New Calcium-Sensitive Ligand for Nuclear Magnetic Resonance Spectroscopy†

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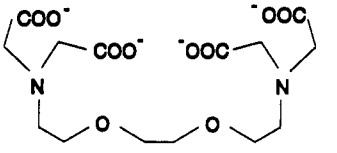
ABSTRACT: Fluorinated calcium-sensitive indicators such as 5,5′-difluoro-1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (difluoro-BAPTA) will often be less sensitive under in vivo conditions than gyromagnetic ratio considerations alone would have predicted. This is due to the very broad line widths displayed by these molecules within the living cell. In order to provide a spectroscopic alternative to these molecules, we have synthesized ^{13}C-enriched 1-(2-aminophenoxy)-2-(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid or AATA. The rationale for the design of this molecule was the increased signal to noise ratio available by selective detection of ^{13}C-attached protons in AATA using proton-observe carbon-edited spectroscopy or multiple-quantum coherence. AATA has the advantage of increased number of detectable nuclei and narrow line widths. As such, it should provide a 6–10-fold improvement in the signal to noise ratio over existing fluorinated indicators. As a hybrid between EGTA and BAPTA, AATA should display intermediate pK_a's, exchange rates, and K_D values. We have measured pK_a values of 5.94 ± 0.05 and 9.03 ± 0.05 for AATA. K_D values of 350 ± 80 nM and 6.6 ± 2.0 mM were obtained for the AATA–Ca^{2+} and AATA–Mg^{2+} interactions, respectively, at 37 °C in 0.1 M KCl. As such, this new ligand displays the expected selectivity for Ca^{2+} over Mg^{2+}. This new approach to detection of intracellular probes with NMR can be readily extended to other probes for intracellular ions, pH, and membrane potential. In addition, the move toward carbon-selected proton spectroscopy should also permit more flexibility in synthetic approaches since the strong electronegativity of fluorine often hampers synthetic design.

Recognizing that EGTA[1] (**1**) (Schmid & Reilley, 1957) displayed a high selectivity for Ca^{2+} over Mg^{2+}, Tsien (1980), with the synthesis of BAPTA, first proposed that EGTA be systematically modified to produce rationally designed Ca^{2+}-specific molecular probes. As a result, a wide array of EGTA-based Ca^{2+}-sensitive probes have now become available to the cellular biologist (Grynkiewicz et al., 1984).

EGTA has a 1:1 stoichiometric relationship with Ca^{2+}. While its K_D value for this ion is in the physiological range, this dissociation constant is very pH sensitive (Harrison & Bers, 1987). Moreover, since two nitrogen-bound protons must be expelled by Ca^{2+} during coordination with EGTA at neutral pH values (Martell & Smith, 1974), this molecule exhibits very slow off rates in Ca^{2+} buffering. In addition, EGTA offers no elegant means of spectroscopic detection within cells. In sharp contrast, the family of molecules proposed by Tsien (1980) have the advantage that they maintain stoichiometric and K_D characteristics, while introducing fast buffering times and pH insensitivity (Harrison & Bers, 1987) due to the very low pK_a values of these molecules. In addition, the fluorescence spectra of these molecules are



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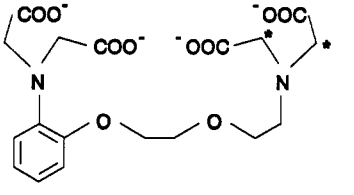

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very sensitive to coordination Ca^{2+}, and, as such, several of these ligands can be used intracellularly.

While the fluorescent characteristics of these molecules make them ideal candidates for the analysis of single cells and cell layers, they prevent their use for the analysis of entire organs, particularly in vivo. As a result, spectroscopists (Smith et al., 1983; Levy et al., 1987) have chosen to synthesize fluorinated derivatives of BAPTA and QUIN2 for use in NMR. The rationale for the incorporation of fluorine labeling was the 100% natural abundance of ^{19}F, its high relative sensitivity, and its low natural background. Unfortunately, fluorination has not risen to its initial promise. This was due in part to the suboptimal signal to noise ratio encountered with fluorinated probes. As such, for feasible NMR detection, it was often necessary to load the cells with quantities of probe which were known to buffer intracellular Ca^{2+} (Barritt & Lee, 1985).

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[1] Abbreviations: AATA, 1-(2-aminophenoxy)-2-(2-aminoethoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid. The systematic name for ^{13}C-enriched AATA is *N*-[2-[2-[2-[bis(carboxymethyl)amino]phenoxy]ethoxy]ethyl]-*N*-(carboxy[^{13}C]methyl)[2-^{13}C]glycine tetrasodium salt; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; DBBAPTA, 5,5′-dibromo-BAPTA; DFBAPTA, 5,5′-difluoro BAPTA; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FURA2: 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; INDO1: 1-[2-amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; QUIN2, 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis(carboxymethyl)-amino]quinoline.

