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Inhibition of Ca^{2+}-Pump ATPase and the Na^+/K^+-Pump ATPase by Iron-Generated Free Radicals

PROTECTION BY 6,7-DIMETHYL-2,4-DI-1-PYRROLIDINYL-7*H*-PYRROLO[2,3-*d*]PYRIMIDINE SULFATE (U-89843D), A POTENT, NOVEL, ANTIOXIDANT/FREE RADICAL SCAVENGER

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ABSTRACT. Preincubation of red blood cell (RBC) membranes with a model system known to generate reactive oxygen species (ROS) and free radicals (200 μM ferrous sulfate and 200 μM EDTA, Fe^{2+}/EDTA) resulted in inhibition of the Na^+/K^+-pump ATPase, the basal Ca^{2+}-pump ATPase, and the calmodulin-activated Ca^{2+}-pump ATPase. Inhibition of the ion pump ATPases was also associated with membrane protein cross-linking and lipid peroxidation, the latter as monitored by the formation of thiobarbituric acid reactive substances (TBARS). Inhibition of the ion transport ATPases, protein cross-linking and formation of TBARS were prevented by U-89843D in a concentration-dependent manner, with half-maximal protection seen at 0.3 μM. U-89843D was more potent than the classical antioxidant butylated hydroxytoluene. Neither U-89843D nor the solvent DMSO had any effect on the assay of TBARS. U-89843D exerted only minimal inhibitory activity on ATPase activities. Thus, U-89843D was potent *in vitro* in preventing a variety of membrane-damaging reactions mediated by ROS. It is suggested that protection of membranes from ROS-mediated damage is of potential usefulness in the prevention and treatment of certain disease processes. BIOCHEM PHARMACOL 51;4:471–476 , 1996.

KEY WORDS. calcium pump; sodium/potassium pump; free radical; membrane; lipid peroxidation; antioxidant

The involvement of ROS† in the pathophysiology of CNS trauma and ischemia/reperfusion is well documented [1–4]. In many cases, a major target for ROS is the lipid bilayer of living cells, resulting in lipid peroxidation [5]. End products produced during the lipid peroxidation process, including MDA, are very reactive, and capable of cross-linking membrane proteins containing amino groups [6]. The cross-linking of membrane proteins by MDA may lead to the inactivation of important membrane-spanning proteins including the ion transport ATPases. Ca^{2+} accumulates in damaged cells [7], and this may be a final common pathway in cell death [8–10]. Low intracellular Ca^{2+} is maintained by a CaM-activated Ca^{2+}-pump ATPase located in the plasma membrane (PMCA) [11–13], the endoplasmic reticulum Ca^{2+}-pump ATPase (SERCA) [14], and Na^+/Ca^{2+} exchange [15] powered indirectly via the Na^+/K^+-pump ATPase [16]. Inadvertent exposure of cells to ROS may lead to inhibition of the Na^+/K^+-pump [17] and the Ca^{2+} pump [18], altered ionic gradients, Ca^{2+} accumulation, and eventually cell death [19]. Therefore, development of drugs that can prevent the lipid peroxidation process from occurring during and/or following an ischemic insult may be useful in the treatment of CNS trauma and related disorders.

U74006F is currently in clinical trials for the treatment of CNS injury and subarachnoid hemorrhage. U74006F is a non-glucocorticoid, 21-aminosteroid [20] that has shown promising activity in experimental models of CNS ischemia/reperfusion [21–29]. U74006F is an antioxidant, and we, as well as others, have shown it to be an effective inhibitor of iron-dependent lipid peroxidation [20, 30, 31]. In the present work, we tested the effectiveness of a novel, free radical scavenger, U-89843D, using a model system of human RBC membranes and Fe^{2+}/EDTA previously described [30]. We found that low concentrations of U-89843D *in vitro* prevented lipid peroxidation and protected ion transport ATPases from free radical-induced damage.

