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Intracellular calcium measurements by ^{19}F NMR of fluorine-labeled chelators

(calcium indicators/mitogenic stimulation/thymocytes)

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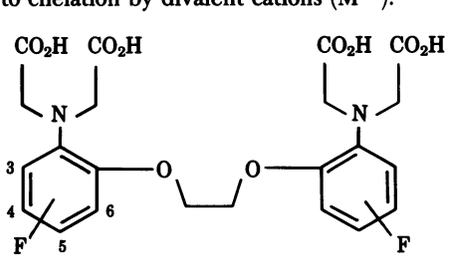
ABSTRACT **Symmetrically substituted difluoro derivatives of 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (*n*FBAPTA) show large ^{19}F NMR chemical shifts on chelating divalent cations. The complexes of Ca^{2+} with 4FBAPTA and 5FBAPTA show fast and slow exchange behavior, respectively, and the chemical shift or the areas of the resonances from the free and complexed forms can be used to determine the free Ca^{2+} concentration. The measurement of the free Ca^{2+} concentration by either ligand is unaffected by free Mg^{2+} concentrations <10 mM, by pH 6–8, or by contaminating divalent ions of high affinity (Zn^{2+}, Fe^{2+}, Mn^{2+}). The tetraacetoxymethyl ester derivative of 5FBAPTA was used to load mouse thymocytes with 5FBAPTA to intracellular concentrations of 1 mM, and the ^{19}F spectrum indicated a free intracellular Ca^{2+} concentration ($[Ca]_i$) of 250 nM. The $[Ca]_i$ was increased to 350 nM by addition of succinylated concanavalin A at mitogenic concentrations, and the addition of A23187 saturated the intracellular chelator with Ca^{2+} from the external medium. The method provides a measurement of $[Ca]_i$ and other divalent cation concentrations with direct identification of the ionic species chelated.**

Fluorescent intracellular chelators have recently been designed by Tsien *et al.* (1–3) to measure the free Ca^{2+} concentration in cells ($[Ca]_i$). Experiments with the prototype Ca^{2+} indicator quin2 illustrated the potential of the technique but also pointed to some of the practical limitations of its use (4). Intracellular concentrations between 0.1 and 1 mM quin2 are required to detect its fluorescence above the autofluorescence from the cells, and we have shown that these concentrations of quin2 cause both metabolic and mitogenic stimulation of lymphocytes (4). Furthermore, the measurement of a single physical parameter (fluorescence intensity) does not provide an unambiguous indication of the identity and amount of the ion(s) bound to the chelator in the cells. It is therefore important to establish independent physical assays to determine the identity and concentration of the ion(s) bound to the chelator.

We report here that the symmetrically substituted difluoro derivatives of 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (*n*FBAPTA) have ^{19}F chemical shifts that are highly sensitive to chelation by divalent cations (M^{2+}).



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(The position of the fluorine atom on each ring is denoted by *n*.)

The ^{19}F chemical shifts and linewidths indicate the identity of the chelated ions and their exchange rates with the *n*FBAPTA analogues. We report the characterization by NMR of the complexes of these new chelators with Ca^{2+}, Mg^{2+}, H^+, Zn^{2+}, Fe^{2+}, and Mn^{2+}, and we show that mouse thymocytes can be loaded with 5FBAPTA by using the tetraacetoxymethyl derivative that is hydrolyzed in the cells to release 5FBAPTA. The estimated $[Ca^{2+}]_i$ is close to the value obtained from quin2 measurements on the same type of cell (2, 4). The ^{19}F NMR chelators have the advantage of providing a direct identification of the bound ions. They may also be useful for studies with opaque cell suspensions and tissues inaccessible to fluorescence measurements, thereby extending the use of intracellular chelators as M^{2+} indicators.

MATERIALS AND METHODS

***n*FBAPTA Analogues.** The *n*FBAPTA analogues were prepared from the corresponding nitrophenols by the method described by Tsien for BAPTA (1). The tetraacetoxymethyl esters of [^{3}H]4FBAPTA (40.5 Ci/mol; 1 Ci = 37 GBq) and [^{3}H]-5FBAPTA (0.44 Ci/mol) were prepared after exchange of the tetraethyl esters with [^{3}H]trifluoroacetic acid in chloroform (4). Affinities of the *n*FBAPTA analogues for Ca^{2+} were measured by back-titration of the fluorescence of Ca–quin2 complexes as described (4).