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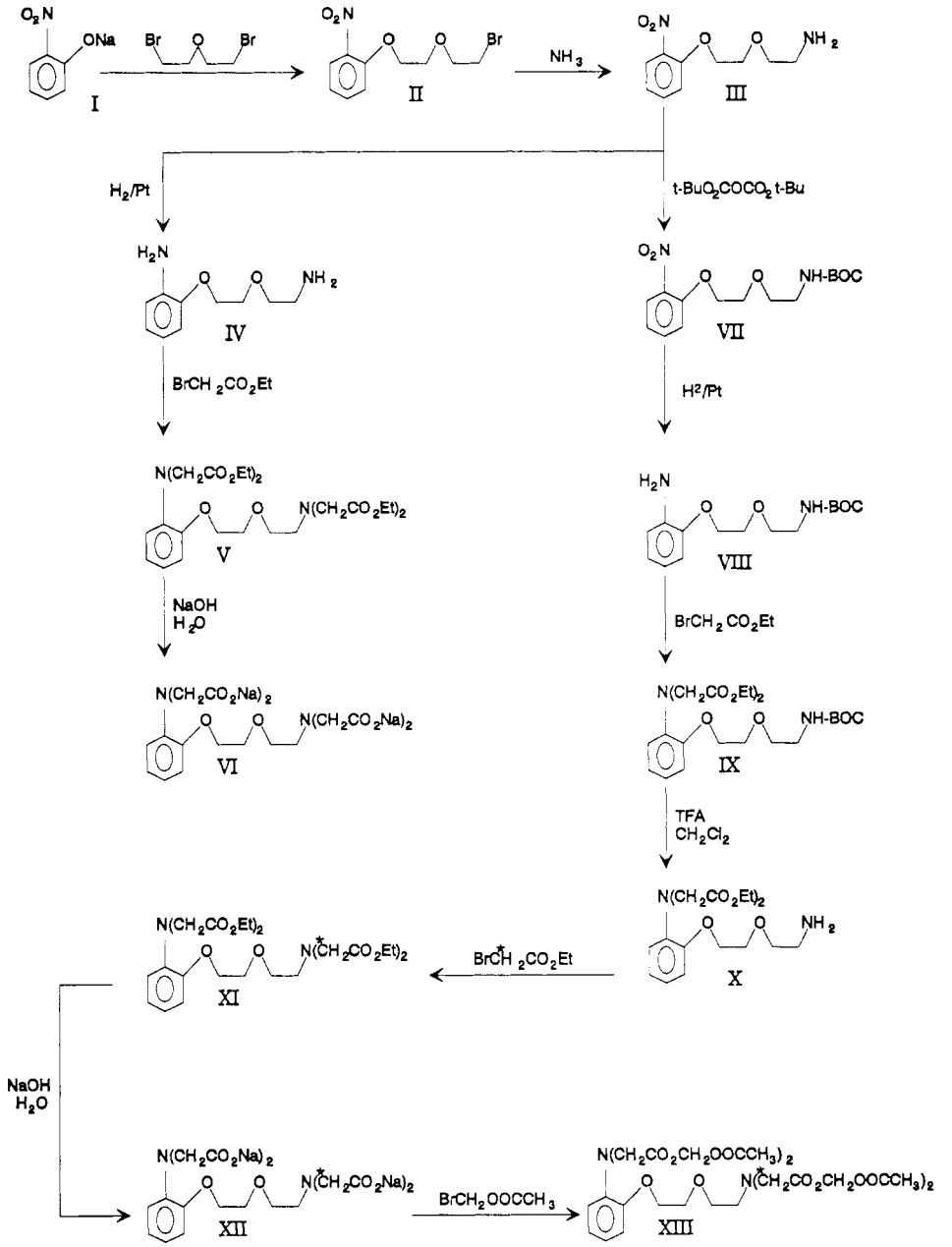

FIGURE 1: Synthetic pathways to AATA and to ^{13}C-enriched AATA.

Early experience with fluorinated molecules revealed that they lacked NMR sensitivity partly due to fluorine's very broad line widths in vivo (Marban et al., 1988). While it is clear that the line widths obtained with molecules like DFBAPTA in solution are primarily related to the rates of Ca^{2+} exchange, exchange rates alone cannot explain the very large line widths observed in the perfused heart (Metcalfe et al., 1985; Marban et al., 1988). It appears that the line widths exhibited by these molecules under in vivo conditions reflect fluorine's large range of chemical shifts. This may account for fluorine's extreme sensitivity to changes in its microscopic environment.

Prompted by the importance of line widths and a desire to improve the signal to noise ratio, we have synthesized ^{13}C-enriched 1-(2-aminophenoxy)-2-(2-aminoethoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, or AATA (**2**). The ^{13}C atoms are denoted with an asterisk and have been incorporated to permit the selective detection of ^{13}C-attached protons using proton-observe carbon-edited spectroscopy (Bendall et al., 1981) or multiple-quantum coherence (Ernst et al., 1987). This replaces the two fluorine atoms of molecules like DFBAPTA with four NMR equivalent ^{13}C-attached protons. As a hybrid between EGTA and BAPTA, AATA should have intermediate exchange rates and K_D values.

EXPERIMENTAL PROCEDURES

The synthetic route to AATA and to ^{13}C-enriched AATA is illustrated in Figure 1. Synthetic methods are as follows:

I→*II*. 12.34 g (0.05 mol) of sodium 2-nitrophenoxide (I), 56.9 g (0.16 mol) of diethylene glycol dibromide, and 30 mL of DMF were heated together at 90–108 °C for 1 h. The bright orange color of the reaction mixture soon faded to pale yellow, and a white precipitate was deposited. After cooling, 150 mL of dichloromethane was added, the precipitate was filtered off, and the dichloromethane was removed with a rotary evaporator. The diethylene glycol dibromide and DMF were removed under vacuum to yield 19.53 g of crude product (87.8%) which can be used directly in the next reaction. NMR ($CDCL_3$, 250 MHz); 3.47 (t, CH_2Br, 2 H); 3.90 (m, C-CH_2-OCH_2-C, 4 H); 4.25 (t, Ar-O-CH_2, 2 H); 6.92–7.85 (m, C_6H_4, 4 H) ppm. ^{1}H NMR spectra are presented in the following

format: chemical shift; spin multiplicity (s = single, d = doublet, dd = doublet of doublet, dt = doublet of triplet, t = triplet, q = quartet, m = complex multiplet, br = broad); fragment of interest; relative number of protons.

II → III. 4.90 g (0.017 mol) of II, 40 mL of ethanol, and 15 g (0.88 mol) of ammonia gas were added to a 120-mL pressure tube at −78 °C. The pressure tube was sealed and heated to 70–80 °C for 4 h. The reaction mixture was then cooled to −10 °C. The pressure was gradually released to atmospheric pressure while allowing the temperature of the reaction mixture to rise to room temperature. 30% of the solvent was then removed with a rotary evaporator. 10 mL of 5% potassium hydroxide solution was then added followed by removal of another 30% of the solvent. 50 mL of ethyl ether and 10 mL of 50% potassium hydroxide solution were added, and the resulting mixture was extracted with dichloromethane to yield 4.0 g of III (97%). NMR ($CDCL_3$, 250 MHz); 1.55 (br, NH_2, 2 H); 2.87 (t, CH_2N, 2 H); 3.59 (t, O-CH_2, 2 H); 3.85 (t, CH_2-O, 2 H); 4.25 (t, Ar-O-CH_2, 2H); 6.98–7.82 (m, Ar, 4 H) ppm.

