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KINETICS OF CALCIUM BINDING TO FLUO-3 DETERMINED BY STOPPED-FLOW FLUORESCENCE

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SUMMARY : The kinetics of Ca^{2+} dissociation from fluo-3 was measured using stopped flow fluorimetry. Analysis of dissociation revealed, in contrast to other commonly used fluorescent Ca^{2+} indicators, a biexponential behaviour with two distinct dissociation rates of 550 s^{-1} and 200 s^{-1} at physiological pH and room temperature. The dissociation rate constant of the fast phase increases to 700 s^{-1} at physiological temperature, whereas that of the slow phase does not change markedly. While the rate constants do not depend on pH between 6.6 and 7.8, the dissociation turns out to be monoexponential at pH 5.86. The association rate of Ca^{2+} to fluo-3 could not be measured within the mixing dead time and is estimated to be above $10^9 M^{-1} s^{-1}$. Since the rate constants of fluo-3 are larger than those of other fluorescent Ca^{2+} indicators, fluo-3 is well suited for investigations of Ca^{2+} oscillations in biological systems.

Fluorescent calcium-sensitive indicators have gained widespread use for measuring cytosolic free Ca^{2+} ($(Ca^{2+})_i$) and have been essential to our understanding of calcium as an intracellular messenger. Stimulus-response coupling has been linked to a sustained rise of $(Ca^{2+})_i$ in some, as well as to periodic spikes and oscillations of $(Ca^{2+})_i$ (1,2) and high intracellularly localized Ca^{2+} gradients derived from intracellular calcium release sites (3-5) in other cells. Since changes in $(Ca^{2+})_i$ on a sub-second timescale in a wide variety of biological preparations, techniques which accurately measure both the time-course of calcium spiking and its amplitude must allow detection of processes faster than biological reactions. Indicators for calcium measurements such as quin-2 (6), fura-2 (7,8), indo-1 (7), azo-1 (8) and antipyralazo III (9), reveal appropriate calcium binding and are therefore well suited to detect rapid calcium changes. Since the acetoxymethlesters of these indicators are trapped and the dyes incoporated into cells rapidly, these indicators are more widely used. However, the lower specificity for calcium and lower fluorescence intensity leading to a larger

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intracellular calcium buffering capacity of quin-2 made the use of fura-2 and indo-1 for many purposes more convenient. On the other hand, a three- to fourfold increase of dissociation constant of fura-2 in the prescence of cytoplasmic proteins raise difficulties in determining biological reactions appropriately (10).

A new group of fluorescent indicators with visible excitation and emission wavelengths for measurement of $(Ca^{2+})_i$ has been described recently (11). Their high specificity for calcium and their fluorescence properties not requiring UV-light for fluorescence microscopy are favourable compared to previous indicators but are not suitable for measurements using ratios at two wavelengths. However, in particular fluo-3, promises advantages for calcium determinations in fibroblasts (12) and platelets (13). The purpose of this study was to obtain a measure of the rates of Ca^{2+} changes that may be reliably estimated using this indicator. The association and dissociation was investigated using stopped-flow techniques.

Materials and Methods

All experiments were performed at least in double and are represented as mean ± SD where analysis of data allows to calculate these values. Fluorescence properties and equilibrium dissociations constants were determined at 37 ± 0.5°C by titration of buffers (100 mM KCl, 10 mM K-MOPS, 10 mM K_2H_2EGTA, 0.1 to 10 uM fluo-3 with free Ca^{2+} values ranging from below 1 nM to 1 mM on a Perkin-Elmer 5LS spectrofluometer, pH 7.4 as detailed elsewhere (11).

Stopped flow measurements were performed with a Durrum-Gibson mixing device equipped with tandem monochromator, a 200 W Hg-Xe lamp (Osram), double beam optics and two photomultipliers (EMI 9558 QA and 9783A) as decribed in detail in (6). The signal was digitized by use of a DATALAB DL950 transient recorder (Canberra Electronics Ltd.) and transients curves were analyzed on-line with a Twix 88-XT personal computer using the programs COLECT1 and COSY (written by one of us, M.E.). Two non-linear least squares algorithms, the ELORMA and the Marquardt procedures (14) were used to fit the transient curves to one or more overlapping exponentials. The mixing dead time of stopped flow instrument was determined as described elsewhere (15) and was 2.3 ms. Fluorescence was excited at 436 nm unless otherwise stated and emission was collected above 490 nm observed at right angle to the incident beam using a GG 490 cut-off filter (Schott u. Gen., Mainz, BRD). In general, unless otherwise detailed, experiments were performed by mixing 1 uM fluo-3 in 50 mM HEPES, 150 mM NaCl and 4 uM $CaCl_2$ with a large excess of EGTA (10^3 to 10^5 excess over fluo-3; vol/vol:1/1).