NMR Measurements. The ^{19}F NMR measurements were made by using the Fourier transform technique on 50 mM solutions of *n*FBAPTA analogues in a buffer of 150 mM KCl/50 mM Hepes, pH 7.1, at 37°C with a Varian XL100 NMR spectrometer at 94.1 MHz or with a Bruker WM200 spectrometer at 188.3 MHz. Typically 200 transients (free induction decays) were accumulated in 8,000 or 16,000 data points by using 5- or 10-Hz spectral widths with a 90° pulse and an acquisition time of 800 msec. Increasing the acquisition time for 5FBAPTA complexes showing slow exchange with Ca^{2+} had no effect on the relative areas of the resonances from the bound and free forms. The acquisition time used did not, therefore, affect the estimation of percent Ca–5FBAPTA or $[Ca]_i$. Measurements of ^{19}F NMR chemical shifts as a function of free Ca^{2+} or Mg^{2+} concentration were made in a citrate buffer (45 mM tripotassium citrate/31 mM NaCl/7.5 mM Hepes, pH 7.1, at 37°C), and the free M^{2+} concentrations in the buffer were computed by using an iterative program similar to that described by Fabiato and Fabiato (5). Binding constants for H^+ and M^{2+} for citrate were obtained from Martell and Smith (6). The ^{19}F NMR

Abbreviations: Con A, concanavalin A; M^{2+}, divalent cation; *n*FBAPTA, symmetrically substituted difluoro derivative of 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid.

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measurements of *n*FBAPTA analogues in thymocytes were made in a Bruker WM200 spectrometer at 188.3 MHz at 37°C. In this case, 1,500 transients (free induction decays) were accumulated in 16,000 data points with a spectral width of 10 kHz and with a recycle time of 800 msec.

Loading Cells with *n*FBAPTA Analogues and Metabolic Effects. Thymocytes from BALB/c mice were prepared and loaded with 4- or 5FBAPTA by using the tetraacetoxymethyl derivatives as described for loading with quin2 (4). Cells (6×10^8 in 30 ml) in RPMI 1640 medium buffered with 10 mM Hepes (pH 7.3) were incubated with 20 μM [^{3}H]5FBAPTA tetraacetoxymethyl ester (0.44 Ci/mol) for 45 min at 37°C. The cells were centrifuged (500 × *g*; 3 min) and suspended in fresh medium for 20 min at 25°C. They were then washed by centrifugation, suspended in 2 ml of a 1:1 (vol/vol) mixture of RPMI 1640 medium and 300 mM Hepes (pH 7.3) and transferred to a 10-mm-diameter NMR tube at a final cell concentration of $0.9–3 \times 10^8$ per ml. The intracellular concentration of [^{3}H]5FBAPTA was 0.9–1.1 mM, assuming an intracellular volume of 105 fl per cell (4).

Measurements of ATP concentration and lactate output were made as described (4). The viability of cells loaded with 1 mM 5FBAPTA was >96% as judged by eosin exclusion, the ATP content was 93 ± 3% of the amount in control cell samples without 5FBAPTA, and the rate of lactate output was increased by 40–70% above that in control cell samples, similar to the effect reported previously for quin2 at the same intracellular concentrations (4). After a typical 90-min NMR experiment [including additions of succinylated concanavalinA (Con A) and the ionophore A23187] the viability was >86% and the ATP level in the cells was 84 ± 9%. The pH of the cell suspension in the NMR tube dropped from 7.3 to 7.2 over 90 min because of metabolic production of lactic acid. Cells loaded with 0.8 mM 5FBAPTA gave 83 ± 7% of the mitogenic stimulation of control cells without 5FBAPTA when treated with Con A at 1 μg/ml and assayed by [^{3}H]thymidine incorporation at 42–48 hr as described (4).

Succinylated Con A was prepared by treatment of Con A with succinic anhydride, according to the method of Gunther *et al.* (7). The succinyl-Con A was purified chromatographically as described by Beppu *et al.* (8), before repeating the succinylation and purification procedures to give a single chromatographic fraction.