III → IV. 2.32 g (10.2 mmol) of III was hydrogenated at ambient temperatures and a pressure of 20 psi using 50 mg of platinum (5% on carbon) catalyst in 40 mL of 95% ethanol. After full hydrogen uptake (5 h), the catalyst was filtered off and the ethanol was removed using a rotary evaporator. The crude product was purified with a silica gel chromatography column. The mobile phase consisted of 6:2:1 cyclohexane/CH_2CL_2/EtOAc. The column was then flushed with 98% methanol/2% ethylenediamine to yield 1.54 g of IV (79%). NMR ($CDCL_3$, 250 MHz); 2.65 (br, s, NH_2, 4 H); 2.87 (t, CH_2-N, 2 H); 3.55 (t, O-CH_2-C-N, 2 H); 3.80 (t, O-CH_2-C-O-Ar, 2 H); 4.14 (t, Ar-O-CH_2, 2 H); 6.64–6.80 (m, Ar, 4 H) ppm.

IV → V. 1.54 g (7.8 mmol) of IV was mixed with 8.88 g (41.3 mmol) of 1,8-bis(dimethylamino)naphthalene, 0.62 g (4.13 mmol) of anhydrous sodium iodide, 6.95 g (41.3 mmol) of ethyl bromoacetate, and 9.5 mL of acetonitrile. The mixture was stirred and heated to reflux under a nitrogen atmosphere for 20 h. After the mixture was cooled and diluted with 60 mL of toluene, the precipitate was filtered off and washed with an additional 20 mL of toluene. The combined organic phase was washed four times with 10-mL portions of phosphate buffer at pH 2 and then washed with water, dried over $MgSO_4$, and evaporated to a residue. The residue was purified using a silica gel chromatography column (1:1 ethyl acetate/cyclohexane) to yield 3.3 g (81.48%) of pure V. NMR ($CDCL_3$, 250 MHz); 1.19–1.25 (dt, CH_3, 12 H); 2.97 (t, CH_2-N-C-COO-, 2 H); 3.60 [s, -C-C-N-$(CH_2)_2$, 4 H]; 3.64 (t, O-CH_2-C-N, 2 H); 3.72 (t, O-CH_2-C-O-Ar, 2 H); 4.07 (t, Ar-O-CH_2, 2 H); 4.09 [s, -Ar-N-$(CH_2)_2$, 4 H]; 4.13 (q, -$COOCH_2$-, 8 H); 6.83 (m, Ar, 4 H) ppm.

V → VI. The tetraester (V) was saponified to the sodium salt using a 1.5% excess of 0.5 N NaOH water solution by refluxing for 10 h under a nitrogen atmosphere. NMR (D_2O, 250 MHz); 2.86 (t, CH_2-N-C-COO-, 2 H); 3.27 [s, -C-C-N-$(CH_2)_2$, 4 H]; 3.68 (t, O-CH_2-C-N, 2 H); 3.83 [s, -Ar-N-$(CH_2)_2$, 4 H]; 3.86 (t, O-CH_2-C-O-Ar, 2 H); 4.19 (t, Ar-O-CH_2, 2 H); 6.95 (m, Ar, 4 H) ppm.

III → VII. In a 50-mL round bottom flask, 1.5 g (6.64 mmol) of III and 30 mL of 50% dioxane/50% water were added. 1.46 g (6.69 mmol) of di-*tert*-butyl dicarbonate was added stepwise while maintaining a reaction mixture pH value of 8–9.5 with the addition of NaOH aliquots. The reaction reached completion in 90 min. The mixture was neutralized and extracted with 40 mL of ethyl acetate. The organic phase was then washed with water and dried over $MgSO_4$. The solvents were removed with a rotary evaporator to yield 2.23 g of VII which could be used directly in the next reaction. NMR ($CDCL_3$, 250 MHz); 1.40 (s, CH_3, 9 H); 1.59 (s, NH, 1 H); 3.31 (q, CH_2-NH-BOC, 2 H); 3.59 (t, O-CH_2-C-N, 2 H); 3.83 (t, O-CH_2-C-O-Ar, 2 H); 4.23 (t, Ar-O-CH_2, 2 H); 7.06–7.40 (m, C_6H_4, 4 H) ppm.

VII → VIII. 2.23 g (approximately 6.64 mmol) of crude VII was hydrogenated at ambient temperatures and a pressure of 20 psi with 75 mg of platinum (5% on carbon) in 40 mL of ethanol. After full hydrogen uptake (5 h) the catalyst was filtered off. The ethanol was removed using a rotary evaporator to yield 2.01 g of VIII which was used directly in the next reaction. NMR ($CDCL_3$, 250 MHz); 1.40 (s, CH_3, 9 H); 1.59 (t, NH, 1 H); 1.95 (br, s, NH_2, 2 H); 3.30 (q, CH_2-NH-BOC, 2 H); 3.57 (t, O-CH_2-C-N, 2 H); 3.79 (t, O-CH_2-C-O-Ar, 2 H); 4.14 (t, Ar-O-CH_2, 2 H); 6.64–6.85 (m, Ar, 4 H) ppm.

VIII → IX. 1.97 g (approximately 6.64 mmol) of VIII was mixed with 9.08 g (42.4 mmol) of 1,8-bis(dimethylamino)-naphthalene, 0.63 g (4.24 mmol) of anhydrous sodium iodide, 7.08 g (42.4 mmol) of ethyl bromoacetate and 7.0 mL of acetonitrile. The mixture was stirred and heated to reflux under a nitrogen atmosphere for 20 h. The mixture was cooled and diluted with 60 mL of toluene. The precipitate was filtered off and washed with an additional 20 mL of toluene. The combined organic phase was washed with four 10-mL portions of phosphate buffer at pH 2, washed with water, dried over $MgSO_4$, and rotary evaporated to give a residue of 3.08 g of crude IX. NMR ($CDCL_3$, 250 MHz); 1.21 (t, CH_3-C-O, 6 H); 1.40 (s, CH_3, 9 H); 1.59 (s, NH, 1 H); 3.31 (q, CH_2-NH-BOC, 2 H); 3.54 (t, O-CH_2-C-N, 2 H); 3.75 (t, O-CH_2-C-O-Ar, 2 H); 4.11 (t, Ar-O-CH_2, 2 H); 4.13 (q, $COOCH_2$, 4 H); 4.17 [s, -Ar-N-$(CH_2)_2$, 4 H]; 6.85 (m, C_6H_4, 4 H) ppm.

