Comparison of potassium and sodium binding *in vivo* and in agarose samples using TQTPPI pulse sequence

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List of abbreviations:

TQ – triple quantum

SQ – single quantum

MQ – multiple quantum

TPPI – time proportional phase increment

FT – Fourier transformation

RF – radio frequency

TQ^A – TQ MR peak area

SQ^A – SQ MR peak area

A_{SQF} – SQ MR signal amplitude, fast relaxing component

A_{SQL} – SQ MR signal amplitude, slowly relaxing component

A_{SQ} – total SQ MR signal amplitude, sum of the fast and slowly relaxing components

A_{TQ} – TQ MR signal amplitude

T_{2F} – T_2 relaxation time, fast component

T_{2L} – T_2 relaxation time, slow component

t-max – position of the TQ signal maximum

ns – number of steps

Abstract

Potassium and sodium specific binding *in vivo* were explored at 21.1 T by triple quantum (TQ) magnetic resonance (MR) signals without filtration to achieve high sensitivities and precise quantifications. The pulse sequence used time proportional phase increments (TPPI). During simultaneous phase-time increments, it provided total single quantum (SQ) and TQ MR signals at single and triple quantum frequencies, respectively. The detection of both TQ and SQ signals was performed at identical experimental conditions and the resulting TQ signal equals $60 \pm 3\%$ of the SQ signal when all ions experience sufficient time for binding. In a rat head *in vivo* the TQ percentage relative to SQ for potassium is $41.5 \pm 3\%$ and for sodium is $16.1 \pm 1\%$. These percentages were compared to the matching values in agarose tissue model with MR relaxation times similar to those of mammalian brain tissue. The sodium TQ signal in agarose samples decreased in the presence of potassium, suggesting a competitive binding of potassium relative to sodium ions for the same binding sites. The TQTPPI signals correspond to almost two times more effective binding of potassium than sodium. *In vivo*, up to $\sim 69\%$ of total potassium and $\sim 27\%$ of total sodium can be regarded as bound or experiencing an association time in the range of several milliseconds. Experimental data analyses show that more than half of the *in vivo* total sodium TQ signal could be from extracellular space, which is an important factor for quantification of intracellular MR signals.

Key words: *in vivo*; rat; triple quantum NMR; potassium; sodium; binding; TQTPPI;

Introduction

Potassium and sodium are major ions *in vivo* and their MR signals have great potential to convey valuable information about cell functioning (1-5). The difference in concentration of potassium and sodium between intracellular and extracellular sites is an important indicator of cellular energy metabolism (6-8) and the concentration gradients give rise to neuronal membrane potentials essential for nerve activity.

In addition to *in vivo* studies of the single quantum (SQ) signals from sodium in brain and heart, the triple quantum (TQ) MR signals from sodium and potassium have come under investigation (9-18) because of their potential to reveal changes in intracellular ion content without using contrast agents. For example, after calibration, the TQ filtered (TQF) sodium MR signal can measure changes in the intracellular sodium concentration in an isolated perfused rat heart without shift reagents (19).

Sodium and potassium nuclei have spin $S = 3/2$, thus these ions interact with the surrounding electric field gradients of the macromolecules *in vivo* with small (< 200 Hz) quadrupole interactions. The effect of such interactions on the signal cannot be observed as separate satellite MR peaks. However, there are exceptions as was detected for potassium in muscle (20), sodium in cartilage (21) and the excised bovine optic nerve (22). The fraction of such MR signals is very small in the rat head relative to the total MR signals of sodium or potassium and was not observed in our experiments. *In vivo* macromolecules provide many electro-negative interaction sites for sodium and potassium. These macromolecules are mainly proteins, nucleic acids and carbohydrates with exposed carboxyl groups ($-\text{COOH}$), hydroxyl groups ($-\text{COH}$) and phosphate groups (PO_4^{3-}). All these groups are involved in interaction with the positively charged ions of potassium and sodium with binding times in the range of several milliseconds. These interactions result in the TQ MR signal.

A widespread and accurate use of TQ signals is currently hindered by the low sensitivity of sodium and particular potassium NMR signals. Historically, the application of TQF pulse sequences was the primary way to detect TQ signals. TQF detection is usually performed by the pulse sequence $90 - \tau - 90 - \delta - 90$ (Fig. 1) using a specially selected phase cycling. The TQF pulse sequence interval “ τ ” is usually maximized for the most prevailing time of binding from a wide range of ion binding times existing *in-*

in-vivo; a typical value for interval “ τ ” is 2-6 ms. The TQF signal *in-vivo*, detected by such pulse sequences is only a small fraction of the total single quantum (SQ) signal.