RESULTS

^{19}F Chemical Shifts with M^{2+} Ions. The ^{19}F chemical shifts (measured relative to 6-fluorotryptophan) of the *n*FBAPTA analogues in the presence of various M^{2+} ions are summarized in Fig. 1. The chemical shifts shown for Ca^{2+}, Zn^{2+}, and Fe^{2+}, which have high affinities for the chelators, were obtained with saturating (equimolar) concentrations of the M^{2+} ions. It can be seen that large chemical shifts are obtained for M^{2+} complexes relative to the shifts in the KCl/Hepes buffer solution in the order $Fe^{2+} > Ca^{2+} > Zn^{2+}$ for the 5FBAPTA molecule, with similar trends for the 3FBAPTA and 4FBAPTA analogues but only small shifts for 6FBAPTA with any of the ions except Fe^{2+}. However, the sign of the shifts for a given M^{2+} ion relative to the shifts in the KCl/Hepes buffer solution varies with the different *n*FBAPTA analogues. Mn^{2+} causes large shifts and line broadening on complex formation with all of the analogues (data not shown). For Mg^{2+}, which has a relatively low affinity for the chelators (1), saturation was not achieved even at high molar ratios of Mg^{2+} to the chelators (see Fig. 5), and shifts relative to those in the KCl/Hepes solution were observed only for the 3FBAPTA and 5FBAPTA analogues.

The magnitudes of the chemical shifts on binding Ca^{2+} relative to the shifts in the KCl/Hepes buffer solution follow qualitatively the affinities of the *n*FBAPTA analogues for Ca^{2+} given in Fig. 1. There was no change in chemical shift at Ca^{2+} concentrations greater than equimolar Ca^{2+}/*n*FBAPTA, consistent with a maximum stoichiometry of binding of 1:1. When the *n*FBAPTA analogues were complexed with 0.33 mol equivalents of Ca^{2+}, the spectra indicated that the free and bound forms in 3FBAPTA and 5FBAPTA complexes with Ca^{2+} were in slow exchange, whereas for 4FBAPTA the two forms were in fast exchange on the NMR time scale at 94.1 MHz (Fig. 2). The two exchange conditions are determined by the dissociation rate (K_d) of Ca^{2+} from the Ca–*n*FBAPTA complexes relative to the chemical shift in Hz(δ) between the resonances from the free and bound forms. For slow exchange ($K_d < \delta$), two