IX → XII. A solution of 1.0 g (approximately 2.2 mmol) of IX in 2 mL of dichloromethane was added dropwise into 5 mL of trifluoroacetic acid at room temperature. Following addition, the mixture was stirred for an additional 15 min, and the solvents were removed, first with a rotary evaporator and then with a vacuum pump. The residue was dissolved in 2 mL of dichloromethane, and 1.5 mL of triethyleneamine was added. The solution was mixed with 1.42 g of 1,8-bis-(dimethylamino)naphthalene, 1.5 mL of acetonitrile, 0.1 g of anhydrous sodium iodide, and 1.0 g of [2-^{13}C]ethyl bromoacetate. The resulting mixture was stirred and heated to reflux under a nitrogen atmosphere for 20 h. The mixture was then cooled and diluted with 15 mL of toluene. The precipitate was filtered off and washed with an additional 5 mL of toluene. The combined organic phase was washed with four 5-mL portions of phosphate buffer at pH 2, washed with water, dried over $MgSO_4$, and evaporated to give a residue. Following purification with a silica gel column using a 1:1 EtOAc/cyclohexane mobile phase, 0.4 g of the pure [^{13}C]ester (XI) was obtained. NMR ($CDCL_3$, 250 MHz); 1.19–1.26 (dt, CH_3, 12 H); 2.99 (t, CH_2-N-*C-COO-, 2 H); 3.35 (d, N-*CH_2-COO, 2 H); 3.65 (t, O-CH_2-C-N, 2 H); 3.72 (t, O-CH_2-C-O-Ar, 2 H); 3.90 (d, N-*CH_2-COO, 2 H); 4.09 (t, Ar-O-CH_2, 2 H); 4.11 (dq, COO-CH_2-, 8 H), 4.16 [s, -Ar-N-$(CH_2)_2$, 4 H]; 6.84 (m, Ar, 4 H) ppm.

The tetraester (XI) was saponified to the sodium salt with a 1.5% excess of 0.5 N NaOH water solution by refluxing for 10 h under a nitrogen atmosphere. Solvent removal and drying at 0.2 mm Hg vacuum degrees and 110 °C for 1 h yielded 0.4 g of pure product XII. NMR (D_2O, 250 MHz); 2.85 [br, t, CH_2-N-(*C-COO-)$_2$, 2 H]; 2.98 (d, N-*CH_2-COO, 2 H);

3.52 (d, N-*CH_2-COO, 2 H); 3.68 (t, O-CH_2-C-N, 2 H); 3.78 [s, Ar-$(CH_2)_2$, 4 H]; 3.82 (t, O-CH_2-C-O-Ar, 2 H); 4.11 (t, Ar-O-CH_2, 2 H); 6.82–7.05 (m, Ar, 4 H) ppm.

XII → XIII. 0.296 g (0.5 mmol) of XII, 0.02 g sodium iodide, 0.612 g (4 mmol) of bromomethyl acetate, 0.01 g of triethyl amine, and 1.5 mL of methyl ethyl ketone were placed in a 15-mL round bottom flask. The reaction mixture was stirred and heated at 95–100 °C for 1 h, to yield 0.41 g of crude product after workup. This product was purified with a silicon gel chromatography column to yield 0.114 g of pure tetraacetoxylmethyl ester (XIII). NMR ($CDCl_3$, 250 MHz); 2.08 (s, CH_3COO-, 12 H); 2.99 [t, CH_2-N-(*C-COO-)$_2$, 2 H]; 3.41 (d, N-*CH_2-COO, 2 H); 3.65 (t, O-CH_2-C-N, 2 H); 3.72 (t, O-CH_2-C-O-Ar, 2 H); 3.96 (d, N-*CH_2-COO, 2 H); 4.08 (t, Ar-O-CH_2, 2 H); 4.16 [s, Ar-N$(CH_2$-C$)_2$, 4 H]; 5.70 (s, -O-CH_2-O, 2 H); 5.74 (s, -O-CH_2-O-, 2 H); 6.84 (m, Ar, 4 H) ppm.

Determination of pKa's and Stability Constants. Proton ionization constants and metal ion binding constants were determined for EGTA and AATA using 0.001 M ligand in 0.1 M KCl at 20 °C. In determining stability constants, 0.001 M $CaCl_2$ was utilized. Titrations were performed under inert atmosphere, and the nitrogen was humidified with a 0.1 M KCl solution. Excess acid employed was determined by the addition of a measured volume of standard acid to the ligand solution. The pH meter was calibrated to pH 4 and 10 using standard solutions (VWR Scientific, Media, PA). Standard base (0.1006 M KOH) was added dropwise using either a buret or a syringe. All constants were determined in triplicate, and each data set was composed of approximately 50–70 data points. All titrations for binding constant determinations were performed by adding at least 4 equiv of base. For accurate constant determination, it was often necessary to use equilibration times of 30–45 min. Constants were evaluated using the PKAS and BEST programs developed by Martell and Motekaitis (1988). Sigma fits ranged from 0.03 to 0.07.

Nuclear Magnetic Resonance. ^{1}H NMR spectra were acquired at 400 or 500 MHz using a 4–8-kHz sweep width, a 4–16K block size, and a 90° excitation. ^{19}F NMR spectra were acquired at 37 °C and 235.36 MHz by summing 80 transients using a 16–32K block size, a 5-kHz sweep width, and a 45° excitation. Samples typically contained 1 mM ligand and 0.1 M KCl at pH 7.2 in 99.8% D_2O. Calcium and magnesium ion concentrations were varied as described in the figure legends. NMR confirmation of pK_a values was accomplished by titrating 15 2-mL samples of 1 mM AATA in a solution 0.1 M KCl and 10% D_2O from pH 2.5 to 11.5. Proton-observed carbon-edited spectra were acquired as previously described (Bendall et al., 1981) using a commercial two-channel [^{33}S–^{31}P/^{1}H] broad-band probe operating in the reverse mode.