Fluo-3 penta-potassium salt was purchased from Molecular Probes Inc. Eugene OR, HEPES (2-(4-(2-hydroxyl)-1-piperazinyl)-ethanesulfonic acid) was purchased from Merck, Darmstadt, BRD. Double destilled water was purified for preparation of buffers to a resistance of 18 MOhm/cm by use of a Barnstead NANOpure cartridge system from SKAN AG, Basel, Switzerland. All other compounds were obtained from Fluka, Buchs, Switzerland.

Results and Discussion

Maximal fluorescence intensity of fluo-3 was observed at 526 nm with an excitation wavelength of 508 nm, at 37 ± 0.5 °C of fluo-3. The same result was reported previously at 22 °C (11). Although discrimination of calcium decreased above 600 nm a specific wavelength could not be determined which could be used easily for calcium measurements without use of trypsination procedures in cells as described for fura-2 (5). The dissociation constants at equilibrium for calcium were 404 ± 5 nM at 22 ± 2 °C, 412 ± 6 nM at 37 ± 0.5 °C and 418 ± 9 nM at 45 ± 2 °C. These constants which are larger than those reported for quin-2, fura-2 and indo-1 make fluo-3 suitable for measurements of higher calcium levels in biological systems.

Due to the use of a Hg/Xe light source most of stopped flow experiments were performed at 436 nm excitation wavelength. In order to investigate the binding kinetics of Ca^{2+} to fluo-3 a series of measurements mixing 500 nM fluo-3 and 2 uM EGTA with micromolar concentrations of Ca^{2+} were performed. However, no interpretable transient curves were found indicating that the increase in fluorescence caused by binding of Ca^{2+} to fluo-3 is too fast to be observed under these conditions. From this the association rate constant must be larger than $10^9\ M^{-1}\ s^{-1}$ and thus faster than that for fura-2 and indo-1 (7) and its precise mechanism of the interaction remains undetermined.

If buffer without EGTA is mixed with 1 uM fluo-3 and 4 uM Ca^{2+}, no fluorescence is observed indicating that the fluo-3/Ca^{2+}-complex does not dissociate significantly within the time of measurement, 36 ms. The dissociation of the Ca^{2+} was measured by mixing 1 uM fluo-3 and 4 uM Ca^{2+} with 10 mM EGTA. A biexponential decrease of fluorescence was observed, with a first rapid phase of rate constant of 550 s^{-1} and a second slower phase of 200 s^{-1}. The slower phase contributes to about one third to the total signal. Therefore the dissociation of the fluo-3/Ca^{2+}-complex is

TABLE 1 : Independence of the apparent dissociation rate constants, k_1 and k_2, of the fluo-3/Ca^{2+} complex from EGTA

EGTA (mM)	k_1 (s^{-1})	k_2 (s^{-1})
0.5	595 ± 26	204 ± 10
1.5	533 ± 33	210 ± 12
5.0	564 ± 38	221 ± 12
15.0	544 ± 30	176 ± 21
50.0	566 ± 20	181 ± 12

TABLE 2 : pH dependence of the apparent dissociation rate constants, k_1 and k_2, of the fluo-3/Ca^{2+} complex

pH	buffer	k_1 (s^{-1})	k_2 (s^{-1})
7.78	H	636 ± 31	211 ± 14
7.46	H	578 ± 26	222 ± 17
7.11	H	581 ± 30	198 ± 20
7.05	H	565 ± 29	180 ± 16
7.05	M	592 ± 24	200 ± 13
6.67	M	588 ± 33	241 ± 11
6.23	M	486 ± 16	140 ± 24
5.86	M	364 ± 3	---
5.84	M	323 ± 7	---

