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In Vitro Inhibition of the Rate of Erythrocyte Sickling by RMI 11,071A and Its Possible Mechanism (40652)

ROBERT D. MACKENZIE, E. MARTIN GLEASON, GERALD L. SCHATZMAN, AND MADISON J. CAWEIN

Merrell Research Center, Merrell-National Laboratories, Division of Richardson-Merrell Inc., Cincinnati, Ohio 45215

The importance of the interaction between the erythrocyte membrane and the abnormal hemoglobin aggregates in sickle cell anemia (SCA) has been well documented in recent years (1, 2). Though the genetic abnormality lies in the hemoglobin molecule, the functional lesion may lie in an abnormal membrane. The membrane of the sickle cell contains a much higher concentration of calcium (Ca) than that of the normal cell (3). It has also been shown that the ionophore A23187 will induce an influx of Ca, which produces bizarre shapes and stabilizes the sickled cell (4, 5). This stabilization will prevent the sickled cell from unsickling on reoxygenation similar but not identical to the irreversible sickled cell (ISC).

In a previous paper (6) we described a simple *in vitro* method for screening compounds for inhibition of the rate of sickling. We also described the activity of a compound that inhibited sickling rate and affected Ca content of the cells.

The purpose of this communication is to report the activity of RMI 11,071A, hexahydro-2-(α-methyl-*p*-phenoxybenzyl)-imino azepine hydrochloride, on the rate of sickling at 100 μg/ml of blood and its influence on Ca fluxes. RMI 11,071A is a member of a series of compounds, several of which have been found to inhibit platelet aggregation (7).

Materials and methods. Rate of sickling. The methods and apparatus for measuring sickling rate have been previously described (6). This procedure involves collecting blood from adult subjects who have SCA into 3.8% sodium citrate (1 part citrate to 9 parts of blood). Samples (3 ml) of the citrated blood were placed in serum bottles and cylinders of various gases (95% O_2 and 5% CO_2 for high oxygen tension or 2.5% O_2, 5% CO_2, and 92.5% N_2 for low oxygen tension) were connected through a manifold and flowmeter system to the blood bottles. The blood was first exposed to high O_2 tension to reduce the sickled cells to a minimum. Then either saline or RMI 11,071A solution was added and high O_2 tension maintained for 15 more min. A blood sample was then taken and fixed in 3% neutral formalin. The gas was then changed to the low O_2 tension. Blood samples were taken after 15, 30, 45, and 60 min and processed. Pictures of cells taken through Normarski interference optics were coded and at least 300 cells counted to determine percentage sickled cells. A cell was counted as a sickled cell if it had one or more points.

^{45}Ca flux. Blood was prepared as previously described except heparin (0.1 ml of 20 mg/ml for every 10 ml of whole blood) was used as the anticoagulant. Whole blood, 3.2 ml, was placed in the serum bottles, 0.4 ml of 50 μCi/ml of $^{45}CaCl_2$. (Final concentration of $^{45}CaCl_2$ was 5 μCi/ml of blood.) The bottle was placed in the apparatus and oxygenated for 30 min with 95% O_2, 5% CO_2. Samples were taken for analysis and deoxygenated for 30 min with 95% N_2, 5% CO_2 and again samples taken for analysis. The blood was then reoxygenated for 30 min and samples were taken for analysis.

Blood, 0.5 ml, was taken from each bottle through the rubber stopper with an 18-gauge needle into a 1-ml syringe. The samples were washed twice with 4.5 ml of N_2-saturated saline, then diluted to 1 ml with saline and the erythrocytes counted with a Coulter counter. One-half milliliter of the diluted cells was added to 4.5 ml of 6.7% TCA mixed, and centrifuged 1250*g* for 10 min at 4°. Two-tenths milliliter of the supernatant was counted in a scintillation counter. The radioactivity of each sample was compared to the total count added to the blood sample and is expressed as a percentage of ^{45}Ca dose/10^6 erythrocytes.

Results. Effects on sickling rate. The effect of RMI 11,071A at 100 μg/ml on sickling rate

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is shown in Fig. 1. The data in this figure are the composite of two experiments of 6 controls and 6 tests each (total of 12 determinations for each group). In experiment 1 inhibition of the sickling rate was 46.5% at 0–15 min and 43.3% at 15–30 min, for an inhibition of 45.1% for the 0- to 30-min period. In the second experiment inhibition of the sickling rate was 30.1% at 0–15 min and 33.8% at 15–30 min for a total inhibition for 0–30 min of 32.5%. The compound inhibited the rate of sickling for the first 30 min in both experiments. By 45 min of low O_2 tension the inhibitory effect had disappeared and the sickling rate of the test had increased enough that the number of sickled cells was approaching the control group by 60 min.