RESULTS

The ^{19}F NMR spectra of 1 mM DFBAPTA in 0.1 M KCl at pH 7.2 in the presence of varying amounts of Mg^{2+} are shown in Figure 2. As free Mg^{2+} concentrations are raised from 0 to 3 mM, the ^{19}F signal displays a 1 ppm shift and experiences a change in line width from 14 to 104 Hz. In sharp contrast, the tetraacetic acid methylene protons and aromatic protons of DFBAPTA shift by less than 0.03 ppm and undergo no appreciable change in line widths in the corresponding ^{1}H NMR spectra. In addition to chemical shift and line width considerations in DFBAPTA, the spectroscopic rationale for the design of AATA can be understood by examining the ^{1}H NMR spectra of EDTA, EGTA, BAPTA,

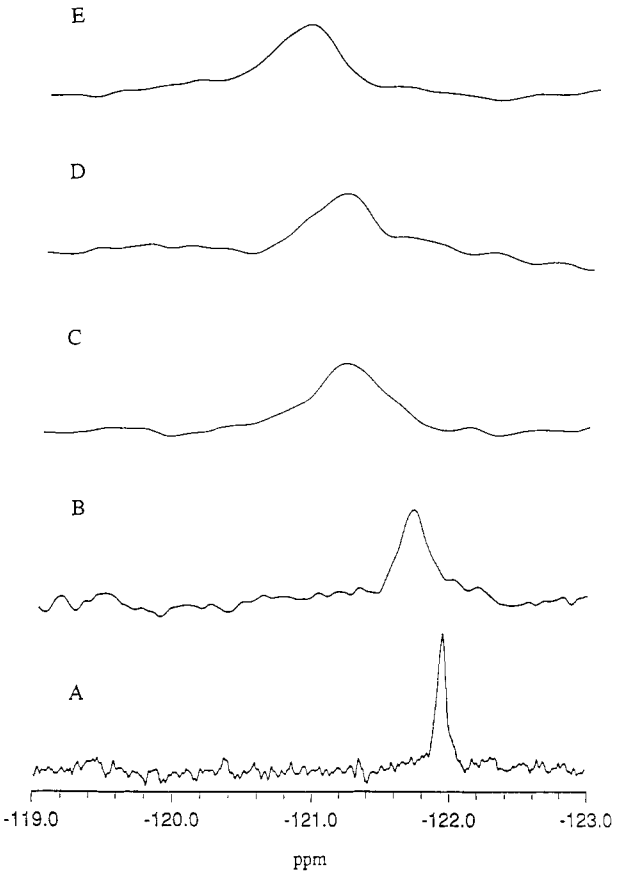

FIGURE 2: Effect of increasing Mg^{2+} concentrations on the ^{19}F spectrum of DFBAPTA in the absence of Ca^{2+} in 0.1 M KCl at 37 °C (pH 7.2). Panels A–E correspond to 0, 0.5, 1.0, 2.0, and 3.0 mM Mg^{2+}, respectively. The ^{19}F resonance experiences a change in line width from 14 to 104 Hz and a 1 ppm change in chemical shift.

and DFBAPTA in the presence and absence of Ca^{2+} as shown in Figure 3.

The ^{1}H NMR spectrum of EDTA is characterized by singlets at 3.77 and 3.40 ppm corresponding to the tetraacetic acid methylene protons and ethylene bridge protons, respectively (see Figure 3A). Upon coordination with Ca^{2+}, the tetraacetic acid methylene protons undergo a shift of –0.53 ppm (Figure 3B). In addition, these protons now form a distorted quartet reflecting the very slow off rates for the EDTA–Ca^{2+} interaction at 37 °C. Similarly, the tetraacetic acid methylene protons of EGTA shift from 3.96 to 3.38 ppm on Ca^{2+} coordination and once again appear as a distorted quartet due to slow Ca^{2+} off rate at 37 °C (Figure 3C–D). In contrast, the analogous protons of BAPTA and DFBAPTA undergo shifts of less than 0.12 ppm upon Ca^{2+} coordination, and although some broadening occurs, these resonances retain their singlet appearance (Figure 3E–H).

The lack of significant chemical shift changes for BAPTA and DFBAPTA upon Ca^{2+} coordination can be explained by the absence of nitrogen-bound protons on these molecules at neutral pH values. In addition, the faster Ca^{2+} off rates of BAPTA and DFBAPTA are sufficient to result in singlet broadening only in these molecules at 37 °C. Therefore, in designing a new Ca^{2+}-sensitive molecular probe for ^{1}H NMR, it would be preferable to combine the shift characteristics displayed in EDTA and EGTA with the singlet behavior of BAPTA and DFBAPTA. This can be accomplished by synthesizing a molecule which is a hybrid between EGTA and BAPTA. The tetraacetic acid methylene protons of the EGTA side of this new molecule should shift by about 0.5 ppm on Ca^{2+} coordination. In addition, since this molecule should

