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Ca^{2+}-activated membrane ATPase: Selective inhibition by ruthenium red

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SUMMARY

1. Ca^{2+}-ATPase, (Na^+-K^+)-ATPase and Mg^{2+}-ATPase activities were determined in isolated red blood cell membranes.

2. The hexavalent dye, ruthenium red, produced concentration-dependent inhibition of Ca^{2+}-ATPase ($I_{50} \cong 10^{-5}$ M) without significantly affecting (Na^+-K^+)-ATPase or Mg^{2+}-ATPase.

3. We suggest that ruthenium red may be a useful tool for selective inhibition of Ca^{2+} transport and/or Ca^{2+}-ATPase.

Since the report by Schatzmann[1] concerning active Ca^{2+} transport in red cell ghosts, there has been increasing evidence for such transport[2,3] and its relationship to a membrane-bound enzyme, *i.e.* a Ca^{2+}-activated, Mg^{2+}-dependent ATPase (Ca^{2+}-ATPase)[4,5]. Ca^{2+}-ATPase of erythrocyte membranes is not inhibited by ouabain[5-7] which inhibits Na^+,K^+-activated, Mg^{2+}-dependent ATPase ((Na^+-K^+)-ATPase)[6,8] and Na^+,K^+ transport in the red cell[8-10]. Oligomycin which also inhibits (Na^+-K^+)-ATPase[6] and Na^+ transport[11] is ineffective as an inhibitor of calcium transport in erythrocytes[2,5]. The search for a specific inhibitor of Ca^{2+}-ATPase and/or Ca^{2+} transport has thus far been unsuccessful. Mersalyl[5], ethacrynic acid[12], and the lanthanides[13] inhibit Ca^{2+}-ATPase and Ca^{2+} transport. However, these substances are rather non-specific in that they inhibit other membrane ATPases such as (Na^+-K^+)-ATPase[5,13].

Recently, ruthenium red, an inorganic dye used in the staining of tissues, cells and organelles[14-16], has been shown to inhibit Ca^{2+} transport in isolated mitochondria[17]. Thus, in searching for a possible inhibitor of Ca^{2+}-ATPase or Ca^{2+} transport, we decided to examine the effects of ruthenium red on Ca^{2+}-ATPase, (Na^+-K^+)-ATPase and Mg^{2+}-dependent ATPase (Mg^{2+}-ATPase) in erythrocyte membranes.

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid.

Isolated human red cell membranes were prepared as described previously[18,19] with the slight modification that outdated (22–28 day old) packed red cells were used instead of whole blood.

ATPase activities were determined as follows; 0.3 ml of red cell membrane suspension was preincubated with 80 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 4 mM ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid (EGTA), 30 mM Tris-HCl and ruthenium red at pH 7.1 in a final volume of 2 ml at 37° for 5 min. Choline chloride (95 mM) was substituted for NaCl and KCl in some experiments. Tris–ATP (3 mM) was added to start the reaction and the mixture was incubated at 37° for 60 min. Mg^{2+}-ATPase was determined with the addition of 0.1 mM ouabain to the incubation medium. (Na^+-K^+)-ATPase, *i.e.* the ouabain-sensitive ATPase, was calculated as the difference between activities in the presence and absence of ouabain. Ca^{2+}-ATPase, known to be insensitive to ouabain[5], was calculated as the difference produced by the addition of Ca^{2+} (pCa^{2+} = 3.92) in the presence of ouabain. EGTA (4 mM) was present in all incubations including the Ca^{2+}-free conditions. Standard Ca^{2+} stock solutions were prepared by complexiметric titration. Aqueous solutions of ruthenium red (K and K Laboratories) were prepared and assayed for purity and concentration according to the method of Luft[20].

The enzyme reaction was terminated by the addition of 1.0 ml of 1.5 M $HClO_4$. After removal of the precipitated protein by centrifugation, an aliquot of the incubation medium was assayed for inorganic phosphate by the method of Fiske and SubbaRow[21] with correction by means of appropriate blanks for non-enzymatic hydrolysis. Ruthenium red did not interfere with the assay of inorganic phosphate. Under the above conditions, ATP hydrolysis was a linear function of incubation time and enzyme concentration. Enzymatic activity was expressed as μmoles of inorganic phosphate liberated per mg protein in 60 min. The enzyme preparations were assayed for protein content according to the method of Lowry *et al.*[22].

