23Na and 39K NMR studies of ion transport in human erythrocytes

(\textsuperscript{Na}\textsuperscript{+}, K\textsuperscript{+}-pump/ouabain/gramicidin/chloride permeability/K\textsuperscript{+}-selective electrode)


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ABSTRACT  Ion transport in human erythrocytes was studied by \textsuperscript{23}Na and \textsuperscript{39}K NMR with an anionic paramagnetic shift reagent, Dy(P\textsubscript{3}O\textsubscript{10})\textsubscript{2}\textsuperscript{−}. The intra- and extracellular \textsuperscript{23}Na and \textsuperscript{39}K NMR signals were well separated (over 10 ppm) at 5 mM concentration of the shift reagent. The NMR visibility of the intracellular Na\textsuperscript{+} and K\textsuperscript{+} was determined to be 100% in human and duck erythrocytes. The intracellular ion concentrations were 8.1 ± 0.8 mM Na\textsuperscript{+} (n = 7) and 110 ± 12 mM K\textsuperscript{+} (n = 4) for fresh human erythrocytes. The ouabain-sensitive net Na\textsuperscript{+} efflux was 1.75 ± 0.08 mmol/l per liter of cells at 37°C (n = 3). The granamicidin-induced ion transport in human erythrocytes was also studied by \textsuperscript{23}Na and \textsuperscript{39}K NMR or by simultaneous measurements of \textsuperscript{23}Na NMR and a K\textsuperscript{+}-selective electrode. The time courses of the Na\textsuperscript{+} and K\textsuperscript{+} transport induced by the ionophore were biphasic. The initial rapid fluxes were due to an exchange of Na\textsuperscript{+} for K\textsuperscript{+}, which were found to occur with a 1:1 stoichiometry. The subsequent slow components were the net Na\textsuperscript{+} and K\textsuperscript{+} effluxes rate-limited by the Cl\textsuperscript{−} permeability and accompanied by a reduction in cell volume. The Cl\textsuperscript{−} permeability determined from the NMR measurements of these slow fluxes was 3.2 ± 0.5 x 10\textsuperscript{−8} cm/sec at 25°C (n = 4).

Concentration gradients of Na\textsuperscript{+} and K\textsuperscript{+} across the erythrocyte (RBC) membrane are primarily maintained by an active transport of Na\textsuperscript{+} and K\textsuperscript{+} by the Na\textsuperscript{+}-K\textsuperscript{+}-pump (1). The kinetic properties of the Na\textsuperscript{+}-K\textsuperscript{+}-pump have been extensively studied in RBC. Alterations in Na\textsuperscript{+} fluxes with resultant abnormal intracellular Na\textsuperscript{+} concentration in RBC have been suggested to exist in a variety of disease processes, such as essential hypertension (2), affective disorders (3), myotonic dystrophy (4), and hyperthyroidism (5).

Conventional methods of analysis for intracellular ions such as flame photometry and radioisotope tracer techniques have the disadvantage of requiring time-consuming destructive methods to achieve physical separation of intra- and extracellular compartments. Furthermore, there are uncertainties associated with the possibility of nonspecific binding of ions to the cell membrane and of ion fluxes occurring during the separation procedure. Clearly, a nondestructive and noninvasive method would be welcome.

NMR spectroscopy offers such an approach. The recent development of anionic paramagnetic shift reagents (SRs) (6, 7) has made it possible to resolve the intra- and extracellular NMR signals of \textsuperscript{23}Na and \textsuperscript{39}K in cells and tissues. This approach has been applied to measure intracellular Na\textsuperscript{+} (7, 8) and K\textsuperscript{+} (9) concentrations and to study the physical state of intracellular Na\textsuperscript{+} in RBC (10). However, no studies of ion transport in RBC have been reported, even though the NMR method would have advantages in the study of ion fluxes (11).

The present study demonstrates the suitability of NMR spectroscopy with the paramagnetic SR Dy(P\textsubscript{3}O\textsubscript{10})\textsubscript{2}\textsuperscript{−} for measuring Na\textsuperscript{+} and K\textsuperscript{+} fluxes as well as ion concentrations in human RBC.