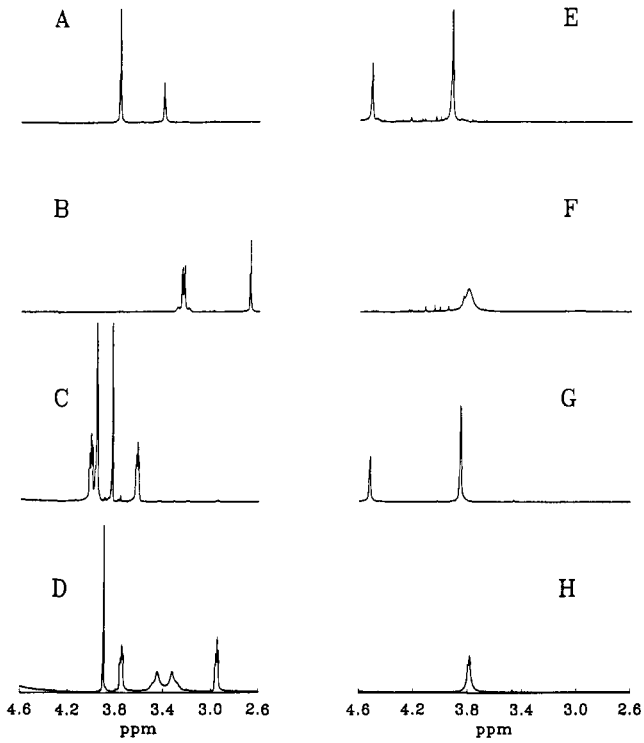

FIGURE 3: 400-MHz ^{1}H NMR spectra of 1 mM EDTA (A and B), EGTA (C and D), BAPTA (E and F), and DFBAPTA (G and H) in a 0.1 M KCl/99.8% D_2O solution at 37 °C (pH 7.2). Spectra were acquired using a standard one-pulse experiment in the presence (B, D, F, H) and absence (A, C, E, G) of 3 mM Ca^{2+}. Ca^{2+} coordination causes the tetraacetic acid methylene protons of EDTA (3.77 ppm) and EGTA (3.96 ppm) to appear as distorted quartets at 3.24 and 3.38 ppm, respectively. Ca^{2+} coordination causes primarily a change in line width for the analogous resonances of BAPTA and DFBAPTA.

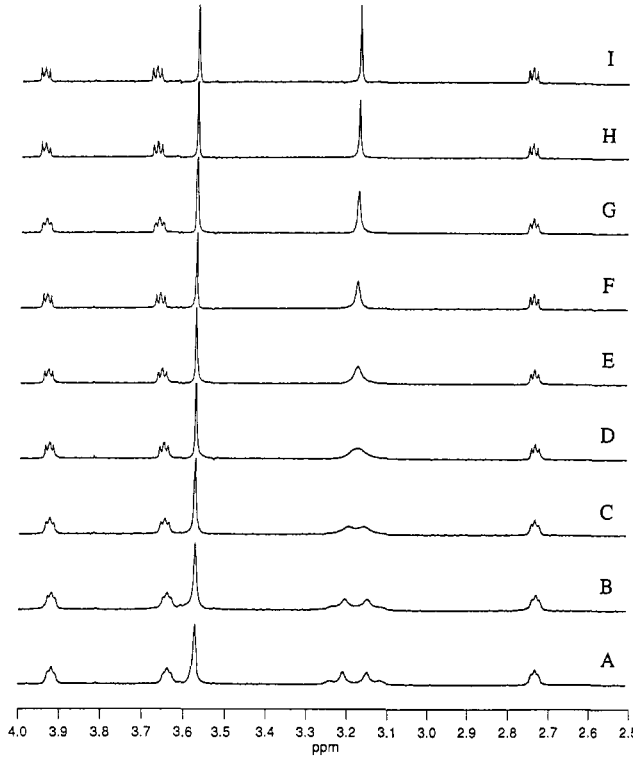

FIGURE 4: 500-MHz ^{1}H NMR spectra of a solution containing 1 mM AATA, 3 mM Ca^{2+}, and 0.1 M KCl in 99.8% D_2O (pH 7.2) acquired using a conventional one-pulse sequence. In this study, the temperature was increased from 7 to 77 °C (A–I). Note that the tetraacetic acid methylene protons on the EGTA side of the molecule (3.19 ppm) initially resonate as a distorted quartet and with temperature enhanced Ca^{2+} exchange, eventually appear as a singlet. Since these resonances actually experience a 0.5 ppm downfield shift with increasing temperature, spectra have been plotted by referencing all chemical shifts to the shifts noted at 7 °C for added clarity.

have only one nitrogen-bound proton at neutral pH, it should have higher Ca^{2+} off rates than EGTA. This should help ensure that the tetraacetic acid methylene protons on the EGTA side of the molecule resonate as singlets at 37 °C.

The ^{1}H NMR spectrum of 1 mM AATA in the presence of 3 mM Ca^{2+} as a function of temperature is shown in Figure 4. The effect of temperature on Ca^{2+} off rates with AATA can be visualized by examining the tetraacetic acid methylene portion of the spectrum at 3.19 ppm. As expected, these protons resonate as a singlet upon Ca^{2+} coordination at 37 °C (Figure 4E). Line width analysis (Harris, 1983) of similar data establishes that AATA has off rates on the order of 43 s^{-1} at 37 °C and 23 s^{-1} at 20 °C.

Using potentiometric titrations, we have measured pK'(pK' = log {[ML]/[M][L]}) and pK''(pK'' = log {[MHL]/[ML][H]}) values of 8.64 ± 0.1 and 5.02 ± 0.1 for AATA–Ca^{2+} at 20 °C and of 8.31 ± 0.1 and 5.26 ± 0.1 at 37 °C. We have also determined pK_a values of 5.60 ± 0.05 and 9.33 ± 0.05 for AATA by these methods and by NMR at 20 °C and obtained pK_a values of 9.03 ± 0.05 and 5.94 ± 0.05 for AATA at 37 °C by potentiometric titration. Tsien (1980) has reported pK_a's of 5.47 and 6.36 for BAPTA at 20 °C, and pK_a's of 8.96 and 9.58 have been reported for EGTA (Martell & Smith, 1974). Consequently, the pK_a's for AATA generally correspond to the third pK_a for BAPTA and the fourth pK_a for EGTA. From the pK', pK'', and pK_a data measured for AATA, we calculate a K_D of 306 ± 80 nM for this molecule and Ca^{2+} at 20 °C and a pH value of 7.2. A value of 350 ± 80 nM was calculated at 37 °C. When these K_D values are combined with the off rates estimated above, this results in calculated on rates of 0.75 × 10^8 M^{-1} s^{-1} and 1.2 × 10^8 M^{-1} s^{-1} for AATA–Ca^{2+} at 20 and 37 °C, respectively.

