NMR measurement of the off rate from the first calcium-binding site of the synaptotagmin I C_2A domain

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Abstract The off rate from the first calcium-binding site of the C_2A domain of synaptotagmin I, a putative calcium receptor in neurotransmitter release, has been determined by ^{15}N-nuclear magnetic resonance relaxation dispersion measurements. The exchange rate was obtained by fitting the dependence of the transverse relaxation rates on the interval between 180° pulses in relaxation-compensated CPMG experiments at 3.2 μM calcium concentration. The measured k_{ex} is 2.0×10^3 s^{-1}. The calcium on rate of $3.5\pm1\times10^7$ s^{-1}, determined from the measured off rate and the dissociation constant (5.3×10^{-5} M), is close to the diffusion limit. These results are consistent with the proposed role of synaptotagmin I as a calcium sensor in release, but suggest that additional factors may help to accelerate the diffusion of Ca^{2+} to the sensor.

Key words: Neurotransmitter release; Synaptotagmin; C_2 domain; Calcium binding; Relaxation; Nuclear magnetic resonance

1. Introduction

The release of neurotransmitters by synaptic vesicle exocytosis is a central step in interneuronal communication. Release occurs very fast (< 1 ms) in response to an action potential and is tightly regulated by Ca^{2+}. The synaptic vesicle protein synaptotagmin I is specifically required for the last, Ca^{2+}-triggered step of fast neurotransmitter release [1] and is the primary candidate to act as a Ca^{2+} sensor in this process [2,3]. Most of the cytoplasmic region of synaptotagmin I is formed by two C_2 domains, which are widespread Ca^{2+}-binding modules with a β-sandwich structure (reviewed in [4]). The first of these C_2 domains (the C_2A domain) binds three Ca^{2+} ions in a tight cluster at the top loops of the β-sandwich [5] (see Fig. 1), and most likely functions via its Ca^{2+}-dependent interactions with negatively charged phospholipids and with SNARE proteins [6–8]. The second C_2 domain (the C_2B domain) has recently been found to bind two Ca^{2+} ions and to also interact with phospholipids in a Ca^{2+}-dependent manner [9,10].

Attempts to correlate the biochemical properties of synaptotagmin I with the electrophysiological characteristics of neurotransmitter release have focused on the equilibrium constants for Ca^{2+} binding. The Ca^{2+}-binding sites of synaptotagmin I have low intrinsic affinities [5,10]. However, the apparent Ca^{2+} affinities in the presence of phospholipids (5–10 μM) [6,10,11] correlate well with the Ca^{2+} concentrations required for release at the calyx of Held, which have recently been found to be ~10 μM [12,13]. These low Ca^{2+} concentrations and the fast speed of release impose stringent kinetic requirements, but much less is known about the ability of synaptotagmin I to meet these requirements. The kinetics of Ca^{2+}-dependent binding of the C_2A domain to phospholipids were studied by stopped-flow fluorescence [8], and the time constant measured for phospholipid dissociation (ca. 4 ms) indicates that an equilibrium may not be reached in the time scale of fast neurotransmitter release. The kinetics of intrinsic Ca^{2+} binding to the C_2A domain were found to be too fast to be measured by this technique.

Because of the high Ca^{2+} cooperativity of neurotransmitter release, current models used to fit electrophysiological measurements to the kinetic constants involved in Ca^{2+} triggering of release assume sequential binding of at least five Ca^{2+} ions to the Ca^{2+} sensor, followed by a fusion-promoting step [12,13]. Whereas the latter step could involve, at least in part, Ca^{2+}-induced binding of synaptotagmin I to phospholipids, which exhibits high Ca^{2+} cooperativity [11], the initial steps of these models represent intrinsic binding of Ca^{2+} ions to individual sites of the sensor. Hence, a detailed comparison between the rate constants of these initial steps derived from electrophysiology and the rate constants of intrinsic Ca^{2+} binding to the presumed sensor is critical to assess whether the sensor can meet the kinetic requirements of neurotransmitter release. As a first step in this direction, we have determined the off rate of the highest-affinity Ca^{2+}-binding site from the synaptotagmin I C_2A domain using nuclear magnetic resonance (NMR) transverse relaxation measurements, which are sensitive to exchange processes that take place in the milli–microsecond time scale.

