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Evaluation of Multiple-Quantum-Filtered ^{23}Na NMR in Monitoring Intracellular Na Content in the Isolated Perfused Rat Heart in the Absence of a Chemical-Shift Reagent

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The feasibility of employing triple-quantum-filtered (TQF) or double-quantum-filtered (DQF) ^{23}Na NMR spectra to monitor intracellular Na (Na_{in}) content in isolated rat hearts perfused in the absence of a chemical-shift reagent (SR) was investigated. This necessitated characterization of the following: first, the pool of Na_{in} represented by the intracellular TQF (TQF_{in}) spectrum; second, the maximum extent to which altered transverse relaxation times affect TQF_{in} spectral amplitudes; and finally, the situations for which the SR-free method can reliably be applied. The rates of increase in peak amplitudes of both intracellular TQF spectra, adjusted for changes in both fast (T_{2f}) and slow (T_{2s}) transverse relaxation times, and intracellular single-quantum (SQ_{in}) spectra were identical during no-flow ischemia, indicating that TQF_{in} and SQ_{in} spectra represent the same Na_{in} population. Addition of an Na/K ATPase inhibitor, ouabain ($\geqslant$500 μM), and no-flow ischemia induced similar rates of increase of Na_{in} content. However, the Na_{in} level for which the T_2 values started to increase was lower for ischemic (<140% of preischemic values) than for ouabain-exposed (>165%) hearts, which is consistent with the known earlier onset of intracellular swelling in ischemic hearts. Exposure of hearts to hyperosmotic perfusate (200 mM sucrose) increased [Na_{in}], due to a decreased cell volume and an unchanged Na_{in} content, but caused a decrease in T_2 values, a trend opposite to that observed with exposure of hearts to ouabain or ischemia. T_2 values therefore consistently correlated only with cell volume, not with Na_{in} content or concentration, indicating an important role for intracellular macromolecule concentration in modulating transverse relaxation behavior. The combined effect of ischemia-induced increases in T_2 values and their inhomogeneous broadened forms was an ~6% overestimation of Na_{in} content from amplitudes of SR-aided TQF_{in} spectra, indicating negligible effect of transverse relaxation-dependent alterations on TQF_{in} spectral amplitudes. Thus, Na_{in} content may be reliably determined from SR-free TQF spectra when the contribution from extracellular Na does not appreciably vary, such as during constant pressure perfusion. Following complete reduction in perfusion pressure, both SR-free TQF and DQF spectra respond to increases in Na_{in} content. However, SR-free DQF NMR provides an estimate of Na_{in} content much closer to that provided by the SR-aided method, due to the appreciable decrease of the extracellular DQF signal resulting from destructive interference between second- and third-rank tensors.
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INTRODUCTION

Measurement of biological intracellular Na (Na_{in}) content in the isolated rat heart may be achieved by acquisition of single-quantum (SQ) or triple-quantum-filtered (TQF) sodium (^{23}Na) NMR spectra. A chemical-shift or relaxation reagent is not necessarily required to allow monitoring of Na_{in} content by ^{23}Na TQF NMR. In the isolated crystalloid-perfused rat heart, a significant portion of the extracellular Na (Na_{ex}) pool is not detected by a TQF NMR pulse sequence, since fast (T_{2f}) and slow (T_{2s}) transverse relaxation times for this pool are equal, so that multiple-quantum transitions do not occur (*1*). In contrast, the signal from Na_{ex} dominates the SQ NMR signal. An important consequence of this selective elimination of the Na_{ex} TQF signal is that the Na_{in} pool makes an appreciable contribution to the amplitude of a TQF spectrum (TQF_{total}) acquired in the absence of a shift reagent (SR), maximally estimated to be 40% (*1*), allowing Na_{in} content to be monitored from SR-free TQF (*1, 2*) or double-quantum-filtered (DQF) spectra (*3*). In systems other than the isolated heart, such as the *in vitro* (*4, 5*) and in *in vivo* brain (*6, 7*), the isolated perfused rat kidney (*8, 9*) and salivary gland (*10*), increases in amplitude of SR-free TQF or DQF spectra have also generally been attributed to increases in the intracellular spectral amplitude. Thus, despite the superior sensitivity of SQ NMR, significant interest exists in applying SR-free multiple-quantum-filtered (MQF) NMR to tissue, primarily because of limitations associated with use of SR [for instance, see Ref. (*1*)], or the difficulties in employing SR *in vivo*.

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The amplitude of a SR-free TQF spectrum acquired in the isolated rat heart during constant perfusion correlates with the intracellular TQF (TQF_{in}) spectral amplitude, since the extracellular spectrum remains constant (*1*). However, it is not yet known whether a change in the TQF_{in} spectral amplitude accurately mirrors Na_{in} content. This question was addressed in the current study by comparing the response of intracellular SQ (SQ_{in}) and TQF_{in} spectral amplitudes to increases in Na_{in} content. Increases in Na_{in} content may be accompanied by alterations in ^{23}Na T_2 values (*3, 11*) in the rat heart, but this relationship needs to be better characterized. TQF_{in} spectral amplitudes also depend on transverse relaxation times, which are not easily evaluated when the intracellular spectrum is not resolved by a chemical-shift reagent. Thus, further development of the SR-free TQF ^{23}Na NMR method requires characterizing those situations which result in maximal changes in transverse relaxation behavior, and then quantitating the resultant alterations in TQF_{in} spectral amplitudes.

The goal of this study was to critically evaluate whether TQF ^{23}Na NMR spectra acquired in the absence of a chemical-shift reagent can accurately monitor a change in Na_{in} content in the crystalloid-perfused isolated rat heart. Two interventions, which altered both Na_{in} content and intracellular ^{23}Na NMR transverse relaxation behavior, were chosen to be representative of applications of general interest. These interventions employed either normal perfusion pressure (with an Na/K ATPase inhibitor, ouabain, present in the perfusate) or zero-pressure perfusion (no-flow ischemia). We first examined whether T_2-adjusted TQF_{in} and SQ_{in} NMR spectra provided identical estimates of changes in Na_{in} content. The interventions employed presumably were severe enough to maximally alter transverse relaxation behavior, which allowed estimation of the maximum extent to which intracellular TQF spectral amplitudes are altered by T_2 values. Comparison of the value of the Na_{in} content for which T_2 values started to change during each of the two interventions studied, along with an examination of intracellular volume-dependent (Na_{in}-independent) changes in T_2 values, provided insight into the mechanism most responsible for altering relaxation behavior. Having demonstrated the equivalence of SR-free ^{23}Na TQF and SR-aided SQ methods in monitoring Na_{in} content during constant-pressure perfusion, we addressed whether SR-free methodology could be extended to no-flow ischemia, which is representative of a situation in which the amplitude of the extracellular TQF spectrum is not constant.

THEORY

A. Comparison of TQF_{in} and SQ_{in} Spectra in Monitoring Na_{in} Content

Development of SR-free TQF or DQF NMR as a method for monitoring a change in intracellular Na content first requires characterization of the intracellular Na pool represented by a TQF_{in} spectrum. This is accomplished in the current study by evaluating whether TQF_{in} and SQ_{in} NMR spectra provide identical estimates of a change in Na_{in} content. The amplitude of a TQF spectrum acquired on-resonance at time t, measured from peak height to baseline, is

$$\mathrm{TQF}_{(t)} = \alpha M_0^{\mathrm{TQF}}(e^{-\tau/T_{2s}} - e^{-\tau/T_{2f}})(T_{2s}^* - T_{2f}^*), \quad [1]$$

where $\alpha = \frac{9}{40}$, M_0^{TQF} is the equilibrium magnetization for the TQF spectrum, τ is the creation time, T_{2s} and T_{2f} are the slow and fast transverse relaxation time constants, respectively, and T_{2s}^* and T_{2f}^* are the corresponding inhomogeneity-broadened forms. In this expression, evolution time-dependent terms were not explicitly included. An identical expression can be written for the amplitude of a DQF spectrum when the quadrupolar splitting factor averages to zero, except $\alpha = \frac{3}{20}$. Due to the insertion of a 180° RF pulse between the first two 90° pulses in the pulse sequence (Eq. [9]), the effects of magnetic field inhomogeneities do not have to be considered during the creation time. On the other hand, due to the lack of a 180° pulse during the acquisition time, magnetic field inhomogeneities must be considered during this time interval.