We have also measured pK' and pK'' values of 10.82 ± 0.1 and 3.89 ± 0.1 for EGTA at 20 °C. When combined with pK_a values from the literature (Martell & Smith, 1974), these pK' and pK'' values results in a calculated K_D of 63 ± 20 nM at a pH value of 7.2. Martell and Smith (1974) have reported pK' and pK'' values of 10.97 ± 0.1 and 3.79 ± 0.1, respectively, for EGTA. These result in a calculated K_D value of 91 ± 20 nM. Therefore, our ability to confirm pK' and pK'' values for EGTA reported by Martell and Smith (1974) gives added weight to the constants determined for AATA in this study.

The pK', pK'', and pK_a data measured above for AATA and data collected from the literature (Tsien, 1980; Harrison & Bers, 1987; Martell & Smith, 1974) were combined to generate Figure 5. From this figure, it is apparent that the K_D values of DFBAPTA and DBBAPTA are pH insensitive in the neutral range as previously reported (Harrison & Bers, 1987). The K_D value for AATA is less sensitive than that of EGTA to changes in pH but more sensitive than the K_D of BAPTA and DBBAPTA.

We have also measured a pK' of 4.06 for the interaction of AATA with Mg^{2+}. This results in a calculated K_D of 6.6 ± 2.0 mM for AATA–Mg^{2+}, neglecting the pK'' term. Martell and Smith report a pK' value of 5.21 for the EGTA–Mg^{2+} interaction at 20 °C. As such, the pK' values of EGTA–Ca^{2+} and EGTA–Mg^{2+} are separated by slightly more than 5 log units at 20 °C. Similar analysis for the pK' values of AATA–Ca^{2+} and AATA–Mg^{2+} shows a separation of nearly 4.5 units at 37 °C. Although this interaction requires further study, it appears that AATA maintains the high selectivity of Ca^{2+} over Mg^{2+} as predicted by its EGTA–BAPTA hybrid structure.

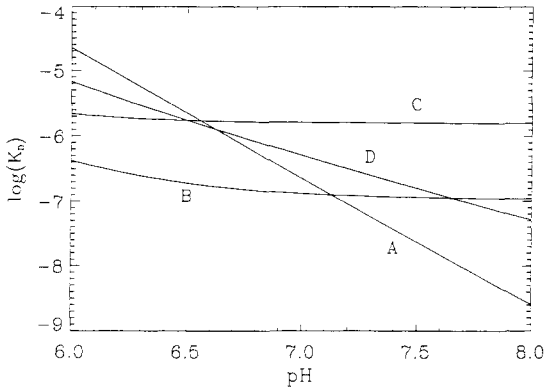

FIGURE 5: Illustration of the pH-induced changes in K_D values for EGTA (A), BAPTA (B), DBBAPTA (C), and AATA (D). The pH insensitivity of K_D for BAPTA and DBBAPTA is readily apparent. AATA is less sensitive than EGTA but much more sensitive than BAPTA and DBBAPTA. Proton and calcium association constants were either determined by potentiometric titration in this study for AATA or taken from the literature (Tsien, 1980; Harrison & Bers, 1987; Martell & Smith, 1974).

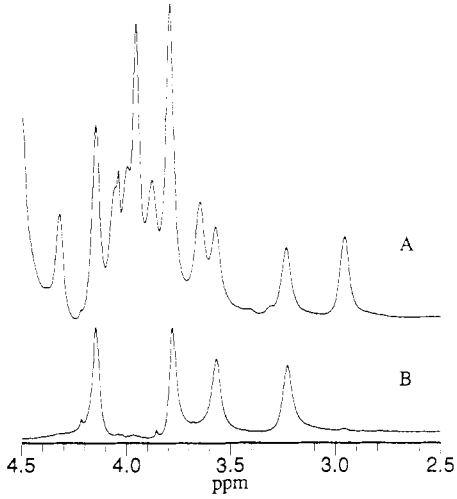

FIGURE 6: (A) 400-MHz ^{1}H NMR spectrum of 1 mM ^{13}C-enriched AATA at pH 7.2 and 37 °C in the presence of 0.3 mM $CaCl_2$ and 0.1 M KCl. This spectrum contains all the proton resonances of AATA in the presence and absence of Ca^{2+} coordination. (B) Same spectrum with ^{13}C editing (no ^{13}C decoupling applied). This spectrum contains two sets of ^{13}C-coupled doublets corresponding to the ^{13}C-attached tetraacetic acid methylene resonances of AATA in the presence and absence of Ca^{2+} coordination.

The ^{1}H NMR spectrum of 1 mM ^{13}C-enriched AATA in the presence of 0.3 mM Ca^{2+} is shown in Figure 6A and the carbon-edited proton spectrum in Figure 6B. The carbon-edited spectrum shows two sets of ^{13}C doublets corresponding to free AATA (4.15 and 3.78 ppm) and Ca^{2+}–AATA (3.57 and 3.22 ppm). Carbon-decoupled and -edited proton spectra of AATA acquired in the presence of varying levels of Ca^{2+} and 2 mM Mg^{2+} at 37 °C are displayed in Figure 7. Each of these spectra contains two resonances corresponding to free AATA (3.96 ppm) and Ca^{2+}–AATA (3.4 ppm). Both the singlet nature and chemical shift characteristics of these two resonances confirm our presynthesis predictions for AATA. The result of varying free Ca^{2+} concentrations in each of these solutions are directly apparent in Figure 7. These results are directly analogous to those previously obtained in the ^{19}F NMR spectra of DFBAPTA (Metcalfe et al., 1985).