RMI 11,071A at 100 μg/ml did not effect pH, pCO_2, or the oxygen dissociation curve. The average pH values (±SEM) with and without compound were 7.324 ± 0.018 and 7.359 ± 0.021 respectively; the pCO_2 values with and without compound were 39.2 ± 2.1 and 39.9 ± 1.4, respectively, and the P_{50} values with and without compound were 33.70 ± 0.55 and 32.95 ± 1.00, respectively.

Effect on calcium fluxes. The results are shown in Table I. The amount of ^{45}Ca incorporated into the erythrocytes on deoxygenation was higher in the test group than in the controls. However, on reoxygenation the control group incorporated slightly more Ca (+6%), while the test groups lost a substantial amount of that previously incorporated (−20%).

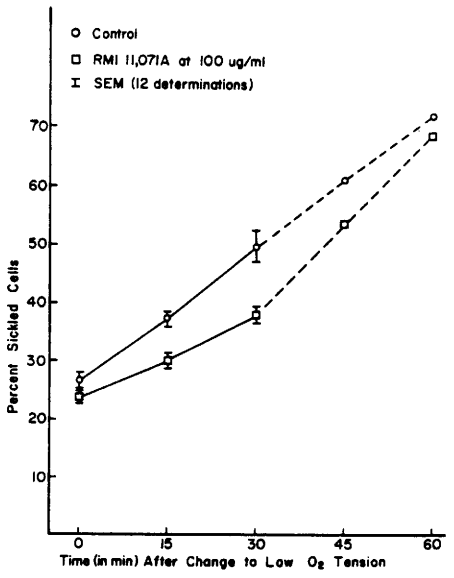

FIG. 1. Effect of RMI 11,071A at 100 μg/ml on sickling rate. Percentage sickled cells versus time after change to low oxygen tension (2.5%). Composite of two experiments with 6 determinations in each (total of 12 determinations) ± SEM.

Discussion. RMI 11,071A at 100 μg/ml inhibited the rate of sickling in an *in vitro* test system. This compound had no effect on pH, pCO_2, or the oxygen dissociation curve, but did affect Ca fluxes, results similar to those found for RMI 6792 reported in our previous paper (6). Since the erythrocytes from patients with SCA contain high levels of Ca, which increases as the cells are repeatedly deoxygenated and reoxygenated (sickled and unsickled) until they will no longer unsickle and become rigid and fragile, the functional lesion may be at or on the cell membrane. Dunn (8) reported that an increase in Ca influx caused an influx of Na and an efflux of K. He attributed the efflux of K to the cell-shape changes to echonocytic spherocytes. Glader and Nathan (9) discussed the relationship of Ca to Na–K shift and the ATP-energy connection but indicated that questions remain unanswered concerning the complete nature of the mechanism to the sickle-cell membrane lesion. The cell membrane in SCA has been shown to have an altered phosphorylation activity (10, 11).

Procaine and PABA (12) and zinc (13, 14) have been reported to inhibit Ca uptake and thereby improve cell filtration or decrease ISC count.

During the initial incubation of the sickled-cell/whole-blood samples with RMI 11,071A at high pO_2, the calcium pool may have decreased due to the effect of the compound on calcium efflux at high pO_2. Thus, the cell would contain less calcium, but more potassium ion (8). This loss of calcium would help explain the results, which showed an increased ^{45}Ca influx on deoxygenation even though the sickling rate was decreased over control. Even though the calcium pool size may be different between treated and untreated blood samples after the initial incubation, this difference would not cause the flux to change direction on reoxygenation. We were unable to measure total calcium

TABLE I. EFFECT OF RMI 11,071A AT 100 μg/ml ON CALCIUM INFLUX AND EFFLUX IN SICKLE CELL ERYTHROCYTES

Group	Percentage calcium ($45 \times 10^{-4}/10^{6}$ erythrocytes ± SEM)		
	Start	After deoxygenation for 30 min	After reoxygenation for 30 min
Control	322 ± 69	325 ± 66	345 ± 40
Change from previous value	—	+3	+20
RMI 11,071A at 100 μg/ml	389 ± 44	503 ± 87	403 ± 68
Change from previous value	—	+114*	−100*

* Significantly different from control.

content in these experiments due to breakdown of instrumentation.

Though the absolute calcium content was not measured, we propose that RMI 11,071A reduces the effect of Ca on the sickle cell system by improving the ability of the cell to decrease the Ca content upon reoxygenation. This may lower the K loss and reduce the rate of sickling initially on deoxygenation.

Summary. RMI 11,071A, one of a series of lactamimides, was found to inhibit the sickling rate in an *in vitro* system. This inhibition was not total or permanent; the sickling rate increased after 30 min at low O_2 tension and the number of sickled cells approached that of the controls by 60 min. There was no effect on pH, pCO_2, or the oxygen dissociation curve. ^{45}Ca fluxes were affected. Though influx of ^{45}Ca increased on deoxygenation, the compound caused a decrease in ^{45}Ca content on reoxygenation. This decrease was absent in the control group.

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