Fig. 1. Comparison of the TQTPPI and TQF pulse sequences. The TQTPPI pulse sequence has simultaneous increments of interval $\tau = ns * \Delta$ and RF phase $\alpha = ns * 45^\circ$ (ns is a number of steps). The RF phase β has values of $+90^\circ$ and -90° for each value of τ and α . Both results are added together to suppress a double quantum signal. The receiver phase R remains unchanged. In the TQF pulse sequence, the interval “ τ ” has a fixed value in the range of 2 – 6 ms. The phase α changes throughout the cycle of 6 steps, while the receiver phase alternates $\pm 180^\circ$ with each RF phase step. A sum of all signals gives the TQF signal. A 180 degree pulse ($180_{\alpha+90^\circ}$) at the middle of interval “ τ ” is not shown.

A dramatic improvement in the efficiency and accuracy of the TQ signal detection can be achieved by avoiding filtration using the TQTPPI method (Fig. 1). In comparison to a typical TPPI pulse sequence (23,24), where the phase increment is 90° , the largest phase increment to detect the TQ signal is 45° . Smaller phase steps are also acceptable, for example 30° . The TQTPPI has the same efficiency for a wide range of ion bindings. The TQ MR signals are acquired concurrently with SQ peak. For example, during the 45° phase increment a full cycle (period of 360°) is covered by 8 phase steps. Thus, after Fourier transform (FT) in the second dimension, the SQ peak

can be found at $1/(8*\Delta\tau)$, which is well separated in the frequency domain from the TQ signal at $3/(8*\Delta\tau)$.

The TQTPPI pulse sequence for sodium was previously shown in the test samples (25,26) and *in vivo* (27). The current study is devoted to the application and analysis of the TQTPPI pulse sequence for potassium and sodium in a rat head at 21.1 T. The results were compared with the corresponding data from conventional TQF experiments. Additionally the same experiments were conducted in a model system of brain tissue consisting of 7.5% agarose. This concentration of agarose gives the MR relaxation parameters close to the *in vivo* results (28). As will be shown, the model system was able to demonstrate a stronger and competitive binding of potassium relative to sodium for the same binding places.

Methods

The experiments were performed on a 21.1T magnet using Bruker MRI Avance III console (PV 5.1) and a 64 mm gradient coil (RR, Inc.). Volume MRI coils for potassium (41.8 MHz) and sodium (^{23}Na , 238 MHz) were approximately the same size with ID/L = 33/54 mm. A double tuned Na/H RF coil was described previously (29). The potassium coil was a single tuned RF coil of Alderman-Grant design (28).

A commonly used TQ filtering pulse sequence $90^\circ_\alpha - \tau - 90^\circ_{\alpha+\beta} - \delta - 90^\circ_0$ was modified so that the phase “ α ” was incremented by 45° ($\alpha = ns*45^\circ$), at each step ns when the time delay ($\tau = ns*\Delta$) was incremented (Fig. 1). Before incrementing τ the phase “ β ” was alternated ($\pm 90^\circ$) and the results were added to suppress the double quantum (DQ) signal. The interval δ is usually selected to be the minimum allowed by the MR scanner hardware ($\sim 100 \mu\text{s}$). The time increment Δ in TQTPPI pulse sequence was 100 - 300 μs , the typical durations of the 90° pulse for sodium was 120 μs , and 140 μs for potassium. In both pulse sequences, TQTPPI and TQF, a $180^\circ_{\alpha+90}$ pulse was used in the middle of “ τ ” interval to compensate for the inhomogeneity of the magnetic field.

Free induction decays were acquired with “ np ” complex points, for potassium $np = 2048$, and for sodium $np = 4092$. The number of increments “ ns ” was selected in the range 128 - 1024. The large numbers of steps (>128) were selected to increase the

accuracy of the spectra in the second dimension in some experiments. The spectral width was 25 KHz for potassium and sodium. The number of accumulations NA and repetition time TR were for potassium NA = 16, TR = 200 ms, for sodium NA = 1 and TR = 300 ms. The FID signals were phased and Fourier transformed (FT) in the first dimension. Then “ns” data points at the maximum of the central peaks of these spectra were selected (at the position of $(np/2 + 1)$). These points were used for a nonlinear fit in the time domain to derive amplitudes of the SQ and TQ signals. The same data were also FT transformed in the second dimension using the TPPI mode in which the phased real part was accompanied by zero filling for the omitted imaginary points. The area of the SQ signal and both positive and negative parts of the TQ peak areas were calculated. The SQ peak was normalized to 100%. Pre-processing of the above and other data was performed using MatNMR software v.3.9.94 (30). All results of the time domain fit and spectral peak areas are presented as mean $\pm$ standard deviation.

