Role of an aprotinin-sensitive protease in the activation of Ca^{2+} -ATPase by superoxide radical ($\text{O}_2^{\cdot-}$) in microsomes of pulmonary vascular smooth muscle

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We have investigated the role of an aprotinin-sensitive protease in regulating Ca^{2+} -ATPase activity and Ca^{2+} uptake (ATP-dependent and Na^+ -dependent) in microsomes of bovine pulmonary vascular smooth muscle during treatment with the $\text{O}_2^{\cdot-}$ -generating system hypoxanthine plus xanthine oxidase. Treatment of the smooth muscle microsomes with the $\text{O}_2^{\cdot-}$ -generating system produced a protease in a gelatin-containing zymogram with an apparent molecular mass of 16 kDa. This 16 kDa proteolytic protein was found to be inhibited by superoxide dismutase (SOD) and aprotinin but not by PMSF. Using polyclonal antiserum to aprotinin, we found that it is an ambient antiprotease of the smooth muscle microsomes. Treatment of the microsomes with the $\text{O}_2^{\cdot-}$ -generating system stimulated protease activity tested with a synthetic substrate *N*-benzoyl-DL-arginine *p*-nitroanilide and also enhanced Ca^{2+} -ATPase activity. It also

stimulated ATP-dependent Ca^{2+} uptake. In contrast, Na^+ -dependent Ca^{2+} uptake was found to be inhibited by the $\text{O}_2^{\cdot-}$ -generating system. Pretreatment of the microsomes with SOD and aprotinin preserved the increase in protease activity, Ca^{2+} -ATPase activity and ATP-dependent Ca^{2+} uptake. In addition, $\text{O}_2^{\cdot-}$ -caused inhibition of the Na^+ -dependent Ca^{2+} uptake which was reversed by SOD and aprotinin. Pretreatment with PMSF did not cause any discernible alteration in the protease activity, Ca^{2+} -ATPase activity, ATP-dependent Ca^{2+} uptake and Na^+ -dependent Ca^{2+} uptake in the microsomes caused by the $\text{O}_2^{\cdot-}$ -generating system. These results suggest that an aprotinin-sensitive protease plays a pivotal role in regulating Ca^{2+} -ATPase and Ca^{2+} -uptake activities in microsomes of pulmonary vascular smooth muscle under oxidant $\text{O}_2^{\cdot-}$ -triggered conditions.

INTRODUCTION

Ca^{2+} plays the role of a second messenger in many biochemical and physiological events [1–3]. An increase in Ca^{2+} levels *in situ* caused by a variety of agonists is due to an influx of extracellular Ca^{2+} and/or release of Ca^{2+} from its suborganelle stores [3–5]. We have previously indicated that oxidants such as H_2O_2 and *t*-butyl hydroperoxide stimulate release of Ca^{2+} from suborganelles of smooth muscle and endothelium of pulmonary vasculature [3,4]. Microsomes sequester a large portion of mobilizable Ca^{2+} and play a pivotal role in regulating Ca^{2+} dynamics in different cells and tissues [6–8]. In many systems, Ca^{2+} -ATPases provide a major line of defence by counteracting agonist-induced increases in Ca^{2+} levels *in situ* [9–12].

Ca^{2+} -ATPases have been shown to be activated by a variety of proteases in different systems [13,14]. Activation of proteases under stimulated conditions has been shown to inactivate ambient protease inhibitors resulting in an alteration of the protease–antiprotease balance in favour of proteases [15,16].

In isolated lung, infusion of the superoxide ($\text{O}_2^{\cdot-}$)-generating system hypoxanthine plus xanthine oxidase has been shown to produce pulmonary hypertension [17]. Previous research has provided evidence that the occurrence of Ca^{2+} overload caused by the oxidant *t*-butyl hydroperoxide in isolated lung plays a critical role in pulmonary hypertension [18]. Pulmonary hypertension often causes substantial morbidity and mortality

