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Inhibition of actin-activated myosin Mg^{2+}-ATPase in smooth muscle by Ruthenium red

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Ruthenium red was found to inhibit actin-activated myosin Mg^{2+}-ATPase in smooth muscle and to bind to myosin heavy chain, but not to F-actin. The inhibition by Ruthenium red of actin-activated Mg^{2+}-ATPase was of the competitive type with respect to actin (K_i 4.4 μM) and of the non-competitive type with respect to ATP (K_i 6.6 μM). However, Ruthenium red scarcely dissociated the acto-heavy meromyosin complex during the ATPase reaction. These results suggest that Ruthenium red interacts directly with the binding site for F-actin on the myosin heavy chain. This site is considered to be necessary not for maintaining the binding affinity of myosin for F-actin, but for activation of the Mg^{2+}-ATPase.

Ruthenium red; Smooth muscle; Myosin ATPase

1. INTRODUCTION

The contractile protein myosin is accepted as an essential energy-transducing enzyme that plays a major role in the contraction of smooth muscle, and the regulation of the actin–myosin interaction and of the ATPase activity is very important for kinetic cellular functions. This regulation is mediated by myosin via the Ca^{2+}-calmodulin-dependent myosin light chain phosphorylation [1–3].

Ruthenium red, $[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_5]Cl_6 \cdot 4H_2O$, is a hexavalent polycationic dye which is known to block Ca^{2+}-induced Ca^{2+} release from sarcoplasmic reticulum in both skeletal and cardiac muscles [4–6], as well as the energy-dependent Ca^{2+} uptake into isolated mitochondria [7–9]. It has also been reported that Ruthenium red inhibits the Ca^{2+} pump of the smooth muscle plasma membrane [10]. However, the effects of Ruthenium red on the contractile proteins in smooth muscle have not been reported.

In this study, we found that Ruthenium red is a potent and selective inhibitor of the actin-activated Mg^{2+}-ATPase of smooth muscle myosin, using a reconstituted system composed of contractile proteins. The mechanism of action of Ruthenium red on the actin-activated Mg^{2+}-ATPase was also examined.

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Abbreviations: HMM, heavy meromyosin; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

2. MATERIALS AND METHODS

2.1. *Preparation of proteins*

Myosin was purified from chicken gizzard as described previously [11]. The following proteins were purified by previously described methods: myosin light chain kinase from chicken gizzard [12], tropomyosin from chicken gizzard [13], actin from rabbit skeletal muscle [14], and calmodulin from bovine brain [15]. Thiophosphorylated smooth muscle myosin was prepared by the previously described method [16,17]. Complete phosphorylation of the 20 kDa myosin light chain was confirmed by urea-glycerol gel electrophoresis as described by Perrie and Perry [18], and the extent of phosphorylation of the 20 kDa myosin light chain was stable throughout the experiments. Heavy meromyosin (HMM) was prepared by proteolysis with α-chymotrypsin from thiophosphorylated smooth muscle myosin by the method described previously [19].

2.2. *Assay of ATPase activities*

ATPase activities were determined by measuring the amount of P_i liberated during 0–10 min at 25°C by the method described previously [20]. Each reaction mixture (final volume, 0.2 ml) included 25 mM Tris-HCl buffer (pH 7.5) that contained 80 mM KCl, 5 mM $MgCl_2$, 1 μM thiophosphorylated myosin or HMM, 10 μM F-actin, in the presence or absence of 1.6 μM tropomyosin, 1 mM [γ-^{32}P]ATP, and various amounts of Ruthenium red for assays of actin-activated Mg^{2+}-ATPase. For assays of K^+-EDTA-ATPase, reaction mixtures included 25 mM Tris-HCl buffer (pH 7.5) that contained 0.5 M KCl, 2 mM EDTA, 1 μM thiophosphorylated myosin or HMM, 1 mM [γ-^{32}P]ATP, and Ruthenium red. For assays of K^+-EDTA-ATPase, reaction mixtures included 25 mM Tris-HCl buffer (pH 7.5) that contained 0.5 M KCl, 10 mM $CaCl_2$, 1 μM thiophosphorylated myosin or HMM, 1 mM [γ-^{32}P]ATP, and Ruthenium red. After the mixtures had been preincubated in the absence of ATP at 25°C for 5 min, the reactions were started by addition of ATP.

