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INHIBITION OF RAT BRAIN MICROSOMAL ($Na^+ + K^+$)-ATPase AND K^+-*p*-NITROPHENYLPHOSPHATASE BY PERIODIC ACID

JOHN M. BERTONI

Division of Neurology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284 (U.S.A.)

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The effects of mild periodate exposure on the kinetics of ($Na^+ + K^+$)-ATPase and K^+-*p*-nitrophenylphosphatase were studied using rat cerebral microsome preparations. Fifty percent inhibition of both enzyme activities was attained near 3 μM periodate concentrations. This inhibition was biphasic with time. Mg^{2+}-ATPase and Mg^{2+}-*p*-nitrophenylphosphatase activities were much less inhibited by periodate. Periodate inhibition was partially reversed by dimercaprol and dithiothreitol but not by dilution. The possible reaction products formic acid, formaldehyde, glyceraldehyde, and acetaldehyde had no inhibitory effects in similar concentrations. Periodate exposure produced no detectable changes in the activation of ($Na^+ + K^+$)-ATPase by Na^+, K^+, Mg^{2+}, or ATP. Residues shared by both ($Na^+ + K^+$)-ATPase and K^+-*p*-nitrophenylphosphatase are both critical to hydrolytic function and sensitive to mild oxidation by periodate.

Introduction

Periodic acid has been applied to the study of structure/function of cellular membranes because its interaction with membrane glycoproteins and other readily oxidizable groups is regarded as relatively specific [1–3]. Various oxidative agents have been used to probe the active sites of ($Na^+ + K^+$)-ATPase, including superoxide radicals [4], and changes in the redox potential [5]. The fact that naturally occurring changes in the oxidation state may control the activity of ($Na^+ + K^+$)-ATPase [5] makes the study of mild controllable oxidation by periodate particularly interesting. We report here that periodic acid at near micromolar concentrations is a potent inactivator of ($Na^+ + K^+$)-ATPase and its partial reaction K^+-*p*-nitrophenylphosphatase in rat cerebral microsomes.

Materials and Methods

Adult (200–300 g body weight) Sprague-Dawley rats of both sexes were obtained from Spartan Research Animals (Haslett, MI) and were fed Purina rat chow. NaI-treated microsomes were prepared as described elsewhere [6]. [γ-^{32}P]ATP was obtained from ICN Chemical and Radioisotope division (Irvine, CA). Periodic acid was purchased from G.F. Smith Chemical company (Columbus, OH). Dimercaprol, dithiothreitol, and 2,4,6-tripyridyl-*S*-triazine were purchased from Sigma Chemical Company (St. Louis, MO). Other reagents were obtained as described earlier [6]. Only distilled water that had been treated in a Super Q system (Millipore Corporation, Bedford, MA) was used for these experiments.

Stock 50 mM solutions of periodic acid were made fresh every two weeks and kept stoppered in

foil-wrapped glassware at 4°C in the dark. Experimental results and spectrophotometric assay [7] indicated no loss of oxidative potency during this time period. Periodic acid solutions were diluted in cold Super Q deionized distilled water just prior to use.

Microsomal protein (0.1 mg/ml) was preincubated with periodic acid for 30 min in the dark at 22°C, in the absence of added buffer. Some experiments included 75 mM imidazole-HCl (pH 7.4) in the preincubation medium. Higher periodic acid concentrations were required for the same amount of inhibition when imidazole-HCl was used due to an unidentified reducing contaminant in the imidazole.

In washing experiments, the microsomal material was centrifuged after the standard exposure to periodic acid at $50000 \times g$ for 1 h in a Beckman Model L5-50 Ultracentrifuge using a swinging bucket rotor and then resuspended. For the control, microsomal material was treated identically except that water was used instead of periodic acid. Protein concentrations were determined in the original and resuspended microsomes according to the method of Lowry et al. [8]. In some experiments, the microsomes previously exposed to periodic acid were treated with 1 mM dithiothreitol or 1 mM dimercaprol at 22°C for 60 min in the dark prior to assay, in an attempt to reverse the effects of periodate oxidation. Controls were obtained by substituting water for the periodic acid and/or thiol reagents.