[19,20]. Because of inadequate understanding of the regulation of Ca^{2+} dynamics under $\text{O}_2^{\cdot-}$ -triggered conditions in pulmonary smooth muscle, it is of interest to investigate the role that $\text{O}_2^{\cdot-}$ plays in Ca^{2+} -ATPase activity and Ca^{2+} -uptake in microsomes of bovine pulmonary vascular smooth muscle and to study the involvement of a protease in this phenomenon. We asked the following questions. Does the $\text{O}_2^{\cdot-}$ -generating system hypoxanthine plus xanthine oxidase stimulate Ca^{2+} -ATPase activity in microsomes of bovine pulmonary artery smooth muscle? Does pretreatment with the protease inhibitor aprotinin prevent $\text{O}_2^{\cdot-}$ stimulation of the Ca^{2+} -ATPase activity? Does treatment of the microsomes with the $\text{O}_2^{\cdot-}$ -generating system stimulate an aprotinin-sensitive protease activity? What role does aprotinin play in ATP-dependent and Na^+ -dependent Ca^{2+} uptake in microsomes of the smooth muscle under basal and $\text{O}_2^{\cdot-}$ -triggered conditions? Our results indicate that an aprotinin-sensitive protease plays a crucial role in regulating Ca^{2+} -ATPase and Ca^{2+} uptake in microsomes of bovine pulmonary artery smooth muscle during treatment with the $\text{O}_2^{\cdot-}$ -generating system.

MATERIALS AND METHODS

Materials

Hypoxanthine, aprotinin, gelatin, Tris/ATP, EGTA, PMSF, BSA, *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), molecu-

Abbreviations used: $\text{O}_2^{\cdot-}$, superoxide radical; HBPS, Hanks buffered physiological saline; BAPNA, *N*-benzoyl-DL-arginine *p*-nitroanilide; SOD, superoxide dismutase.

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lar-mass markers, superoxide dismutase (SOD), LaCl_3 and CaCl_2 were obtained from Sigma Chemical Co. Xanthine oxidase was purchased from Boehringer-Mannheim. $^{45}\text{Ca}^{2+}$ was from ICN. All other chemicals used were of analytical grade.

Isolation of bovine pulmonary artery smooth muscle tissue

Bovine pulmonary artery collected from a slaughterhouse was washed several times with Hanks buffered physiological saline (HBPS) (pH 7.4). The washed pulmonary artery was used for further processing within 2 h of collection. The intimal and external portions were removed and the tunica media, i.e. the smooth muscle tissue, was collected and used for the present studies.

Preparation of smooth muscle tissue microsomes

The smooth muscle tissue was homogenized with a cyclomixer in ice-cold medium containing 0.25 M sucrose and 10 mM Tris/HCl buffer, pH 7.4. The homogenate was centrifuged at 600 *g* for 15 min at 4 °C. The resulting supernatant was centrifuged at 15000 *g* for 20 min to sediment mitochondria and lysosomes [21]. The supernatant was centrifuged at 100000 *g* for 1 h. The pellet was suspended in 10 % (w/v) sucrose containing 10 mM Tris/HCl buffer, pH 7.4, and was layered on a discontinuous gradient consisting of 40 % (w/v) and 20 % (w/v) sucrose both containing 10 mM Tris/HCl buffer, pH 7.4. The gradient was centrifuged at 105000 *g* for 2 h. The fraction collected at the 20–40 % sucrose interface was used as the plasma membrane [22] and the pellet was used as the microsomal fraction. The microsomal pellet was suspended in homogenizing buffer. All operations were carried out at 4 °C. Microsomes were stored under liquid N_2 and thawed before use.

Assay of rotenone-insensitive NADPH–cytochrome *c* reductase activity

Rotenone-insensitive NADPH–cytochrome *c* reductase activity was measured as previously described [23].

Assay of cytochrome *c* oxidase activity

Cytochrome *c* oxidase was assayed by the procedure of Wharton and Tzagoloff [24].

Assay of acid phosphatase activity

Acid phosphatase activity was determined at pH 5.5 using *p*-nitrophenyl phosphate as the substrate [25].

Assay of 5'-nucleotidase activity

Release of P_i from 5'-AMP, an index of 5'-nucleotidase activity, was determined by the method of Chen et al. [26].

Assay of protease activity

Protease activity was assessed by determining the hydrolysis of the synthetic substrate BAPNA as previously described [27]. Briefly, 43.5 mg of BAPNA was dissolved in 1 ml of DMSO and the solution was brought to 100 ml with 0.05 M Tris/HCl buffer, pH 8.0, containing 0.02 M CaCl_2 . Then 4.9 ml of the substrate stock solution was allowed to equilibrate in a thermostatically controlled water bath at 25 °C for 5 min. The reaction was initiated by adding 0.1 ml of bovine pulmonary artery smooth

muscle microsomal suspension (50 μg of protein) and the reaction was run for 30 min. Subsequently, 1 ml of 30 % acetic acid was added to terminate the reaction and the quantity of *p*-nitroanilide was estimated spectrophotometrically at 410 nm. A blank without enzyme activity was included to monitor spontaneous hydrolysis of the substrate.