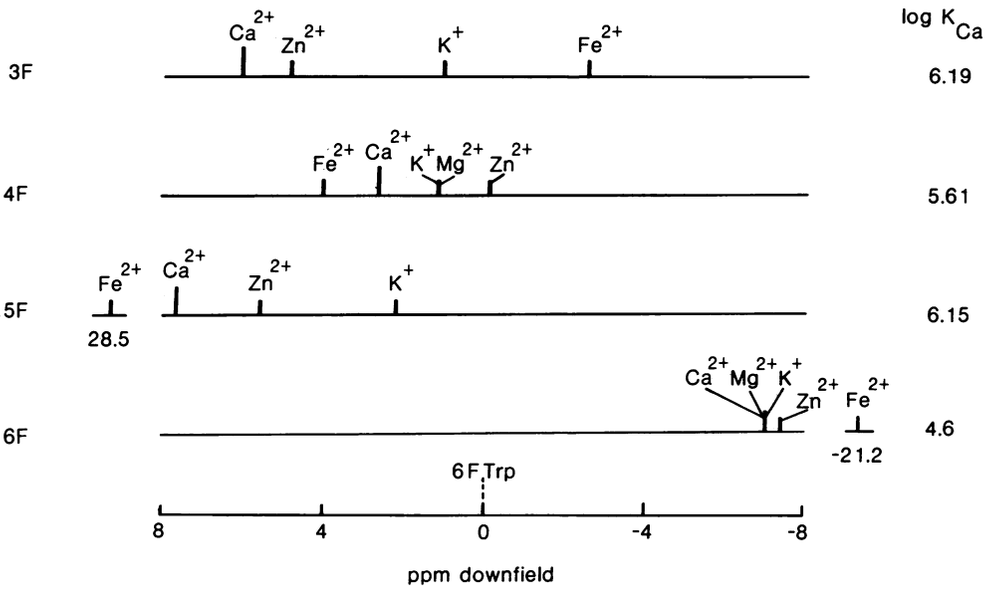

FIG. 1. ^{19}F chemical shifts in ppm (downfield positive) from an external reference of 6-fluorotryptophan (6F Trp) for complexes of *n*FBAPTA with M^{2+} ions at pH 7.1 and 37°C. Shifts labeled K^+ denote positions of the resonances in a buffer containing 150 mM KCl and 10 mM Hepes at pH 7.1.

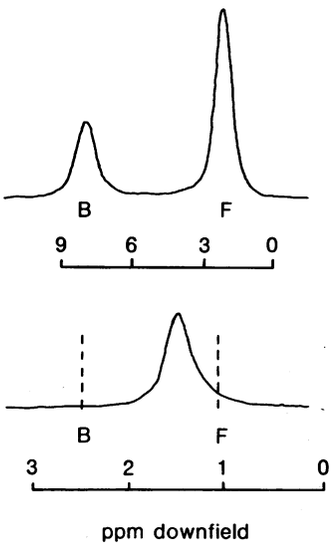

FIG. 2. ^{19}F spectra of 5FBAPTA (*Upper*) and 4FBAPTA (*Lower*) with Ca^{2+} (molar ratio 3:1) at 37°C and 188.3 and 94.1 MHz, respectively. Chemical shifts are measured downfield from an external reference of 6-fluorotryptophan (6F Trp). Positions of the resonances of 4FBAPTA without Ca^{2+} (F) and saturated with Ca^{2+} (B) are indicated.

separate resonances are observed, weighted in area according to the proportions of the free and bound forms of the chelator, as shown for 5FBAPTA in Fig. 2. For fast exchange ($K_d > \delta$), a single resonance is observed at a chemical shift between the positions of the free and bound forms weighted according to their proportions as shown for 4FBAPTA. The different exchange conditions offer independent NMR assays of $[Ca]_i$ in different concentration ranges and were therefore characterized further for the 5FBAPTA and 4FBAPTA analogues.

Slow-Exchange Complex: Ca–5FBAPTA. At 94.1 MHz, the ^{19}F resonances of the free and the bound forms of the Ca^{2+}/5FBAPTA (1:2) mixture in Fig. 2 are broadened compared with the resonances from the all-bound or all-free chelators. This exchange broadening is decreased when the observing frequency is increased to 188.3 MHz and δ (in Hz) is increased 2-fold compared with K_d. Confirmation that the spectrum of the

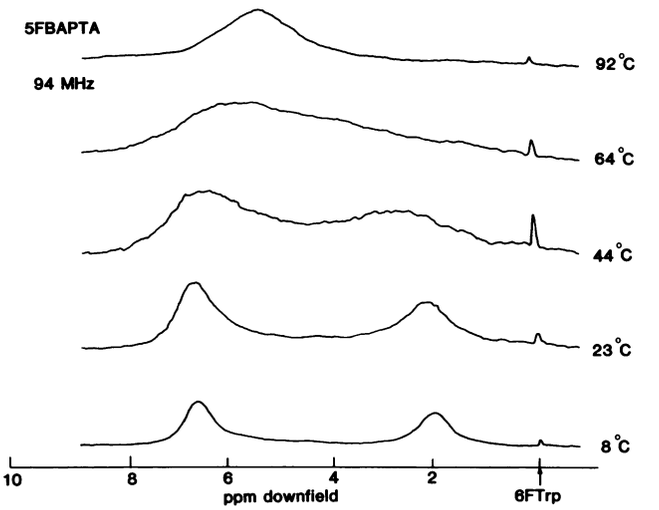

FIG. 3. Effect of temperature on spectra of 5FBAPTA with Ca^{2+} (2:1 molar ratio) at 94.1 MHz. Note that there is a downfield shift of the 5FBAPTA resonances with respect to the external standard of 6-fluorotryptophan (6F Trp) as the temperature increases.