The ratio, $\Delta\mathrm{TQF}(t)$, between the peak amplitudes of a TQF spectrum acquired at time t and at $t = 0$, is given by

$$\Delta\mathrm{TQF}(t) = \frac{\mathrm{TQF}(t)}{\mathrm{TQF}(t=0)} = \frac{M_0^{\mathrm{TQF}}(t)}{M_0^{\mathrm{TQF}}(t=0)} \times \frac{(e^{-\tau/T_{2s}(t)} - e^{-\tau/T_{2f}(t)})}{(e^{-\tau/T_{2s}(t=0)} - e^{-\tau/T_{2f}(t=0)})} \times \frac{(T_{2s}^*(t) - T_{2f}^*(t))}{(T_{2s}^*(t=0) - T_{2f}^*(t=0))}. \quad [2]$$

The peak intensity of an SQ spectrum acquired on-resonance at time t is

$$\mathrm{SQ}(t) = M_0^{\mathrm{SQ}}(0.4T_{2s}^* + 0.6T_{2f}^*), \quad [3]$$

where M_0^{SQ} is the SQ equilibrium magnetization. The ratio, $\Delta\mathrm{SQ}(t)$, between the peak amplitudes of an SQ spectrum acquired at time t and at $t = 0$ is

$$\mathrm{SQ}(t) = \frac{\mathrm{SQ}(t)}{\mathrm{SQ}(t=0)} = \frac{M_0^{\mathrm{SQ}}(t)}{M_0^{\mathrm{SQ}}(t=0)} \times \frac{(0.4T_{2s}^*(t) + 0.6T_{2f}^*(t))}{(0.4T_{2s}^*(t=0) + 0.6T_{2f}^*(t=0))}. \quad [4]$$

As will be shown, it can be assumed that $T_{2s}^* \gg T_{2f}^*$, with the result that the ratio of amplitudes of TQF and SQ spectra

acquired at time t, measured relative to those acquired at $t = 0$, contains no terms arising from the inhomogeneous broadened forms of T_{2s} and T_{2f}; i.e.,

$$\frac{\Delta TQF}{\Delta SQ}(t) = \frac{M_0^{TQF}(t)/M_0^{TQF}(t=0)}{M_0^{SQ}(t)/M_0^{SQ}(t=0)} \times \left(\frac{(e^{-\tau/T_{2s}(t)} - e^{-\tau/T_{2f}(t)})}{(e^{-\tau/T_{2s}(t=0)} - e^{-\tau/T_{2f}(t=0)})} \right). \quad [5]$$

Thus, following compensation for any change in T_2 values, the relationship between M_0 values for TQF and SQ spectra can be determined solely from a change in amplitudes of TQF and SQ spectra.

B. Monitoring Na_{in} Content by TQF_{in} Spectra: Effect of T_2 and T_2^* Values on Amplitudes

The next stage in developing SR-free TQF (or DQF NMR) as a method for monitoring a change in intracellular Na content requires evaluation of the effect of T_{2f}, T_{2s} and their inhomogeneous broadened forms on $\Delta TQF_{in}(t)$ (Eq. [2]). Maximal changes in the transverse relaxation times and their inhomogeneous broadened forms were induced by subjecting hearts to severe pathophysiological conditions, in order to provide an upper limit of the extent to which TQF_{in} amplitudes are affected by these terms.

Since T_2 is related to T_2^* by

$$1/T_2^* = 1/T_2 + \gamma\Delta B_0, \quad [6]$$

where $\gamma\Delta B_0$ is a factor resulting from magnetic field inhomogeneity, the ratios of terms from T_{2s}^* and T_{2f}^* in Eq. [2] can be expressed as

$$\frac{(T_{2s}^*(t) - T_{2f}^*(t))}{(T_{2s}^*(t=0) - T_{2f}^*(t=0))} = \frac{(T_{2s}(t) - T_{2f}(t))}{(T_{2s}(t=0) - T_{2f}(t=0))} \times \frac{(1 + \gamma\Delta\beta_0 T_{2s}(t=0))(1 + \gamma\Delta\beta_0 T_{2f}(t=0))}{(1 + \gamma\Delta\beta_0 T_{2s}(t))(1 + \gamma\Delta\beta_0 T_{2f}(t))}. \quad [7]$$

As will be shown for all conditions examined in the current study, $\gamma\Delta B_0 T_{2f} \ll 1$, so Eq. [7] simplifies to

$$\frac{(T_{2s}^*(t) - T_{2f}^*(t))}{(T_{2s}^*(t=0) - T_{2f}^*(t=0))} = \frac{(T_{2s}(t))}{(T_{2s}(t=0))} \frac{(1 + \gamma\Delta\beta_0 T_{2s}(t=0))}{(1 + \gamma\Delta\beta_0 T_{2s}(t))}. \quad [8]$$

Thus, in contrast to the situation for $(\Delta TQF/\Delta SQ)(t)$ (Eq. [5]), computation of a relative change in Na_{in} content from a relative change in TQF_{in} intensities, $\Delta TQF(t)$ (Eq. [2]), requires evaluation of intracellular T_{2f}, T_{2s}, as well as of T_{2f}^* and T_{2s}^*. This approach contrasts with that employed by others (*11*), in which inhomogeneous-broadened forms of T_2 values were ignored when evaluating a relative change in TQF_{in} intensities. In the current study, justification is provided for the assumptions that $T_{2f} \ll T_{2s}$ and $T_{2f}^* \ll T_{2s}^*$, so only the slow forms of these relaxation times need to be considered. The effect of T_{2s}^* in Eq. [2] was evaluated by measurement of linewidths of SQ_{in} spectra acquired using a Hahn spin-echo sequence, with the spin-echo time set to longer than $3T_{2f}$, so that signal only from the T_{2s} transition is acquired (*12*).

EXPERIMENTAL

Perfusion of Hearts

The protocol for preparation of isolated rat hearts was similar to a previously published method (*1*). Briefly, non-fasting male Wistar rats, weighing ~400 grams, were anesthetized with ketamine (60 mg/ml) and xylazine (20 mg/kg). Following extraction of the heart and cannulation of the aorta, the heart was perfused in a retrograde manner to a constant perfusion pressure of ~90 mm Hg. Left ventricular pressure was monitored via a water-filled balloon (Hugo Sachs Elektronic, March-Hugstetten, Germany) placed within the left ventricle. Balloon volume was adjusted by delivery of a calibrated volume by a Gilmont syringe to produce a constant end-diastolic pressure of ~10 mm Hg. Isolated rat hearts were perfused with modified Tyrode's solution (pH 7.4) containing (in millimolar) NaCl 144, KCl 5, $MgCl_2$ 0.9, HEPES 6, $CaCl_2$ 1.5, and dextrose 15 that was bubbled with 95% O_2. Perfusate reservoirs, lines leading to the heart and the bubble trap, were enclosed within a water jacketing system heated to 35 ± 1°C. To maintain this temperature during no-flow ischemia, the bore of the magnet was continually flushed with a temperature-controlled air stream. A latex balloon encasing the heart minimized the contribution from sodium in the perfusate to the extracellular SQ spectrum. The perfused heart was inserted into an NMR tube (outer diameter of 20 mm), which was filled with a static pool of water to just below the height of the balloon encasing the heart. This improved the heat transfer between the heated air outside the NMR chamber and the heart during no-flow ischemia and aided in tuning the receiver. A suction tube located above the heart, but still within the balloon enclosing the heart, collected perfusate effluent.

To resolve intra- and extracellular ^{23}Na spectra, the sodium salt of the paramagnetic shift reagent, thulium (III) 1,4,7,10-tetraazacyclododecane-*N,N′,N″,N‴*tetra(methylenephosphonate) [$Na_5Tm(DOTP)$] was employed (4.5

mM), with corresponding adjustment of the NaCl level in the perfusate. Due to chelation of divalent cations by Tm(DOTP)$^{5-}$, Ca^{2+} in the perfusate was increased by 3.0 mM in order to maintain a free Ca^{2+} level of 1 mM, which was confirmed by a Ca^{2+} sensitive electrode (Orion). Ouabain was purchased from Sigma.

NMR Methods

All experiments were performed at a resonant frequency of 79.4 MHz for ^{23}Na on a Bruker WB-AM 300 spectrometer. Double- and triple-quantum-filtered spectra were acquired using the pulse sequence

$$90°—\frac{\tau}{2}—180°—\frac{\tau}{2}—\theta—\delta—\theta—\mathrm{Acq}(t_1), \quad [9]$$

where τ and δ denote the creation and evolution times, respectively, and t_1 is the acquisition time (150 ms). The radiofrequency flip angle, θ, was set to either 90° or 54.7° for acquisition of DQF spectra and to 90° for acquisition of TQF spectra. An RF angle of $\theta = 54.7°$ in DQF acquisitions selects only the contribution from a second-rank tensor and suppresses that from a third-rank tensor (*13*); however, setting $\theta = 90°$ in DQF acquisitions selects contributions from both types of tensors. Thirty-two (*3*) and 12-step (*14*) phase-cycling schemes were employed for detection of double- and triple-quantum coherences, respectively. Free-induction decays were exponentially multiplied with a line broadening of 10 Hz prior to Fourier transformation and amplitudes of spectra were measured from baseline to peak.