These techniques have been applied to study conformational changes [14], domain motions [15] and ligand-binding processes [16]. Calcium-binding rates to calmodulin have been studied by Malmendal et al. using the exchange contribution to ^{15}N transverse relaxation [17].

2. Materials and methods

2.1. Theory

The apparent transverse relaxation rate in the presence of exchange

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is a function of τ_{cp}^{-1}, the reciprocal of the time separation between consecutive π pulses in the CPMG train in a relaxation-compensated Carr–Purcell–Meiboom–Gill (RC-CPMG) experiment [18]. As an alternative to measuring the complete decay for each τ_{cp}^{-1} value, relaxation rates can be determined from the ratio of two points in a constant-time RC-CPMG experiment (CT-RC-CPMG) [19]. This faster method allows a much better sampling of the relaxation dispersion profile even though each particular transversal rate is less well determined.

The rank of application of the method is limited by the condition $k_{ex}\tau_{cp} > 1$. Thus, CPMG experiments are eligible for processes in the slow exchange and for those in the fast exchange regime provided that $k_{ex} < 2000$–$2500\ s^{-1}$. The time scale of the process can be determined by exploration of the static magnetic field dependence of the dispersion profile [20].

The exchange contribution to transverse relaxation for a two-state system in the fast exchange limit is given by

$$R_{ex} = p_a\, p_b\, \Delta\omega^2/k_{ex} \quad (1)$$

where p_a and p_b are the populations of the two states, $\Delta\omega$ is the frequency difference between the chemical shifts of the two sites and k_{ex} is the sum of the pseudo-first order rate constants for the direct and reverse processes. In bimolecular processes, such as calcium binding, the pseudo-first order rate constant for the binding process depends on the concentration of calcium:

$$k_{ex} = k_{on}[Ca^{2+}] + k_{off} \quad (2)$$

At low calcium concentrations, the NMR-measurable k_{ex} for calcium binding approaches k_{off} and the calcium-free form is usually the only observable species (i.e. $p_a \gg p_b$).

Assuming exchange occurring only between two states, the exchange rate constant and the population of each conformer can be determined by fitting the experimental dispersion profile to the relevant equation. Carver and Richards derived an approximate equation for systems composed of two exchanging sites with arbitrary populations, which is valid for all time scales [21]. Ishima and Torchia have suggested a simpler empirical function that approximates the complete expression and that is valid over all time scales provided that $p_a \gg p_b$ [22]. In the fast exchange limit, dispersion curves can be fitted to the formula [23]:

$$R_2\,(\tau_{cp}^{-1}) = R_2 + R_{ex}\,[1 - 2\tanh(k_{ex}\,\tau_{cp}/2)/(k_{ex}\,\tau_{cp})] \quad (3)$$

2.2. NMR experiments

Measurements of k_{ex} to determine the off rate of the first Ca^{2+}-binding site of the C_2A domain were performed with a [u-^{15}N]D232N C_2A domain sample that was expressed as a glutathione *S*-transferase fusion protein, cleaved with thrombin and purified as described previously [5]. The sample used contained 0.93 mM protein, 0.198 $CaCl_2$, 0.198 mM EDTA, 20 mM Tris, 100 mM NaCl, pH 7.4. The concentration of the relevant species was calculated using a dissociation constant for calcium-bound D232N C_2A of 53 μM and for Ca-EDTA of 1.26 μM [24]. The concentration of free calcium is 0.7 μM and the population of the calcium-free form is 0.987. The temperature of the experiments was 298 K and was calibrated before each measurement using a sample of ethylene glycol. Assignments of ^{1}H–^{15}N-HSQC cross-peaks were obtained from a 3D ^{1}H–^{15}N-NOESY-HSQC experiment carried out on a Varian Inova 600 spectrometer, by comparison with the assignments of the wild type C_2A domain [25]. Chemical shifts of the calcium-free and calcium-bound forms were correlated by calcium titration, which also afforded the dissociation constant. RC-CPMG experiments were carried out using a Bruker Avance 800 spectrometer. For each value of τ_{cp}^{-1} the decay of the signal was measured using eight data points with two duplicate points per curve. Relaxation rates and their errors were obtained using the CurveFit program. Up to six values of τ_{cp}^{-1} (2000, 1000, 500, 250, 166.7, 125 s^{-1}) were used to characterize the relaxation dispersion curves. CT-RC-CPMG experiments were performed using 16 relaxation points including two duplicates at frequencies: 50, 100, 150 (2), 200, 250, 300, 350, 400, 500 (2), 600, 700, 800, 900, 1000. Fitting of the relaxation dispersion curves was done using Microcal Origin (Microcal Software) or Mathematica (Wolfram Research).