MATERIALS AND METHODS

Vanadium-free ATP was purchased from Boehringer Mannheim (Indianapolis, IN). BHT, deferoxamine, ferrous sulfate, thiobarbituric acid, 1,1,3,3-tetraethoxypropane, EDTA, BSA, anti-mouse IgG (Fab specific) peroxidase conjugate, and non-

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† *Abbreviations:* BHT, butylated hydroxytoluene; CaM, calmodulin; LP, lipid peroxidation; MDA, malondialdehyde; RBC, red blood cell; ROS, reactive oxygen species including H_2O_2, superoxide radical and hydroxyl radical; TBARS, thiobarbituric acid reactive substances; U74006F, tirilazad mesylate; and U-89843D, 6,7-dimethyl-2,4-di-1-pyrrolidinyl-7*H*-pyrrolo[2,3-*d*]pyrimidine sulfate.

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fat dried milk were all purchased from the Sigma Chemical Co. (St. Louis, MO). Tetramethyl benzidine membrane peroxidase substrate (1 component) was purchased from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD). Polyvinylidine difluoride (PVDF) membranes (0.2 μM) were purchased from Novel Experimental Technology (San Diego, CA). Monoclonal (mouse) anti-plasma membrane Ca^{2+}-pump ATPase antibody (IgG2a) (clone 5F10) was a gift from Dr. John Penniston. U-89843D was provided by Dr. Edward Hall of the Upjohn Co. (Kalamazoo, MI). All chemicals were of the highest purity grade available.

Membrane Preparation

As previously described [30], isolated plasma membranes were prepared from outdated human RBCs. The membranes were given a final wash with 9 mL (per original 2.5 mL of packed RBCs) of 40 mM/histidine 40 mM/imidazole buffer, pH 7.1 (H/I). After removal of the supernate, an approximately equal volume of H/I was added to the white and apparently hemoglobin-free pellet. Membrane protein content was determined by the bicinchoninic acid method [32] using BSA as a standard. The membranes were stored on ice in the refrigerator until used.

Preincubation

To minimize free radical scavenging by H/I, membranes (0.5 to 1 mL) were first spun at 5000 *g* for 25 min in a Fisher 235B microcentrifuge, and the supernate was removed. Membranes were then resuspended in an equal volume of distilled water and immediately diluted in the preincubation medium. Specific activities in membranes suspended in water were comparable to those obtained using membranes suspended in buffer (data not shown). Other have used a saline solution to preincubate RBC membranes and have not reported any adverse effects from such treatment [33]. Unbuffered membranes were then added to reaction vessels with the following reagents: 500 μL of 1.8% NaCl, with or without ferrous sulfate/EDTA in nominally equimolar amounts, and other reagents as specified. When present, Fe^{2+}/EDTA was thus at a final concentration of 200 μM. Distilled water and 10 μL of DMSO, or drug dissolved in 10 μL of DMSO, were added to a final volume of 1 mL. U-89843D or BHT was dissolved in DMSO at a concentration of 10 mM. The stock solution was stored at −20°. On the day of an experiment, the stock solution was serially diluted in DMSO to achieve the desired drug concentration. The presence of 1% DMSO had no effect on any of the parameters monitored (data not shown). Protein concentration was 1 mg/mL and pH was 6.8 to 7.0. Incubations were carried out in a Dubnoff shaking incubator at 37°. At the conclusion of the preincubation period, 4 mM deferoxamine was added to "stop" the iron-mediated reactions.

TBARS Measurements

TBARS were assayed as previously described by Buege and Aust [34] with some modification. The absorbance of the supernate was read at 535 nm, and quantification was based upon a molar extinction coefficient of 1.21×10^5 mol · cm obtained from standard curves generated using 1,1,3,3-tetraethoxypropane.

ATPase Activities

ATPase activities were assayed by incubations carried out in triplicate in covered 96-well microtiter plates at 37° in a Dubnoff shaking incubator as previously described [35]. The buffer provided final concentrations of 18 mM histidine, 18 mM imidazole, 80 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, and 0.1 mM EGTA, pH 7.1. When added, CaM was 30 nM, $CaCl_2$ was 0.2 mM, and ouabain was 0.1 mM. All wells contained isolated human RBC membranes equivalent to approximately 0.75 μg of membrane protein. The reaction mixture, 90 μL, was preincubated for 10 min. Following this preincubation, the enzymatic ATPase reaction was initiated by the addition of 10 μL ATP (final concentration of 3.0 mM). ATPase activity was determined by a colorimetric method of Fiske and Subbarow [36] using a plate reader with an 810 nm filter.