Experimental Protocols

A. Comparison of TQF_{in} and SQ_{in} Spectra in Monitoring Na_{in} Content

(i) Na_{in} T_{2f} and T_{2s} measurements. A change in transverse relaxation times can affect the amplitude of a TQF spectrum (Eq. [1]). To determine T_{2f} and T_{2s} for the intracellular Na pool, amplitudes of SR-aided TQF_{in} spectra acquired at 10 different τ were fitted to the expression $[\exp(-\tau/T_{2s}) - \exp(-\tau/T_{2f})]$. To compensate for the increase in Na_{in} content during the measurement, the order of the τ values was randomized and the sequence was repeated four times with 72 transients per τ value (for a total of 288 transients for each τ in a cycle). The interpulse delay time was 150 ms and the acquisition time was 250 ms, so the total duration of time required for each T_{2f} and T_{2s} value was 20 min.

(ii) Comparison of TQF_{in} and SQ_{in} spectra. To determine the relationship between the TQF_{in} and SQ_{in} equilibrium magnetizations, M_0^{TQF} and M_0^{SQ}, respectively, SR-aided TQF_{in} and SQ_{in} spectra were acquired before and following discontinuation of perfusate flow or following application of an Na/K ATPase inhibitor, ouabain (500 or 750 μM). For both sets of interventions, a series of intracellular TQF spectra were acquired in an interleaved manner in the presence of an SR at τ equal to 0.8 and 14.0 ms, with a total acquisition time of ~6 min per set of two τ values. Following acquisition of two sets of TQF spectra, an SQ spectrum (using a simple 90° pulse and collect sequence) was acquired in less than one minute. Amplitudes of TQF_{in} spectra were adjusted for the accompanying increases in T_{2s} and T_{2f} (Eq. [5]), in order to allow direct comparison of M_0^{TQF} and M_0^{SQ}.

(iii) Effect of cell volume on Na_{in} T_{2f} and T_{2s}. Intracellular volume was decreased by inclusion of 200 mM sucrose in perfusate (which also contained SR) and Na_{in} T_2 values were measured as described above in Section (*i*) over 20 min.

B. Monitoring Na_{in} Content by TQF_{in} Spectra. Effect of T_2 and T_2^* Values on Amplitudes

The amplitude of a TQF spectrum depends on terms which include T_2 and T_2^* values (Eqs. [3] to [6]). T_{2s}^* and T_{2f}^*, as well as $\gamma\Delta B_0$, were determined as described previously (*12*).

C. Monitoring Na_{in} Content during No-Flow Ischemia by SR-Free TQF or DQF Spectra

(i) Comparison of SR-aided rank 3 (TQF_{ex}) and rank 2 (DQF_{ex}) spectra during no-flow ischemia. One of the conditions that simplify the calculation of a change in Na_{in} content from an SR-free spectrum is the presence of a constant contribution from extracellular Na. The validity of this condition for TQF and DQF NMR spectra during no-flow ischemia was evaluated by interleaving acquisitions of SR-aided spectra derived from both a third-rank (e.g., TQF_{ex}) and a second-rank (e.g., DQF_{ex}, with $\theta = 54.7°$ in Eq. [9]) tensor during no-flow ischemia, at $\tau = 3$ ms every ~9.5 min. The receiver was set to the extracellular resonance and changed as necessary during the experiment to compensate for the small upfield shift of the extracellular spectrum during no-flow ischemia.

(ii) Acquisition of SR-free TQF spectra during no-flow ischemia. To monitor Na_{in} content during no-flow ischemia, TQF spectra were acquired in the absence of an SR (TQF_{total}) for hearts during no-flow ischemia at five different τ. To compensate for the temporal changes in Na_{in} content, the sequence was repeated four times with 96 transients per τ value (for a total of 384 transients for each τ in a cycle), and a total acquisition time of ~12 min per set of five τ values.

(iii) Acquisition of SR-free DQF spectra during no-flow ischemia. To monitor Na_{in} content during no-flow ischemia, three different sets of SR-free spectra [TQF, DQF ($\theta = 90°$), and DQF ($\theta = 54.7°$)] were concurrently acquired for hearts at $\tau = 3$ ms every 6 min.

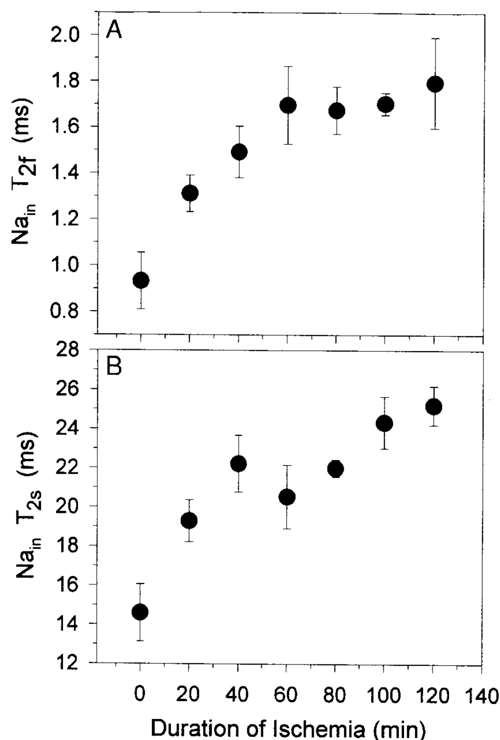

FIG. 1. Intracellular [23]Na (A) T_{2f} and (B) T_{2s} times, determined in the presence of SR during acquisition of TQF spectra, following discontinuation of perfusate flow to the isolated rat heart. Data points are the mean values ± the standard deviation (s.d.) ($n = 5$). Ischemia caused a ~90 and ~70% increase in intracellular T_{2f} and T_{2s} times, respectively, from values obtained during normal baseline perfusion conditions.

RESULTS

A. Comparison of SR-Aided TQF$_{in}$ and SQ$_{in}$ Spectra in Monitoring Na$_{in}$ Content

Na$_{in}$ T_{2f} and T_{2s} measurements during no-flow ischemia. To determine the transverse relaxation times for intracellular Na during no-flow ischemia, a complete set of SR-aided TQF spectra were acquired for different τ, every 20 min. T_{2f} and T_{2s} steadily increased following discontinuation of perfusate flow to ~190 and ~170% of preischemic values, respectively, following 2 h of no-flow ischemia (Figs. 1A and B). For longer durations of ischemia, amplitudes of TQF$_{in}$ and SQ$_{in}$ spectra in some hearts markedly decreased, likely due to leakage of SR into cells.

Comparison of TQF$_{in}$ and SQ$_{in}$ spectra acquired during no-flow ischemia. To determine whether intracellular TQF and SQ NMR spectra provide identical estimates of an ischemia-induced increase in Na$_{in}$ content in the heart, acquisition of TQF (simultaneously acquired at τ equal to 0.8 and 14 ms) and SQ spectra were interleaved in the presence of an SR during no-flow ischemia (Fig. 2A). Discontinuation of perfusate flow caused hearts to stop beating, and following a small delay, amplitudes of TQF$_{in}$ and SQ$_{in}$ spectra steadily increased. The increases in amplitudes of TQF$_{in}$ spectra (ΔTQF$_{in}$) acquired at both short and long τ, measured relative to preischemic levels, were adjusted for the increases in T_{2s} and T_{2f} (Eq. [5]) in order to isolate the TQF equilibrium magnetization terms. Adjustment of the TQF$_{in}$ amplitudes for the increases in T_2 values caused TQF$_{in}$ and SQ$_{in}$ amplitudes to essentially overlap for the entire duration of ischemia investigated (Fig. 2B). Since the ratio of ΔTQF$_{in}$ (T_2-adjusted):ΔSQ$_{in}$ is equal to the ratio of the relative changes in the TQF and SQ equilibrium magnetizations in Eq. [5],

$$\frac{M_0^{\mathrm{TQF}}(t)}{M_0^{\mathrm{TQF}}(t=0)} \Big/ \frac{M_0^{\mathrm{SQ}}(t)}{M_0^{\mathrm{SQ}}(t=0)},$$

the equality of T_2-adjusted ΔTQF$_{in}$ (computed for either τ) and ΔSQ$_{in}$ indicate that M_0^{TQF} and M_0^{SQ} represent the same