EXPERIMENTAL

Blood was drawn from healthy donors into a syringe containing sodium heparin (10 units/ml). The RBC were separated from the plasma and buffy coat by aspiration and washed three to five times by centrifugation at 1500 x g for 10 min in a cold isotonic saline solution with 10 mM glucose at 4°C. The cells were kept on ice until used. Immediately before the NMR measurements, the cells were washed once and resuspended at 50–60% hematocrit in NMR buffer containing 65 mM NaCl, 20 mM KCl, 1 mM Na\textsubscript{2}H\textsubscript{2}PO\textsubscript{4}, 2 mM MgCl\textsubscript{2}, 10 mM glucose, 5 mM Na\textsubscript{2}Dy(P\textsubscript{3}O\textsubscript{10})\textsubscript{2}, 50 mM Hepes at pH 7.4 (the total Na\textsuperscript{+} concentration of the buffer was 100 mM). Na\textsuperscript{+} free choline buffer contained 85 mM choline chloride, 10 mM glucose, 5 mM choline-Dy(P\textsubscript{3}O\textsubscript{10})\textsubscript{2}, and 50 mM Hepes at pH 7.4 (the total choline concentration was 120 mM). Cell water determined by drying a sample of packed cells to constant weight was 0.68 ± 0.02 ml per ml of cells (n = 4). Hemolysis during the NMR measurements was measured by A\textsubscript{415} in supernatants and found to be negligible.

A Bruker WH-360WB NMR spectrometer was used operating at 145.78 MHz for \textsuperscript{31}P, 95.26 MHz for \textsuperscript{23}Na, and 16.81 MHz for \textsuperscript{39}K. For \textsuperscript{31}P, 45° pulses were used with pulse intervals of 1.0 sec. For \textsuperscript{23}Na and \textsuperscript{39}K, 90° pulse intervals were 0.1 and 0.07 sec, respectively. Bruker 20-mm probes were used for \textsuperscript{31}P and \textsuperscript{23}Na. \textsuperscript{39}K NMR measurements were performed with our home-built solenoidal coil probe (11).

During the NMR measurements, the cells were oxygenated and gently mixed with a propeller driven by an air turbine. In several experiments using \textsuperscript{23}Na NMR, a K\textsuperscript{+}-selective electrode (Microelectrodes, Londonderry, NH) was used to measure extracellular K\textsuperscript{+} concentration. In this way, simultaneous net fluxes of Na\textsuperscript{+} and K\textsuperscript{+} could be determined. The electrode was calibrated in the magnet under the same conditions of the NMR measurements. All experiments were performed at 37°C unless otherwise noted.

RESULTS

Since a SR is required to distinguish NMR peaks from extracellular Na\textsuperscript{+} and K\textsuperscript{+}, effects of the SR on energy metabolism in RBC were studied. Fig. 1 shows the \textsuperscript{31}P NMR spectra of RBC resuspended in the NMR buffer with and without the SR. The phosphorus signals from the SR were not observed (Fig. 1). Note that there is no significant alteration in the \textsuperscript{31}P NMR spectrum of RBC in the presence of the SR except that the peaks are slightly broadened, probably due to the increased susceptibility difference between the peaks.

Abbreviations: RBC, erythrocyte(s); SR, shift reagent.
intra- and extracellular compartments produced by the paramagnetic SR. The phosphorus profile, which is characteristic of well-energized cells, was stable over 1 hr. Cellular ATP levels in the presence of the SR as determined from the integrated intensity of β-ATP$^{13}$P signal was $96\% \pm 6\% (n = 8)$ of the levels in controls without the SR. This result suggests that the SR has no deleterious effect on energy metabolism in RBC. Moreover, no hemolysis was observed in the presence of the SR.

Figs. 2 and 3 show $^{23}$Na and $^{39}$K NMR spectra of RBC suspensions in the presence of the SR. In both spectra, the peaks shifted upfield about 10 ppm correspond to the extracellular ions, whereas the unshifted peaks correspond to the intracellular ions. The chemical shifts of the intracellular $^{23}$Na and $^{39}$K signals were stable during the experiments, indicating that the SR does not enter the RBC.

