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The kinetics of calcium binding to fura-2 and indo-1

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The kinetics of Ca^{2+} dissociation from fura-2 and indo-1 were measured using a stopped-flow spectrofluorimeter. The dissociation rate constants were 84 s^{-1} and 130 s^{-1}, respectively, in 0.1 M KCl at 20°C. The rate constants were insensitive to pH over the range 7.0 to 8.0. The second order association rate constants were estimated indirectly to be in the region of 5×10^8 $M^{-1} \cdot s^{-1}$ and thus approach the diffusion-controlled limit. The results demonstrate that these new generation indicators are well-suited to measure rapid changes in concentration of intracellular Ca^{2+}.

Fura-2; Indo-1; Ca^{2+}; Mg^{2+}; Stopped-flow spectrometry; Fluorescence

1. INTRODUCTION

Changes in intracellular Ca^{2+} on a sub-second timescale occur in a wide variety of biological preparations, often as a step in a stimulus-response coupling mechanism. In order to investigate the role of Ca^{2+} under such circumstances, techniques which accurately measure both the amplitude and time-course of the Ca^{2+} concentration changes are required. Any reactions between Ca^{2+} and the sensor must therefore be faster than the transient changes in Ca^{2+} concentration to be measured.

The synthesis of the fluorescent Ca^{2+} indicators fura-2 and indo-1, which show 1:1 Ca^{2+} binding stoichiometry, have a high quantum yield and are available as cell-permeable acetoxy-methyl derivatives [1], have provided a major advancement as a technique for the detection of intracellular Ca^{2+} changes. Their use is now well-established in preparations ranging from mast cells [2] to striated muscle [3] and cardiac muscle [4]. The high quantum yield of the indicators also makes them ideally suited for fluorescence imaging and provide a spatially resolved picture of intracellular Ca^{2+} concentration changes, an application recently reviewed by Poenie and Tsien [5].

In order to obtain a measure of the rates of change of Ca^{2+} that may be reliably estimated using these indicators, the kinetics of Ca^{2+} binding and dissociation were investigated using stopped-flow techniques.

This paper is dedicated to Professor H. Gutfreund FRS in recognition of his contribution to the theory and practice of kinetic methods

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2. MATERIALS AND METHODS

Fura-2 free acid and indo-1 free acid (fura-2, indo-1; Molecular Probes, Eugene, OR 97448, USA) were dissolved in a solution containing 0.1 M KCl, 10 mM Tes (pH 7.0 or 8.0). The final indicator concentration in the mixing chamber was 10 μM. The fluorescence stopped-flow apparatus, to be described in detail elsewhere (A.P.J. and C.R.B., unpublished), had a dead-time of 1 ms. Reactions were followed by excitation at 335 nm using a Hg lamp. Fura-2 fluorescence was detected

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by means of a 420 nm glass long-pass filter (Ealing-Beck Ltd, Watford, England) and a light green Celloid filter (23A; Rank Stroud Ltd, Brentford, England) with peak transmission at 510 nm. Indo-1 fluorescence was recorded using a 370 nm glass long-pass filter.

Ca^{2+} concentrations refer to added Ca^{2+} and do therefore not include contaminant Ca^{2+}, estimated at 7 μM. All experiments were carried out at 20°C.

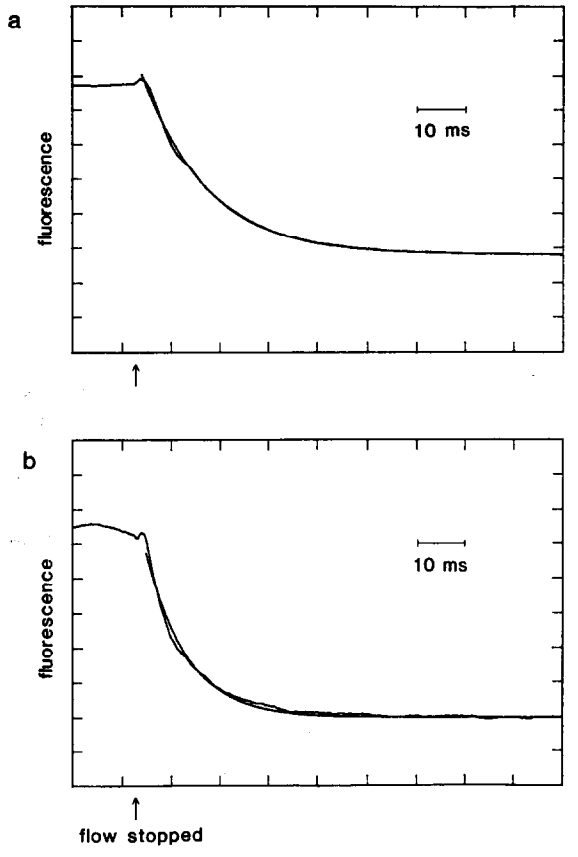

Fig.1. (a) Decrease in fura-2 fluorescence after mixing 20 μM Ca^{2+}-saturated fura-2 with 1.6 mM EDTA (syringe concentration) at pH 7.0 and the fit of this change by a single exponential (k = 75.7 s^{-1}), superimposed upon the recorded decrease. Horizontal axis: 10 ms/div after the arrow (flow-stop); vertical: fluorescence, 20% change/div. (b) Decrease in indo-1 fluorescence on mixing 20 μM Ca^{2+}-saturated indo-1 with 3.2 mM EGTA (syringe concentrations) at pH 8.0. A least-squares fit to a single exponential is superimposed (k = 117 s^{-1}). Horizontal axis: 10 ms/div after arrow; vertical: fluorescence, 10% change/div.