The pulse sequence was first tested using model samples containing agarose gel 5% and 7.5% with added NaCl or KCl at concentration of 154 mM. The agarose (Carl Roth, Karlsruhe, Germany) was dissolved in the above solutions and heated up to 90°C with stirring. All model samples were placed in a plastic cylindrical container (diameter = 27 mm and length = 60 mm; volume = 25 mL). A competition between K^+ and Na^+ ions was observed when both NaCl (154 mM) and KCl (154 mM) were added to the agarose samples at the same time.

The TQTPPI potassium MRI signals were also acquired from polycrystalline potassium chloride (KCl). The results served as a reference emulating a 100% binding of potassium. In this specific case of a solid sample, a 180 degree pulse at the middle of “ τ ” interval was not used.

The TQTPPI/TQF pulse sequences were applied to detect potassium and sodium TQ signals in five male Fisher 344 rats (weight ~ 200 g). The animals were anesthetized by breathing a 1.5% v/v isoflurane/air gas mixture. The rat head was reproducibly positioned in the RF coil to within ± 1 mm using a bite bar (29). The TQ signal maximum amplitude and its position t-max were calculated using a time fit of the TQ bi-exponential decay.

Animal experiments were conducted according to the protocols approved by the Florida State University ACUC.

Results

Comparison of potassium and sodium TQTPPI and TQF MR signals

One way to process the TQTPPI data is to perform a non-linear fit of the total TQTPPI signal (Fig. 2) using the fitting function (31):

$$Y = A_{SQL}\sin(\omega t)\exp(-t/T_{2L}) + A_{SQF}\sin(\omega t)\exp(-t/T_{2F}) + A_{TQ}\sin(3\omega t)(\exp(-t/T_{2L}) - \exp(-t/T_{2F})) + DC \quad (1)$$

- where Y - amplitude of the fid signal, t - time, ω - frequency of the phase modulation = $1/(8\Delta)$, Δ - time step increment, DC is a base line shift, containing imperfect settings of the RF pulse durations and the accuracy of the phase corrections.

Fig. 2. Time course of potassium TQTPPI MR magnetization from a rat head in the second dimension. Note that the TQ MR signal of potassium appears early near the base line and slowly decays thereafter, during decay of the strong SQ potassium signal

The parameters of the time domain fit of the TQTPPI signals from the rat head as well as a corresponding TQF results are presented in Table 1. It is important to note that the single quantum signal has two exponential relaxation components (Fig. 3).

Table 1. *In vivo* potassium and sodium TQ MR signal parameters in the rat head. The MR signal amplitudes are presented in percent relative to the total SQ signal. Note a less efficient sodium binding and the similarity of the TQTPPI and TQF parameters.

TQ parameters	Potassium TQTPPI	Potassium TQF	Sodium TQTPPI	Sodium TQF
TQ^A/SQ^A , %	41.5 ± 3		16.1 ± 1	
A_{TQ}/A_{SQ} , %	35.7 ± 2.8		15.4 ± 1	
A_{SQF}/A_{SQ} , %	55.3 ± 0.9		41.6 ± 0.9	
T_{2L} , ms	13.3 ± 0.4	13.8 ± 0.8	30.6 ± 2	28.9 ± 1
T_{2F} , ms	0.79 ± 0.03	0.85 ± 0.14	2.3 ± 0.2	2.2 ± 0.5
t-max, ms	2.37 ± 0.07	2.5 ± 0.3	6.5 ± 0.6	6.0 ± 0.9

- TQ^A/SQ^A - ratio for the TQ peak area to the SQ peak area in the TQTPPI spectrum. A_{SQ} - total SQ signal amplitude, $A_{SQ} = A_{SQL} + A_{SQF}$; A_{TQ} – TQ signal amplitude (A_{SQL} , A_{SQF} and A_{TQ} are from TQTPPI time domain fit, formula (3)). T_{2L} and T_{2F} are slow and fast components of the bi-exponential relaxation of the SQ and TQ signals, t-max is a time position of the TQ signal maximum.