ATPase activity was measured after incubation at 37°C for 10 min in media containing 2 μg of microsomal protein in (final concentrations) 75 mM imidazole-HCl (pH 7.4), 3 mM Tris-[γ-^{32}P]-ATP ($(8–25) \cdot 10^5$ cpm/mol), 3 mM $MgCl_2$, 10 mM KCl, in the presence and absence of 80 mM NaCl in a final volume of 40 μl. The remaining steps were carried out as described earlier [6]. $(Na^+ + K^+)$-ATPase activity was taken as the difference between product formed in the presence and absence of Na^+. Mg^{2+}-ATPase was taken as the difference between product formed in the absence of Na^+ and the absence of microsomes.

p-Nitrophenylphosphatase activity was assayed by a spectrophotometric method [9] after incubation at 37°C for 20 min in media containing 2 μg of microsomal protein in (final concentrations) 75 mM imidazole-HCl (pH 7.4), 5 mM *p*-nitrophenylphosphate, 5 mM $MgCl_2$, in the presence and absence of 20 mM KCl in a final volume of 40 μl. K^+-*p*-Nitrophenylphosphate was calculated from the difference in absorbance obtained in the presence and absence of K^+. Mg^{2+}-*p*-Nitrophenylphosphatase was derived from the difference between the absence of K^+ and the absence of microsomes.

Periodic acid concentrations were determined using the method of Avigad [7] which utilizes 2,4,6-tripyridyl-*S*-triazine. It was determined that 5 mM MgATP or 6 mM Tris completely consumed 25 μM periodic acid within 60 s. These agents had no effect on the absorbance when added to 2,4,6-tripyridyl-*S*-triazine alone. Attempts to measure the amount of periodic acid remaining after exposure to microsomal protein were unsuccessful due to varying turbidity of the suspension and complexing of the color reagent with microsomal protein during centrifugation which was done to remove turbidity.

Results

Periodic acid inhibits the $(Na^+ + K^+)$-ATPase activity of rat cerebral microsomes in a dose-dependent manner (Fig. 1) under the standard

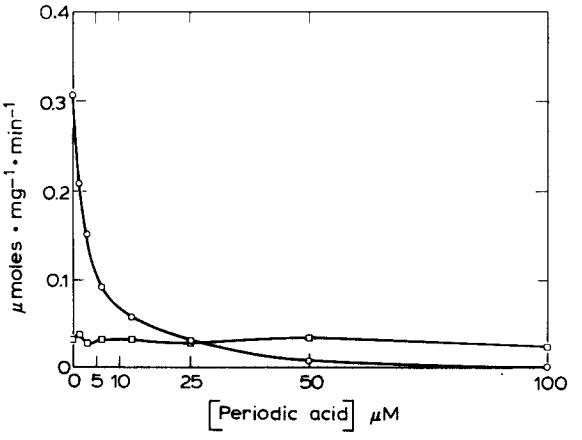

Fig. 1. Effects of periodic acid on rat cerebral $(Na^+ + K^+)$-ATPase (○) and Mg^{2+}-ATPase (□) activities. Microsomes, 0.1 mg/ml protein, were exposed to varying concentrations of periodic acid for 30 min at 22°C prior to assays (see Methods). Half-maximal inhibition of $(Na^+ + K^+)$-ATPase occurs with 3 μM periodic acid. There is little effect on Mg^{2+}-ATPase activity under these conditions.

conditions in the absence of buffer. Fifty percent inhibition is attained near 3 μM periodic acid concentration and inhibition is virtually complete with 50 μM periodic acid. Little if any inhibition of Mg^{2+}-ATPase is found under these conditions (Fig. 1). In some experiments 75 mM imidazole-HCl, pH 7.4, was included in the preincubation mixture to standardize the pH and half-maximal inhibition was attained at 25 μM periodic acid. The presence of a reducing contaminant in the imidazole accounted for the apparent lessening of the potency of periodic acid.