Control values for Ca^{2+}-ATPase, (Na^+-K^+)-ATPase and Mg^{2+}-ATPase were 0.90 ± 0.19, 0.25 ± 0.01 and 0.13 ± 0.02 μmoles P_i per mg protein per h, respectively. Ruthenium red, a hexavalent cation, inhibited Ca^{2+}-ATPase without significantly affecting (Na^+-K^+)-ATPase or Mg^{2+}-ATPase (Fig. 1). The inhibitory effect on Ca^{2+}-ATPase was observed at all concentrations of ruthenium red tested.

Several workers[23–25] recently suggested that there are a number of Ca^{2+}-ATPases present in red cell membranes. Schatzmann and Rossi[25] described an operational subdivision of erythrocyte membrane Ca^{2+}-ATPase based on the presence or absence of Na^+ and/or K^+ in the incubation media. Part of the membrane Ca^{2+}-ATPase is dependent on the presence of either one of the alkali cations; part of the Ca^{2+}-ATPase appears to be independent of Na^+ and/or K^+. The functions of the various Ca^{2+}-ATPases, as defined by Schatzmann, are not yet clear, although it was suggested that the Na^+/K^+-independent fraction is sufficient to support Ca^{2+} transport[25]. Since in our study ruthenium red specifically inhibited Ca^{2+}-ATPase rather than (Na^+-K^+)-ATPase or Mg^{2+}-ATPase, the effects of ruthenium red were examined on each of the above described Ca^{2+}-ATPase fractions. The alkali-cation-independent fraction of Ca^{2+}-ATPase was measured in the manner described above for Ca^{2+}-ATPase but with the use of 95 mM choline in place of omitted Na^+ and K^+. The alkali-cation-dependent fraction of Ca^{2+}-ATPase was calculated as the difference

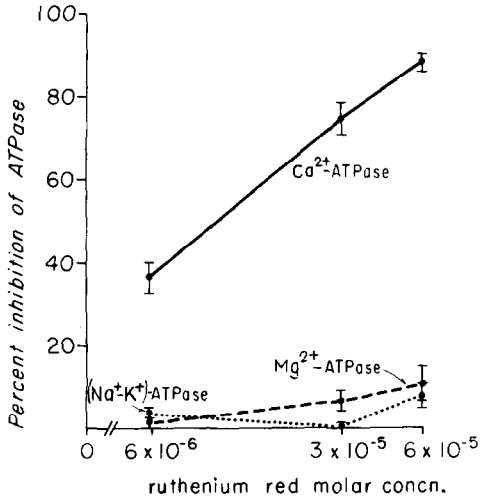

Fig. 1. ATPase activity of erythrocyte membranes in the presence of ruthenium red. Ca^{2+}-ATPase, $(Na^+\text{-}K^+)$-ATPase and Mg^{2+}-ATPase activities of human red cell membranes were measured as described in text. Values are means (± S.E. expressed as vertical bars) of three or five experiments.

between Ca^{2+}-ATPase and alkali-cation-independent Ca^{2+}-ATPase. Both types of Ca^{2+}-ATPase were measured in the presence of ouabain thereby eliminating any contribution by $(Na^+\text{-}K^+)$-ATPase.

The results in Table I show that, at a concentration of $6 \cdot 10^{-6}$ M, ruthenium red inhibited each fraction by approximately one-third and was almost completely inhibitory at $6 \cdot 10^{-5}$ M. Thus, there was no apparent difference in the inhibition by ruthenium red of these two operationally defined Ca^{2+}-ATPases. Since there was no difference in the sensitivity of these ATPases to ruthenium red, they will be considered together hereafter simply as Ca^{2+}-ATPase. $(Na^+\text{-}K^+)$-ATPase and Mg^{2+}-ATPase again were not affected by ruthenium red as shown earlier in Fig. 1.