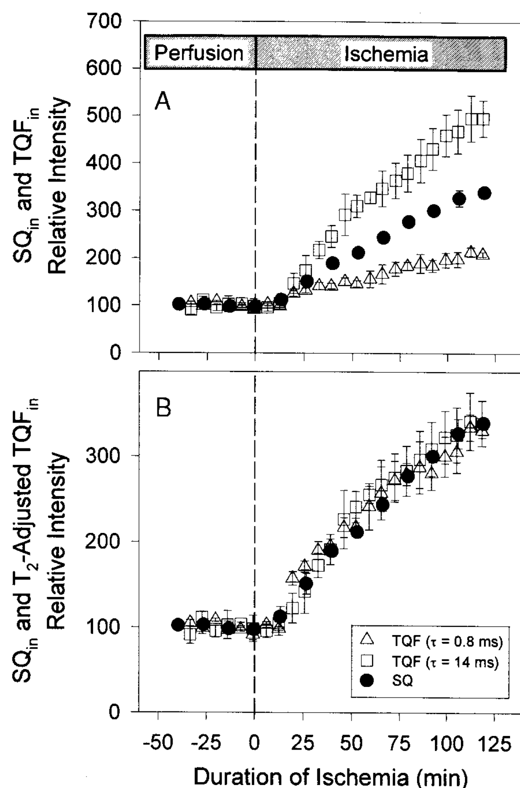

FIG. 2. Dependence of the relative amplitudes of SR-aided SQ$_{in}$ and TQF$_{in}$ spectra (with creation times equal to 0.8 and 14 ms) acquired simultaneously that (A) were not adjusted and (B) were adjusted for the increases in T_2 values with duration of no-flow ischemia. Data points are the mean values ± the s.d. ($n = 3$). Amplitudes of all spectra acquired prior to ischemia were normalized to a value of 100. Adjustment of the amplitudes of TQF$_{in}$ spectra for the increases in T_{2s} and T_{2f} plotted in Fig. 1 allowed direct comparison of M_0^{SQ} and M_0^{TQF}. The equality of ΔSQ$_{in}$ and ΔTQF$_{in}$ (T_2-adjusted) computed for either τ indicates that M_0^{TQF} and M_0^{SQ} represent the same Na$_{in}$ pool within the rat heart.

population of Na_{in}. Thus, even under severe conditions, TQF_{in} and SQ_{in} spectra provide identical estimates of Na_{in} content. This equivalence of the change in TQF_{in} and SQ_{in} equilibrium magnetization constants justified further investigation into employing SR-free TQF NMR as a method for monitoring Na. In particular, the degree to which transverse relaxation-dependent factors alter TQF_{in} spectral amplitudes was determined.

Determination of the Na_{in} content at which T_2's start to increase during no-flow ischemia. It is recognized that acquisition of TQF_{in} spectra at any single τ value, with correction of amplitudes for the increases in T_2 values, would have sufficed for the comparison of SQ_{in} and TQF_{in} M_0 values (Eq. [5]). However, an advantage of acquiring TQF_{in} spectra at τ that differ significantly from τ_{max}, where τ_{max} is the creation time for which the amplitude is maximized [e.g., τ was chosen to be much less (0.8 ms) or greater (14 ms) than τ_{max}, which itself increases from 2.7 to 5.1 ms following 2 h of ischemia due to the increase in T_2 values], is improved temporal resolution of the change in T_2 values during ischemia. For instance, an increase in ΔTQF_{in} (not corrected for changes in T_2 values) for spectra acquired at $\tau \neq \tau_{max}$ will no longer be independent of τ when the T_2 values start to increase (Eq. [2]) and the temporal resolution is improved to ~6 min (the time required for acquisition of 2 TQF_{in} spectra), compared to the 20 min employed for actual determination of the T_2 values. Thus, ΔTQF_{in} values first started to differ for spectra acquired at the two different τ following ~13–19 minutes of ischemia, representing when T_2 values started to increase (Fig. 2A). The continued increase in difference between ΔTQF_{in} values with continued ischemia was due to continued increases in T_{2s} and T_{2f}.

Besides providing a direct comparison of M_0^{TQF} and M_0^{SQ}, concurrent acquisition of TQF_{in} (at $\tau \neq \tau_{max}$) and SQ_{in} spectra also allowed calculation of the value of ΔSQ_{in} for which T_2 values started to increase. Determination of these values of ΔSQ_{in} for different interventions thus allowed an evaluation of the role of the Na_{in} level in modulating transverse relaxation behavior. As a result of the equality of the changes in M_0^{TQF} and M_0^{SQ} during ischemia, ΔTQF_{in} determined at any creation time will match ΔSQ_{in}, but only for as long as the T_2 values do not vary (Eq. [5]). ΔTQF_{in} (not corrected for changes in T_2 values) determined at $\tau \ll \tau_{max}$ and $\tau \gg \tau_{max}$ will display negative and positive deviations, respectively, from ΔSQ_{in} when T_2 values start to increase for concurrently acquired TQF_{in} and SQ_{in} spectra. Thus, following extrapolation of the TQF_{in} spectral amplitudes to take into account the interleaved nature of SQ and TQF spectral acquisitions, the data from Fig. 2A were replotted to allow direct comparison of ΔTQF_{in} and ΔSQ_{in} (Fig. 3A). Values of ΔTQF_{in} matched ΔSQ_{in} up to <140% of preischemic values (Fig. 3A) and then began to diverge. Assuming that the actual change in Na_{in} content equals ΔSQ_{in}, it can be concluded that no-flow ischemia first induces a change in T_2 values when Na_{in} content is <140% of preischemic levels.

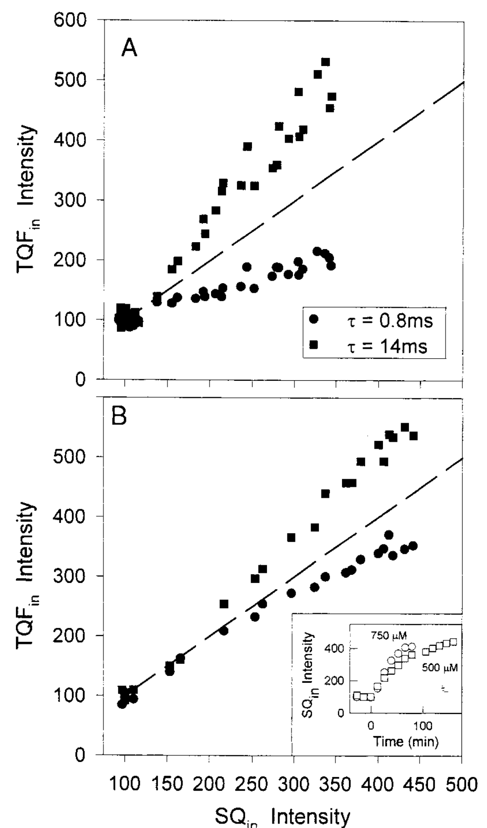

FIG. 3. Relationship of amplitudes of TQF_{in} spectra with amplitudes of SQ_{in} spectra, acquired in the presence of SR, for hearts exposed to (A) no-flow ischemia and (B) 500 and 750 μM ouabain. Data points in (A) represent the amplitudes of those TQF_{in} and SQ_{in} spectra (plotted in Fig. 2A) that were acquired in a near-simultaneous manner (the amplitude of each set of TQF_{in} spectrum was extrapolated to take into account the small difference in time between acquisition of TQF_{in} and SQ_{in} spectra [<1 min]). This format allowed resolution of the value for ΔSQ_{in} for which T_2 values start to diverge. The inset in (B) depicts the dependence of the amplitudes of SQ_{in} spectra on the duration of exposure of the hearts to ouabain, demonstrating that ΔSQ_{in} was at least as great for ouabain as for ischemia. The value of ΔSQ_{in} for which ΔTQF_{in} starts to show a negative and positive deviation for spectra acquired at τ equal to 0.8 and 14.0 ms, respectively (and therefore the value for which T_2 values start to increase) was <140% of normal levels during ischemia and >165% following exposure of hearts to ouabain.