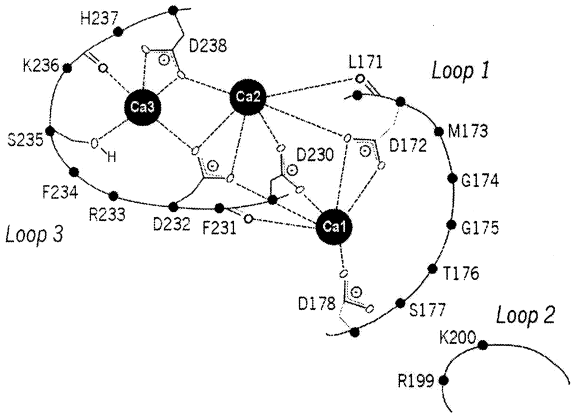

Fig. 1. Diagram summarizing the Ca^{2+}-binding sites of the synaptotagmin I C_2A domain (see [5]). Only the side chains and backbone carbonyl groups involved in Ca^{2+}-binding are shown. The ligands for the three sites arise from two loops located at the top of a β-sandwich (loops 1 and 3), but another loop (loop 2) is also close to the binding sites. Mutation of Asp232 to Asn (D232N C_2A mutant) abolishes Ca^{2+} binding to sites Ca2 and Ca3 while preserving binding to site Ca1 [5].

3. Results

The kinetics of Ca^{2+} binding to the C_2A domain are complicated by the fact that this domain binds three Ca^{2+} ions in a tight cluster (Fig. 1) with intrinsic affinities of 54 μM, 530 μM and >10 mM [3,5]. In order to focus on the off rate of the highest-affinity Ca^{2+}-binding site of the C_2A domain, we studied the D232N C_2A mutant in which only this site is preserved [5]. As in the wild type C_2A domain, the D232N C_2A mutant binds calcium without a substantial conformational change [26,27], and the equilibrium Ca^{2+} dissociation constant of D232N C_2A (53 μM; our unpublished results) is analogous to that of the high-affinity site of the wild type C_2A domain. Hence, the wild type kinetic constants for Ca^{2+} binding to the highest-affinity site can be assumed to be comparable to those of the same site in this mutant.

Fig. 2A shows the transverse relaxation curves, measured at 18.79 T with τ_{cp} values of 1 and 6 ms, for the backbone ^{15}N signals of R199 and Q209 from D232N C_2A. The former is representative of a residue close to the calcium-binding site while the second is not affected by calcium. The exchange contribution manifests itself in the faster relaxation and in the dependence of the relaxation rates on the value of τ_{cp}. Fig. 2B shows the difference between the relaxation rates measured with the same τ_{cp} values. Only residues close to the calcium-binding loops show significant differences between the two rates, in agreement with the absence of substantial conformational changes upon calcium binding by the C_2A domain of synaptotagmin.

Relaxation dispersion curves for all residues showing calcium-dependent exchange processes measured with the RC-CPMG method were fitted individually to Eq. 3, leaving the chemical shift differences between the calcium-free and calcium-bound forms as adjustable parameters. The chemical shift differences that provide the best fit to the experimental data are in good agreement with the ones determined experimentally by titration (Fig. 3), providing further confirmation that calcium binding is the observed exchange process.