2.3. *Binding experiments*

The binding of Ruthenium red to smooth muscle actomyosin was determined by staining with Ruthenium red after SDS-polyacrylamide gel electrophoresis [21], according to the method described previously [22]. Gels were stained with 25 mg/l Ruthenium red in 25 mM Tris-HCl buffer (pH 7.5) that contained 80 mM KCl, 5 mM $MgCl_2$, and 1 mM ATP.

2.4. *Other techniques and chemicals*

Protein concentrations were determined by the method of Bradford [23] with bovine serum albumin as the standard. Ruthenium red was purchased from Fluka Chemical Company Ltd. and the concentration of Ruthenium red was measured spectrophotometrically at 533 nm by the method described previously [24]. [γ-^{32}P]ATP was purchased from Amersham Corp. ATPγS was purchased from Boehringer Mannheim GmbH. α-Chymotrypsin was purchased from Worthington Biochemical Corp. All other chemicals were of the highest grade commercially available.

3. RESULTS AND DISCUSSION

We examined the effects of Ruthenium red on the various types of thiophosphorylated myosin and HMM ATPase activities of smooth muscle, namely, those of the actin-activated Mg^{2+}-ATPase, K^+-EDTA-ATPase, and Ca^{2+}-ATPase. Fig. 1A shows the dependence on the concentration of Ruthenium red of these activities of thiophosphorylated myosin. The actin-activated Mg^{2+}-ATPase activities, both in the presence and in the absence of tropomyosin, decreased in a similar fashion with increasing concentrations of Ruthenium red, while the K^+-EDTA-ATPase and the Ca^{2+}-ATPase activities were not significantly inhibited by Ruthenium red. Moreover, Ruthenium red did not inhibit the Mg^{2+}-ATPase activity of thiophosphorylated myosin alone (data not shown). In the case of thiophosphorylated HMM, only the actin-activated Mg^{2+}-ATPase activities, measured both in the presence and the absence of tropomyosin, were inhibited by Ruthenium red, as was true in the case of thiophosphorylated myosin (Fig. 1B). These results indicate that Ruthenium red is a potent and selective inhibitor of the actin-activated Mg^{2+}-ATPase of myosin and HMM in smooth muscle. Moreover, the inhibitory effect of Ruthenium red on the actin-activated Mg^{2+}-ATPase was not significantly influenced by the presence of tropomyosin.

To clarify the molecular mechanism of action of Ruthenium red, we examined the ability of Ruthenium red to bind to myosin, F-actin, and tropomyosin by the staining with Ruthenium red. Fig. 2 shows that Ruthenium red can bind to the heavy chain region of myosin and tropomyosin, but not to F-actin or to myosin light chains. These results indicate that Ruthenium red inhibits the actin-activated Mg^{2+}-ATPase of myosin via a direct effect on myosin heavy chain and not via an effect on F-actin. The interaction between Ruthenium red and tropomyosin may not be involved in the mechanisms of inhibition by Ruthenium red of the actin-activated Mg^{2+}-ATPase activity, because the presence of tropomyosin did not influence the inhibitory effect of Ruthenium red.

The kinetics of the inhibition by Ruthenium red were examined with respect to F-actin and ATP for the actin-activated Mg^{2+}-ATPase of thiophosphorylated smooth muscle myosin. The effects of Ruthenium red on the actin-activated Mg^{2+}-ATPase with respect to F-actin, in the absence of tropomyosin, are shown in Fig. 3A. From the double-reciprocal plot, the mode of inhibition by Ruthenium red of the actin-activated Mg^{2+}-ATPase was found to be of the competitive type with respect to F-actin. By contrast, Ruthenium red inhibited the actin-activated Mg^{2+}-ATPase in a non-competitive manner with respect to ATP. The values of K_i were 4.4 ± 1.2 μM (n = 3) with respect to F-actin and 6.6 ± 1.3 μM (n = 3) with respect to ATP, respectively. These results suggest that Ruthenium red directly interacts with the actin binding site of the myosin heavy chain to accelerate the release of P_i, but not with the ATP binding or the cata-