Similarly, periodic acid inhibits the K^+-*p*-nitrophenylphosphate activity (Fig. 2) of the same rat cerebral microsomal preparations by 50% at 3 μM period acid and over 94% at 50 μM under the standard conditions in the absence of added buffer. In comparison, Mg^{2+} -*p*-nitrophenylphosphate activity is only 20% inhibited at 3 μM and only 50% inhibited at 50 μM periodic acid.

When residual $(Na^+ + K^+)$-ATPase activity is plotted as a function of the time of preincubation with periodic acid (Fig. 3), the biphasic character of period acid inhibition is apparent. There is a rapid phase of inhibition followed by a slower phase at each periodic acid concentration tested. The ATP present in the incubation medium rapidly removes any periodic acid oxidative potential, so that exposure to periodic acid ceases at the time the assay begins.

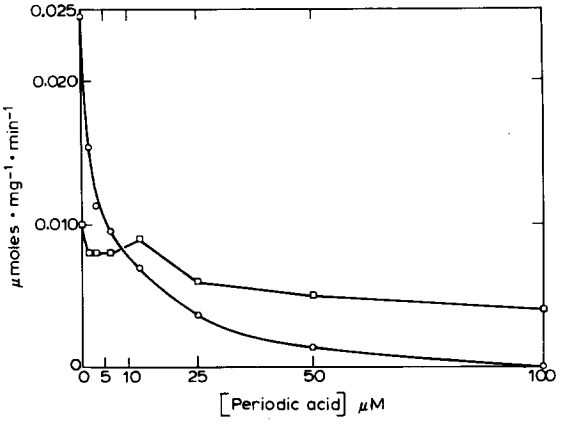

Fig. 2. Effects of periodic acid on rat cerebral K^+-*p*-nitrophenylphosphatase (○) and Mg^{2+}-*p*-nitrophenylphosphatase (□) activities. Microsomes, 0.1 mg/ml protein, were exposed to varying concentrations of periodic acid for 30 min at 22°C prior to assays (see Methods). Half-maximal inhibition of K^+-*p*-nitrophenylphosphatase occurs with about 3 μM periodic acid. Much less inhibition of Mg^{2+} -*p*-nitrophenylphosphatase is found under these conditions.

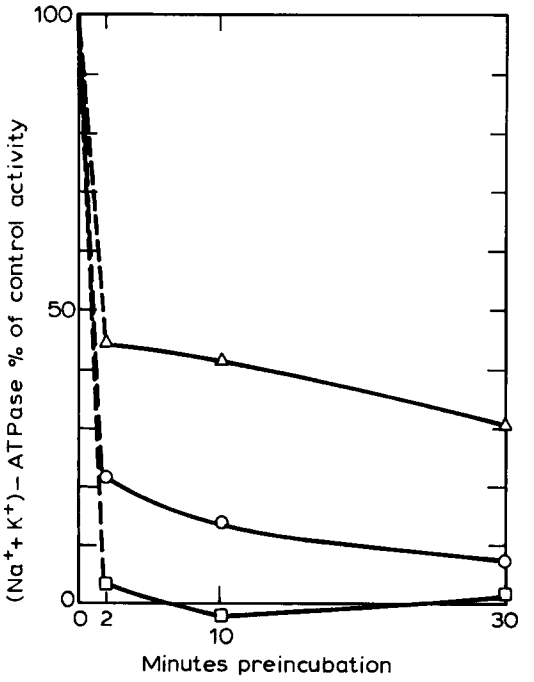

Fig. 3. Effect of time of preincubation on periodic acid inhibition of rat cerebral $(Na^+ + K^+)$-ATPase. Preincubation and assay conditions are as in Figs. 1 and 2 and Methods. A rapid partial inactivation occurs within 2 min and is followed by a slower inactivation phase for 3.125 μM (△), 25 μM (○), and 100 μM (□) periodic acid.