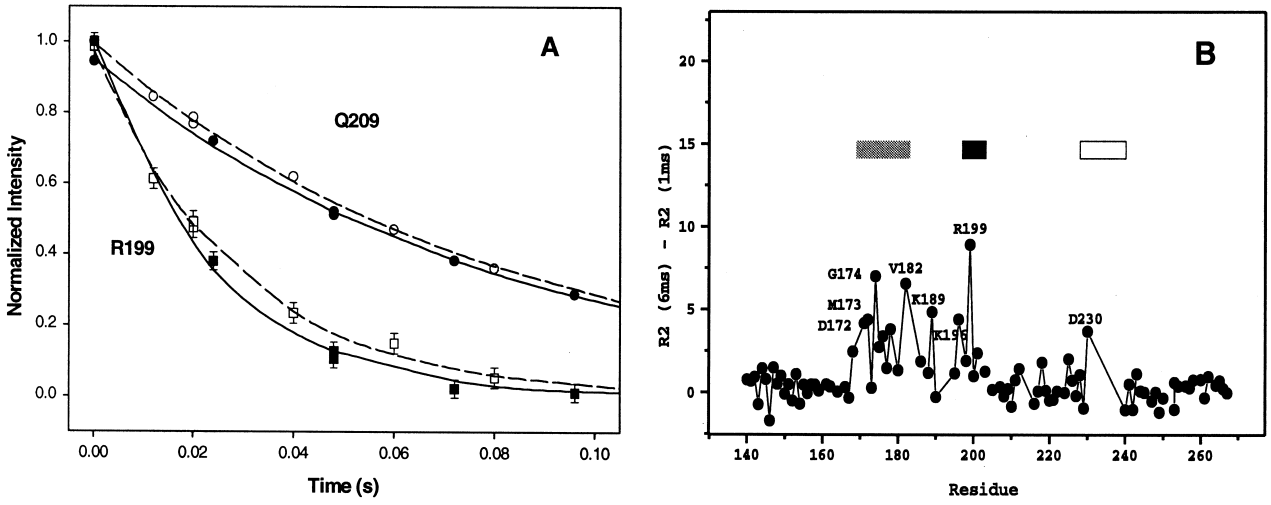

Fig. 2. A: Typical relaxation curves obtained with CPMG sequences with an interpulse delay of 1 ms (dashed line, open symbols) and 6 ms (solid line, filled symbols) for a residue involved (R199) or not involved (Q209) in chemical exchange. B: Difference between the apparent relaxation rates determined with interpulse times of 6 ms and 1 ms. Calcium-binding loops are indicated.

Finally, the complete set of relaxation dispersion curves was fitted simultaneously using Mathematica (Wolfram Research). The experimentally determined chemical shift differences were used for these calculations. The uncertainty in the calculated exchange rate was determined by a Monte Carlo simulation. One thousand artificial data sets were produced by assigning to the relaxation rates random values according to a Gaussian distribution centered in the measured value and with the standard deviation given by the fitting error provided by CurveFit. The average rate constant and the standard deviation are 2017 ± 580 s^{-1}. A second set of data collected using the CT-RC-CPMG technique led to a value of 2147 ± 237 s^{-1} when all exchanging residues were simultaneously fitted to Carver and Richards equation. The agreement between both values validates the experimental methods as well as the equations used in the fitting procedure. Assuming that the exchange rate constant measured reflects primarily the off rate at the Ca^{2+} concentrations used, the on rate calculated from the off rate and the equilibrium dissociation constant is $3.9 \times 10^7 \pm 0.8 \times 10^7$ M^{-1} s^{-1}, which approximates a diffusion-controlled rate.