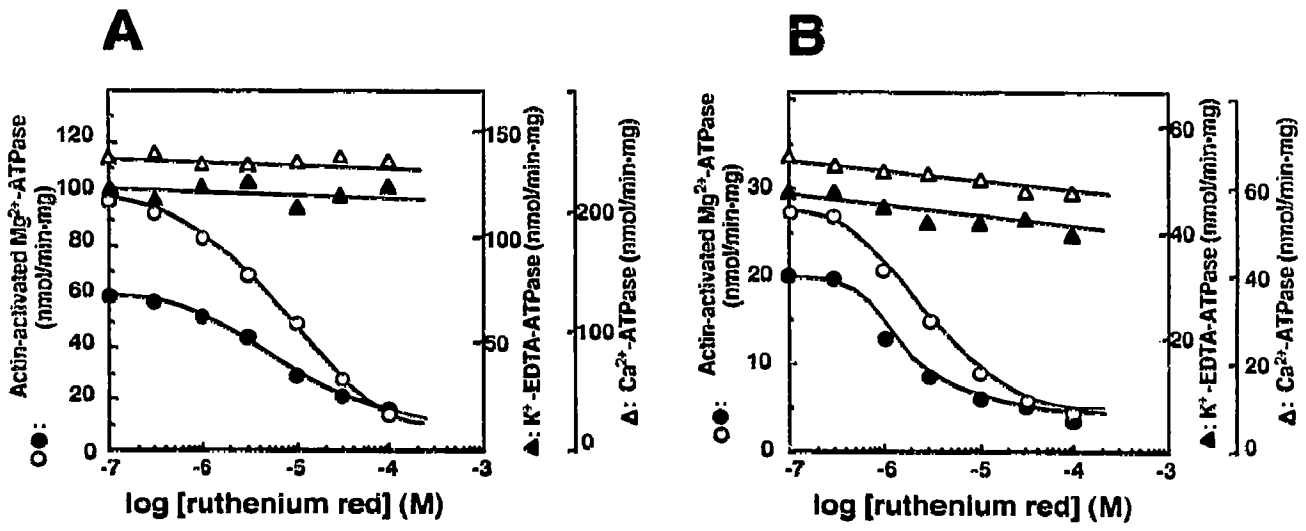

Fig. 1. Effects of Ruthenium red on the ATPase activities of smooth muscle thiophosphorylated myosin and HMM. ATPase activities of myosin (A) and HMM (B) were measured as described in section 2, in a reconstituted system in the presence of various concentrations of Ruthenium red. Actin-activated Mg^{2+}-ATPase activity, in the presence (○), and in the absence (●) of 1.6 μM tropomyosin; Ca^{2+}-ATPase activity, (△); K^+-EDTA-ATPase activity, (▲). Each point represents the mean of triplicate determinations.