Ca–5FBAPTA complex is close to the intermediate exchange condition at 94.1 MHz is provided by the effect of temperature on the spectra shown in Fig. 3, in which the resonances of the free and bound forms merge to a single broad peak at 90°C when $K_d \simeq \delta$.

Fast-Exchange Complex: Ca–4FBAPTA. The ^{19}F chemical shift of the 4FBAPTA resonance at 94.1 MHz is directly proportional to the molar ratio of Ca^{2+} to 4FBAPTA (Fig. 4), and the resonance is broadened by exchange when the two forms are present (Fig. 4 *Inset*). The affinity of Ca^{2+} for 4FBAPTA was determined by making chemical shift measurements as a function of free Ca^{2+} concentration in a citrate buffer. Sips plots (9) of the data $\{\log(\delta_{bound} - \delta)/(\delta_{free} - \delta)$ vs. $\log[Ca^{2+}]_{free}\}$ gave

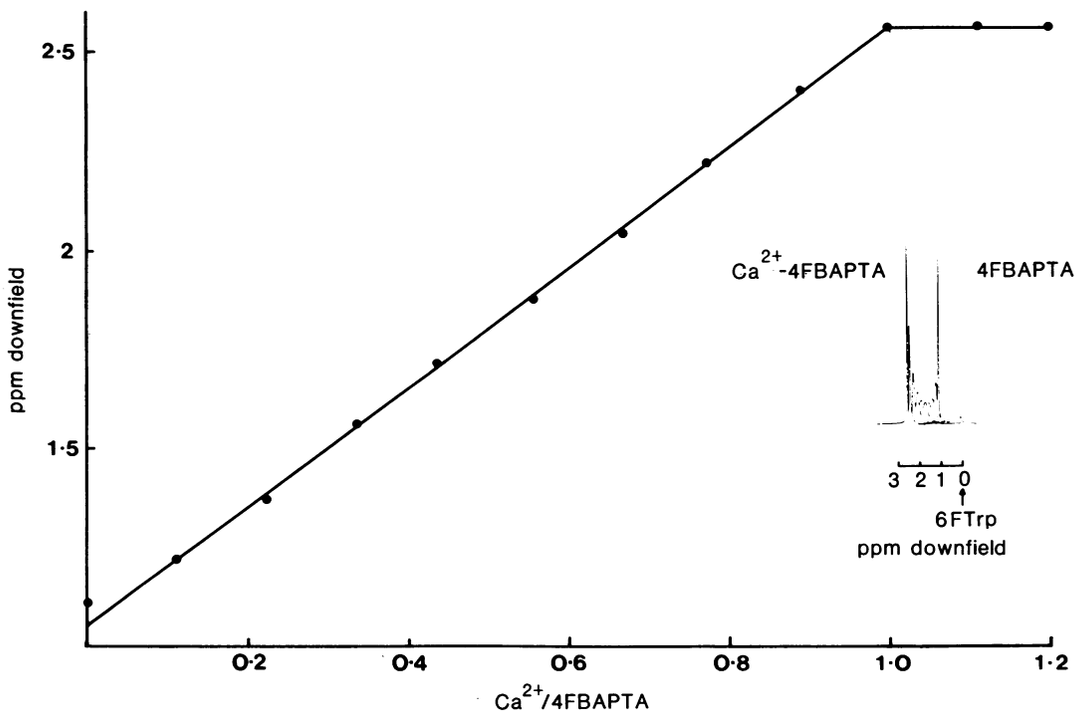

FIG. 4. Downfield shift at 37°C and 94.1 MHz of 4FBAPTA resonance as a function of the Ca^{2+}/4FBAPTA molar ratio. (*Inset*) Spectra at 37°C and 94.1 MHz of 4FBAPTA as a function of the Ca^{2+}/4FBAPTA molar ratio.

