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EFFECTS OF RUTHENIUM RED ON ACTIVATION OF Ca^{2+}-DEPENDENT CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

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Ruthenium red inhibited Ca^{2+}-dependent phosphodiesterase (Ca^{2+}-PDE) selectively with an IC_{50} value of 15 μM. Increasing calmodulin concentration in the presence of both 100 μM and 4000 μM Ca^{2+} completely reversed the inhibition of Ca^{2+}-PDE activity by ruthenium red. Ruthenium red-induced inhibition of Ca^{2+}-PDE activity was also overcome by increasing the concentration of Ca^{2+} in the presence of both 200 ng and 2000 ng calmodulin, in sharp contrast to fluphenazine-induced inhibition of Ca^{2+}-PDE. These results indicate that ruthenium red has distinct inhibitory mechanism which differs from that of calmodulin antagonists previously reported.

Ruthenium red, an inorganic dye, has been used as a stain to demonstrate anionic carbohydrates (1). Ruthenium red inhibits Ca^{2+} uptake in mitochondria (2) and Ca^{2+} release from sarcoplasmic reticulum in skeletal and cardiac muscles (3). Ruthenium red has also been shown to inhibit Ca^{2+}-ATPase and ATP independent Ca^{2+} binding with rat heart sarcolemma (4) and prevent capsaicin-induced transmitter release from primary afferent nerve terminals (5). The Ca^{2+}-induced aldosterone response was entirely blocked by either ruthenium red or W-7 (6). The mechanisms by which these various influences occur are not fully understood.

We now report the findings of ruthenium red, which inhibits Ca^{2+}-PDE by a mechanism differing from those related to reported

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calmodulin antagonists. Some of these pharmacological actions may be explained by its selective interaction with calmodulin.

MATERIALS AND METHODS

Preparation of Phosphodiesterase and Calmodulin

A low Km cyclic guanosine monophosphate (cGMP) phosphodiesterase (cGMP-PDE) and a low Km cyclic adenosine monophosphate (cAMP) phosphodiesterase (cAMP-PDE) were partially purified from human heart as previously described (7). Briefly, human heart was obtained within 1 hour of autopsy following a written informed consent from the family. Left ventricular muscle was homogenized in 10 volumes of 250 mM sucrose, 10 mM Tris-HCl (pH 7.8), 5mM $MgCl_2$, 0.2 mM EGTA, and 100 µM phenylmethyl-sulfonylfluoride (PMSF). The homogenate was centrifuged at 100,000xg for 40 min. The supernatant was applied to a DEAE-cellulose column and was eluted using a continuous 0.07-1.0 M sodium acetate gradient.

Calmodulin deficient calcium-calmodulin dependent cyclic nucleotide phosphodiesterase (Ca^{2+}-PDE) was partially purified from rat brain as previously reported (8). Calmodulin was isolated from bovine brain and purified by precipitation with trichloroacetic acid, DEAE-cellulose chromatography and phenyl-Sepharose chromatography according to Yazawa and colleagues (9).

Preparation of Trypsin-treated Ca^{2+}-PDE

Ca^{2+}-PDE was digested with 100 ng/ml trypsin in the presence of 50 mM Tris-HCl (pH 8.0) and 1 mM EGTA. After a 10 min incubation at 30°C, the reaction was terminated by the addition of soybean trypsin inhibitor at a weight ratio to trypsin of 10 to 1. Ca^{2+}-PDE could be stimulated by limited tryptic digestion up to 94% of activity induced by calmodulin.

Measuring Phosphodiesterase Activity

Cyclic nucleotide phosphodiesterase (PDE) activity was measured by the two-step assay as described (10). Unless otherwise noted, the enzymatic reaction was in a total volume of 0.5 ml containing buffer (50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 50µg of bovine serum albumin, and 1 mM EGTA or 100 µM $CaCl_2$/ 200 ng calmodulin), substrate (0.4 µM [^{3}H]cAMP or 0.4 µM [^{3}H]cGMP), and appropriate dilutions of the enzyme. The reaction was run at 30°C for 15 min before the termination by boiling for 5 min. 5'-[^{3}H]AMP or 5'-[^{3}H]GMP formed by the PDE is converted to [^{3}H]adenosine or [^{3}H]guanosine by the action of nucleotidase. The product isolated by cation-exchange resin was counted in a liquid scintillation counter.

Reagents

[^{3}H]cAMP and [^{3}H]cGMP were obtained from Amersham Co. Unlabeled cAMP, cGMP, Trypsin (bovine pancreas), trypsin inhibitor (soybean) and snake venom (Crotalus atrox) were purchased from Sigma Chemical Co. (St.Louis, MO, U.S.A.). Ruthenium red $[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5]Cl_6{:}4H_2O$ was purchased from Wako Pure Chemical Industries Ltd.(Tokyo, Japan) and stored in the dark at -20°C. All other chemicals were of reagent grade or the best commercially available.