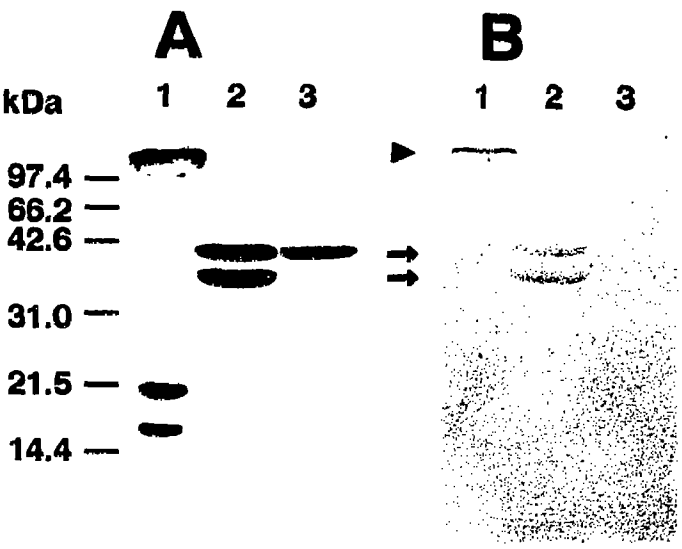

Fig. 2. Staining of smooth muscle myosin, tropomyosin, and actin with Ruthenium red. Samples containing approximately equal mol of amounts of purified proteins were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and then stained with Coomassie blue (A) or Ruthenium red (B) as described in section 2. Lane 1, myosin; lane 2, tropomyosin; lane 3, actin. The positions of molecular-weight markers (kDa) are as indicated. The arrowhead indicates the position of the myosin heavy chain, and arrows show the position of tropomyosin.

lytic site. We also studied the effects of Ruthenium red on the binding of HMM to F-actin by the method described previously [25]. The effects of Ruthenium red on the binding ability of HMM for F-actin were determined in parallel with the assay for the actin-activated Mg^{2+}-ATPase activity. Ruthenium red scarcely dissociated the acto-HMM complex during the ATPase reaction (data not shown). These findings indicate that the inhibitory effects of Ruthenium red are not due to a dissociation of the acto-HMM complex during the ATPase reaction.

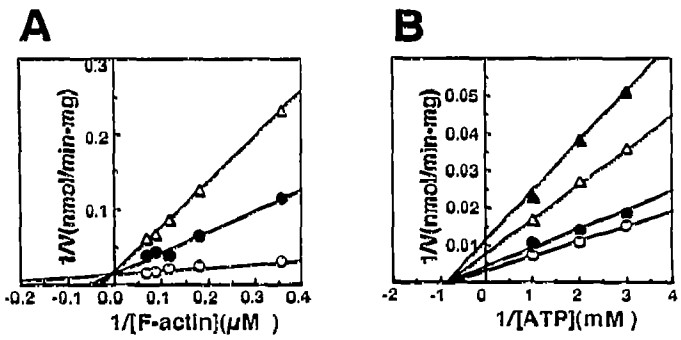

Fig. 3. Kinetic analysis of inhibition of the actin-activated Mg^{2+}-ATPase of smooth muscle thiophosphorylated myosin by Ruthenium red. The conditions used for the measurements of the actin-activated Mg^{2+}-ATPase activity are described in section 2. (A) Double-reciprocal plots of the initial velocity of the ATPase activity vs. the concentration of F-actin; the concentrations of Ruthenium red were 0 μM (○), 7.5 μM (●), and 15 μM (△). (B) Double-reciprocal plots of the initial velocity of the ATPase activity vs. the concentration of ATP; the concentrations of Ruthenium red were 0 μM (○), 5 μM (●), 10 μM (△), and 15 μM (▲).

In this study, we found that (i) Ruthenium red can bind heavy chain region of the myosin molecule; (ii) Ruthenium red inhibits the activation of Mg^{2+}-ATPase by F-actin in a competitive fashion with respect to F-actin, without affecting the binding affinity of the acto-HMM complex during the ATPase reaction; and (iii) Ruthenium red does not directly affect the catalytic site or the ATP-binding site of myosin heavy chain. The evidence presented here suggests that the Ruthenium red binding site in the heavy chain region of the myosin molecule is necessary for the transient interaction with F-actin that is required for activation of myosin Mg^{2+}-ATPase. Moreover, the site on the myosin heavy chain, that is necessary to maintain the affinity for F-actin during steady-state ATPase activity appears to be different from the Ruthenium red-binding site. These results are consistent with the previous report that smooth muscle S_1 heavy chain contains two putative actin-binding surface loops, the A1 site and the A2 site [26]. Moreover, in rabbit skeletal S-1 it was reported that the S-1 interacts with actin at two sites, and that the one site maintains the acto-S-1 link during steady-state ATPase activity while the rebinding of the other site to F-actin is necessary for activation of the ATPase [27]. In addition, we speculate that there may be at least two myosin-binding sites on F-actin, because a previous report suggests that the interface of S-1-actin appears to be composed of three different contact regions on actin [28].