DISCUSSION

In the presence of increasing Mg^{2+} concentrations, DFBAPTA exhibits a 7-fold increase in fluorine line widths and

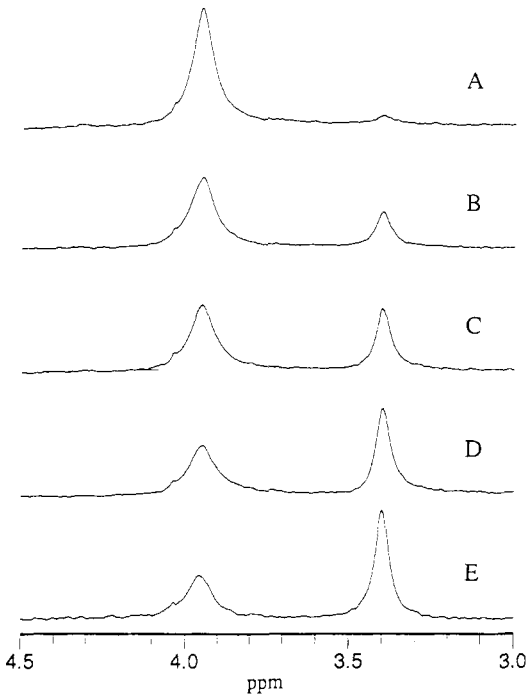

FIGURE 7: Carbon-edited 400-MHz ^{1}H NMR spectra of 1 mM ^{13}C-enriched AATA in the presence of 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mM Ca^{2+} (A–E) in 0.1 M KCl at 37 °C and pH 7.2 ± 0.1. Each spectrum was acquired with ^{13}C decoupling in 100 scans. Resonances correspond to the ^{13}C-attached tetraacetic acid methylene protons of free AATA (3.96 ppm) and Ca^{2+}-AATA (3.4 ppm). These resonances correspond to the resonances found in Figure 6B and appear as singlets due to carbon decoupling during acquisition.

a 1 ppm change in chemical shifts. Under the same conditions, the tetraacetic acid methylene protons experience no noticeable change in line widths or chemical shifts. These difference between proton and fluorine line width and chemical shifts in response to increasing Mg^{2+} can perhaps be partially attributed to electronic differences within this molecule. However, since the aromatic protons of DFBAPTA experience little change in the presence of increasing Mg^{2+}, a significant fraction of the observed changes in line width should be primarily linked to the nature of the ^{19}F nucleus itself, namely, its very large range of chemical shifts. The more dramatic response of fluorine line widths and chemical shifts in DFBAPTA to microenvironment change relative to the proton constitute the principle disadvantage of utilizing this type of fluorinated indicators under in vivo conditions. Fluorine line widths and chemical shift will also be much more sensitive than hydrogen to dynamic changes in the cell, which may act to lower the sensitivity of the measurement of interest. For this reason, fluorinated indicators will often be less sensitive than gyromagnetic ratio considerations alone would have predicted.

Since AATA proton line widths should be much less sensitive to microenvironmental changes and since AATA has a 2-fold increase in detectable spins, a 6–10 fold signal to noise improvement can be expected with this molecule even with the 1.5–2.7-fold loss in sensitivity expected from carbon editing. The Mg^{2+} interaction will also slightly broaden the resonance corresponding to free AATA in the edited proton spectrum of this molecule. These effects have been taken into account in calculating the increased sensitivity provided by AATA over DFBAPTA. As a result, AATA should allow the experimentalist to lower intracellular probe levels, helping to alleviate problems with Ca^{2+} buffering (Barritt & Lee, 1985), or enable the use of lower acquisition times and smaller sample volumes.

As with AATA, the EGTA-based Ca^{2+} probes described thus far have very fast on rates for Ca^{2+}, and their speed of response to physiological changes is generally governed by the dominating off rates (Bennett & Bagshaw, 1986; Smith et al., 1984; Jackson et al., 1987; Quast et al., 1984; Smith et al., 1983). Off rates between 0.3 and 1.0 s^{-1} have been reported for EGTA (Bennett & Bagshaw, 1986; Smith et al., 1984). As a result of these very slow off rates, EGTA itself is unsuitable for the dynamic study of Ca^{2+} changes in excitable tissue. In contrast, off rates between 84 and 130 s^{-1} have been reported for FURA2, INDO1, and QUIN2 (Jackson et al., 1987; Quast et al., 1984). DFBAPTA has very fast off rates on the order of 570 s^{-1} (Smith et al., 1983). Since fluorescent techniques are essentially instantaneous, very rapid off rates provide an advantage in applying these methods. This advantage is not shared by NMR. In light of typical NMR acquisition times, the off rate of 43 s^{-1} estimated for AATA at 37 °C is adequate for the measurement of dynamic changes in excitable tissue such as the myocardium.

The increased sensitivity of AATA over DFBAPTA arises both from the lower sensitivity of the proton chemical shifts to increasing Mg^{2+} concentrations and from a decreased Ca^{2+} off rate. Indeed, in designing AATA, we deliberately lowered off rates to improve line widths. The advantage/disadvantage ratio of such a move must be assessed for each biological problem of interest.

As expected, the K_D value for AATA with Ca^{2+} is intermediate between those for BAPTA and EGTA. As with these parent molecules, AATA has a K_D value in the physiologically relevant range. However, when using AATA as an indicator of intracellular free Ca^{2+} concentrations, it is important that the intracellular pH be accurately determined. As an example, AATA has a K_D of 488 ± 100 nM at a pH value of 7.0 and 306 ± 80 nM at 7.2.

Fluorinated probe studies require only the application of simple one-pulse experiments for detection. Unfortunately, AATA requires much more sophisticated NMR methods and hardware for detection. As such, cell perfusion studies with AATA will require the manufacture of rather complex NMR probes. This is because ^{13}C-edited proton approaches require both excellent water suppression and the use of multinuclear excitation. These approaches may or may not involve the use of B_0 gradient coils, further complicating experimental aspects. In addition, the experimentalist must now contend with natural abundance ^{13}C-attached protons in their spectra. However, this situation can probably be reasonably addressed by acquiring baseline spectra prior to ligand loading. Alternatively, the use of ^{15}N–^{13}C–^{1}H multiple-quantum selection may resolve these background issues with little additional signal loss from the added selection.

In spite of increased experimental difficulties, the move away from fluorination and the shift to spectral editing techniques should result in the creation of a new class of compounds which are specifically designed for increased sensitivity in NMR studies. This should also permit more flexibility in synthetic approaches since the strong electronegativity of fluorine often hampers synthetic design. Moreover, these editing based approaches can be readily extended to other probes for intracellular ions, pH, and membrane potential.

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