To determine the intracellular Na$^+$ and K$^+$ concentrations in RBC from the NMR signals, it is necessary to determine the fractions of intracellular Na$^+$ and K$^+$ that are NMR visible. The observation of separate signals for intra- and extracellular Na$^+$ and K$^+$ as shown in Figs. 2 and 3 enabled us to determine directly their NMR visibility in RBC without physically isolating the intra- and extracellular compartments by inducing ion fluxes and comparing the changes in the NMR signals of intra- and extracellular Na$^+$ and K$^+$ (11). In this experiment, RBC were resuspended into a Na$^+$-free choline buffer at 25°C and an ionophore, gramicidin (15 μg/ml), was added to facilitate Na$^+$ and K$^+$ fluxes across the RBC membrane. As shown in Fig. 4, the time course of the Na$^+$ flux mediated by the ionophore was biphasic—an initial rapid influx and a subsequent slow efflux. The integrated changes in the $^{23}$Na NMR signal of the intracellular

![Fig. 1. $^{31}$P NMR spectra (145.78 MHz) of human RBC resuspended in the NMR buffer at 37°C with (A) and without (B) SR. In the experiment of B, the SR was replaced by the equivalent total amount of NaCl in the NMR buffer. Hematocrit was 58.8% (A) and 60.4% (B), respectively. Exponential filter (20 Hz) was applied to accumulated free induction decays (5 min). Peak assignments: peaks 1 and 2 are 3-P and 2-P of 2,3-diphosphoglycerate. Peaks 3, 4, and 5 are γ-P, α-P, and β-P of ATP.](image)

![Fig. 2. $^{23}$Na NMR spectrum (95.26 MHz) of human RBC resuspended in the NMR buffer at 37°C. Na$_i$ and Na$_o$ represent the intracellular and extracellular sodium peaks, respectively. Hematocrit was 52.2%. Exponential filter (10 Hz) was applied to accumulated free induction decays (2 min). One division in the spectrum is equal to 10 ppm.
](image)

![Fig. 3. $^{39}$K NMR spectrum (16.81 MHz) of human RBC resuspended in the NMR buffer at 37°C. K$_i$ and K$_o$ represent the intracellular and extracellular potassium peaks, respectively. Hematocrit was 61.2%. Exponential filter (10 Hz) was applied to accumulated free induction decays (5 min). One division in the spectrum is equal to 50 ppm.
](image)
Na⁺ were plotted against those of the extracellular Na⁺ (Fig. 4 Inset) and the slope indicated the fractional visibility of the intracellular Na⁺. Linear-regression analysis of these data gave a slope of 1.00 ± 0.01, indicating that the intracellular Na⁺ is 100% NMR visible relative to the extracellular Na⁺. Furthermore, following addition of a known amount of Na⁺ to the cell suspension and to the buffer, comparison of relative increases in the 23Na NMR signal intensities (99.5% ± 0.8%, n = 3) indicated that the extracellular Na⁺ was 100% NMR visible in RBC suspensions. The transported Na⁺ is therefore 100% NMR visible. Finally, upon addition of 1% Triton X-100 to the cell suspension, which completely lysed the RBC, the intra- and extracellular 23Na NMR peaks collapsed into a single peak whose intensity was 100.8% ± 1.5% (n = 3) of the total signal intensity before the addition, confirming that the intracellular 23Na NMR signal represents the entire intracellular Na⁺ pool. On the basis of this observation, the intracellular Na⁺ content was 5.5 ± 0.5 mmol/liter of cells (n = 7) for fresh RBC. The NMR visibility of the intracellular K⁺ was determined in the same way. Linear-regression analysis of the integrated changes in the intra- and extracellular 39K NMR signals induced by gramicidin, as previously described for the 23Na experiment, gave a slope of 0.99 ± 0.02, indicating that the intracellular K⁺ is also 100% NMR visible since the extracellular K⁺ was found to be all NMR visible (98.8% ± 2.8%, n = 3). The intracellular K⁺ content measured by NMR was 74.8 ± 8.2 mmol/liter of cells (n = 4) for fresh RBC.