The smooth muscle microsomal suspension was treated with an $\text{O}_2^{\cdot-}$ -generating system (2 mM hypoxanthine plus 0.03 unit/ml xanthine oxidase) for 30 min, and protease activity was measured.

Aprotinin (1 mg/ml), SOD (80 μg /ml) and PMSF (1 mM) with or without aprotinin (1 mg/ml) were added to the microsomal suspension for 15 min and this was followed by treatment with the $\text{O}_2^{\cdot-}$ -generating system for 30 min; protease activity was then measured.

Zymogram of protease activity

Polyacrylamide gels (12 %; 0.75 mm thick) were cast containing 0.1 % gelatin. Gelatin solution was made up as a 2 % stock solution in distilled water and dissolved by heating. Bovine pulmonary artery smooth muscle microsomal suspension (50 μg of protein) was applied to the gel in standard SDS-loading buffer containing 0.1 % SDS but lacking 2-mercaptoethanol; it was not boiled before loading. The gels were run at 200 V for 1 h in a Bio-Rad Mini protean II apparatus and then soaked in 200 ml of 2 % Triton X-100 in distilled water in a shaker for 1 h at 20 °C. Next, the gels were soaked in reaction buffer (100 mM Tris/HCl, pH 8.0) for 12 h at 37 °C and then stained with Coomassie Brilliant Blue, and this was followed by destaining in methanol/acetic acid/water (5:5:1, by vol.) [28].

The smooth muscle microsomal suspension was treated with the $\text{O}_2^{\cdot-}$ -generating system for 30 min and a zymogram protease assay was performed.

Aprotinin (1 mg/ml), SOD (80 μg /ml) and PMSF (1 mM) with or without aprotinin (1 mg/ml) were added to the microsomal suspension for 15 min and this was followed by treatment with the $\text{O}_2^{\cdot-}$ -generating system for 30 min; a zymogram protease assay was performed.

Assay of Ca^{2+} -ATPase activity

Ca^{2+} -ATPase activity was determined colorimetrically by measuring Ca^{2+} -dependent release of P_i [29]. Briefly, the assay mixture contained (in a total volume of 1 ml): 100 mM Tris/HCl buffer, pH 7.4, 200 nmol of EGTA with or without CaCl_2 and 0.1 mmol of Tris/ATP. The reaction was initiated by the addition of 20 μl of bovine pulmonary artery smooth muscle microsomal suspension (50 μg of protein). CaCl_2 concentration was varied to give the required free Ca^{2+} concentrations as described by Pershadsingh and McDonald [30] based on the experimental value obtained earlier by Sillen and Martell [31]. The amount of contaminating Ca^{2+} was determined by atomic absorption spectrometry to be 5–8 μM . This value was included in the calculations of free Ca^{2+} . After 30 min of incubation, the reaction was terminated by the addition of 20 % trichloroacetic acid. After removal of the precipitate, released P_i was measured by the method of Lowry and Lopez [32]. Ca^{2+} -ATPase activity was determined by subtracting values obtained with chelator alone from those obtained with Ca^{2+} plus chelator.

Bovine pulmonary artery smooth muscle microsomal suspension was treated with the $\text{O}_2^{\cdot-}$ -generating system for 30 min, and Ca^{2+} -ATPase activity was determined.

Aprotinin (1 mg/ml), SOD (80 μg /ml) and PMSF (1 mM) with or without aprotinin (1 mg/ml) were added to the smooth

muscle microsomal suspension for 15 min and this was followed by treatment with the $\text{O}_2^{\cdot-}$ -generating system for 30 min; Ca^{2+} -ATPase activity was determined.