RESULTS

Table 1 summarizes the concentrations of ruthenium red producing 50% inhibition (IC_{50}) of various PDE. Although IC_{50}

Table 1. Effects of ruthenium red on cyclic nucleotide phosphodiesterase

Phosphodiesterase (PDE)	IC_{50}(μM)*
Cyclic GMP-PDE (PDEI)	>1000
Ca^{2+}-dependent PDE (PDEII)	
in the presence of Ca^{2+}-Calmodulin	15
in the absence of Ca^{2+}-Calmodulin	600
trypsin treated PDEII	550
Cyclic AMP-PDE (PDEIII)	120

* IC_{50} value is defined as the concentration of drug required to produce 50% inhibition of enzyme activity. The activity was assayed with 0.4 μM nucleotide as described in Materials and Methods.

values of ruthenium red on both cGMP-PDE (PDEI) and cAMP-PDE (PDEIII) were higher than 100 μM, that on Ca^{2+}-PDE was 15 μM. There was no significant effect on basal Ca^{2+}-PDE (in the absence of Ca^{2+}-calmodulin), up to a concentration of 100 μM. There was about a 40-fold difference in the IC_{50} values between calmodulin-activated and basal Ca^{2+}-PDE. Trypsin treated Ca^{2+}-PDE was suggested to be a good tool for elucidating the effect of compounds on the basal catalytic activity of Ca^{2+}-PDE (11). The inhibitory effect of ruthenium red on trypsin-treated Ca^{2+}-PDE was almost the same as that on Ca^{2+}-PDE in the absence of Ca^{2+}-calmodulin. Thus, ruthenium red inhibits the specific calmodulin-dependent stimulation of Ca^{2+}-PDE. Reversibility of inhibition by ruthenium red was confirmed by dialysis method. No significant effect of $RuCl_3$ on Ca^{2+}-PDE activity was observed.

Calmodulin antagonists antagonize the calmodulin stimulation of the PDE. Whether ruthenium red is a calmodulin antagonist was tested further by examining the ability of ruthenium red to alter the activation of Ca^{2+}-PDE by Ca^{2+} and calmodulin in comparison with that of fluphenazine.

Inhibitions of Ca^{2+}-PDE by both ruthenium red and fluphenazine were prevented by the addition of calmodulin in a