Extensive studies of myosin ATPase in smooth and skeletal muscle have been reported and some have involved the inhibition of the myosin ATPase activity of smooth muscle [25,29]. To our knowledge, no drug has previously been reported to be a selective inhibitor of the actin-activated Mg^{2+}-ATPase activity of smooth muscle myosin via a direct effect on the myosin heavy chain, in the absence of any effect on the binding affinity of myosin for F-actin. Thus, Ruthenium red may be a useful chemical tool for studies of the molecular mechanisms of actomyosin contractile systems in smooth muscle.

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REFERENCES

[1] Adelstein, R.S. and Eisenberg, E. (1980) Annu. Rev. Biochem. 49, 921–956.

[2] Kamm, K.E. and Stull, J.T. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 593–620.
[3] Hartshorne, D.J. (1987) in: Physiology of the Gastrointestinal Tract, 2nd Ed. (Johnson, L.R., Ed.) pp. 423–482. Raven Press, New York.
[4] Salviati, G. and Volpe, P. (1988) Am. J. Physiol. 254, C459–C465.
[5] Smith, J.S., Coronado, R. and Meisner, G. (1986) Biophys. J. 50, 921–928.
[6] Chamberlain, B.K., Volpe, P. and Freischer, S. (1984) J. Biol. Chem. 259, 7547–7553.
[7] Ash, G.R. and Bygrave, F.L. (1977) FEBS Lett. 78, 166–168.
[8] Sordahl, L.A., Asimakis, G.K., Dowell, R.T. and Stone, H.L. (1977) J. Appl. Physiol. 42, 426–431.
[9] Moor, C.L. (1971) Biochem. Biophys. Res. Commun. 42, 298–305.
[10] Missiaen, L., Smedt, H.D., Droogmans, G., Wuytack, F., Raeymaekers, L. and Casteels, R. (1990) Biochim. Biophys. Acta 1023, 449–454.
[11] Persechini, A. and Hartshorne, D.J. (1983) Biochemistry 22, 470–476.
[12] Walsh, M.P., Hinkins, S., Dabrowska, R. and Hartshorne, D.J. (1983) Methods Enzymol. 99, 279–288.
[13] Bretscher, A. (1984) J. Biol. Chem. 259, 12873–12880.
[14] Driska, S. and Hartshorne, D.J. (1975) Arch. Biochem. Biophys. 167, 203–212.
[15] Yazawa, M., Sakuma, M. and Yagi, K. (1980) J. Biochem. (Tokyo) 87, 1313–1320.
[16] Heaslip, R.J. and Chacko, S. (1985) Biochemistry 24, 2731–2736.
[17] Chacko, S., Conti, M.A. and Adelstein, R.S. (1977) Proc. Natl. Acad. Sci. USA 74, 129–133.
[18] Perrie, W.T. and Perry, S.V. (1970) Biochem. J. 119, 31–38.
[19] Kaminski, E.A. and Chacko, S. (1984) J. Biol. Chem. 259, 9104–9108.
[20] Ikebe, M. and Hartshorne, D.J. (1985) Biochemistry 24, 2380–2387.
[21] Laemmli, U.K. (1970) Nature 227, 680–685.
[22] Charuk, J.H.M., Pirraglia, C.A. and Reithmeier, R.A.F. (1990) Anal. Biochem. 188, 123–131.
[23] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[24] Luft, J.H. (1971) Anat. Rec. 171, 347–368.
[25] Horiuchi, K.Y., Samuel, M. and Chacko, S. (1991) Biochemistry 30, 712–717.
[26] Yanagisawa, M., Hamada, Y., Katsuragawa, Y., Imamura, M., Mikawa, T. and Masaki, T. (1987) J. Mol. Biol. 198, 143–157.
[27] Katoh, T. and Morita, F. (1984) J. Biochem. (Tokyo) 96, 1223–1230.
[28] Labbe, J.P., Boyer, M., Roustan, C. and Benyamin, Y. (1992) Biochem. J. 284, 75–79.
[29] Takito, J., Nakamura, H., Kobayashi, J. and Ohizumi, Y. (1986) J. Biol. Chem. 29, 13861–13865.