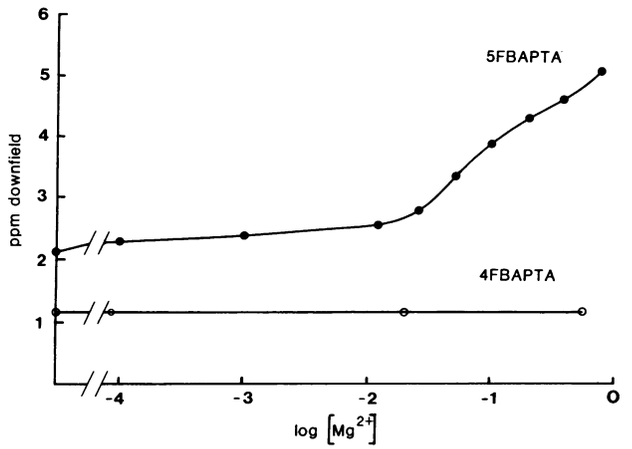

FIG. 5. ^{19}F chemical shifts at 37°C and 94.1 MHz of 5FBAPTA and 4FBAPTA as a function of free Mg^{2+} concentration in citrate buffer.

an affinity of log K_{Ca} = 5.66 compared with the value of 5.61 (Fig. 1) obtained by back-titration of the fluorescence of Ca–quin2. The slopes of the Sips plots from NMR shift measurements or the fluorescence titrations were 1.0 ± 0.02 (data not shown), indicating a homogeneous population of binding sites for Ca^{2+} with a 1:1 stoichiometry.

^{19}F Chemical Shifts with Mg^{2+}. All *n*FBAPTA complexes with Mg^{2+} gave single averaged resonances for bound and free species characteristic of fast NMR exchange and as expected from the low affinity of BAPTA for Mg^{2+} (1). The 3FBAPTA and 5FBAPTA analogues did not show limiting chemical shifts even at very large molar excesses of Mg^{2+}, and the chelators appear to bind a second Mg^{2+} ion with very low affinity (1). The chemical shifts of 5FBAPTA and 4FBAPTA titrated with Mg^{2+} are shown in Fig. 5. 5FBAPTA shows chemical shifts at 2 M Mg^{2+} of about 5 ppm, comparable with those obtained for Ca–5FBAPTA complexes. From the data in Fig. 5, it can be seen that changes in intracellular free Mg^{2+} concentration up to 10 mM will produce chemical shifts of <0.2 ppm, which is <5% of the shift of the Ca–5FBAPTA resonance. Thus, changes in free Mg^{2+} concentration in the cells will not affect the resolution of the resonances of the Ca–5FBAPTA from the free form of the chelator in fast exchange with Mg^{2+}. The 4FBAPTA analogue is insensitive to titration with Mg^{2+} (Fig. 5).

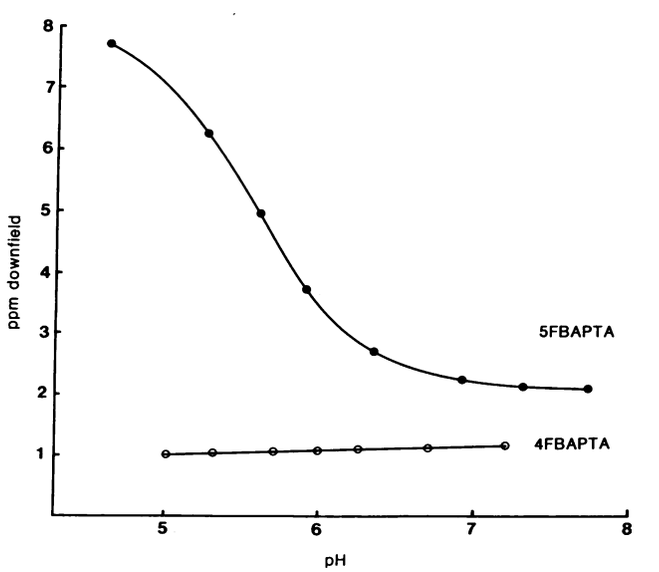

FIG. 6. ^{19}F chemical shifts at 37°C and 94.1 MHz of 5FBAPTA and 4FBAPTA as a function of pH.