Determination of the Na_{in} content at which T_2 values start to increase during constant pressure perfusion with ouabain. To determine if the T_2 values change at the same value of ΔSQ_{in} for a different intervention, spectra were acquired in an identical fashion from hearts that were perfused at constant aortic pressure with Tyrodes that contained SR and ouabain, a Na/K ATPase inhibitor. Very high concentrations of ouabain (>500 μM) were employed in order to provide a rate and amplitude of increase of ΔSQ_{in} that was equal or greater than that for ischemia (inset in Fig. 3B). At these

TABLE 1
Effect of Decreasing Cell Volume on Na_{in} Transverse Relaxation Times

	T_{2f} (ms)	T_{2s} (ms)
Control perfusion	0.8 ± 0.1	16.1 ± 1.3
200 m*M* sucrose	0.7 ± 0.1	10.6 ± 1.1

doses, the Na/K pump was inhibited to such an extent that the total amount of Na that flows into the cells with each beat was not removed. Hearts failed to beat ~25 and ~10 minutes following application of 500 and 750 μM ouabain, respectively, which correlated with a ΔSQ_{in} of at least 150% of baseline levels in both instances. A plot of ΔTQF_{in} (not corrected for changes in T_2 values) versus ΔSQ_{in} displayed negative and positive deviations from a slope of unity for short and long τ, respectively, at a value of $\Delta SQ_{in} > 165\%$ of pre-ouabain levels (Fig. 3B), significantly greater than the ΔSQ_{in} (<140%) for which T_2 values started to change during ischemia. The magnitudes of the deviations of ouabain-induced ΔTQF_{in} from linearity were not as great as those observed during no-flow ischemia (i.e., compare Fig. 3A with 3B), reflecting a smaller increase in T_2 values with ouabain (data not shown).

Effect of Cell Volume on Na T_2 values. Despite the similarities in the temporal profiles of ΔSQ_{in} for ischemia and constant perfusion with ouabain, T_2 values started to increase at different values of ΔSQ_{in} for these two interventions, indicating that T_2 values depend at least in part on a factor(s) other than Na_{in} content. The two interventions investigated, ischemia and addition of ouabain, depolarize the plasma membrane, and the resulting ion influx was quite likely accompanied at some point with osmotically driven water. To investigate only the effect of a change in cell volume on transverse relaxation times, Na_{in} T_2 values were determined on hearts exposed to 200 m*M* sucrose in the perfusate for 20 min. It was expected that sucrose did not readily enter cells (*15*), consistent with just a minor decrease in ΔSQ_{in} being observed (25 ± 11%). Furthermore, heart function was minimally affected, since addition of sucrose to the perfusate caused only 25 ± 10 and 20 ± 4% sustained decreases in ventricular pressure and heart rate, respectively. Sucrose-induced cell shrinkage decreased both T_{2f} (by ~10%) and T_{2s} (by ~65%) (Table 1), with minimal change in Na_{in} content. This indicated that transverse relaxation behavior in the rat heart can be significantly altered independent of Na_{in} content.

B. *Monitoring Na_{in} Content by TQF_{in} Spectra. Effect of T_2s and T_2^* Values on Amplitudes*

Having shown that TQF_{in} and SQ_{in} spectra respond in an identical manner in rat cardiac tissue, we next quantitate the error introduced by maximum alterations in transverse relaxation times when using TQF_{in} spectra to monitor increases in Na_{in} content. T_{2f}, T_{2s}, and their inhomogeneous broadened forms were measured from TQF_{in} spectra acquired in the presence of an SR during severe pathophysiological interventions (i.e., no-flow ischemia and exposure to high [ouabain]), since these were the maximum possible T_2-related changes likely to be observed. As a result of the increases in both T_2 values, the creation time for which the TQF_{in} intensity was maximized ($\tau_{max}(t)$) shifted from 2.7 during normal baseline conditions to 5.1 ms during 120 min of ischemia. The effect of this shift in τ_{max} values on the creation time-dependent part of the TQF_{in} signal amplitude will be an overestimation of the increase in Na_{in} content of ~7 and ~10% for durations of no-flow ischemia of 40 and 90 min, respectively.

To determine the effect of T_2^* values on ΔTQF_{in} (Eq. [2]), it was assumed that $T_{2f}^* \ll T_{2s}^*$, based on the experimental finding that $\gamma\Delta B_0 = 105\ s^{-1}$ during normal baseline perfusion, and $\gamma\Delta B_0$ decreased at most by 7% from this value following either 90 min of no-flow ischemia or exposure of hearts to 500 μM ouabain (data not shown). This value of $\gamma\Delta B_0$ was significantly less than the value of 330 s^{-1} reported by Allis *et al.* (*12*), presumably due to our use of $Tm(DOTP)^{5-}$ rather than $Dy(PPP)_2$ employed in that study. Thus, the effect of T_{2f}^* on ΔTQF_{in} could be neglected. T_{2s}^*, as measured from the linewidths, increased by 22 ± 14% following 90 min of no-flow ischemia (data not shown). Taking into account the increases in the T_2 (Fig. 1) and T_{2s}^* values, ΔTQF_{in} is calculated from Eq. [2] to overestimate an increase in Na_{in} content by ~6% following 90 min of no-flow ischemia (for spectra acquired at τ_{max}). If TQF_{in} spectra are acquired at a τ value midway between the range of τ_{max} (i.e., at 4 ms), as has been previously suggested in order to eliminate the effect of ischemia-induced increases in T_2 values on ΔTQF_{in} (*11*), this error increases to ~19% due to a lack of compensation for the increase in T_{2s}^*. Thus, since most interventions of general interest will not result in similar magnitudes of increases in cell volume and/or $[Na_{in}]$, the effect of altered T_2 values on TQF_{in} spectral amplitudes acquired at the preinterventional τ_{max} is negligible.

C. *Monitoring Na_{in} Content during No-Flow Ischemia by SR-Free TQF or DQF Spectra*

Comparison of SR-aided rank 3 (TQF_{ex}) and rank 2 (DQF_{ex}) spectra during no-flow ischemia. To be certain that a change in amplitude of a TQF or DQF spectrum acquired in the absence of an SR (ΔTQF_{total} or ΔDQF_{total}, respectively) is due solely to a change in Na_{in} content, it is necessary to ensure that the extracellular spectra remains constant. To characterize the response of extracellular spectra to no-flow ischemia, DQF spectra derived solely from a

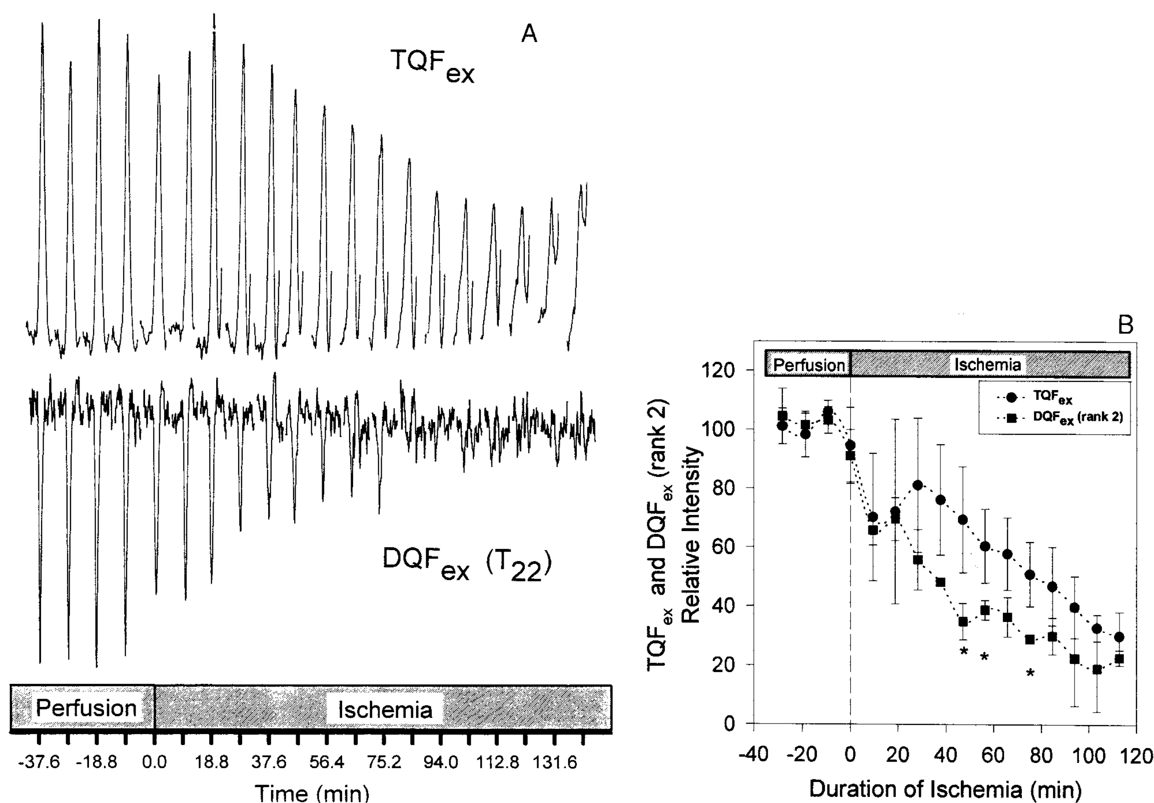

FIG. 4. (A) Extracellular TQF and DQF (with $\theta = 54.7°$ in Eq. [9], so only the contribution from a second-rank tensor was selected) spectra concurrently acquired in the presence of SR, both before and during no-flow ischemia in the isolated rat heart. The widths of each spectrum were 2.8 and 4.9 ppm for TQF_{ex} and second-rank DQF_{ex} spectra, respectively. Second-rank DQF_{ex} were opposite in phase to TQF_{ex} spectra. (B) Amplitudes of TQF_{ex} and second-rank DQF_{ex} spectra decreased with increasing duration of ischemia, measured relative to those spectra acquired during normal baseline conditions, and only significantly differed at three times ($p < 0.05$). Data points are the mean values ± the s.d. ($n = 3$).