Determination of ATP-dependent Ca^{2+} uptake

ATP-dependent Ca^{2+} accumulation was measured as previously described [33]. Briefly, aprotinin (1 mg/ml), SOD (80 $\mu\text{g}/\text{ml}$) and PMSF (1 mM) with or without aprotinin (1 mg/ml) were added to bovine pulmonary artery smooth muscle microsomes (50 μg of protein) at 37 °C for 15 min in 0.5 ml of medium containing 140 mM KCl, 10 mM Mops/Tris buffer, pH 7.4 and 2 mM MgCl_2 , and this was followed by treatment with the $\text{O}_2^{\cdot-}$ -generating system for 30 min. Then 40 μM $^{45}\text{Ca}^{2+}$ (0.6–0.8 Ci/mmol) and 50 μM CaCl_2 were added, and Ca^{2+} uptake was initiated by adding 4 mM Tris/ATP. After 30 min of incubation at 37 °C, 250 μl aliquots were immediately filtered through Millipore filters (0.45 μM pore size), and radioactivity in the filters was determined as previously described [33].

Determination of Na^+ -dependent Ca^{2+} uptake

Na^+ -dependent Ca^{2+} -uptake measurements were carried out as previously described [34]. Briefly, aprotinin (1 mg/ml), SOD (80 $\mu\text{g}/\text{ml}$) and PMSF (1 mM) with or without aprotinin were added to bovine pulmonary artery smooth muscle tissue microsomes (50 μg of protein) and preincubated at 37 °C for 15 min in medium containing 140 mM NaCl and 20 mM Mops/Tris buffer, pH 7.4. This was followed by treatment with the $\text{O}_2^{\cdot-}$ -generating system for 30 min. Aliquots (0.5 ml) of the Na^+ -loaded vesicles with or without the stated agents were added to a series of tubes containing 15 ml of medium consisting 140 mM KCl buffered with 20 mM Mops/Tris buffer, pH 7.4, plus 40 mM $^{45}\text{Ca}^{2+}$ (0.6–0.8 Ci/mmol) and 50 μM CaCl_2 for 30 min. Ca^{2+} uptake was terminated by adding 5 ml of ice-cold stopping solution (140 mM KCl, 1 mM LaCl_3 , 20 mM Mops/Tris buffer, pH 7.4). Aliquots (250 μl) were filtered through Millipore filters (0.45 μm pore size), and radioactivity in the filters was determined as previously described [33].

Identification of aprotinin in the smooth muscle microsomes

Aprotinin was identified in the smooth muscle microsomes by immunodiffusion with polyclonal antiserum to aprotinin [35].

Determination of protein

Protein was measured by the method of Lowry et al. [36] with BSA as standard.

Statistical analysis

Data were analysed by unpaired *t* test and analysis of variance followed by the test of least significant difference [37] for comparisons within and between the groups.

RESULTS

Treatment of the smooth muscle microsomes with the $\text{O}_2^{\cdot-}$ -generating system produced several proteolytic activities in the gelatin-containing zymogram, with molecular masses ranging between 16 and 80 kDa (Figure 1, lane 2). Pretreatment of the microsomal suspension with SOD prevented the appearance of all of the proteolytic activities (Figure 1, lane 4). Pretreatment with PMSF resulted in one band with proteolytic activity (Figure 1, lane 3) with an apparent molecular mass of 16 kDa. Pre-

Figure 1 Effect of pretreatment with PMSF, aprotinin and SOD on protease activity caused by the $\text{O}_2^{\cdot-}$ -generating system hypoxanthine plus xanthine oxidase in microsomes of bovine pulmonary artery smooth muscle

Lane 1, untreated (control) microsomes; lane 2, microsomes treated with the $\text{O}_2^{\cdot-}$ -generating system for 30 min; lane 3, microsomes pretreated with PMSF (1 mM) for 15 min followed by treatment with the $\text{O}_2^{\cdot-}$ -generating system for 30 min; lane 4, microsomes pretreated with SOD (80 $\mu\text{g}/\text{ml}$) for 15 min followed by treatment with the $\text{O}_2^{\cdot-}$ -generating system for 30 min; lane 5, microsomes pretreated with aprotinin (1 mg/ml) for 15 min followed by treatment with the $\text{O}_2^{\cdot-}$ -generating system for 30 min; lane 6, microsomes pretreated with PMSF (1 mM) in combination with aprotinin (1 mg/ml) for 15 min followed by treatment with the $\text{O}_2^{\cdot-}$ -generating system for 30 min.

treatment with aprotinin prevented the 16 kDa proteolytic activity (Figure 1, lane 5), although other proteolytic activities appeared to be uninhibited. In contrast, pretreatment with PMSF in combination with aprotinin prevented all of the proteolytic activities caused by the $\text{O}_2^{\cdot-}$ -generating system (Figure 1, lane 6).