^{19}F Chemical Shifts with pH. The 5FBAPTA resonance shifts by about 5.7 ppm between pH 7.5 and pH 4.5 (Fig. 6), and the chelator is insoluble at lower pH values in the protonated form(s). The chemical shift is about 0.1 ppm between pH 6.9 and 7.3 [i.e., ±0.2 pH unit about the estimated intracellular pH of 7.15 for mouse thymocytes (10)], which is <2% of the shift obtained for the Ca–5FBAPTA resonance. The complete resolution of the resonances from 5FBAPTA and Ca–5FBAPTA necessary to measure the free Ca^{2+} concentration is maintained between pH 6 and 8; hence, intracellular pH changes will not affect the estimation of $[Ca]_i$ with this chelator. The 4FBAPTA resonance is insensitive to pH changes (<0.25 ppm shift from pH 5 to pH 8 and <0.03 ppm shift from pH 6.9 to pH 7.3).

Estimation of $[Ca]_i$ in Thymocytes. The ^{19}F NMR spectrum at 188.3 MHz from thymocytes loaded with ≈1 mM [^3H]-5FBAPTA consists of two resonances at the positions corresponding to free 5FBAPTA and Ca–5FBAPTA (Fig. 7*a*). The chemical shift of the free 5FBAPTA resonance indicates that the free intracellular Mg^{2+} concentration must be <1 mM and that the intracellular pH must be >6.9. The relative areas of the two resonances correspond to 20% Ca–5FBAPTA, equivalent to a $[Ca]_i$ of ≈250 nM. The estimated percentage of Ca–5FBAPTA did not change in successive 20-min determinations on the same cell suspension, which indicates a stable $[Ca]_i$ under the conditions of the NMR experiment. Addition of 5 mM

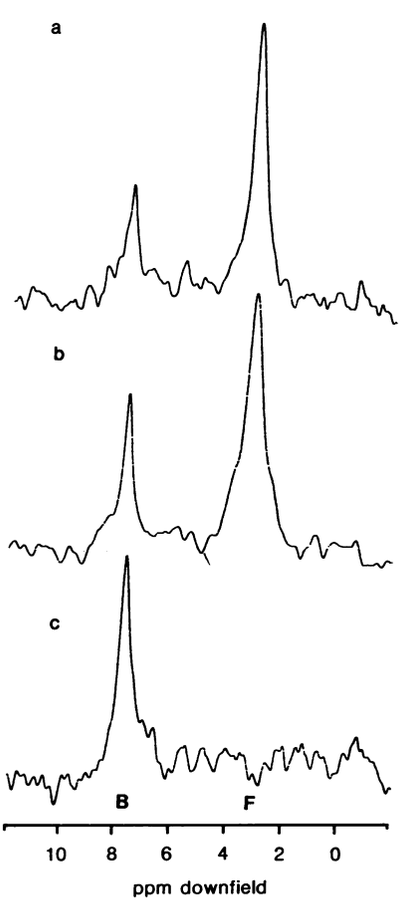

FIG. 7. (*a*) ^{19}F spectrum at 37°C and 188.3 MHz of [^3H]5FBAPTA in BALB/c mouse thymocytes accumulated in 20 min at 37°C. (*b*) Effect of addition of succinyl Con A at 100 μg/ml. (*c*) Effect of addition of 50 μM A23187 to the same cell preparation. The intracellular 5FBAPTA is saturated with Ca^{2+} (B) and a single resonance at the Ca–5FBAPTA position is observed. F, resonance of 5FBAPTA without Ca^{2+}.

sodium azide to decrease the cellular ATP level to 50% of the level in untreated cells (11) had no detectable effect on the estimated percentage of Ca–5FBAPTA, consistent with previous evidence from quin2 experiments that the normal $[Ca]_i$ is maintained when the cellular ATP concentration is substantially reduced by metabolic inhibitors (12).

As found in the model experiments the 5FBAPTA resonances are broadened by exchange, and in some experiments there was an additional minor resonance between the two main resonances. On addition of succinylated Con A at 50 μg/ml or 100 μg/ml, there was a consistent and significant increase in the proportion of Ca–5FBAPTA, corresponding to an increase in $[Ca]_i$ to about 350 nM (Fig. 7*b*). The identity of the Ca–5FBAPTA resonance was confirmed by the addition of 50 μM A23187 to equilibrate the cells with Ca^{2+} from the external medium. A single resonance at the chemical shift of Ca–5FBAPTA was observed (Fig. 7*c*), indicating that all of the chelator is saturated by Ca^{2+}.