To assess the reversibility of the inhibition by periodic acid of $(Na^+$-$K^+)$-ATPase and K^+-*p*-nitrophenylphosphatase activities, microsomal protein was first exposed to period acid under the usual conditions (50 pmol of period acid per μg microsomal protein) and then the material was diluted 100-fold, centrifuged, resuspended and finally assayed for activity (see Methods). Appropriate corrections were made for any microsomal protein lost during the washes. $(Na^+ + K^+)$-ATPase activity was inhibited 48% prior to centrifugation and 49% after one wash. A similar test for reversibility of periodic acid inhibition of K^+-*p*-nitrophenylphosphatase showed inhibition of 46% prior to washing and 58% inhibition after both washes 1 and 2.

As another test for the reversibility of periodic

acid inhibition of $(Na^+ + K^+)$-ATPase activity, microsomes that had been preincubated with periodic acid were subsequently exposed to dithiothreitol or dimercaprol or water for 1 h. This resulted in enhancement of both Mg^{2+}-ATPase and $(Na^+ + K^+)$-ATPase activities with or without prior preincubation with periodic acid. The $(Na^+ + K^+)$-ATPase activity was 0.45, 0.49, and 0.64 μmol/mg per min for the control, for 1 mM dithiothreitol, and for 1 mM dimercaprol, respectively, after exposure of rat cerebral microsomes to water for 30 min at 22°C. However, after exposure to 25 μM periodic acid for 30 min in the presence of 75 mM imidazole-HCl, pH 7.4, at 22°C, the final $(Na^+ + K^+)$-ATPase activities were 0.14, 0.30, and 0.46 μmol/mg per min in the control, and with 1 mM dithiothreitol and dimercaprol, respectively. Thus, the final percentage of inhibition with periodic acid was 68, 39, and 27%, respectively. This indicates that roughly half of the periodic acid inactivation was reversed by these thiol reagents.

Formic acid, formaldehyde, glyceraldehyde, and acetaldehyde were tested as likely candidates for the agent(s) directly responsible for the periodic acid inhibition effect. These agents and periodic acid at 25 μM concentrations were each preincubated with microsomal protein in the presence of imidazole buffer. K^+-*p*-Nitrophenylphosphatase activity was found significantly higher ($P < 0.001$, Student's two tailed *t*-test) after preincubation with them than after preincubation with periodic acid but there was no difference from controls (data not shown).

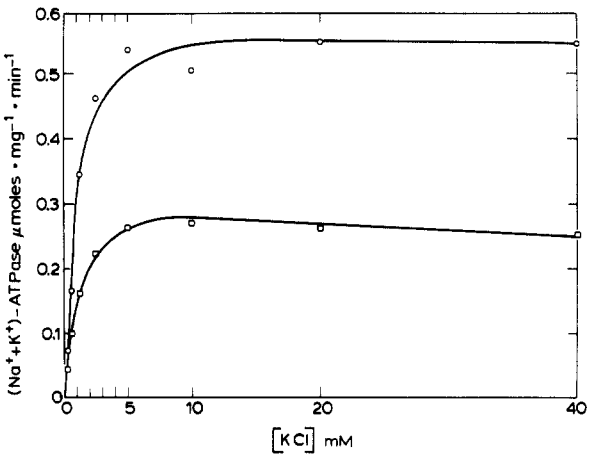

Fig. 5. K^+ activation of rat cerebral microsomal $(Na^+ + K^+)$-ATPase. Microsomes were exposed to either 25 μM periodic acid (□) in 75 mM imidazole-HCl (pH 7.4) buffer or buffer alone (○) under the standard conditions prior to assay. The activation by K^+ is similar in both situations, except for the expected reduction in activity. Other final concentrations were: 80 mM NaCl, 3 mM $MgCl_2$ and 3 mM ATP.