We characterized the microsomal fraction by measuring at different steps in the preparation process the activities of cytochrome *c* oxidase (a mitochondrial marker [38]), acid phosphatase (a lysosomal marker [39]), NADPH–cytochrome *c* reductase (a microsomal marker [40]) and 5'-nucleotidase (a plasma-membrane marker [39]). Compared with the 600–15000 *g* pellet and plasma-membrane fractions, the microsomal fraction showed respectively 14-fold and 24-fold increases in the specific activity of the rotenone-insensitive NADPH–cytochrome *c* reductase (Table 1). The microsomal fraction showed a 26-fold decrease in the specific activity of cytochrome *c* oxidase compared with the 600–15000 *g* pellet, a 16-fold decrease in the specific activity of acid phosphatase compared with the 600–15000 *g* pellet and a 26-fold decrease in the specific activity of 5'-nucleotidase compared with plasma-membrane fraction (Table 1).

The immunodiffusion study of the smooth muscle microsomal suspension with polyclonal aprotinin antiserum revealed that aprotinin is an ambient antiprotease of the smooth muscle microsomes (Figure 2).

Using BAPNA as a synthetic substrate for the protease, we found that treatment of the smooth muscle microsomes with the $\text{O}_2^{\cdot-}$ -generating system stimulated hydrolysis of the substrate (Table 2). Treatment of the microsomes with the $\text{O}_2^{\cdot-}$ -generating system also stimulated Ca^{2+} -ATPase activity (Table 2). Pretreatment of the microsomes with SOD markedly reduced the $\text{O}_2^{\cdot-}$ -stimulation of Ca^{2+} -ATPase activity and the protease activity (Table 2). Pretreatment of the smooth muscle microsomal suspension with PMSF did not cause any discernible alteration in $\text{O}_2^{\cdot-}$ -stimulated protease or Ca^{2+} -ATPase activity (Table 2). In contrast, pretreatment with aprotinin prevented the $\text{O}_2^{\cdot-}$ -stimulated protease and Ca^{2+} -ATPase activity (Table 2). The changes

Table 1 Specific activities of cytochrome *c* oxidase, acid phosphatase, rotenone-insensitive NADPH–cytochrome *c* reductase and 5′-nucleotidase at different steps in the preparation of microsomes

Cytochrome *c* oxidase activity is expressed as μmol of cytochrome *c* utilized/30 min per mg of protein. Acid phosphatase activity is expressed as μmol of *p*-nitrophenol/30 min per mg of protein. NADPH–cytochrome *c* reductase (rotenone-insensitive) activity is expressed as reduction of cytochrome *c* at 550 nm/30 min per mg of protein. 5′-Nucleotidase activity is expressed as μmol of P_i/30 min per mg of protein. Values are presented as means $\pm$ S.E.M. ($n = 4$). The values in parentheses indicate the activity as a percentage of that of the 600–15 000 *g* pellet (values of the 600–15 000 *g* pellet are set at 100%).

Fraction	Cytochrome <i>c</i> oxidase	Acid phosphatase	Rotenone-insensitive NADPH–cytochrome <i>c</i> reductase	5′-Nucleotidase
600–15 000 <i>g</i> pellet	3.16 $\pm$ 0.18	3.65 $\pm$ 0.22	0.29 $\pm$ 0.01	0.17 $\pm$ 0.01
15 000–100 000 <i>g</i> pellet	0.32 $\pm$ 0.02	0.44 $\pm$ 0.03	3.12 $\pm$ 0.20	2.02 $\pm$ 0.14
	(10)	(12)	(1075)	(1188)
Microsomes	0.12 $\pm$ 0.01	0.22 $\pm$ 0.01	4.04 $\pm$ 0.28	0.11 $\pm$ 0.10
	(4)	(6)	(1393)	(65)
Plasma membrane	0.15 $\pm$ 0.01	0.36 $\pm$ 0.02	0.17 $\pm$ 0.01	2.85 $\pm$ 0.19
	(5)	(10)	(58)	(1676)

Figure 2 Immunodiffusion for the determination of the presence of aprotinin in bovine pulmonary vascular smooth muscle microsomes using aprotinin antiserum raised in rabbit against purified bovine lung aprotinin

Centre well, aprotinin antiserum; A, homogenate; B, microsomes; C, control serum; D, standard aprotinin.

in the smooth muscle microsomal Ca^{2+} -ATPase activity correlate directly with the changes in protease activity (Figure 3).