4. Discussion

Extensive evidence indicates that synaptotagmin I acts as a Ca^{2+} sensor in neurotransmitter release [1–4]. While the apparent Ca^{2+} affinities of the synaptotagmin I C_2 domains in the presence of phospholipids [5,10,11] are consistent with such a role, much less attention has been paid to assess whether synaptotagmin I can meet the stringent kinetic requirements imposed by the fast speed of release, and the rates of intrinsic Ca^{2+} binding to the synaptotagmin I C_2 domains had not been studied. The results described here provide the first such measurements, focussing on the highest-affinity Ca^{2+}-binding site of the C_2A domain, and allow a comparison with the rates of intrinsic Ca^{2+} binding to the Ca^{2+} sensor deduced by fitting the time and Ca^{2+} dependence of neurotransmitter release observed by electrophysiological measurements [12,13]. Such comparisons should be made with caution since specific features of the release machinery may affect the kinetic parameters and there could be more than one Ca^{2+} sensor. Moreover, the electrophysiological models are necessarily oversimplified, for instance by assuming that all Ca^{2+}-binding sites in the sensor are equivalent, and differ in some details. In one study, where a cooperativity factor for Ca^{2+} binding to five sites was introduced, the on and off rates derived for intrinsic Ca^{2+} binding were 9×10^7 M^{-1} s^{-1} and 9500 s^{-1}, respectively [13], whereas another study that did not introduce such a factor yielded values of 3×10^8 M^{-1} s^{-1} and 3000 s^{-1} for the on and off rates, respectively [12]. The off rate for the highest-affinity Ca^{2+}-binding site of the synaptotagmin I C_2A domain obtained from our data (2.0×10^3 s^{-1}) is similar to that derived in the latter study and, taking into account the variability between the two studies, can be considered fully consistent with a role as a Ca^{2+} sensor in neurotransmitter release. The on rate deduced from our data (ca. 4×10^7 M^{-1} s^{-1}) approaches that expected for a diffusion-controlled pro-

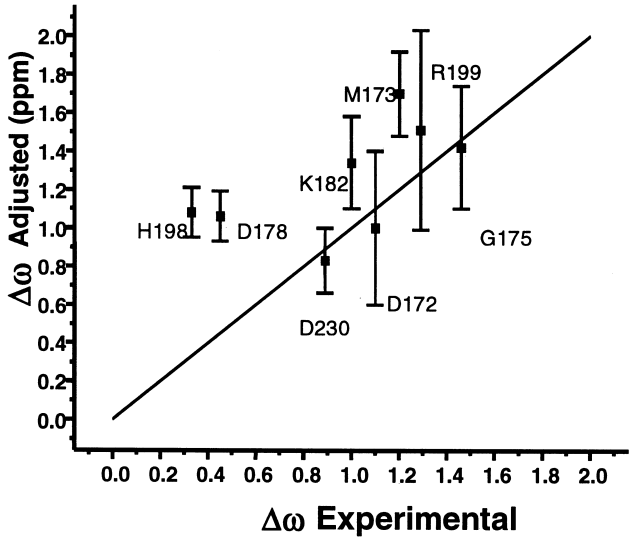

Fig. 3. Plot of chemical shift differences between the calcium-bound and apo forms obtained by titration with the values that provide the best fit to the relaxation dispersion curves.

cess but is somewhat slower than the on rates obtained in both electrophysiological studies. It is possible that this slight discrepancy arises from oversimplification of the models or other considerations mentioned above. Alternatively, it is plausible that, in vivo, access of Ca^{2+} to synaptotagmin I may be facilitated by proximity to Ca^{2+} channels or may be accelerated by specific features of the release apparatus. For instance, the Ca^{2+}-dependent interactions of the C_2A domain involve negatively charged targets such as phospholipids and SNARE proteins, and the specific spatial arrangement of the targets and the C_2A domain may create a negative electrostatic potential that accelerates diffusion of Ca^{2+} even before the Ca^{2+}-dependent interaction occurs. Determination of the kinetic constants for Ca^{2+} binding to the other sites of synaptotagmin I, and analysis of the electrophysiological data assuming non-identical Ca^{2+}-binding sites, will help to distinguish between these possibilities. The kinetic constants described here for the highest-affinity site of the C_2A domain will facilitate application of the same methodology to analyze the other two sites in the wild type C_2A domain and other mutants, and an analogous approach can yield the kinetic constants for the C_2B domain Ca^{2+}-binding sites.

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