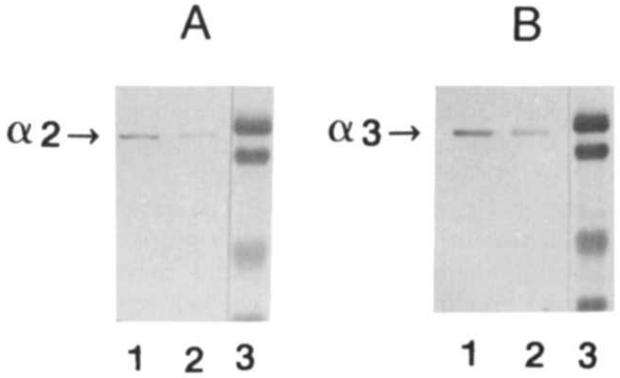

Fig. 5. Oxidative degradation of α_2 and α_3 isoforms of the rat axolemma Na^+/K^+-ATPase. The enzyme was incubated with 0.5 mM H_2O_2, 1 mM ascorbic acid, 20 μM $FeCl_2$, 50 mM Tris-HCl (pH 7.4) at 37°C for 5 min. Control (lane 1) and the oxidized enzyme (lane 2) were then subjected to electrophoresis on 7.5% Laemmli gels and immunostained with α_2-specific antibody (A) and α_3-specific antibody (B) as described in Methods. Lane 3 shows protein markers (106 kDa, 80 kDa, 49.5 kDa, and 32.5 kDa).

remaining α_2 and α_3 bands with isoform specific antibodies. The results (Fig. 5) showed that α_2 and α_3 bands were decreased by about the same extent, indicating that the sensitivities of α_2 and α_3 to the hydroxyl radicals generated by this system are not greatly different.

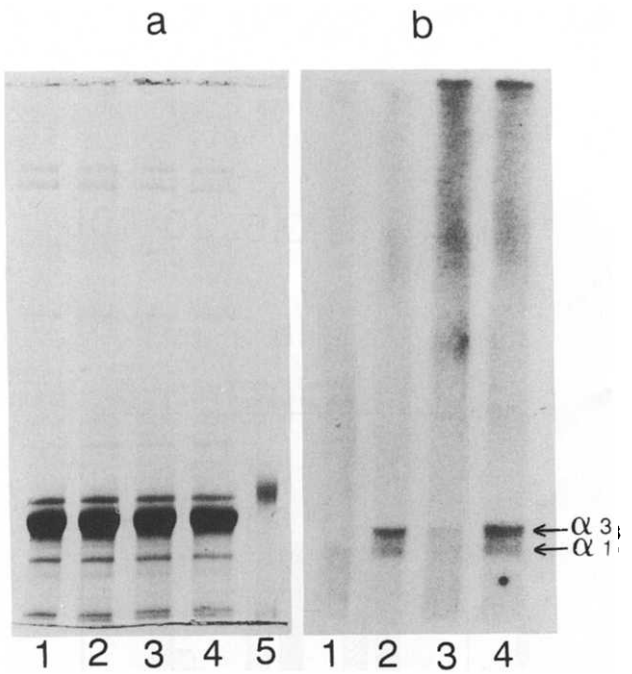

Fig. 6. Demonstration of two α isoforms in the ferret heart Na^+/K^+-ATPase. The crude enzyme preparation was reacted with 20 μM [^{32}P]P_i (1 and 2) or 500 μM [^{32}P]P_i (3 and 4) in the absence of ouabain (1 and 3) and in the presence of 1.5 mM ouabain (2 and 4) as described in Methods; and then subjected to electrophoresis on 5% Laemmli gels, stained (a), and autoradiographed (b). Lane 5 is the protein marker (106 kDa).

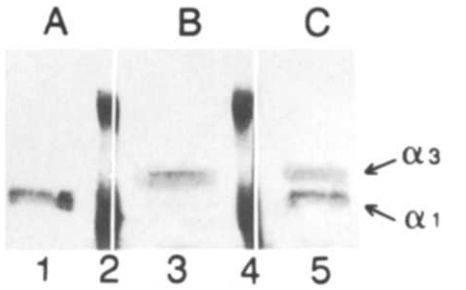

Fig. 7. Identification of α_1 and α_3 isoforms in the ferret heart Na^+/K^+-ATPase. The enzyme was subjected to electrophoresis on 5% gels, and immunostained with isoform specific antibodies (A, α_1 antibody; B, α_3 antibody; C, α_1 and α_3 antibodies) as described in Methods. The position of the markers (2 and 4) were used to match the panels.