second-rank tensor (θ was set to 54.7° in Eq. [9]) were acquired concurrently with TQF spectra (which are derived solely from a third-rank tensor) in the presence of an SR. As discussed below, a direct comparison between extracellular spectra derived solely from a third- (TQF) and a second-rank [DQF, with ($\theta = 54.7°$)] tensor was necessary for the evaluation of which amplitudes of SR-free spectra, ΔTQF_{total} or ΔDQF_{total} ($\theta = 90°$), correlate better with ΔTQF_{in} during no-flow ischemia. As expected (*16*), extracellular DQF spectra (DQF_{ex}) derived from a second-rank tensor were opposite in phase to extracellular TQF (TQF_{ex}) spectra (Fig. 4A), and second-rank tensor DQF_{in} spectra were not detected. TQF_{ex} and TQF_{in} spectra could be resolved for ~110 minutes of ischemia, after which the decrease in chemical shifts between these spectra did not allow accurate measurement of TQF_{ex} amplitudes (Fig. 4A). This progressively poorer resolution of intra- and extracellular TQF spectra can be attributed to a decrease in the molar ratio of $SR:Na_{ex}$ ions, likely due to influx of Na into the cells, and a reduction of the extracellular Na pool due to vascular collapse. The absolute amplitudes of the TQF_{ex} and second-rank DQF_{ex} spectra steadily decreased during ischemia ($n = 3$; Fig. 4B), although the rate of decline was slightly faster for DQF_{ex} spectra.

Monitoring Na_{in} content from SR-free TQF spectra during no-flow ischemia. The degree to which the decline in TQF_{ex} amplitudes causes ΔTQF_{total} to underestimate Na_{in} content during no-flow ischemia was determined by concurrent acquisition of SR-free TQF_{total} spectra over a range of creation times. These creation times included τ_{max} for the Na_{in} TQF signal but, since previous results (*1*) indicated minimal change in ΔTQF_{total} for spectra acquired at τ_{max} for short durations of ischemia, the range $\tau_{max} \leqslant \tau \leqslant 14$ ms was also examined. In addition, TQF_{total} spectra were also concurrently acquired at $\tau = 80$ ms in order to characterize the TQF_{ex} spectral amplitude [since the Na_{in} pool does not contribute to the SR-free TQF_{total} signal at this delay time (*1*)]. At the conclusion of the experiment, values for ΔTQF_{total} were only ~130 to ~160% of preischemic amplitudes for spectra acquired near or at τ_{max} (Fig. 5). These increases were greater than we have previously reported (*1*), due to improved temperature regulation during ischemia, but they still represented only ~25% of the actual increase observed in ΔTQF_{in} for SR-aided TQF spectra acquired under identi-

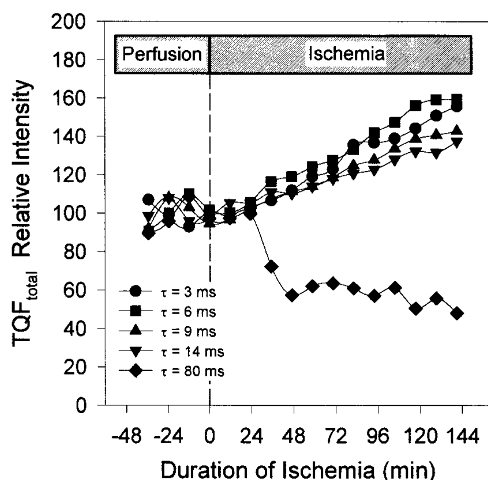

FIG. 5. Dependence of the amplitudes of TQF spectra (ΔTQF_{total}), concurrently acquired in the absence of SR at five different creation times, with duration of no-flow ischemia. Amplitudes of all spectra acquired prior to ischemia were normalized to a value of 100. ΔTQF_{total} varied very little over the range $\tau = 3$ to 14 ms primarily because the higher contributions from TQF_{in} to the TQF_{total} spectrum at short τ were offset by lower magnitudes of ΔTQF_{in} (due to the increase in T_2 values). ΔTQF_{total} decreased to <50% of normal baseline levels for spectra acquired at $\tau = 80$ ms, due to a significant ischemia-induced decrease in ΔTQF_{ex}.

cal conditions (Fig. 3). The substantial decrease in the amplitude of TQF_{total} spectra acquired at $\tau = 80$ ms correlates with the decrease observed in ΔTQF_{ex} for spectra acquired in the presence of an SR (Fig. 4). Thus, Na_{in} content is underestimated from SR-free ΔTQF_{total}, primarily due to a substantial decline in the extracellular TQF amplitude during no-flow ischemia.

Monitoring Na_{in} content from SR-free DQF spectra during no-flow ischemia. In view of the limitations of employing SR-free TQF spectra to monitor Na_{in} content during no-flow ischemia, the feasibility of using SR-DQF spectra was investigated. A DQF_{ex} spectrum (with $\theta = 90°$ in Eq. [9]) acquired from an isolated rat heart can be considered to consist of a spectrum derived from a third-rank tensor, as well as a spectrum of similar shape but opposite phase derived from a second-rank tensor, while a DQF_{in} spectrum contains a contribution solely from a third-rank tensor (*16*). The rates of decrease in amplitude of oppositely phased second- and third-rank extracellular spectra are very similar (Fig. 4B), and the shapes do not appreciably differ [Fig. 4A; (*16*)], so the contribution from Na_{ex} to a DQF_{total} spectrum acquired in the absence of an SR is essentially constant, unlike the situation for SR-free TQF NMR. The important implication of this finding is that a change in Na_{in} content during no-flow ischemia can be estimated from acquisition of SR-free DQF_{total} spectra, provided that the degree to which the extracellular contribution is initially attenuated is known. The degree of attenuation of a DQF_{ex} spectrum can be determined during normal baseline conditions simply from determination of the ratio of amplitudes of SR-free TQF_{total} to DQF_{total} spectra (with $\theta = 90°$ Eq. [9]). For instance, this ratio equals 3.75:1 for complete nulling of the Na_{ex} amplitude for spectra acquired at τ_{max}, computed from the theoretical ratio of 1.5:1 for TQF:DQF signals derived from a third-rank tensor, and by assuming that 40% of the DQF_{total} spectrum (rank 3) represents Na_{in}. Thus, a ratio of TQF_{total}:DQF_{total} spectral amplitudes equal to 3.75 indicates the DQF_{total} spectrum represents solely the DQF_{in} spectrum. It is important to note that complete nulling of the contribution from Na_{ex} to an SR-free DQF_{total} spectrum is not required in order to apply this technique. For instance, an initial TQF_{total}:DQF_{total} ratio less than 3.75 simply indicates the presence of a residual in-phase spectrum from Na_{ex} to the SR-free DQF_{total} spectrum. A ratio greater than 3.75 indicates the presence of a residual oppositely phased spectrum from Na_{ex}, so that the SR-free DQF_{total} signal arising from Na_{in} is attenuated.