To study the relationship of the initial rapid fluxes of Na⁺ and K⁺ induced by gramicidin, simultaneous measurements of the intracellular Na⁺ by NMR and the extracellular K⁺ by an ion-selective electrode were performed. Unfortunately, as shown in Fig. 5A, mechanical mixing of the RBC suspension caused vibration of the electrode, introducing noise into the output. A computer-controlled time-sharing system was developed to circumvent this problem; sample mixing was stopped for 10 sec every minute and the K⁺ concentration was measured in a time-sharing mode with 23Na NMR. Fig. 5B shows the time course of the K⁺ efflux induced by the ionophore measured by the electrode. The net ion movement in the initial rapid K⁺ efflux was 61 µmol and that in the simultaneous Na⁺ influx determined by 23Na NMR was 54 µmol on the same sample. The small discrepancy was mainly due to the time-average nature of NMR analysis. A contribution from H⁺ influx (13) is likely due to small ΔpH at physiological pH (14) and because Na⁺ in the buffer inhibits the H⁺ influx (15). Within experimental errors, gramicidin caused the initial, rapid K⁺ efflux, which was balanced by the Na⁺ influx with a stoichiometry of 1:1.

We have also measured the ouabain-sensitive Na⁺ flux in human RBC. In this experiment, the net flux of Na⁺ was measured by 23Na NMR in Na⁺-loaded RBC suspension before and after addition of ouabain (0.2 mM), an inhibitor of the Na⁺,K⁺-pump (1). As shown in Fig. 6, the difference between these net fluxes was a measure of the ouabain-sensitive active Na⁺ efflux by the Na⁺,K⁺-pump prior to the addition, since ouabain has no effect on influx when the plasma K⁺ is normal (16). This net efflux was 1.75 ± 0.08 mmol/hr per liter of cells (n = 3). The ouabain-insensitive Na⁺ flux was 0.99 ± 0.05 mmol/hr per liter of cells (n = 3). Rubython et al. (5) have reported that the ouabain-sensitive net efflux for Na⁺ in normal human RBC is 1.80 mmol/hr per kg of cells using flame photometry, which is in good agreement with that determined by NMR.

**DISCUSSION**

The Dy(P₃O₁₀)₅²⁻ SR has made it possible to observe the intra- and extracellular Na⁺ and K⁺ in intact cells and tissues. 23Na and 39K NMR have the advantages over conventional...
techniques such as flame photometry and radioisotope tracer of being nondestructive, noninvasive, and continuous so that net fluxes can be followed with a reasonably fast time resolution and with simultaneous measurements of intracellular concentrations, avoiding possible artifacts of the isolation procedures. However, an important question concerning the use of the SR is its possible toxic effects on ion transport and metabolism. The following observations suggest that the SR is not toxic to RBC under the present conditions: (i) The SR has no adverse effect on energy metabolism in RBC, as demonstrated by the ATP levels measured by \(^{31}\text{P}\) NMR; (ii) the ouabain-sensitive Na\(^+\) efflux measured by \(^{23}\text{Na}\) NMR was consistent with reported values using other methods (5); (iii) the SR did not cause hemolysis, as previously noted in an erythrocyte morphology study (10). However, it is interesting to note that in rabbit proximal tubule (17), the SR reduced cellular respiration and ATP levels and that these adverse effects can be overcome by addition of Ca\(^{2+}\) into the medium. It was also reported that the SR produced slight increases in the transcellular short-circuit current and conductance of epithelial tissue but the electrophysiological responses of the tissue were not impaired (18). These observations emphasize the importance of establishing the viability of each preparation before the SR approach is applied.