3.2. H_2O_2 and ouabain sensitivities of the ferret heart enzyme

One could argue that the different oxidant sensitivities of the rat kidney and axolemma enzymes noted above may be due to different states of purity of these membrane preparations, or because of the presence of tissue-specific factors other than the enzyme subunits. To test this possibility we examined the effects of H_2O_2 on a preparation of the ferret heart Na^+/K^+-ATPase which was reported to contain nearly equal amounts of α_1 and α_3 isoforms with different ouabain sensitivities [11,18].

First, we confirmed previous observations by showing that the crude microsomal preparation obtained from ferret heart does in fact contain α_1 and α_3 isoforms. Since phosphorylation of α subunit by suboptimal concentrations of P_i is stimulated by ouabain [7], the preparation was exposed to [^{32}P]P_i in the presence or absence of ouabain, and then subjected to electrophoresis on 5% gels, and subsequent autoradiography. The results showed the presence of two distinct

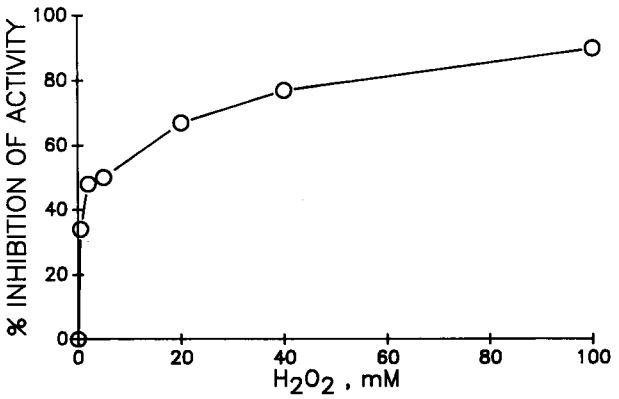

Fig. 8. Irreversible inhibition of ferret heart Na^+/K^+-ATPase by various H_2O_2 concentrations. The enzyme was preincubated with H_2O_2 as indicated in Fig. 3 and the text, and assayed as described in Methods.

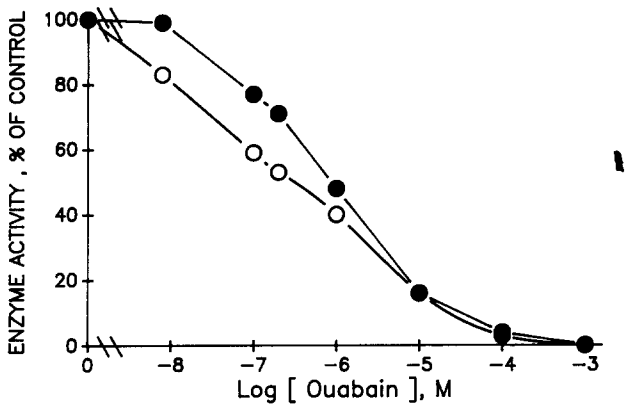

Fig. 9. Effects of various ouabain concentrations on Na^+/K^+-ATPase activities of the native (○) and the H_2O_2-treated (●) ferret heart enzymes. The enzyme was irreversibly inhibited by H_2O_2 to the extent of about 50% as described in Fig. 8. The control and the oxidized enzymes were then assayed in the presence of various ouabain concentrations as described in Fig. 2.

ouabain-sensitive α bands (Fig. 6). Using isoform specific antibodies, the bands were identified as α_1 and α_3, with α_1 having the higher mobility (Fig. 7).

In experiments of Fig. 8 the ferret heart enzyme was preincubated for 20 min with different concentrations of H_2O_2, and irreversible inhibition of Na^+/K^+-ATPase activity was determined. Based on this information, a sample of the enzyme was first inhibited irreversibly by H_2O_2 to the extent of about 50%, and then the ouabain sensitivity of its remaining Na^+/K^+-ATPase activity was compared with that of the control enzyme not exposed to H_2O_2. The results (Fig. 9) showed that (a) in agreement with previous observations [11], inhibition of the control enzyme activity occurred over five log units of ouabain concentration, consistent with the existence of a mixture of α_3 and α_1 and the higher affinity of α_3 for ouabain; and (b) the H_2O_2-treated enzyme was less sensitive than the control enzyme to lower range of ouabain concentrations, consistent with the higher oxidant sensitivity of α_3. To test this conclusion further, in experiments of Fig. 10 the Na^+-dependent phosphorylation of the ferret heart enzyme by [γ-^{32}P]ATP was conducted in the absence of ouabain and in the presence of different concentrations of ouabain. After SDS-gel electrophoresis, the levels of phosphorylated α_1 and α_3 were measured. In agreement with previous observations [11,18], phosphorylation of α_3 was more sensitive to inhibition by