Treatment of the smooth muscle microsomes with the $\text{O}_2^{\cdot-}$ -generating system stimulated ATP-dependent Ca^{2+} uptake whereas Na^+ -dependent Ca^{2+} uptake was found to be inhibited (Table 3). Pretreatment with SOD and aprotinin prevented the stimulation of ATP-dependent Ca^{2+} uptake, and the inhibition of Na^+ -dependent Ca^{2+} uptake was found to be reversed (Table 3). Total Ca^{2+} uptake (ATP- and Na^+ -dependent) during treatment of the microsomes with the $\text{O}_2^{\cdot-}$ -generating system was found to

Figure 3 Regression analysis of Ca^{2+} -ATPase activity (y) and protease activity (x) based on the observed data (Table 2)

be decreased compared with that under basal conditions (153.54 and 218.18 nmol of $^{45}\text{Ca}^{2+}$ /30 min per mg of protein respectively; percentage change from basal value: -30) (Table 3).

Table 2 Effect of SOD, PMSF and aprotinin on protease activity (measured by hydrolysis of BAPNA) and Ca^{2+} -ATPase activity caused by the $\text{O}_2^{\cdot-}$ -generating system in microsomes of bovine pulmonary vascular smooth muscle

Results are means $\pm$ S.E.M. ($n = 4$). ^a $P < 0.001$ compared with basal condition; ^b $P < 0.001$ compared with treatment with the $\text{O}_2^{\cdot-}$ -generating system alone; ^c $P < 0.01$ compared with basal condition; ^d $P < 0.05$ compared with basal condition; ^e $P < 0.001$ compared with respective control. ^f $P < 0.001$ compared with respective control.

Treatment	Protease activity (ΔA_{410} /30 min per mg of protein)	Change (% versus basal value)	Ca^{2+} -ATPase activity (μmol of P _i /30 min per mg of protein)	Change (% versus basal value)
Basal	0.40 $\pm$ 0.04		2.96 $\pm$ 0.20	
$\text{O}_2^{\cdot-}$ -generating system	2.23 $\pm$ 0.22 ^a	+ 460	8.68 $\pm$ 0.44 ^a	+ 193
SOD (80 $\mu\text{g}/\text{ml}$)	0.34 $\pm$ 0.03	+ 15	2.82 $\pm$ 0.18	− 5
SOD (80 $\mu\text{g}/\text{ml}$) + $\text{O}_2^{\cdot-}$ -generating system	0.48 $\pm$ 0.05 ^b	+ 20	3.16 $\pm$ 0.22 ^b	+ 7
PMSF (1 mM)	0.38 $\pm$ 0.04	− 5	2.84 $\pm$ 0.19	− 4
PMSF (1 mM) + $\text{O}_2^{\cdot-}$ -generating system	2.22 $\pm$ 0.18 ^a	+ 455	8.42 $\pm$ 0.42 ^a	+ 184
Aprotinin (1 mg/ml)	0.22 $\pm$ 0.02 ^c	− 45	2.25 $\pm$ 0.14 ^d	− 24
Aprotinin (1 mg/ml) + $\text{O}_2^{\cdot-}$ -generating system	0.62 $\pm$ 0.05 ^{be}	+ 55	3.62 $\pm$ 0.31 ^{bf}	+ 22

Table 3 Effect of SOD, PMSF and aprotinin on ATP-dependent and Na^{+} -dependent Ca^{2+} uptake caused by the $\text{O}_2^{\cdot-}$ -generating system in microsomes of bovine pulmonary vascular smooth muscle

Results are means $\pm$ S.E.M. ($n = 4$). ^a $P < 0.001$ compared with basal condition; ^b $P < 0.001$ compared to treatment with the $\text{O}_2^{\cdot-}$ -generating system alone; ^c $P < 0.01$ compared with basal condition; ^d $P < 0.001$ compared with respective control.