To directly compare the DQF and TQF techniques, three different sets of spectra [TQF, DQF ($\theta = 90°$ in Eq. [9]) and DQF ($\theta = 54.7°$)] were concurrently acquired at τ_{max} before and during no-flow ischemia in the absence of an SR ($n = 4$; Fig. 6). The latter type of DQF spectrum represents only extracellular Na (*16*) and were acquired solely to confirm that Na_{ex} content decreased. Following 80 min of ischemia, ΔDQF_{total} was ~280% of preischemic levels (Fig. 6A). Preischemic ratios of TQF_{total}:DQF_{total} spectral amplitudes varied from 3.55 to 4.50 for the hearts studied (data not shown), indicating variable degrees of attenuation of Na_{ex} from DQF_{total} amplitudes, which has been previously reported (*16*). ΔDQF_{total} for each heart was adjusted to take into account any contributions from Na_{ex}, based on deviations of these preischemic ratios of TQF_{total}:DQF_{total} spectral amplitudes from 3.75, yielding an average value of ~300% of baseline values. This resulted in a generally better correlation in ΔDQF_{total} between hearts, as shown by the reduced standard deviations for most time points (Fig. 6A). ΔDQF_{total} (rank 2 only) decreased ~75% from preischemic levels, confirming a substantial decrease in Na_{ex} content. Thus, the destructive interference of the extracellular signals derived from second- and third-rank tensors resulted in a correlation with SR-aided ΔTQF_{in} that was far better for SR-free ΔDQF_{total} compared to ΔTQF_{total} during no-flow ischemia.

DISCUSSION

Identical Responses of Intracellular TQF and SQ Spectra to Intracellular Na

The aim of the current study was to investigate whether SR-free TQF ^{23}Na NMR spectra provides an estimate of Na_{in} content similar to that provided by SR-aided SQ NMR spectra during constant- or zero-pressure conditions. Measurement of T_2 values was necessary to directly compare

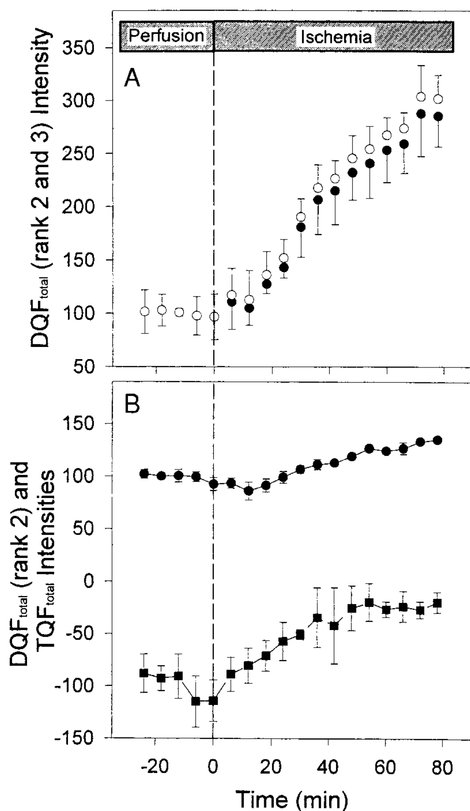

FIG. 6. Dependence of the amplitudes of (A) DQF_{total} spectra (acquired with $\theta = 90°$ in the DQF pulse sequence) not adjusted (●; negative error bars) and adjusted (○; positive error bars), when necessary, for incomplete nulling of the DQF_{ex} contribution (this contribution was nulled in hearts only when the spectral ratio $TQF_{total}:DQF_{total} = 3.75$); (B) TQF_{total} (●) and DQF_{total} (■; with $\theta = 54.7°$ in the DQF pulse sequence, so only the contribution from a second-rank tensor was selected) concurrently acquired with the above spectra in (A). All spectra were acquired at $\tau = 3$ ms in the absence of an SR. Data points are the mean values ± the s.d. ($n = 4$), and amplitudes of all spectra acquired prior to ischemia were normalized to a value of 100. ΔDQF_{total} ($\theta = 54.7°$) represents only Na_{ex} and decreased to <75% of normal baseline levels. Adjusted SR-free ΔDQF_{total} (~300% of normal baseline levels; acquired with $\theta = 90°$) correlated much better than ΔTQF_{total} (~135%) with SR-aided T_2-adjusted ΔTQF_{in} (~280%) for 80 min. of ischemia, primarily due to destructive interference of DQF_{ex} spectra derived from second- and third-rank tensors.

intracellular TQF and SQ equilibrium magnetizations T_2 values recorded in the present study (Fig. 1) were similar to those previously reported for isolated hearts, determined under normal baseline conditions from either MQF (*11, 12*) or SQ (*17*) NMR. Prolonged ischemia resulted in transverse relaxation times similar to those reported here, as determined using DQF NMR (*3*). Also in agreement with the current study (Figs. 3A and 3B), relaxation times have been reported to be independent of minor increases in Na_{in} content of ~150% (induced by hypoxia) (*18*) and ~140% (ischemia) (*19*) of normal baseline levels. In contrast, NMR relaxation times were reported to be independent of a ~500% increase in Na_{in} content (*20*); however, the SR employed in that study markedly impaired baseline heart function, so perhaps baseline relaxation times were already elevated. Ischemia has been reported to induce a rate of increase in transverse relaxation times in isolated rat hearts much higher than that reported in the current study, particularly for T_{2f} values, although the rate of increase in the amplitude of the TQF_{in} spectrum was similar to that observed in the current study (*11*).

Compensation for increases in T_2 values during no-flow ischemia (Fig. 1) allowed the relationship between equilibrium magnetizations, M_0^{TQF} and M_0^{SQ}, to be directly compared (Fig. 2). The equality of SR-aided T_2-adjusted ΔTQF_{in} (computed for both τ examined) and ΔSQ_{in}, and therefore of changes in M_0^{TQF} and M_0^{SQ} (Eq. [5]) at any point during ischemia (Fig. 2), indicate that TQF_{in} and SQ_{in} spectra are sensitive to the same pool of intracellular Na within the rat heart. An identical conclusion was reached in an analogous study on a simpler biological cell system, human erythrocytes (*21*).

Lack of Correlation between T_2 Values and Na_{in} Content or Concentration: The Importance of Cell Volume

The equality of changes in intracellular TQF and SQ equilibrium magnetizations was exploited to determine if either Na content or concentration correlated with T_2 values for different interventions. The value of ΔSQ_{in} for which T_2 values started to increase depended on the nature of the intervention, being <140% of baseline levels during no-flow ischemia (Fig. 3A) and >165% for hearts exposed to ouabain (Fig. 3B). Since the increases in Na_{in} content were similar in both interventions (inset of Fig. 3B), this demonstrates that transverse relaxation times are not determined solely by intracellular Na content. Consideration of this data in view of the differing extents to which cells swell further suggests that transverse relaxation times are not determined solely by intracellular [Na] content either. No-flow ischemia induces acute cardiac cell swelling (*22–26*), attributed primarily to hydrolysis of high-energy phosphates and glycogenolysis (*27*), with only a relatively minor contribution to cellular osmolarity from the accompanying influx of Na (*23*). In contrast, inhibition of the Na^+/K^+ ATPase alone by high concentrations of ouabain causes an initial decrease in volume of isolated myocardial cells (*28*) and no change in intracellular volume in isolated rat hearts (*23*), attributed to the accompanying increase in Ca^{2+} concentration activating Na-dependent (Na^+/Ca^{2+} exchange) and Na-independent mechanisms to limit the rise in intracellular osmolytes (*28*). Thus, the higher Na_{in} content (>165% versus <140%) in swelling-retarded hearts exposed to ouabain implies a much higher [Na_{in}] was attained compared to that of ischemic hearts before initiation of the change in T_2 values. Thus, the correlation between T_2 values and [Na_{in}] was even poorer than that between T_2 values and Na_{in} content. Although cell swelling may be delayed, the well-established ‘‘pump-

leak'' model of cell volume maintenance dictates that inhibition of the Na^+/K^+ pump, whether by pharmacological inhibition (such as by application of ouabain) or by metabolic inhibition (such as by ischemia), eventually results in cell swelling (*29*).

It is important to note that initial changes in T_2 values and cell volume are correlated; i.e., the delayed onset of the increase in T_2 values correlated with a delayed increase in cell volume in ouabain-exposed hearts, relative to ischemic hearts. The sucrose-induced decrease in T_2 values confirmed that manipulations of intracellular volume can alter transverse relaxation behavior, independent of Na_{in} content or concentration (Table 1). This positive correlation between intracellular volume and T_2 values does not appear to be limited to cardiac tissue, having previously been reported for human erythrocytes (*30*). Quite possibly, the effect of cell volume on T_2 values was mediated though alterations in the concentration of those membrane-impermeant macromolecules that affect ^{23}Na relaxation behavior. Thus, for all three interventions investigated in this study—ischemia, inhibition of the Na/K ATPase pump, and hyperosmolarity—T_2 values were proportional to cell volume and were therefore inversely proportional to intracellular macromolecular concentration.