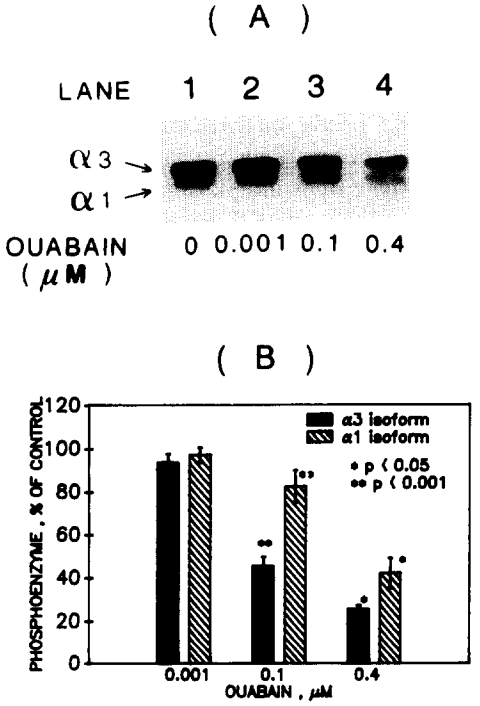

Fig. 10. Inhibitory effects of ouabain on Na^+-dependent phosphorylation by ATP of α_1 and α_3 isoforms of the ferret heart enzyme. The enzyme was exposed to the indicated ouabain concentrations in the presence of 0.1 mM P_i and 2 mM Mg^{2+} for 30 min before it was exposed to [γ-^{32}P]ATP and subjected to electrophoresis on 5% gels as described in Methods. Autoradiograms from one experiment are shown in (A), and the results of five separate experiments are summarized in (B). Values are means ± S.E.

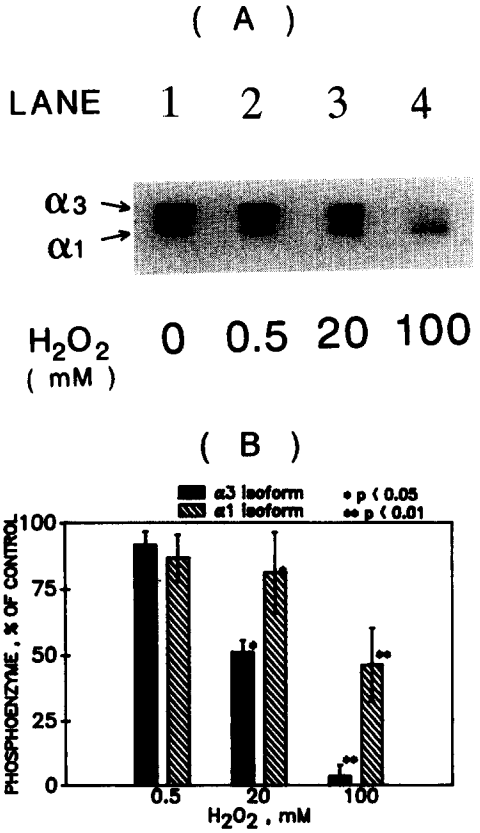

Fig. 11. Inhibitory effects of H_2O_2 on Na^+-dependent phosphorylation by ATP of α_1 and α_3 isoforms of the ferret heart enzyme. The enzyme was preincubated with the indicated H_2O_2 concentrations as indicated in Fig. 2, and then phosphorylated and analyzed as indicated in Fig. 10.