Fig. 3. The results of non-linear fit in the time domain of potassium TQTPPI magnetization from the rat head (from Fig. 2). Note the two-exponential decay for the SQ signal, which represents a total MR potassium signal from a rat head.

The other way to process the TQTPPI data is to perform Fourier transformation in the second dimension of the TQTPPI data. The results are demonstrated for potassium and sodium in a normal rat head from *in vivo* studies (Fig. 4). The sodium SQ peak and the TQ peak are well separated having frequencies of 1.25 kHz or $1/(8 \cdot \Delta)$ and 3.75 kHz or $3/(8 \cdot \Delta)$ respectively. The TQ peak areas relative to the corresponding SQ peak areas (TQ^A/SQ^A) were $41.5 \pm 3\%$ for potassium and $16.1 \pm 1\%$ for sodium.

Fig. 4. TQTPPI spectra for potassium ($\Delta = 0.1$ ms, $ns = 320$, scan time = 30 min) and sodium ($\Delta = 0.15$ ms, $ns = 760$, scan time = 7 min) in the rat head at 21.1T. The percentages (TQ^A/SQ^A) peak area for potassium was $41.5 \pm 3\%$, and for sodium $16.1 \pm 1\%$.

It is important to note that SQ MR signal represents a total MR signal, that is, both the bound and free ions of potassium and sodium. In contrast, the TQ signal represents only specifically bound ions.

For comparison, in the saline solution (0.9% NaCl), the TQTPPI pulse sequence does not give a sodium TQ signal. The base line shows only noise level signals at the expected place for the TQ signal. Another example of the absence of a TQ signal is during sodium binding to glycerol. No TQ signal was detected despite the fact that binding of sodium to the glycerol molecule can shorten sodium MR relaxation times T_1 and T_2 to about 8 ms.

For quantification of the level of binding, solid polycrystalline KCl was used, because all potassium ions are in a bound state. The defects in the crystals are usually enough to produce small quadrupolar interactions at the position of potassium, which are fixed due to the limited mobility of potassium in the crystal. The TQTPPI spectrum gives the TQ peak area of $59.9 \pm 3\%$ ($n = 6$) relative to the SQ peak area (Fig. 5). This

percentage is very close to a theoretical expectation for the intensity of the satellite transitions $1/2 \leftrightarrow 3/2$ of 60% (32,33).

Fig. 5. Potassium TQTPPI spectrum from polycrystalline KCL serves as a reference for the one hundred percent bound potassium system with (TQ^A/SQ^A) peak area's ratio of $59.9 \pm 3\%$ ($\Delta = 0.05$ ms, $ns = 400$, $TR=30$ sec, scan time = 6.5 h). Thus, the TQ signal in the TQTPPI pulse sequence represents the intensity of the MR quadrupole satellite transitions.

The comparison of potassium and sodium binding was also performed in the test samples containing 5% and 7.5% agarose with 154 mM KCl or 154 mM NaCl (Table 2). The percentages of the peak areas of TQ signal to SQ signal for potassium alone was $33.5 \pm 0.1\%$ ($n = 3$) in 5% agarose and increasing to $45.8 \pm 1\%$ ($n = 3$) in 7.5% agarose. The corresponding value for sodium were $25.7 \pm 0.7\%$ ($n = 4$) and $27.3 \pm 0.3\%$ ($n = 4$). The other parameters of the TQTPPI time domain fit are presented in Table 2.

Table 2. TQTPPI detection of the competitive potassium binding over sodium for the same binding places in 5% (A) and 7.5% agarose (B). MR signal amplitudes are presented in percent relative to the total SQ signal. Note a decrease of sodium TQ signal in the presence of potassium in both cases:

Table 2A. *TQTPPI, Agarose 5%*

Potassium	KCl (154 mM)	KCl(154 mM)+NaCl(154 mM)
$(TQ^A/SQ^A)_K$, %	33.5 ± 0.1	33.9 ± 0.9
$(A_{TQ}/A_{SQ})_K$, %	32.5 ± 0.3	33.3 ± 0.6
$(A_{SQF}/A_{SQ})_K$, %	58.9 ± 0.4	58.3 ± 0.2
$T_{2L\ K}$, ms	25.3 ± 0.4	23.8 ± 1
$T_{2F\ K}$, ms	1.96 ± 0.1	1.8 ± 0.3
$t\text{-max}_K$, ms	5.4 ± 0.1	5.0 ± 0.3
Sodium	NaCl (154 mM)	NaCl(154 mM)+KCl(154 mM)
$(TQ^A/SQ^A)_{Na}$, %	25.7 ± 0.7	20.3 ± 0.7 *
$(A_{TQ}/A_{SQ})_{Na}$, %	25.3 ± 0.6	21.7 ± 0.7 *
$(A_{SQF}/A_{SQ})_{Na}$, %	61 ± 0.5	60.2 ± 1
$T_{2L\ Na}$, ms	42.1 ± 0.9	43.4 ± 1.7
$T_{2F\ Na}$, ms	5.0 ± 0.1	6.6 ± 0.4 *
$t\text{-max}_{Na}$, ms	12.2 ± 0.2	14.7 ± 0.8 *

Table 2B. TQTPPI, Agarose 7.5%

Potassium	KCl (154 mM)	KCl(154 mM)+NaCl(154 mM)
$(TQ^A/SQ^A)_K$, %	45.8 ± 0.9	47.4 ± 1.3
$(A_{TQ}/A_{SQ})_K$, %	42.4 ± 0.5	44.5 ± 1.5
$(A_{SQF}/A_{SQ})_K$, %	57.8 ± 0.9	57.2 ± 1.4
$T_{2L K}$, ms	14.5 ± 0.5	13.5 ± 1.3
$T_{2F K}$, ms	1.2 ± 0.1	1.07 ± 0.1
$t\text{-max}_K$, ms	3.3 ± 0.1	2.9 ± 0.1
Sodium	NaCl (154 mM)	NaCl(154 mM)+KCl(154 mM)
$(TQ^A/SQ^A)_{Na}$, %	27.3 ± 0.3	22.3 ± 0.7 *
$(A_{TQ}/A_{SQ})_{Na}$, %	25.0 ± 0.4	22.3 ± 0.7 *
$(A_{SQF}/A_{SQ})_{Na}$, %	61.1 ± 0.5	61.2 ± 0.7
$T_{2L Na}$, ms	36.4 ± 2.5	37.4 ± 3.8
$T_{2F Na}$, ms	3.9 ± 0.2	4.9 ± 0.4 *
$t\text{-max}_{Na}$, ms	9.7 ± 0.6	11.4 ± 1 *

- $(TQ^A/SQ^A)_K$, $(TQ^A/SQ^A)_{Na}$ - ratios for the TQ peak area to the SQ peak area in the TQTPPI spectrum for potassium and sodium signals respectively. A_{SQ} - total SQ signal amplitude, $A_{SQ} = A_{SQL} + A_{SQF}$; A_{TQ} – TQ signal amplitude (A_{SQL} , A_{SQF} and A_{TQ} are from TQTPPI time domain fit, formula (3)). T_{2L} and T_{2F} are slow and fast components of the bi-exponential relaxation of the SQ and TQ signals, $t\text{-max}$ is a time position of the TQ signal maximum. * - difference is statistically significant.

The process of competition between potassium and sodium was detected by the TQTPPI method when both KCl and NaCl were simultaneously present with the same concentration of 154 mM. At both concentrations of agarose, the TQ/SQ ratio for sodium decreased significantly in the presence of potassium (Table 2). For the agarose concentration of 5%, the sodium TQ peak area signal decreased from $25.7 \pm 0.7\%$ ($n = 4$) to $20.3 \pm 0.7\%$ ($n = 6$). In the samples having 7.5% agarose, the corresponding decrease of sodium TQ signals is from $27.3 \pm 0.3\%$ ($n = 4$) to $22.3 \pm 0.7\%$ ($n = 3$).

These changes correlate with an increase of the sodium fast relaxing component $T_{2F Na}$ in the presence of potassium from 5.0 ± 0.1 ms ($n = 4$) to 6.6 ± 0.4 ms ($n = 6$) for 5% agarose samples and from 3.9 ± 0.2 ms ($n = 4$) to 4.9 ± 0.4 ms ($n = 3$) for 7.5%

agarose samples. The consistent increase is also observed for the position of sodium TQ signal maximum in the presence of potassium from 12.2 ± 0.2 ms ($n = 4$) to 14.7 ± 0.8 ms ($n = 6$) for 5% agarose samples. In the samples with 7.5% agarose the corresponding increase is from 9.7 ± 0.6 ms ($n = 4$) to 11.4 ± 1 ms ($n = 3$) (Table 2).