TABLE I

EFFECTS OF RUTHENIUM RED ON ATPases OF ERYTHROCYTE MEMBRANES

Values are expressed as μmoles of inorganic phosphate liberated per mg protein in 1 h. See text for procedural details.

ATPases	*ATPase activity*		
	Ruthenium red concentration (M):		
	0	$6 \cdot 10^{-6}$	$6 \cdot 10^{-5}$
Ca^{2+}-ATPase	0.88	0.55	0.09
alkali-cation-dependent	0.32	0.22	0.04
alkali-cation-independent	0.56	0.33	0.05
$(Na^+\text{-}K^+)$-ATPase	0.28	0.26	0.25
Mg^{2+}-ATPase	0.11	0.11	0.10

The mechanism of the inhibition of Ca^{2+}-ATPase is not defined by our results although several possibilities can probably be ruled out. Moore[17] substituted $RuCl_2$ for ruthenium red and found $RuCl_2$ to be much less effective when compared with ruthenium red, thereby ruling out ruthenium ion (Ru^{3+}) as being responsible for his results. Since ruthenium red is a hexavalent cation, chelation of Ca^{2+} also appears unlikely. The presence in our system of a large reservoir of Ca^{2+} chelated with EGTA also makes chelation an unlikely basis for the effects of ruthenium red which were observed in this study.

Schatzmann recently studied the effects of the lanthanides, holmium (Ho^{3+}) and praseodymium (Pr^{3+}) as inhibitors of Ca^{2+} transport in human red cell ghosts and of Ca^{2+}-ATPase in isolated red cell membranes[13]. The concentrations for 50% inhibition of Ca^{2+} transport and Ca^{2+}-ATPase were approximately 10^{-4} M. Concentrations of lanthanides that inhibited Ca^{2+}-ATPase of isolated membranes also inhibited (Na^+-K^+)-ATPase, but did not inhibit Mg^{2+}-ATPase. Thus, while the lanthanides were offered as a tool for inhibition of Ca^{2+} transport, they would also be expected to directly inhibit Na^+,K^+ transport. Our findings demonstrate that, in contrast to the lanthanides, ruthenium red inhibits Ca^{2+}-ATPase without significantly inhibiting (Na^+-K^+)-ATPase. The concentration for 50% inhibition by ruthenium red was approximately 10^{-5} M. On the basis of the ATPase model for cation transport and because ruthenium red specifically inhibits erythrocyte membrane Ca^{2+}-ATPase, we suggest that ruthenium red would inhibit Ca^{2+} transport without directly affecting Na^+,K^+ transport. Some indirect alteration of Na^+,K^+ transport by ruthenium red might be expected with accumulation of internal Ca^{2+} (ref. 19). It is also noteworthy that ruthenium red did not affect Mg^{2+}-ATPase at concentrations which significantly inhibited Ca^{2+}-ATPase. Duncan[26] and Bowler and Duncan[27] have suggested that Mg^{2+}-ATPase is involved in control of passive permeability of excitable cells and erythrocytes. Thus agents which, by virtue of their non-specificity, would inhibit Mg^{2+}-ATPase as well as Ca^{2+}-ATPase, might be expected to alter membrane permeability to various ions, including Ca^{2+}. This has been noted for mersalyl[5]. Since ruthenium red did not inhibit Mg^{2+}-ATPase, we have no *a priori* reason to expect that it would alter membrane permeability in transport studies.

The site of action of ruthenium red as an inhibitor of Ca^{2+}-ATPase is not defined by these experiments. With the isolated erythrocyte membranes used in this study, both inside and outside surfaces are available to the incubation medium. It is therefore not clear with which one, or both, surfaces ruthenium red must interact to inhibit Ca^{2+}-ATPase. Since ruthenium red is essentially an extracellular marker in histological work[20], it would not be expected to reach inner cell surfaces readily if simply added to a suspension of erythrocytes. However, if necessary in the course of transport experiments, ruthenium red could be added to the inside of erythrocytes by reversible hemolysis[5].

We suggest that the relatively high potency and specificity of ruthenium red as an inhibitor of Ca^{2+}-ATPase may allow its use as a tool for studying Ca^{2+} transport in much the same manner that ouabain is used for studying Na^+, K^+ transport.

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