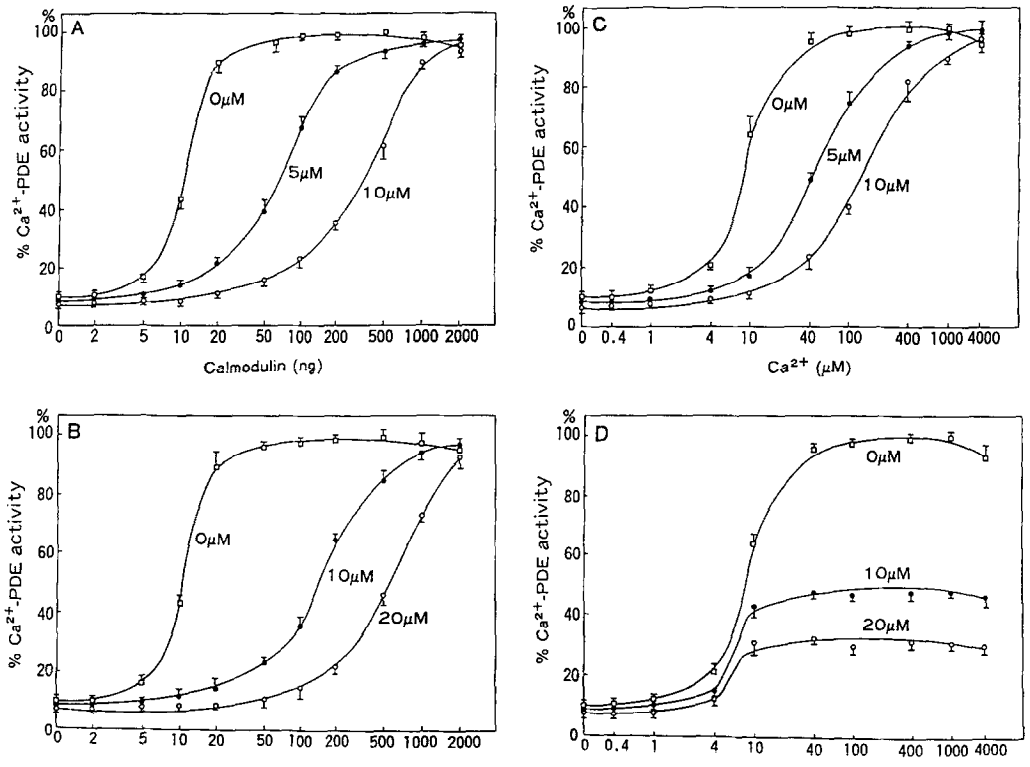

Figure 1: Influence of calmodulin (A) and Ca^{2+} (C) on ruthenium red-induced inhibition or of calmodulin (B) and Ca^{2+} (D) on fluphenazine-induced inhibition of Ca^{2+}-dependent phosphodiesterase. The dose-response curves for calmodulin and Ca^{2+} were measured in the presence of 100 μM Ca^{2+} and 200 ng calmodulin, respectively. Each point represents the mean±S.E. of eight experiments. Ruthenium red at the concentrations of 0(□), 5(●), and 10(○) μM and fluphenazine at the concentrations of 0(□), 5(●), and 10(○) μM were added to assay solutions.

dose-dependent fashion as shown in Fig. 1A and B. Ruthenium red-induced inhibition of Ca^{2+}-PDE activity was also overcome by increasing the concentration of Ca^{2+} in the presence of 200 ng calmodulin (Fig. 1C), in sharp contrast to fluphenazine-induced inhibition of Ca^{2+}-PDE (Fig. 1D).

These inhibitions of Ca^{2+}-PDE by ruthenium red were further analyzed by means of Lineweaver-Burk and Dixon plots. As shown in Fig. 2A and B, ruthenium red inhibited Ca^{2+}-PDE competitively with respect to calmodulin concentration at a constant Ca^{2+} concentration. Fully competitive inhibition for Ca^{2+} was also demonstrated on Ca^{2+}-PDE by ruthenium red in the presence of a

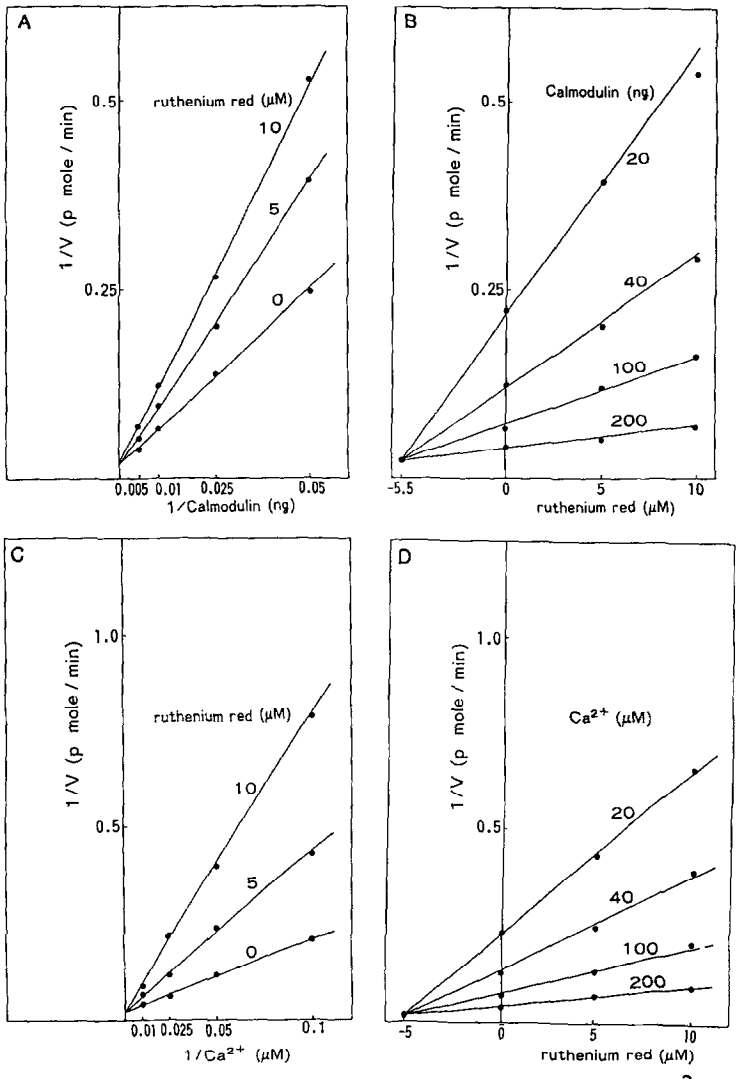

Figure 2: Kinetic analysis of Calmodulin or Ca^{2+} on the inhibition of Ca^{2+}-dependent phosphodiesterase by ruthenium red. (A), Lineweaver-Burk plot of calmodulin; (B), Dixon plot of calmodulin; (C), Lineweaver-Burk plot of Ca^{2+}; (D), Dixon plot of Ca^{2+}. Each point represents the mean of duplicate assays.

constant calmodulin concentration (Fig. 2C and D). At constant Ca^{2+} and calmodulin concentrations, the inhibition by ruthenium red appeared to be uncompetitive with respect to cGMP as substrate when analyzed by Lineweaver-Burk analysis (data not shown).