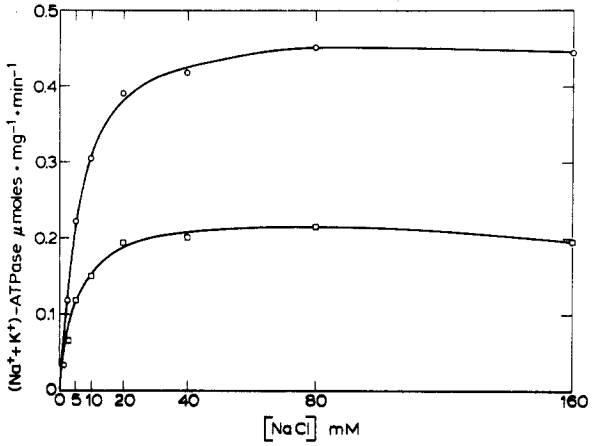

Fig. 4. Na^+ activation of rat cerebral microsomal $(Na^+ + K^+)$-ATPase. Microsomes were exposed to either 25 μM periodic acid (□) in 75 mM imidazole-HCl (pH 7.4) buffer or buffer alone (○) under the standard conditions prior to assay. The activation by Na^+ is similar in both situations, except for the expected reduction in activity. Other final concentrations were: 10 mM KCl, 3 mM $MgCl_2$ and 3 mM ATP.

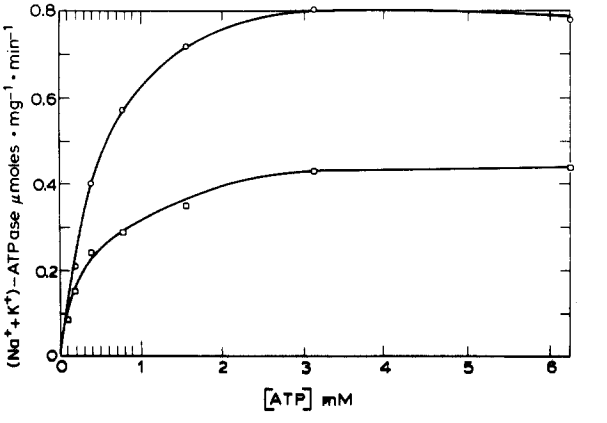

Fig. 6. ATP activation of rat cerebral microsomal $(Na^+ + K^+)$-ATPase. Microsomes were exposed to either 25 μM periodic acid (□) in 75 mM imidazole-HCl (pH 7.4) buffer or buffer alone (○) under the standard conditions prior to assay. The activation by ATP is similar in both situations, except for the expected reduction in activity. Other final concentrations were: 80 mM NaCl, 10 mM KCl, and 3 mM $MgCl_2$.

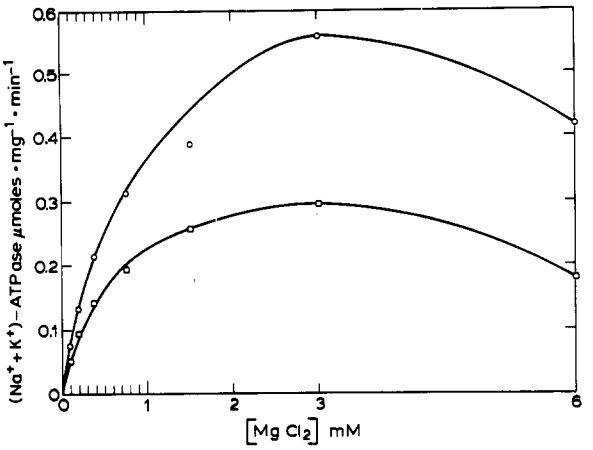

Fig. 7. Mg^{2+} activation of rat cerebral microsomal ($Na^+ + K^+$)-ATPase. Microsomes were exposed to either 25 μM periodic acid (□) in 75 mM imidazole-HCl (pH 7.4) buffer or buffer alone (○) under the standard conditions prior to assay. The activation by Mg^{2+} is similar in both situations, except for the expected reduction in activity. Other final concentrations were: 80 mM NaCl, 10 mM KCl, and 3 mM ATP.

To determine whether periodic acid treated ($Na^+ + K^+$)-ATPase molecules exhibit any alteration in affinity for the known ligands, activation of ($Na^+ + K^+$)-ATPase by Na^+, K^+, Mg^{2+}, and ATP was studied. However, the ($Na^+ + K^+$)-ATPase remaining after 50% inhibition by periodic acid was activated normally by Na^+, K^+, Mg^{2+}, and ATP (Figs. 4–7). These experiments included the use of imidazole buffer.