Discussion

The polycrystalline KCl was selected to serve as a reference system where all potassium ions are bound. The application of the TQTPPI pulse sequence (Fig. 5) demonstrates that the experimental TQ peak area is very close to the theoretically expected value of 60% of the SQ peak area (32,33). Thus, empirically, the observed TQ signal in the TQTPPI pulse sequence represents the intensity of the quadrupole satellite transitions.

It is expected that small quadrupole interactions can decrease the intensity of the SQ peak signal (Fig. 5). In the TQTPPI sequence the same SQ signal is used to detect both SQ and TQ signal in the second dimension. Thus, the ratio of the TQ/SQ signals will represent the intensity of the satellite transition with a good accuracy.

Using the above result for polycrystalline KCl (Fig. 5) as a model for 100% binding, we can estimate from Table 1 that *in vivo* in the rat head approximately $41.5 \times 100 / 60 = 69\%$ of total potassium has long term binding. The corresponding data for sodium *in vivo* suggests that much less, approximately 27%, of sodium has long term binding.

The large difference in the relative TQ signal intensity between potassium and sodium raises a question if this is relevant to the predominant intracellular location of potassium. In efforts to find the answer, the TQTPPI signals of potassium and sodium were compared in the test samples containing 5% and 7.5% agarose (Table 2). The results of experiments, first of all, demonstrate that potassium, at the same binding places, gives a larger TQ/SQ ratio than sodium. In 5% agarose the percentage of TQ peak area relative to the SQ peak area for potassium $(TQ^A/SQ^A)_K = 33.5\%$, while that for sodium $(TQ^A/SQ^A)_{Na} = 25.7\%$. The results for 7.5% agarose show for potassium $(TQ^A/SQ^A)_K = 45.7\%$ and for sodium $(TQ^A/SQ^A)_{Na} = 27.3\%$. Thus, both results *in vivo* and in the agarose samples show that potassium has a stronger TQ signal than sodium. This indicates a stronger binding of potassium.

TQTPPI experiments offer additional and more direct evidence that potassium has stronger binding. Potassium can compete with sodium for the binding places as observed when both potassium and sodium were added together (Table 2). The results demonstrate that there is practically no change in potassium binding in the presence of sodium. For example, in the presence of sodium or without it, the percentage of the TQ peak area relative to the SQ peak area $(TQ^A/SQ^A)_K$ for potassium remains at the same level of 33.7% for the 5% agarose sample and at a level of 46.5% for the 7.5% agarose sample. However, the TQ signal of sodium is decreased in presence of potassium. The TQ peak area relative to SQ peak area for sodium $(TQ^A/SQ^A)_{Na}$ decreased from $25.7 \pm 0.7\%$ to $20.3 \pm 0.7\%$ for the 5% agarose samples in the presence of potassium and from $27.3 \pm 0.3\%$ to $22.3 \pm 0.7\%$ for the 7.5% agarose. Thus, it appears that potassium binding competes with bound sodium. These results correlate with experiments of others on changes of sodium T_1 relaxation times in the presence of phosphatidylserine vesicles due to the partial displacement of sodium by potassium (34). The preference of potassium binding over sodium was also detected in rat liver cell microsomes (35).

It is important to note that there is a difference for potassium between the ratio of the peak areas (TQ^A/SQ^A) and the ratio for the amplitudes (A_{TQ}/A_{SQ}) obtained from the time domain fit (Eq. 3) of the results of TQTPPI pulse sequence (Table 1). However, there is almost no such difference of these ratios for sodium. These results suggest that the two exponential fit of the TQ signal is only a first order of approximation. It is working for sodium and approximately for potassium in 5% agarose samples (Table 2A) and for sodium *in vivo* (Table 1). In all other cases the deviation between (TQ^A/SQ^A) and (A_{TQ}/A_{SQ}) can be observed suggesting more than two or a distribution of the T_2 relaxation times for bound ions.

Another important observation is that the fast decaying components of the SQ signal $(A_{SQF}/A_{SQ})_K$ and $(A_{SQF}/A_{SQ})_{Na}$ in the TQTPPI pulse sequence are of 57 - 61% for agarose samples (Table 2). These values are very close to a theoretical expectation of 60% for the intensity of the MR satellite peaks during quadrupole interaction. However, as it can be seen in the Table 2, the TQ signals in both cases are much less than 60%, the expected value if all of ions experience quadrupole interaction. Thus, a short component of the SQ signal includes both ions with long time binding, represented by

TQ signal and by ions bound to small molecules which may not yield contribution to the TQ signal.