It has been reported that only about 40% of the total Na\(^+\) present is NMR visible in a number of different tissues (19). This has also been found recently in yeast by Ogino et al. (11). However, Yeh et al. (20) found only a slightly reduced value (8%) of Na\(^+\) concentration in RBC determined by NMR when compared with flame photometry analysis. Brophy et al. (9) reported that the intracellular K\(^+\) concentration in RBC determined by NMR was about 8% lower than by flame photometry. More recently, Pike et al. (8) have also observed small but significant discrepancies (5–10%) in the measurements of Na\(^+\) concentrations in RBC by the two methods. These differences are too small to ascribe to a first-order quadrupolar interaction (19). Possible mechanisms suggested by Pike et al. (8) for this discrepancy are some binding of Na\(^+\) and K\(^+\) to the RBC membrane and/or Na\(^+\) and K\(^+\) fluxes occurring during the separation procedures before analysis.

Our strategy for determining NMR visibility was to induce an efflux or influx of intracellular Na\(^+\) and K\(^+\) by addition of gramicidin and to compare the concomitant signal changes in intra- and extracellular Na\(^+\) and K\(^+\) (11), thereby avoiding possible artifacts related to isolation procedures. We observed 100% NMR visibility for the transported Na\(^+\) and K\(^+\). Furthermore, the possibility of binding of Na\(^+\) and K\(^+\) to the cell surface was ruled out by the observation that there was no net change in the total intensity of signals after treatment with Triton X-100. We conclude that there is no evidence for an NMR invisible component of intracellular Na\(^+\) and K\(^+\) in RBC. This result indicates that the NMR properties of ions inside the RBC are not different from those in the external medium. Based on the measured 100% NMR visibility of the intracellular Na\(^+\) and K\(^+\), intracellular contents were 5.5 mmol of Na\(^+\) per liter of cells and 74.8 mmol of K\(^+\) per liter of cells for fresh RBC. Based upon the cell water of 0.68 ml/ml of cells, these values become 8.1 mM Na\(^+\) and 110 mM K\(^+\), which are well within reported values (5, 9, 21) ranging from 7 to 20 mM Na\(^+\) and from 100 to 140 mM K\(^+\).

We have also found that the NMR visibility of intracellular Na\(^+\) in duck RBC (not shown), which, unlike human RBC, are nucleated and contain mitochondria, is 100%. This experiment indicates that existence of a cell nucleus and mitochondria does not necessarily affect the \(^{23}\text{Na}\) NMR visibility. In this context, we have recently found that rabbit proximal tubules do not show a decreased visibility of Na\(^+\) (17).

It is interesting to note that the time courses of the Na\(^+\) and K\(^+\) fluxes induced by gramicidin are biphasic, as shown in Figs. 4 and 5B, which was previously observed for Na\(^+\) and Li\(^+\) fluxes in large unilamellar vesicles of egg lecithin (22). The biphasic nature can be interpreted assuming that the Na\(^+\) and K\(^+\) permeabilities induced by gramicidin are much higher than the net Cl\(^-\) permeability. The initial rapid fluxes are due to an exchange of K\(^+\) for Na\(^+\), both ions moving down their concentration gradients with respect to Cl\(^-\) flux in a typical experiment, initial concentrations (mM) before addition of the ionophore were 7.7 Na\(^+\), 15 Na\(^+\), 112 K\(^+\), and 3 K\(^+\) in the 120 mM choline buffer. Thus, a K\(^+\) gradient still exists when the Na\(^+\) gradient is dissipated. Therefore, this fast ion movement continues until the same diffusion potentials for Na\(^+\) and K\(^+\) (inside negative) are reached, creating a new Na\(^+\) gradient (overshoot) at the expense of dissipating the K\(^+\) gradient. Passive, net Cl\(^-\) transport out of the RBC then occurs. The subsequent slow fluxes are the net Na\(^+\) and K\(^+\) effluxes, rate-limited by the Cl\(^-\) permeability and accompanied by a reduction in cell volume, and they continue until a true equilibrium is reached. This reduction in cell volume was confirmed by measurement of lower hemocrit at the end of the experiment despite no hemolysis.