Discussion

Periodic acid is a reagent that is diagnostic for the presence of vicinal hydroxyl groups [2], especially those found in sugars, including sialic acids. However, it also reacts with other groups. Fleury et al. [10] reported that certain amino acids may also be oxidized by periodic acid, in the general reaction $RCNH_2HCOOH + IO_4^- \rightarrow RCHO + NH_3 + CO_2 + IO_3^-$, which requires an unsubstituted H atom on the N for oxidation [3]. Oxidation is facilitated by neighboring electron releasing groups, and in general occurs more rapidly with higher pH. Clamp and Hough [11] reported that all α-amino acids are oxidized by periodic acid, but with differing rates. Serine, threonine, cysteine, cystine, methionine, proline, hydroxylproline, tryptophan, tyrosine, and histidine are rapidly oxidized. When substituted in the carboxyl and amino groups (as in a polypeptide chain) cysteine, cystine, methionine, tryptophan, tyrosine, and histidine are susceptible to oxidation by periodic acid [11].

Periodic acid causes the oxidation of some unknown group(s) of ($Na^+ + K^+$)-ATPase which is (are) sensitive to micromolar concentrations of periodic acid and also critical to hydrolytic activity of the enzyme. It is possible that this effect is at an active site, acting directly, or at a distant site, acting allosterically. The actual sensitivity of the critical group(s) to periodic acid is masked by the presence of other polypeptides present in these microsomal preparations. Even if the enzyme were completely purified, however, other amino acid or sugar groups on the enzyme molecule could compete for periodic acid and falsely raise the concentration of periodate at which 50% inhibition occurs.

There is a striking specificity of this periodic acid oxidation effect on ($Na^+ + K^+$)-ATPase and K^+-*p*-nitrophenylphosphatase relative to the effect on Mg^{2+}-ATPase and Mg^{2+}-*p*-nitrophenylphosphatase activities, which were much less affected under the experimental conditions. This indicates that a residue (or residues) shared by both ($Na^+ + K^+$)-ATPase and K^+-*p*-nitrophenylphosphatase activities is (are) both relative sensitive to mild conditions of periodic acid oxidiation and critical to a specific hydrolytic function.

Recent evidence indicates that the larger chain of ($Na^+ + K^+$)-ATPase is a glycoprotein [12]. It is therefore tenable that oxidation of a sugar residue on the larger chain by periodic acid might be responsible for the inactivation of enzyme activity reported here. The amino acid residues on the ($Na^+ + K^+$)-ATPase molecule that have a known role near the catalytic site include tyrosine [13,14] and cysteine [15,16]. Further studies are required to identify the particular amino acid residue(s) affected by periodic acid. It is likely that there is more than one type of oxidizable residue that is sensitive to the concentrations of periodic acid used in these experiments.

It is unlikely that oxidation of sialic acid re-

sidues by periodic acid which would occur under these conditions is crucial to $(Na^+ + K^+)$-ATPase hydrolytic function since Perrone et al. [17] demonstrated no significant loss of hydrolytic activity after removal (by neuraminidase) of 37% and essentially 100% of the total sialic acids of $(Na^+ + K^+)$-ATPase preparations of rectal gland and electric organ, respectively. However, it remains possible that the presence of oxidized sialic acid residues is more inhibitory than absence of sialic acid residues.

The significance of the partial reversal by thiol agents of the periodic acid induced inactivation is not immediately apparent. Since some of the periodic acid inactivation is apparently not reversible (by dilution or by dithiothreitol and dimercaprol) it may be that multiple sites or families of sites are affected simultaneously.

It is possible that oxidation and subsequent reduction of residues of $(Na^+ + K^+)$-ATPase may play a physiological role in the regulation of hydrolytic function [5]. These results add further support to this possibility since such mild conditions of exposure to such a small amount of oxidant is shown to be a very effective and at least partially reversible means of inhibiting $(Na^+ + K^+)$-ATPase.

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