SDS–PAGE

SDS–polyacrylamide slab gels were prepared according to the method of Laemmli [37], using an SE 250 mini-gel unit. A 10% polyacrylamide separating gel and a 4% polyacrylamide stacking gel were used for resolving proteins denatured with SDS. Gels were stained with Coomassie brilliant blue.

Western Blot Analysis

Western blot analysis was performed as previously described [38] with some modification. SDS–PAGE was performed (each lane being loaded with 20 μg of total membrane protein) using a 4% stacking gel and a 7.5% separating gel. Following electrophoresis, the wet slab gels were placed on PVDF filter sheets, and the proteins were electroblotted (100 V, 200–600 mA) for 2 hr at 4° in a transfer buffer containing 8.6 g glycine, 1.8 g Tris base, 1 g SDS, and 200 mL methanol in a final volume of 1 L. Blots were blocked with 3% (w/v) nonfat dry milk with 0.02% Tween 20. All washes were performed using PBS with 0.02% Tween 20. The first antibody, a monoclonal antibody to the plasma membrane Ca^{2+}-pump ATPase, was incubated with the blot overnight at 4°. Following four washes (5 min each), the second antibody was incubated with the blot for 1 hr with agitation at room temperature. This antibody consisted of horseradish peroxidase conjugated anti-mouse IgG (Sigma). Following a washing step, peroxidase staining was developed using tetramethyl benzidine (TMB) peroxidase substrate (component 1). The molecular weights of subsequent bands were ascertained using pre-stained SDS–PAGE standards (broad range, Bio-Rad).

RESULTS AND DISCUSSION

In this study, we tested the ability of a novel agent, U-89843D, to prevent iron-mediated LP, protein cross-linking, and inhi-

bition of the ion pump ATPases of RBC membranes. The ability of U-89843D to act as a functional antioxidant and/or free radical scavenger is based upon its structure, which is shown in Fig. 1. Based on the structure of U-89843D, one would predict that it is amphipathic. Therefore, in our model system, it presumably localized to a significant extent near the water/lipid interface of the cell membranes, an advantage for a compound that acts as an inhibitor of LP.

In a previous study in our laboratory, we showed that U74006F attenuated iron-mediated LP, protein cross-linking and inhibition of ion pump ATPases in isolated RBC membranes [30]. In that report, we found that although U74006F prevented iron-induced changes from occurring, it was not very potent [30]. Thus, our ability to test U74006F in this model system was limited due to the fact that it exhibited inhibitory activity on the ion pump ATPases at concentrations of greater than 10 μM.

In the present study, we first tested if U-89843D had any inherent inhibitory activity on the ion pump ATPases. As shown in Fig. 2, U-89843D did not inhibit either the Na^+/K^+- or the basal Ca^{2+}-pump ATPase at any concentration up to 100 μM. However, there was a slight inhibitory effect (10–20%) of U-89843D on the CaM-activated Ca^{2+}-pump ATPase at concentrations exceeding 1 μM (Fig. 2). The reason for this inhibition by U-89843D is unknown to us. It may represent an anti-CaM effect of U-89843D. Anti-CaM actions are exerted by a wide variety of amphipathic cations [39].