In vivo, in the rat head, the A_{SQF}/A_{SQ} values are much smaller for sodium; it is 41.6%, and it is a little less for potassium at 55.3% (Table 1). A value below 60% could be an indication that *in vivo*, a large fraction of sodium and a small fraction of potassium are in a “free” state, i.e. not experiencing significant long time quadrupole interactions.

Estimation of sodium intracellular and extracellular TQ signals

Using the above results for potassium and sodium TQ signals one can estimate the TQ signals from *in vivo* intracellular and extracellular spaces. The intracellular volume is assumed to be 0.85 of total tissue volume and the sodium intracellular and extracellular concentrations are assumed to be 15 mM and 140 mM, respectively, which is close to estimations of others (36). In this case intracellular sodium content would be $0.378Na_T$, and extracellular sodium content is $0.622Na_T$, where Na_T is the total sodium content in tissue. The difference between sodium and potassium TQ signals can be taken from the TQTPPI results in samples with 7.5% agarose (Table 2). These samples have T_1 , T_2 MR relaxation parameters very close to sodium and potassium *in vivo* (28). Thus, from Table 2 sodium has $47.4/22.3 = 2.1$ times less TQ signal than potassium. The intracellular potassium TQ signal can be with high accuracy presented by the measured total potassium $(TQ^A/SQ^A)_K = 41.5\%$ (Table 1) since contribution from extracellular potassium is very small. Assuming the concentration of potassium are 140 mM intracellular and 4 mM extracellular with intracellular volume of 85%, only 0.5% of total potassium content is in extracellular space. Thus, it can be estimated that for intracellular sodium $TQ^A_{Na_i}/SQ^A_{Na_i} = 41.5/2.1 = 19.8\%$. The total $(TQ^A/SQ^A)_{Na}$ signal from the rat head is 16.1%, which was derived from our *in vivo* experiments. This value has respective contributions from extracellular and intracellular compartments as shown in equation 2

$$(TQ^A_{Na_i} + TQ^A_{Na_{ex}})/SQ^A_{Na} = 16.1\% \quad (2)$$

From this equation one can get the following value for extracellular sodium $(TQ^A_{Na_{ex}}/SQ^A_{Na_{ex}}) = 13.9\%$. During calculation it was used that $SQ^A_{Na} = SQ^A_{Na_i} +$

SQ_{Naex}^A , $SQ_{Nai}^A/SQ_{Naex}^A = 0.378/0.622 = 0.608$. This indicates that TQ binding of sodium in intracellular space is stronger $(TQ_{Nai}^A/SQ_{Nai}^A)/(TQ_{Naex}^A/SQ_{Naex}^A) = 19.8/13.9 = 1.4$.

Thus, the total TQ^{Na} signal of sodium is composed of two parts:

$$TQ_{Nai}^A/SQ_{Na}^A = 7.5\% \quad (3)$$

$$TQ_{Naex}^A/SQ_{Na}^A = 8.6\% \quad (4)$$

All percent values here are given relative to the total sodium SQ signal. These results indicate that TQ signals in the rat head from the extracellular space could be ~ 53% relative to the total TQ signal. This estimation is compatible with the results of sodium TQ signal quantification performed in the rat heart (19), where it was found that extracellular TQ signal in the rat heart model can be approximately stable and is in the range of 50-64% of the total TQ signal from the normal rat heart. If in the estimation the intracellular volume is taken as 0.8 of the total tissue volume (due to CSF contribution) and sodium intracellular concentration is 15 mM, the TQ signal coming from extracellular space could be up to 64% of the total TQ signal. Thus, one can see the significant contribution of extracellular interactions to the TQ signal which was also noticed by others (37,38). It is an important factor for quantification of intracellular sodium using the TQ signal (19).

A short relaxation time is not necessary associated with or give evidence of TQ related binding. Sodium in a solution of glycerol and saline (1:1, v/v) has short relaxation times $T_1 = 8.04 \pm 0.06$ ms and $T_2 = 7.54 \pm 0.04$ ms at 21.1 T and room temperature. However, the tumbling time of glycerol molecule is very short and not long enough to create any detectable TQ signal (27). Such types of binding can be present *in vivo*, which decreases sodium and potassium relaxation times without detectable TQ signals (32). Thus, the part of the fast SQ T_2 component *in vivo* may have this type of origin. Consequently, the amplitude of the SQ short relaxing component and the TQ signal can be different.