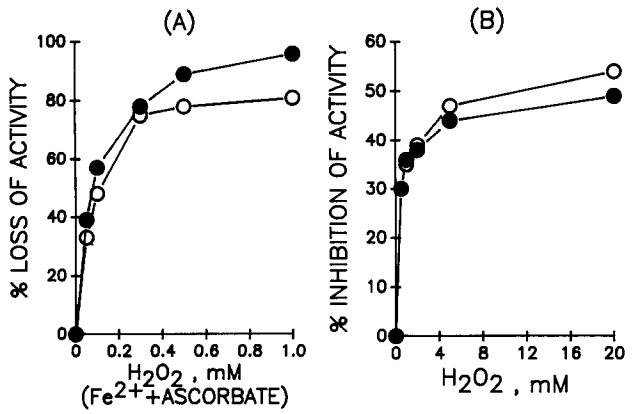

Fig. 12. Comparison of the oxidant sensitivities of the rat kidney (○) and the canine kidney (●) Na^+/K^+-ATPase preparations. In (A) the enzymes were preincubated at 37°C with the indicated H_2O_2 concentrations in the presence of 1 mM ascorbate, 20 μM $FeCl_2$, and 50 mM Tris-HCl (pH 7.4) for 30 s. The samples were then diluted 10-fold and assayed for activity as described in Methods. In (B) the enzymes were incubated with the indicated H_2O_2 concentrations and assayed as described in Fig. 3.

ouabain than that of α_1. Similar experiments with enzyme preparations that were pretreated with different H_2O_2 concentration showed that phosphorylation of α_3 was also more sensitive than that of α_1 to H_2O_2 (Fig. 11).

3.3. Comparison of the oxidant sensitivities of the rat kidney and the canine kidney enzymes

The results of the above experiments showed that those α isoforms that have higher oxidant sensitivity also have higher ouabain sensitivity. To explore the extent of correlation between oxidant and ouabain sensitivities, the effects of H_2O_2 and hydroxyl radicals on the rat and the canine kidney enzymes were compared. While the predominant isoform of both preparations is α_1 [3], their ouabain sensitivities differ by about three orders of magnitude [9,19]. As the data of Fig. 12 show, the oxidant sensitivities of the two preparations are nearly identical, suggesting that oxidant sensitivity is related to structural features that distinguish α_1 from α_2 and α_3 rather than to the structural determinants of ouabain sensitivity.

4. Discussion

A large body of recent work shows that Na^+/K^+-ATPase and the ion movements catalyzed by it are inhibited by various partially reduced oxygen metabolites [6,16,20,21, and references therein]. There is also substantial evidence to suggest that inhibition of this enzyme by reactive oxygen species contributes significantly to ischemia-reperfusion injury in the heart [22,23]. It is important, therefore, to characterize the nature of the interactions of these oxidants with Na^+/K^+-ATPase. Our initial studies in this direction showed irreversible inhibitory effects of several oxidants on the purified canine kidney enzyme, and some structural changes associated with these effects [16]. In the present study we have examined the interactions of H_2O_2 and the hydroxyl radicals with several enzyme preparations of known isoform compositions, and we have demonstrated that the oxidant sensitivities of the α_2 and α_3 isoforms are greater than those of several variants of α_1 isoform.

The existence of multiple genes of the α-subunit that exhibit tissue-specific and developmental pattern of expression is now well established [2,3]. If the oxidant-induced damage to this plasma membrane enzyme is indeed significant to the development of reperfusion injury, or to other pathological conditions caused by reactive oxygen [5], the nature of the predominant isoform of the various tissues may be the prime determinant of their relative susceptibilities to oxidant stress. Consider, for example, the recent findings indicating that the predominant isoforms of the conducting system of the rat heart are α_2 and α_3 [24], whereas the adult rat myocardium contains mostly the α_1 isoform [25]. This suggests that the rapidly developing reperfusion arrhythmias that precede eventual injury to myocytes in the rat heart [26,27] may indeed be due to the higher oxidant sensitivities of the α_2 and α_3 isoforms of the conducting tissue.

Although a good deal is known about the mechanisms involved in the oxidations of the various amino acid residues of proteins by reactive oxygen species [28,29], at this time little information is available about the detailed mechanisms of the irreversible inhibition of Na^+/K^+-ATPase by H_2O_2 and related oxygen metabolites. The unpublished results of such studies that are in progress in our laboratory indicate, however, that oxidant inhibition of Na^+/K^+-ATPase occurs through a metal-catalyzed site-specific mechanism [29]. This and the fact that primary structures of the various isoforms are known, make it reasonable to expect the eventual identification of the specific sites of oxidant interactions with the enzyme, and the clarification of the molecular determinants of its oxidant sensitivity.

5. Acknowledgement

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