In the absence of Fe^{2+}/EDTA in the preincubation medium, activities were 5.89 ± 0.79, 12.0 ± 3.02, and 46.2 ± 6.23 nmol/min/mg membrane protein for the Na^+/K^+-, basal Ca^{2+}-, and CaM-activated Ca^{2+}-pump ATPase, respectively (N = 4, mean ± SEM). Preincubation of isolated RBC membranes in the presence of 200 μM Fe^{2+}/EDTA for 4 hr resulted in complete inhibition of the Na^+/K^+-, basal Ca^{2+}-, and the CaM-activated Ca^{2+}-pump ATPase (see Fig. 3, 0 μM U-89843D). Inhibition of the ion pump ATPases is thought to occur via an iron-generated superoxide-dependent mechanism as previously shown [30]. With the additional presence of U-89843D at concentrations exceeding 0.2 μM, inhibition of the ion pump ATPases by Fe^{2+}/EDTA was diminished. Half-maximal protection of ATPase activities was observed at approximately 0.3

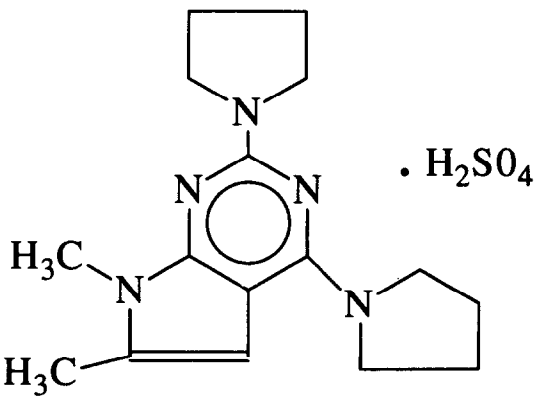

FIG. 1. Structure of 6,7-,dimethyl-2,4-di-1-pyrrolidinyl-7*H*)-pyrrolo[2,3-*d*]pyrimidine sulfate (U-89843D).

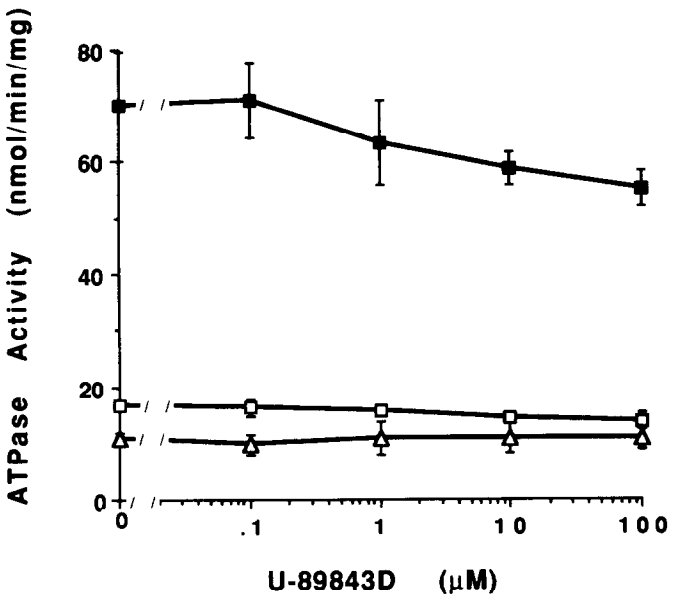

FIG. 2. Effect of U-89843D on ATPase activities. RBC membranes (1 mg protein/mL) were preincubated for 10 min without Fe^{2+}/EDTA in the presence of various concentrations of U-89843D. Following preincubation, activities for the Na^+/K^+-pump ATPase (△), basal Ca^{2+}-pump ATPase (□), and CaM-activated Ca^{2+}-pump ATPase (■) were determined. Data represent the mean ± SEM of 3 experiments.

μM U-89843D (Fig. 3). U-89843D (0.5 μM) provided greater than 88% protection of both the Na^+/K^+- and the basal Ca^{2+}-pump ATPase, and 84% protection of the CaM-activated Ca^{2+}-pump ATPase from iron-mediated inhibition. The slight, direct inhibitory effect of U-89843D on the CaM-acti-