During *in vivo* investigations, changes in tissue such as pH or oxygen partial pressure can be expected to result in a new distribution of binding strength. For

example, during cell death in a rat heart, a maximum TQ signal occurs at an inter-pulse delay shorter than that for a normal heart (39). The deviation from an optimum TQ signal detection can be significant and if not taken into consideration, may lead to a wrong quantification. The TQTPPI pulse sequence does not have this problem. The changes in binding during interventions will be reflected by changes in the shape of the TQ signal peak, while the integral of the peak will be proportional to the amount of bound sodium. There is no need to perform an optimization of the inter-pulse delay " τ ". A small tissue RF power deposition relative to the spin-lock version of the pulse sequence is another valuable benefit. No extra reference is needed as the SQ signal can serve as a reference. In conclusion, the ratio of TQ/SQ signals is a very sensitive parameter for detection of minor changes in binding.

The TQTPPI and TQF methods have different time efficiency. For a proper evaluation of the relative efficiency, it is important to include all the steps needed to conduct the TQF experiment. For the TQF strategy, one must first find the value between the first and second RF pulses that gives the maximum TQ signal. Thus, we need to acquire a number of delays depending on the required accuracy. Additionally, a separate experiment is needed to acquire a SQ signal. In the TQTPPI pulse sequence all these stages are combined, and there is no need for any extra experiments. When the inter-pulse delay is approximately known, the number of steps in the TQTPPI sequence can be as low as 8. In this case, TQTPPI still preserves its capability to separate the TQ signal. The actual difference in acquisition time between TQTPPI and the TQF pulse sequences may depend on the *in vivo* model we use in the TQ experiments. If the inter-pulse delay is known and we do not expect it to change during the experiments, the TQF method may take less time. However, due to filtration, the TQF technique has less sensitivity to detect small TQ signals.

It is important to note that *in vivo* the majority of the binding sites are the negatively charged carbonyl and carboxyl groups, while in agarose samples the binding sites are carboxyl groups. Thus, the agarose model is not a complete model representative of the brain. The validity of the agarose tissue model is based on the fact that the relaxation times of potassium and sodium in agarose samples match those from the *in vivo* rodent head study (28). Additionally, the observed large TQ MR signals of potassium relative to sodium in agarose samples are comparable to the results *in*

vivo. Thus, we expect that the agarose model system can reflect the main features of the TQ MR signals of the *in vivo* environment.

Finally, in the current study, the *in vivo* the MR signals from the whole rat head were used. There were no other localizations, except for the RF coil dimensions. The observed MR signals were a sum of the potassium or sodium MR signals from a rat brain and the surrounding muscles. The results have a value as it shows the total signal we obtain before using any localization technique. The MR imaging using TQTPPI approach is under development now, which could help in the localization of the signals from different parts of the head.

Conclusion

Simultaneous detection of SQ and TQ signals using the TQTPPI method provides an efficient method for evaluation of MR signals for potassium and sodium bound *in vivo* to macromolecules. The TQTPPI pulse sequence avoids the need for a separate SQ pulse sequence as it does not cancel the SQ signal, which is the case for the conventional TQF. The bound potassium and sodium signals are detected as separate peaks at the triple quantum frequency relative to the SQ signal. The experimental conditions for SQ and TQ signals are the same which provide a high accuracy of the measurements. In comparison to the TQF methods, TQTPPI detects bound ion signals optimally for a wide range of binding strengths, which is important for evaluating ion concentration studies associated with diseases or interventions. Furthermore, the changes in sodium bindings can be monitored with an internal SQ reference signal, which is a valuable feature for *in vivo* applications. The TQ signal and its changes can be detected more sensitively, as there is no extra noise due to the filtration procedure. The quantification of the TQTPPI signals showed almost two times more effective binding of potassium than sodium, as well as a competitive binding of potassium relative to sodium for the same binding site. *In vivo* studies of the rat head showed up to ~69% of total potassium and ~27% of total sodium can be regarded as long-term bound or experience an association of several milliseconds during binding. Analysis of the results support the model that sodium ions in the extracellular space can contribute significantly to the total TQ signal. This specific feature of the TQ

signals is little recognized but must be considered for studies that involve quantification of intracellular ions.

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