It is interesting to determine a stoichiometry of the initial rapid Na\(^+\) influx and K\(^+\) efflux. A double-tuned probe for \(^{23}\text{Na}\) and \(^{39}\text{K}\) could be used to measure Na\(^+\) and K\(^+\) fluxes simultaneously. However, the low sensitivity of \(^{39}\text{K}\) NMR hampers simultaneously rapid measurements. An alternative approach is to use an ion-selective electrode to measure extracellular \(^{39}\text{K}\) during the \(^{23}\text{Na}\) NMR measurement, since the K\(^+\) electrode is considerably more sensitive than \(^{39}\text{K}\) NMR. However, it should be noted that the electrode measures the activity of K\(^+\) in the buffer. We have developed a computer-controlled time-sharing system that allows simultaneous measurements of intracellular Na\(^+\) by \(^{23}\text{Na}\) NMR and extracellular K\(^+\) by an ion-selective electrode on the same sample (Fig. 5). Using this system, we found that the initial rapid Na\(^+\)–K\(^+\) exchange mediated by the ionophore occurs with a 1:1 stoichiometry.

Another interesting aspect of the time courses is that the Cl\(^-\) permeability can be determined by NMR measurements of an initial slope of slow fluxes of Na\(^+\) and K\(^+\). Since the RBC membrane is much less permeable to cations than anions and due to the electroneutrality restriction, it is not normally possible to measure the true anion flux, but only the rate of anion–anion exchange (23). Net flux of Cl\(^-\) occurs only if the membrane potential differs from the chloride equilibrium potential. This was accomplished by addition of gramicidin, which can render the RBC membrane highly permeable to cations and eliminate the restricting effects on net Cl\(^-\) flux. Hunter showed that these kinetics
could be analyzed by the constant field equations of Goldman (24). In particular, at high ionophore concentrations, the total slow fluxes of Na\(^+\) and K\(^+\) in Figs. 4 and 5B are equal to the Cl\(^-\) flux and the membrane potential is equal to the cationic diffusion potential. Therefore, the Cl\(^-\) permeability can be calculated from ion concentrations and an initial slope of the slow fluxes of Na\(^+\) and K\(^+\) (25), which is free from an effect of cell volume change. Using cell area = 133 \(\mu\)m\(^2\) and cell volume = 87 \(\mu\)m\(^3\) (23), the Cl\(^-\) permeability determined in this way was 3.2 \(\pm\) 0.5 \times 10\(^{-8}\) cm/sec (n = 4) at 25\(^\circ\)C, which is well within reported values ranging 2–12 \(\times\) 10\(^{-8}\) cm/sec at 37\(^\circ\)C (23).

Despite the fact that human RBC contain an ouabain-sensitive Na\(^+\),K\(^+\)-pump, no \(^{23}\)Na NMR measurement of Na\(^+\) flux has been reported under physiological conditions, presumably because of the unusually low cationic permeabilities of the RBC membrane (1). As shown in Fig. 6, we demonstrated that the high sensitivity of \(^{23}\)Na NMR made it possible to measure the ouabain-sensitive net Na\(^+\) efflux of the Na\(^+\),K\(^+\)-pump in human RBC. This net efflux was 1.75 mmol/hr per liter of cells, which is in good agreement with a reported value of 1.80 mmol/hr per kg of cells determined by flame photometry (5).

In conclusion, there is no evidence for an NMR invisible component of the intracellular Na\(^+\) and K\(^+\) in RBC.\(^{23}\)Na NMR can be used to measure the ouabain-sensitive Na\(^+\) efflux of the Na\(^+\),K\(^+\)-pump in human RBC. In particular, a combination of \(^{23}\)Na NMR and a K\(^+\)-selective electrode could open up the possibility to measure simultaneously net fluxes of Na\(^+\) and K\(^+\) on the same sample with a reasonably fast time resolution and thereby to study the Na\(^+\),K\(^+\)-pump in normal and diseased RBC.

\(^{2}\)Gupta et al. (26) have recently reported an \(\approx\)30% increase in the \(^{23}\)Na NMR signal when packed human RBC are lysed. We have found that the extracellular Na\(^+\) in the packed cells has the decreased visibility but not the intracellular Na\(^+\). These results do not interfere with our conclusion obtained in the RBC suspension.

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