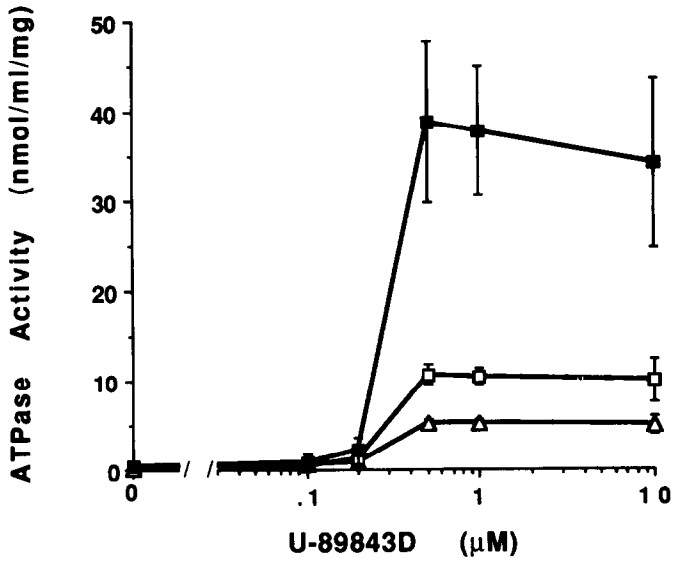

FIG. 3. Protection of ATPase activities by U-89843D. RBC membranes were preincubated with Fe^{2+}/EDTA (200 μM) and increasing concentrations of U-89843D. Following preincubation, ATPase activities were determined as in Fig. 2. Key: Na^+/K^+-pump ATPase (△), basal Ca^{2+}-pump ATPase (□), and the CaM-activated Ca^{2+}-pump ATPase (■). Data represent the means ± SEM of 4 experiments.

vated Ca^{2+}-pump ATPase (as seen in Figs. 2 and 3) may account for the peak activity being less than the respective control value.

U-89843D was also found to be very effective in preventing LP in isolated RBC membranes. TBARS formation, as promoted by Fe^{2+}/EDTA, was inhibited completely by U-89843D in a concentration-dependent fashion. Half-maximal protection was observed at 0.3 μM (Fig. 4), which corresponds to the IC_{50} for protecting the ion pump ATPases.

It may be pertinent to compare the potency of U-89843D with other known antioxidants. Our laboratory has shown previously that BHT, a typical antioxidant "gold standard," prevents TBARS formation initiated by Fe^{2+}/EDTA in RBC membranes with half-maximal protection at approximately 0.5 μM [40]. In addition, previous studies have shown that U74006F prevents iron-mediated LP in rat brain homogenates with calculated IC_{50} values between 8 and 59 μM [20, 41]. Using the same model system, Hall *et al.* [41] demonstrated that a second generation aminosteroid, U78517F, prevents iron-mediated LP with an IC_{50} of 0.6 μM. Taken together, these results indicate that U-89843D was 1.7 times as potent as BHT, twice as potent as U78517F, and roughly 27 times more potent than U74006F.

An interesting feature of U-89843D in our model system, was the exceedingly steep concentration–effect curves obtained for both protecting the ion pump ATPases and preventing TBARS formation (Figs. 3 and 4). In this regard, U-89843D showed a dynamic range between 0.2 and 0.5 μM. The reasons for the steep concentration–effect relationship of U-89843D are unknown to us, but may reflect the conditions of our assay. For example, no added soluble protein carrier was included in the preincubation mixture. Being amphipathic, it is possible that the effects of U-89843D occurred only after it reached a critical micelle concentration where it no longer associated with water (or DMSO), but instead partitioned into the lipid phase of the RBC membrane.