Treatment	ATP-dependent $^{45}\text{Ca}^{2+}$ uptake (nmol/30 min per mg of protein)	Change (% versus basal value)	Na^{+} -dependent $^{45}\text{Ca}^{2+}$ uptake (nmol/30 min per mg of protein)	Change (% versus basal value)
Basal	72.92 $\pm$ 2.56		145.26 $\pm$ 3.24	
$\text{O}_2^{\cdot-}$ -generating system	107.26 $\pm$ 2.78 ^a	+ 47	46.28 $\pm$ 2.06 ^a	- 68
SOD (80 $\mu\text{g}/\text{ml}$)	68.48 $\pm$ 2.76	- 6	150.44 $\pm$ 3.72	+ 3
SOD (80 $\mu\text{g}/\text{ml}$) + $\text{O}_2^{\cdot-}$ -generating system	74.24 $\pm$ 2.88 ^b	+ 2	142.15 $\pm$ 3.34 ^b	- 2
PMSF (1 mM)	70.86 $\pm$ 2.44	- 2	146.98 $\pm$ 3.42	+ 1
PMSF (1 mM) + $\text{O}_2^{\cdot-}$ -generating system	105.94 $\pm$ 2.62 ^a	+ 45	47.82 $\pm$ 2.22 ^a	- 67
Aprotinin (1 mg/ml)	62.88 $\pm$ 2.04 ^c	- 14	158.42 $\pm$ 3.78 ^c	+ 12
Aprotinin (1 mg/ml) + $\text{O}_2^{\cdot-}$ -generating system	83.45 $\pm$ 2.12 ^{b,d}	+ 14	131.56 $\pm$ 2.35 ^{b,d}	- 9

DISCUSSION

Our results indicate that treatment of bovine pulmonary artery smooth muscle microsomes with the $\text{O}_2^{\cdot-}$ -generating system hypoxanthine plus xanthine oxidase causes stimulation of Ca^{2+} -ATPase activity (Table 2). This is apparently in contrast with previous observations that oxidants depress Ca^{2+} -pump activity in sarcoendoplasmic reticulum [41] and heart sarcolemma [33]. The differences between these studies and our present observations using pulmonary smooth muscle microsomes may possibly be explained by considering the differences in biochemical characteristics and functional responsiveness of pulmonary vessels and other vascular and non-vascular systems. Because of their different functions, pulmonary vessels and systemic vessels respond differently to stimuli such as hypoxia, α -adrenergic activators and prostaglandin $\text{F}_{2\alpha}$ [42,43]. Pulmonary vessels also differ from other types of blood vessels in their receptor types and surface enzymes [44]. Therefore, to understand the role that the pulmonary smooth muscle microsomes play in Ca^{2+} -ATPase activity, under conditions of oxidant stimulation caused by the $\text{O}_2^{\cdot-}$ -generating system, we have carried out experiments on pulmonary vascular smooth muscle microsomes rather than extrapolate findings from other types of tissue.

In the present study, we tested the hypothesis that a protease plays an important role in regulating Ca^{2+} -ATPase activity by determining its possible involvement in the enhanced Ca^{2+} -ATPase activity caused by an $\text{O}_2^{\cdot-}$ -generating system. Activation of erythrocyte Ca^{2+} -ATPase by selective proteolytic cleavage of the calmodulin-binding domain has been demonstrated by previous researchers [14]. A previous study [13] with human erythrocyte membrane has also demonstrated that proteolysis of Ca^{2+} -ATPase causes activation of its ATP-hydrolytic activity. In addition, earlier reports provided evidence that proteolysis of endogenous protease inhibitors causes an imbalance between protease and antiprotease with the resultant shift of the equilibrium towards protease [15,16,45].

Several pieces of evidence indicate that a protease plays a crucial role in the enhanced Ca^{2+} -ATPase activity caused by the $\text{O}_2^{\cdot-}$ -generating system in microsomes of pulmonary smooth muscle. First, treatment of the smooth muscle microsomes with the $\text{O}_2^{\cdot-}$ -generating system produces a band with proteolytic activity in a gelatin-containing zymogram (Figure 1, lane 2). This protease has an apparent molecular mass of 16 kDa (Figure 1). The identity of the 16 kDa protease is unknown at present, but it is inhibited by aprotinin (Figure 1, lanes 5 and 6), an ambient

protease inhibitor of smooth muscle microsomes (Figure 2). Interestingly, this 16 kDa protease is not inhibited by PMSF (Figure 1, lane 3). Recent research has demonstrated that a protease in mitochondria of rat submaxillary gland is inhibited by aprotinin but not by PMSF [46]. Secondly, treatment of the smooth muscle microsomes with the $\text{O}_2^{\cdot-}$ -generating system not only increases the protease activity but also stimulates Ca^{2+} -ATPase activity (Table 2). Thirdly, aprotinin prevents the protease and Ca^{2+} -ATPase activities in the microsomes caused by the $\text{O}_2^{\cdot-}$ -generating system (Table 2). Fourthly, changes in protease activity correlate directly with changes in Ca^{2+} -ATPase activity (Figure 3). These four pieces of evidence strongly support the concept that an aprotinin-sensitive protease plays a pivotal role in stimulating Ca^{2+} -ATPase activity in smooth muscle microsomes under treatment with the $\text{O}_2^{\cdot-}$ -generating system hypoxanthine plus xanthine oxidase.