Effects of ruthenium red on the specific calmodulin dependent stimulation of Ca^{2+}-PDE in the presence of excess Ca^{2+}

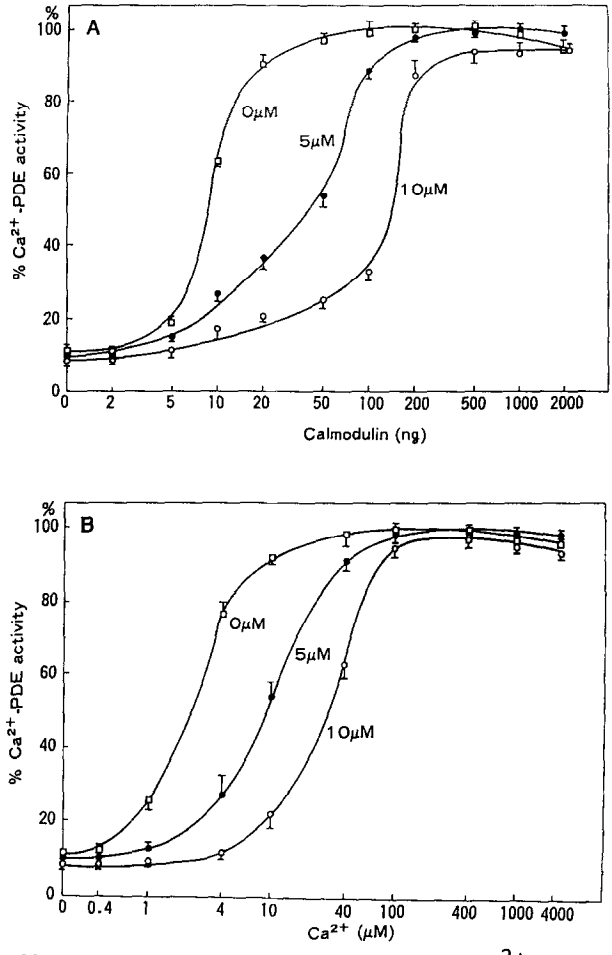

Figure 3: Influence of calmodulin (A) or Ca^{2+} (B) on ruthenium red-induced inhibition of Ca^{2+}-dependent phosphodiesterase. Each activity was measured in the presence of 4000 μM Ca^{2+} (A) or 2000 ng calmodulin (B). Each point represents the mean±S.E. of eight experiments. Ruthenium red at the concentrations of 0(□), 5(●), and 10(○) μM were added to assay solutions.

or excess calmodulin were also investigated. Increasing calmodulin concentration in the presence of 4000 μM Ca^{2+} (Fig. 3A) and increasing Ca^{2+} concentration in the presence of 2000 ng calmodulin (Fig. 3B) completely reversed the inhibition of Ca^{2+}-PDE by ruthenium red.

DISCUSSION

Our results indicate that ruthenium red inhibits Ca^{2+}-PDE selectively by a mechanism differing from that related to fluphenazine. Ca^{2+} antagonized competitively the ruthenium red-

induced inhibition of activation of Ca^{2+}-PDE, suggesting that the inhibition of Ca^{2+}-PDE by ruthenium red is due to its effect on Ca^{2+} binding. Increasing calmodulin concentration also completely reversed the inhibition of Ca^{2+}-PDE by ruthenium red. Kinetic analysis of ruthenium red-induced inhibition of activation of Ca^{2+}-PDE revealed that ruthenium red inhibits this activation in a competitive manner with both calmodulin and Ca^{2+} and not an interaction with the enzyme. A competitive inhibition of Ca^{2+}-PDE with calmodulin by ruthenium red was demonstrated even in the presence of excess Ca^{2+}, suggesting that the antagonism of the ruthenium red-induced inhibition of Ca^{2+}-PDE is not due to its secondary effect on Ca^{2+} concentration such as chelating action. Fluphenazine inhibits the formation of the catalytically active complex and its inhibition was not reversed by addition of excess Ca^{2+} (12). But increasing Ca^{2+} concentration completely reversed the inhibition of Ca^{2+}-PDE by ruthenium red. These results suggest that the inhibition of Ca^{2+}-PDE by ruthenium red may be due to its effects on both Ca^{2+} binding and phosphodiesterase binding to calmodulin. Ruthenium red may have distinct binding sites which differ from those occupied by previously reported calmodulin antagonists.

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