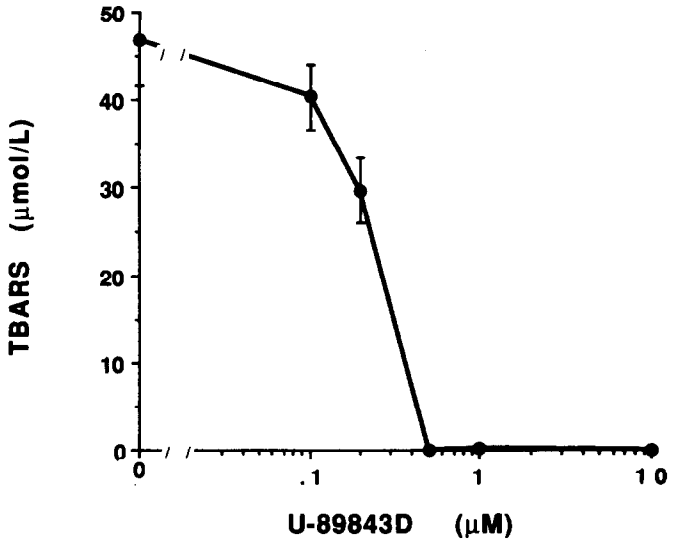

FIG. 4. Inhibition of lipid peroxidation by U-89843D. RBC membranes were preincubated for 4 hr in the presence of 200 μM Fe^{2+}/EDTA and increasing concentrations of U-89843D, as in Fig. 2. Following preincubation, TBARS were determined as described in Materials and Methods. Data represent the means ± SD of 4 experiments.

As demonstrated previously, there appears to be a good correlation between LP and inhibition of ion pump ATPases using chelated iron [30]. One end product of LP is MDA [42], which is capable of cross-linking membrane components containing amino groups [6]. We have hypothesized that one potential mechanism for iron-mediated damage of the Ca^{2+}-pump ATPase in this model system is through cross-linking [43] by MDA. We have shown that preincubation of RBC membranes with Fe^{2+}/EDTA resulted in non-specific cross-linking of RBC membrane proteins that was diminished in the additional presence of 10 μM U74006F [30].

In the present study, we tested the ability of U-89843D to prevent membrane protein cross-linking and compared its effects with those of BHT. Fe^{2+}/EDTA promoted cross-linking of membrane proteins, as shown in Fig. 5A. SDS-PAGE revealed the presence of a high molecular weight, proteinaceous material (lane 2), in RBC membranes preincubated with Fe^{2+}/EDTA. The appearance of this cross-linked material with molecular weight sufficiently high that it barely entered the stacking gel was also associated with the general loss of known RBC proteins (Fig. 5A). However, in the additional presence of 0.5 μM U-89843D (lane 3), membrane protein cross-linking was prevented almost completely. By contrast, BHT (0.5 μM) provided little or no protection from iron-induced protein cross-linking (Fig. 5A, compare lanes 3 and 4).

Data presented in Fig. 5A do not provide direct evidence that the Ca^{2+}-pump ATPase was cross-linked, since it is present in insufficient amounts to be visualized by Coomassie staining of SDS gels. Therefore, to determine possible cross-linking of the Ca^{2+}-pump ATPase, western blot analysis was performed. Figure 5B shows the results of such an experiment, with representative lanes being identical to those presented in Fig. 5A. A major band running at 140 kDa was recognized by the monoclonal antibody in the control (lane 1) in which RBC membranes were preincubated alone. This band represents the Ca^{2+}-pump ATPase. In addition to this band at 140 kDa, there were two additional minor bands running at approximately 200 kDa, which most likely represent natural aggregation products of the Ca^{2+}-pump ATPase. Fe^{2+}/EDTA promoted cross-linking of the Ca^{2+}-pump ATPase. Compared with control membranes, membranes exposed to Fe^{2+}/EDTA demonstrated a loss in the band representing the Ca^{2+}-pump ATPase, with the appearance of a high molecular weight complex at the top of the stacking gel, as well as at the interface between the stacking and separating gels (Fig. 5B, lane 2). However, in the additional presence of U-89843D (0.5 μM), cross-linking of the enzyme was prevented completely (lane 3), whereas BHT, at the same concentration, provided little protection from iron-induced cross-linking of the Ca^{2+}-pump ATPase (lane 4).

Taken together, these data demonstrate that U-89843D protected ion pump ATPases from iron-mediated inhibition, presumably by limiting the degree of LP and levels of LP by-products, including MDA, capable of cross-linking mem-