150 mM NaCl, 50 mM either HEPES (H) or MES (M) was used as buffer, 25°C.
-- indicates that reaction is strictly monoexponential.

much faster than that of quin-2 (6), indo-1 and fura-2 (7,8) and is comporable to azo-1 (8). In contrast to fluo-3, the quin-2/Ca^{2+}-complex dissociates with monoexponential kinetics (6). The two rate constants of fluo-3 do not depend on EGTA concentrations (table 1) nor on the concentrations of the buffer constituents (data not shown), at 22 ± 2°C, pH 7.40. Therefore the dissociation of the fluo-3/Ca^{2+}-complex is rate limiting under these conditions.

The dependence of dissociation rate constants on pH was investigated from pH 5.84 to pH 7.78, at 25°C (table 2). These experiments show that the dissociation is biphasic with little effect on the rate constants around neutral pH and within its physiological range, while the second, slower phase disappears at low pH. In contrast to the increase of the quin-2 dissociation rate constant (6), the fast dissociation rate constant of fluo-3 decreases at lower pH, although this rate is still faster than that of quin-2.

The temperature dependence of the apparent dissociation rate constant k_1 are summarized in table 3. While the rate constant of the rapid phase

TABLE 3 : Temperature dependence of apparent dissociation rate constant, k_1, at pH 7.00

°C	k_1 (s^{-1})	°C	k_1 (s^{-1})
10.6	286 ± 17	30.0	678 ± 30
15.1	354 ± 33	34.3	710 ± 20
20.4	488 ± 57	35.1	702 ± 36
25.4	526 ± 39	39.9	735 ± 34

pH was adjusted every 10°C.

TABLE 4 : Independence of apparent dissociation rate constants, k_1 and k_2, from excitation wavelength

Excitation (nm)	k_1 (s^{-1})	k_2 (s^{-1})
436	578 ± 26	195 ± 14
470	552 ± 24	172 ± 23
480	577 ± 40	203 ± 20
495	536 ± 27	176 ± 19

increases, k_1, no marked temperature dependence was observed for the slower dissociation rate constant, k_2 (data not shown). Linear regression analysis of ln k_1 versus 1/T yields a slope of -2818 K and correlation coefficient of 0.997. From this, using the van't Hoff equation ($d\ln k/dt = H/RT^2$, where R is the gas constant and T the absolute temperature) an apparent activation energy E_A of 23.4 ± 1.9 kJ/mol is calculated. A similar value was reported for quin-2 (6).

In biological system calcium transport rates from and into calcium storage sites and changes of time-course and amplitude of calcium transients vary significantly depending on the organelles investigated, and conditions and assumptions used. The dissociation rate constants of fluo-3 determined in this study afford a high time resolution which proves the suitability of fluo-3 as calcium sensitive probe to analyze rapid kinetics. Both, the estimated value for the association and the measured dissociation rates are faster than those determined for fura-2 and indo-1 (7,8). However, the biexponential kinetic of calcium dissociation for fluo-3 at physiological conditions might raise difficulties in the interpretation of data, in particular if possible interactions with cytosolic proteins have to be considered as for fura-2 (10). This biexponential dissociation does not allow the assumption of a simple bimolecular mechanism as reported for other fluorescent dyes. Various factors have to be considered to explain the two distinct dissociation rates. Among others this might be explained due effects at the excitation wavelength used or

TABLE 5 : Independence of the apparent dissociation rate constants, k_1 and k_2, from fluo-3 concentration at pH 7.0

Fluo-3 (nM)	k_1 (s^{-1})	k_2 (s^{-1})
100	530 ± 170	165 ± 170
250	553 ± 30	245 ± 85
500	557 ± 51	201 ± 32
1000	587 ± 27	186 ± 17

alternatively by a stacking mechanism. Since the dissociation could be best analyzed by the two different rate constants at various excitation wavelengths (table 4) and since the two rate constants are independent from fluo-3 concentrations itself (table 5), there is no evidence for these explanations for two rate constants. Furthermore, the linear relation between fluorescence intensities of saturated fluo-3 solutions and fluo-3 concentrations between 0.03 to 30 uM (data not shown), is additional evidence against a stacking mechanism.

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