DISCUSSION

Line-shape analysis of the ^{19}F spectra of the 4- and 5FBAPTA complexes with Ca^{2+} at 94.1 MHz and 188.3 MHz allows the approximate K_d of the Ca^{2+} complexes to be estimated (13) as 9.8×10^2 sec^{-1} and 5.7×10^2 sec^{-1}, respectively. From the affinities given in Fig. 1, the association rate constants for Ca^{2+} are calculated as $\approx 4.0 \times 10^8$ liter/mol per sec for 4FBAPTA and 8.1×10^8 liter/mol per sec for 5FBAPTA, which are approaching the diffusion rate limit. Chelators of the BAPTA series are therefore capable of rapid responses (within 10 msec) to changes in free Ca^{2+} concentration >0.1 μM.

The fast and slow NMR exchange conditions for the different Ca^{2+}–*n*FBAPTA complexes provide two NMR assays of $[Ca]_i$ over a wide range of concentrations. The fast-exchange 4FBAPTA chelator has the advantage that $[Ca]_i$ is indicated by the measurement of the chemical shift, which is inherently more sensitive than the measurement of the areas of resonances from the free and bound forms for slow-exchange complexes. The accessible range of $[Ca]_i$ is from $\approx$300 nM to 30 μM, assuming shift measurements are accurate to 0.05 ppm for the exchange-broadened peaks. Furthermore, the chemical shift of the 4FBAPTA resonance is very insensitive to $[Mg^{2+}]$ and pH and the shift, therefore, provides a direct measurement of the proportion of the chelator complexed with Ca^{2+}, and hence $[Ca]_i$, without correction for $[Mg^{2+}]$ and pH. Minor intracellular ions (e.g., Zn^{2+}, Fe^{2+}, Mn^{2+}, etc.) have higher affinities than Ca^{2+} for 4FBAPTA and are in slow exchange. These ions will not, therefore, affect the Ca–4FBAPTA resonance that indicates $[Ca]_i$. In thymocytes the chemical shift of 4FBAPTA is very close to the shift of the free form, indicating a limit to $[Ca]_i$ of $<$300 nM, which is consistent with the value from the slow-exchange 5FBAPTA assay of 250 nM. The 4FBAPTA chelator should be particularly useful as an indicator for $[Ca]_i \simeq 1$ μM.

To assess the validity of the $[Ca]_i$ values obtained with the *n*FBAPTA indicators, they may be compared with those obtained by independent techniques. The measurement of $[Ca]_i$ by aequorin injection (14) or by Ca^{2+} microelectrodes (15) in large cells is subject to uncertainties associated with cell penetration. Although aequorin is highly selective for Ca^{2+} and is very sensitive to changes in $[Ca]_i$, the calibration of absolute $[Ca]_i$ values with aequorin is not very precise. Microelectrode determinations of $[Ca]_i$ suffer from lack of absolute specificity, drift, and other problems. The $[Ca]_i$ values obtained by these techniques in various large cells generally lie between 50 and 300 nM and the error in absolute $[Ca]_i$ is estimated as at least 50% and usually larger. The values of $[Ca]_i$ obtained with quin2 in a range of cells lie between 80 and 150 nM and are therefore consistent with the values from the independent techniques. The slow-exchange Ca–5FBAPTA complex indicated a $[Ca]_i$ in mouse thymocytes of 250 nM compared with 120 nM using quin2 in the same cells, so that the fluorescence and NMR indicators give similar $[Ca]_i$ values, at least within the accuracy of current independent methods. The effect of succinylated Con A in increasing $[Ca]_i$ is also consistent with data for this ligand obtained using quin2 (4).

We have noted previously that quin2 fluorescence is quenched by several minor intracellular M^{n+} ions with high affinity for quin2 and that this may account for the apparent decrease in $[Ca]_i$ as the intracellular quin2 concentration is decreased (4). In this respect, the resolution of the M^{n+}–5FBAPTA components in the NMR spectra represents a substantial advantage of the NMR indicator over quin2. We conclude that further development and comparison of the NMR and fluorescence indicators under nonperturbing conditions (i.e., at intracellular concentrations <0.1 mM) will improve the accuracy and range of $[Ca]_i$ measurements substantially.

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