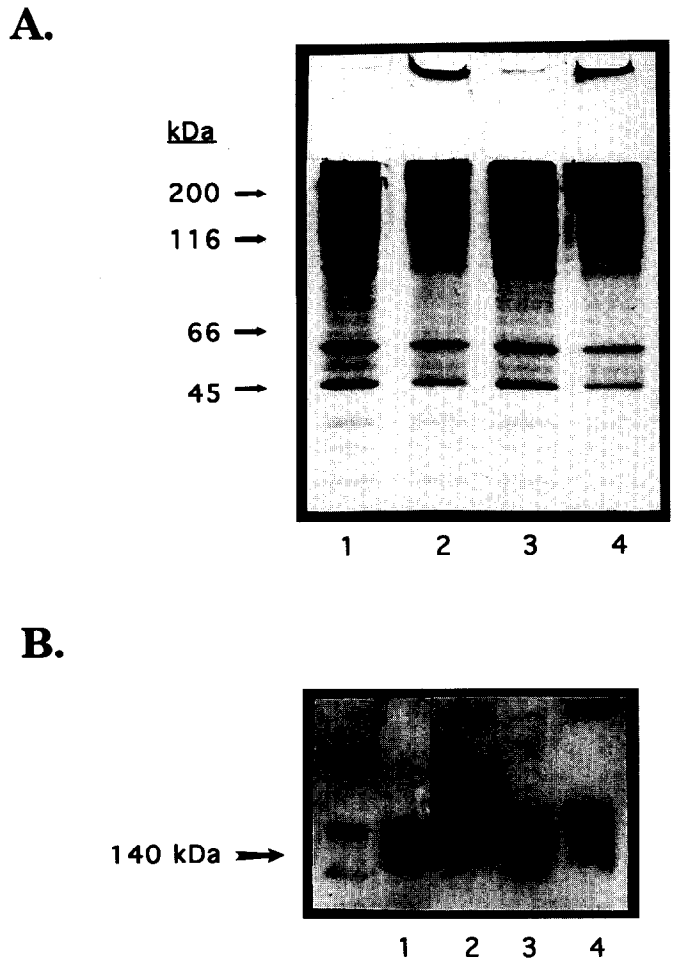

FIG. 5. SDS–PAGE and western blot analysis of RBC membranes incubated in the presence of Fe^{2+}/EDTA alone or in the presence of Fe^{2+}/EDTA and 0.5 U-89843D or BHT. Following preincubation, RBC membrane proteins from each reaction were analyzed on an SDS-gel (panel A), or in separate experiments, run on SDS-gel, transferred to PVDF filters, and then incubated with a monoclonal antibody to the Ca^{2+}-pump ATPase (panel B). See Materials and Methods for details. Samples are identical for both panels A and B and are: lane 1, control, RBC membranes only; lane 2, membranes plus Fe^{2+}/EDTA; lane 3, membranes plus Fe^{2+}/EDTA and U-89843D; and lane 4, membranes plus Fe^{2+}/EDTA and BHT.

brane proteins. In addition, our data demonstrate that exposure of RBC membranes to Fe^{2+}/EDTA-generated ROS can result in the direct modification of the Ca^{2+}-pump ATPase, which contributes to the inhibition of the enzyme as seen in ATPase assays. This result in itself is important considering that inhibition of the Ca^{2+}-pump ATPase *in vivo* may lead to increased Ca^{2+} in the cell. Increased Ca^{2+} has perilous results that contribute to the pathophysiology seen in CNS trauma and ischemia/reperfusion, among a host of other conditions [44]. Therefore, protection afforded by U-89843D of the plasma membrane ion transport ATPases may help ensure that extrusion of Ca^{2+} from Ca^{2+}-loaded cells occurs following restoration of circulation in compromised tissues.

In summary, recent research has demonstrated a role for ROS in numerous pathological conditions, including CNS trauma and ischemia/reperfusion. Aminosteroids, that have antioxidant activity, such as U74006F, may be useful for the early treatment of certain of these CNS disorders. We have found previously that U74006F protects ion transport ATPase activities and inhibits lipid peroxidation in isolated RBC membranes. U-89843D appears to have similar properties. We found that U-89843D is a potent *in vitro* inhibitor of iron-induced LP and membrane protein cross-linking. U-89843D protected ion pump ATPase activities from iron-mediated inhibition at nanomolar concentrations. Thus, U-89843D is a potent and efficacious antioxidant with potential usefulness to protect biological membranes from ROS-mediated damage.

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