A pertinent question that may arise at this juncture is whether the increase in Ca^{2+} -ATPase activity reflects an increase in ATP-dependent Ca^{2+} uptake or is merely the result of membrane-associated changes. To address this question, we determined ATP-dependent Ca^{2+} uptake under basal and $\text{O}_2^{\cdot-}$ -stimulated conditions in the microsomes. Our results show that ATP-dependent $^{45}\text{Ca}^{2+}$ uptake is stimulated during $\text{O}_2^{\cdot-}$ treatment (Table 3). Our results also show that pretreatment with SOD and aprotinin prevents the increase in ATP-dependent Ca^{2+} uptake caused by the $\text{O}_2^{\cdot-}$ -generating system (Table 3), suggesting that Ca^{2+} -pump activity is indeed associated with ATP-dependent Ca^{2+} uptake under basal and $\text{O}_2^{\cdot-}$ -triggered conditions in smooth muscle microsomes.

It is evident from Table 3 that Na^{+} -dependent Ca^{2+} uptake is depressed by $\text{O}_2^{\cdot-}$ in the microsomes compared with basal conditions. The inhibition of the Na^{+} -dependent Ca^{2+} uptake appears to overpower the ATP-dependent Ca^{2+} uptake in the presence of $\text{O}_2^{\cdot-}$ (Table 3). Our present study also indicates that pretreatment with aprotinin prevents the stimulation of the ATP-dependent Ca^{2+} uptake and reverses the inhibition of the Na^{+} -dependent Ca^{2+} uptake caused by the $\text{O}_2^{\cdot-}$ -generating system (Table 3). Aprotinin therefore appears to prevent Ca^{2+} overload in pulmonary smooth muscle tissue. The mechanisms by which the protease causes stimulation of Ca^{2+} -pump activity and ATP-dependent Ca^{2+} uptake, and also inhibition of Na^{+} -dependent Ca^{2+} uptake, are currently under investigation.

Proteolytic processes play an important role in experimentally induced and physiologically occurring changes in cells and tissues. $\text{O}_2^{\cdot-}$ has been demonstrated to produce pulmonary hypertension

in isolated lung [17]. In isolated lung, aprotinin has been shown to prevent pulmonary hypertension caused by the Ca^{2+} -mobilizing agent A23187 [47]. Conceivably, $\text{O}_2^{\cdot-}$ induces protease activity on inactivating the ambient antiprotease aprotinin thereby causing an alteration in the protease-antiprotease balance in favour of protease, resulting in Ca^{2+} overload in pulmonary smooth muscle and subsequent pulmonary hypertension. The mechanism(s) by which the protease stimulates Ca^{2+} -ATPase activity in smooth muscle microsomes is currently not known. It may act directly on Ca^{2+} -ATPase [48] or indirectly via factors that influence Ca^{2+} -ATPase activity such as calmodulin [49], protein kinase C [50], cyclic AMP [51], cyclic GMP [52] and the proteolipid phospholamban [53].

In conclusion, our present study suggests that (i) treatment of bovine pulmonary smooth muscle microsomes with an $\text{O}_2^{\cdot-}$ -generating system stimulates Ca^{2+} -ATPase activity through involvement of an aprotinin-sensitive protease; (ii) aprotinin is an ambient antiprotease in smooth muscle microsomes; (iii) treatment of the microsomes with an $\text{O}_2^{\cdot-}$ -generating system stimulates ATP-dependent Ca^{2+} uptake but inhibits Na^+ -dependent Ca^{2+} uptake; and (iv) the inhibition of Na^+ -dependent Ca^{2+} uptake appears to overpower the stimulation of ATP-dependent Ca^{2+} uptake in microsomes treated with an $\text{O}_2^{\cdot-}$ -generating system.

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