The importance of cell volume, relative to Na_{in} content or concentration, in modifying transverse relaxation behavior is consistent with a wide range of previous studies. An inverse correlation between concentration of macromolecules and ^{23}Na T_2 values at constant Na content has previously been demonstrated in model systems of biological macromolecules (*31*). Previous reports of the independence of Na_{in} content in erythrocytes to both the ratio of MQF:SQ intensities (*21*) and ^{23}Na T_2 values (*32–34*) are also consistent with a limited role for Na_{in} content in modifying transverse relaxation behavior. The reported independence of T_2 values to increased Na_{in} content may be accounted for by a compensatory decrease in intracellular potassium content (*34*), so intracellular osmolarity (and therefore volume) did not change. The limited role of Na_{in} content or concentration, but an important role for cell volume, appears to be most consistent with a model of transverse relaxation behavior outlined by Rooney and Springer (*35, 36*), in which a distribution of correlation times are used to describe aqueous Na. The data in the current study are inconsistent with other models which generally assume rapid exchange of Na between a discrete number of binding sites. In discrete-exchange models, T_2 values are generally assumed to be dependent on [Na] and any inconsistencies in this relationship, such as the collective observations in the current study, are usually explained by assuming a paucity in the number of sites able to bind Na and/or competition from other cations for these sites. Thus, experimental and theoretical analyses are consistent with cellular volume-induced alterations in macromolecular concentration, and not Na_{in} content or concentration, in being most important in modulating Na transverse relaxation behavior. However, this does not exclude other non-Na-related mechanisms altering Na T_2 values in cardiac or other tissue. For instance, acidosis has been reported to increase T_2 values in erythrocytes (*37*) and decrease ^{23}Na DQF intensities in brain homogenate (*7*), at constant Na_{in} content. These results have been explained as H^+ ions effectively competing with Na^+ ions for a discrete number of binding sites, however, severe acidosis-induced alterations in macromolecular conformation may also account for the changes in T_2 values.

Monitoring Cardiac Na_{in} Content Using SR-Free MQF Spectra

SR-free TQF spectra acquired during no-flow ischemia. One of the conditions that must be fulfilled in order to monitor Na_{in} content by acquisition of SR-free TQF spectra is a constant, or at least quantifiable, Na_{ex} spectral amplitude. The substantial decline in the amplitudes of the DQF_{ex} (rank 2) and TQF_{ex} spectra (rank 3; Fig. 4) was most likely due to vascular collapse. More than 70% of the water in the extracellular space (the interstitium, chambers, and vasculature) of isolated rat hearts perfused with a crystalloid solution moves to the surrounding bath within 60 min of no-flow ischemia (*38, 39*). The difference in chemical shifts between intra- and extracellular TQF spectra slightly declined during ischemia (see TQF spectra in Fig. 4A), indicating a decreased molar ratio of SR:Na_{ex}, so some of the reduction in the amplitudes of both the TQF and DQF extracellular resonances (Fig. 4A) can be attributed to signal quenching caused by the SR. However, this formed only a minor role in the decline in the Na_{ex} TQF amplitudes, since amplitudes of SR-free TQF spectra acquired at 80 ms (Fig. 5), and of SR-free DQF (second-rank only) spectra (Fig. 6), which are both representative of solely Na_{ex}, also substantially declined.

The near-independence of ΔTQF_{total} values to creation time during ischemia (reaching a maximum ranging from ~130 to ~160% of preischemic levels; Fig. 5) can primarily be attributed to two opposing influences: ΔTQF_{in} in general increases as the creation time increases, due to the influence of the increase in T_2 values (Fig. 2A), but the contribution from Na_{in}, relative to Na_{ex}, pools to the TQF_{total} signal decreases with increasing creation time. For instance, the contribution from the Na_{in} pool to a TQF_{total} signal is greatest at short τ; however, the increase in the amplitude attributable to the Na_{in} pool (ΔTQF_{in}) is smallest at short τ (Fig. 2A).

ΔTQF_{total} was ~150% of baseline values for SR-free TQF spectra acquired at τ_{max} following 90 min of no-flow ischemia (Fig. 5), compared to ~300% for ΔTQF_{in} computed from SR-aided spectra (Fig. 3). The substantial decline in the amplitudes of extracellular TQF spectra acquired at τ_{max} during no-flow ischemia (Fig. 4) was primarily responsible for the ~75% underestimation of Na_{in} content by SR-free

TQF NMR in this experiment. This underestimation may also result from misestimation of the relative contributions from intra- and extracellular Na to the TQF_{total} spectrum during normal baseline conditions, lack of compensation for any changes in Na_{ex} T_2 or T_2^* values, or a more deleterious insult in the presence of an SR. Until the actual decrease in extracellular content is better defined, the SR-free TQF NMR technique is less accurate in monitoring intracellular Na content during zero-pressure compared to constant-pressure crystalloid perfusion.

In contrast to the data in the current study, Schepkin *et al.* (*40*) recently reported that the extracellular Na contribution to the TQF signal was not altered by cessation of flow and, on this basis, argued that SR-TQF spectra accurately reflect Na_{in} content. However, that result was based on experiments in which hearts were reperfused at selected intervals during no-flow ischemia. Reperfusion may have reversed vascular collapse in that study, since we have shown that the amplitude of the extracellular TQF spectrum does not change during constant-pressure perfusion, even when the heart is severely compromised (*1*).

SR-free DQF spectra acquired during no-flow ischemia. In contrast to SR-free TQF NMR, it is not necessary to compensate for the decline in extracellular Na content during no-flow ischemia using SR-free DQF spectra, since this decline is largely negated due to the destructive interference of extracellular spectra derived from second- and third-rank tensors (Fig. 4). However, ΔDQF_{total} determined in the absence of an SR overestimates ΔDQF_{in} by a minor amount, since the destructive interference between the second- and third-rank tensors was not complete [i.e., the extracellular amplitude for the opposite-phased DQF_{ex} (rank 2) spectrum decreased slightly faster than that for the normal-phased TQF_{ex} (rank 3) spectrum (Fig. 4), thereby imparting a small net normal-phased spectrum]. The slightly higher rate of decline in the DQF_{ex} (rank 2) resonance may be due to (i) a decrease in the quadrupolar splitting factor, ω_q, (ii) greater quenching of the third-rank TQF_{ex} signal compared to the second-rank DQF_{ex} signal or; (iii) different sensitivities of DQF_{ex} (rank 2) and TQF_{ex} spectra to extracellular Na. Following adjustment to take into account the variability in the ratio of TQF_{total}:DQF_{total} spectral amplitudes, SR-free ΔDQF_{total} (~300% of baseline levels) much more closely approximated SR-aided ΔTQF_{in} (~275%) than ΔTQF_{total} (~135%), measured 80 min. following cessation of perfusate flow (Fig. 6). Thus, despite the uncertainties associated with employing a method based on destructive interference of signals which are not fully defined, the SR-free DQF method provides an estimate of Na_{in} content similar to that provided by the SR-aided method.

CONCLUSIONS

It is concluded that SR-free ^{23}Na TQF NMR may be employed to monitor Na_{in} content under constant perfusion pressure in the isolated crystalloid-perfused rat heart. Under this condition, Na_{in} content will be overestimated by ~6% from SR-free TQF spectra. This ''error'' will only be experienced for changes in Na_{in} content that are accompanied by substantial changes in cell volume. Potential applications of this technique during constant-pressure perfusion are many and include monitoring the effects of pathophysiogical interventions and pharmacologic agents on Na_{in} content. In the case of no-flow ischemia, both SR-free TQF and DQF spectra respond to increases in Na_{in} content, but DQF NMR provides an estimate of Na_{in} content much closer to that provided by the SR-aided method. The SR-free DQF NMR method should be useful in those situations in which Na_{in} content is expected to be further altered in some manner during no-flow ischemia, such as by addition of a pharmacologic agent.

Some predictions on the feasibility of using SR-free multiple-quantum-filtered NMR to monitor Na content in biological samples other than the isolated rat heart are warranted. In isolated perfused organs, T_{2f} and T_{2s} will be identical for Na in the perfusate not in contact with tissue, so the contribution from intracellular Na should be substantially greater to the TQF than to the SQ spectrum. This appears to be the case for the isolated liver (*41*). If changes in relaxation-dependent terms are not any greater than those observed here with rat hearts, SR-free TQF NMR should allow accurate monitoring of Na_{in} content in isolated organs during perfusion with crystalloid buffer. A recent report of a substantial decrease in the extracellular SQ intensity during no-flow ischemia in *in vivo* liver (*42*) suggests that SR-free TQF NMR spectra will underestimate Na_{in} content. SR-free TQF spectra can be used to study no-flow conditions in isolated cells, provided that care is taken to minimize the contribution from perfusate Na during the experiment (*5*).

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