3. RESULTS

3.1. *Ca^{2+}-fura-2 kinetics*

The dissociation kinetics of the fura-2-Ca^{2+} complex were measured by mixing 20 μM fura-2 and 20 μM Ca^{2+} in one syringe with various concentrations of EDTA or EGTA in a second syringe. These competing chelators were chosen on the basis of their known kinetics for the reactions with Ca^{2+} and Mg^{2+} [6,7].

Fig.1a shows the decrease in fura-2 fluorescence upon mixing Ca^{2+}-saturated fura-2 with a solution containing 1.6 mM EDTA. This decrease is very well fitted by a single exponential (k = 75.7 s^{-1}), superimposed upon the recorded decrease.

Fig.2 shows the time constants of fitted exponentials to the fura-2-Ca^{2+} dissociation measurements at different EDTA (pH 7.0) and EGTA (pH 8.0) concentrations. Hyperbolic fits to the data show that the asymptotes for the EDTA and EGTA data are not significantly different (84.5 s^{-1} and 84.3 s^{-1}, respectively) indicating a value for the Ca^{2+} dissociation rate from fura-2 of 84 s^{-1}, which is pH independent over the range 7.0 to 8.0.

The association rate constant (k_1) for the Ca^{2+}-fura-2 complex may be estimated in two ways. Firstly, from the equilibrium constant. The apparent dissociation constant (K_d') of the fura-2-Ca^{2+} complex was measured by Grynkiewicz et al. [1] as 135 nM in 0.1 M KCl at

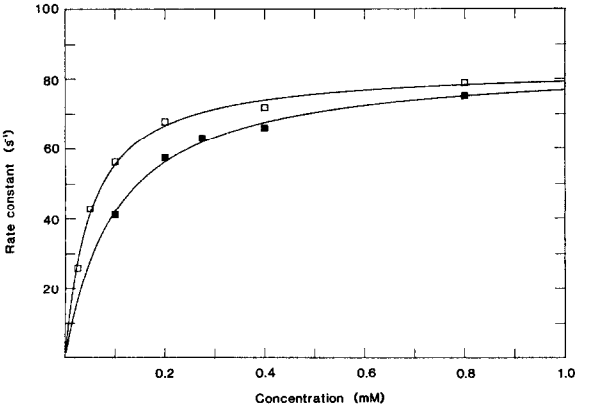

Fig.2. Rate constants of the fitted exponentials to the Ca^{2+}-fura-2 fluorescence changes (see e.g. fig.1) on mixing with different EDTA (pH 7.0; ■) and EGTA (pH 8.0; □) reaction chamber concentrations. The curves are hyperbolic least-square fits to the data.

20°C, pH 7.1–7.2. The association rate constant is therefore calculated as 6.2×10^8 $M^{-1} \cdot s^{-1}$, under these conditions.

The second method makes use of the known kinetics of the EGTA-Ca^{2+} reactions at pH 8.0. EGTA (E) competes with fura-2 (F) for Ca^{2+}:

$$Ca^{2+} + F \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CaF \quad (1)$$

$$Ca^{2+} + E \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} CaE \quad (2)$$

The rate of EGTA-Ca^{2+} complex formation is given by:

$$\frac{d[CaE]}{dt} = k_2 \cdot [Ca^{2+}] \cdot [E] - k_{-2} \cdot [CaE] \quad (3)$$

The rate of change of free Ca^{2+} is:

$$\frac{d[Ca^{2+}]}{dt} = -k_1 \cdot [Ca^{2+}] \cdot [F] + k_{-1} \cdot [CaF] - k_2 \cdot [Ca^{2+}] \cdot [E] + k_{-2} \cdot [CaE] \quad (4)$$

Provided the chelators involved have a high affinity and are in excess concentration over the total Ca^{2+}, the free Ca^{2+} concentration remains close to zero throughout the reactions. Accordingly the rate of change of free Ca^{2+} remains close to zero relative to that of other species. Therefore to a good approximation:

$$k_1 \cdot [Ca^{2+}] \cdot [F] + k_2 \cdot [Ca^{2+}] \cdot [E] = k_{-1} \cdot [CaF] + k_{-2} \cdot [CaE] \quad (5)$$

Substituting into eqn 3:

$$\frac{d[CaE]}{dt} = k_2 \cdot \left\{ \frac{k_{-1} \cdot [CaF] + k_{-2} \cdot [CaE]}{k_1 \cdot [F] + k_2 \cdot [E]} \right\} \cdot [E] - k_{-2} \cdot [CaE] \quad (6)$$

$$[Ca_0] = [CaF] + [CaE]$$

$$\frac{d[CaE]}{dt} = \frac{k_2 \cdot k_{-1} \cdot [E] \cdot [Ca_0]}{k_1 \cdot [F] + k_2 \cdot [E]} - \left\{ \frac{k_2 \cdot k_1 \cdot [E] + k_{-2} \cdot k_1 \cdot [F]}{k_1 \cdot [F] + k_2 \cdot [E]} \right\} \cdot [CaE] \quad (7)$$

The formation of CaE (or the disappearance of CaF) is therefore a single exponential function with rate constant:

$$k_{obs} = \frac{k_{-1}}{1 + (k_1 \cdot [F])/(k_2 \cdot [E])} + \frac{k_{-2}}{1 + (k_2 \cdot [E])/(k_1 \cdot [F])} \quad (8)$$

A plot of k_{obs} against [E] therefore yields a value (or limit) of k_{-2} as the Y intercept and k_{-1} as the limiting value of k_{obs} at infinite [E]. This is in accord with the data of fig.2, where k_{-1} is 84 s^{-1} and k_{-2} very close to zero. Independent measurements of Ca-EGTA kinetics at pH 8.0 gave a value for k_{-2} of 0.3 s^{-1} [7], confirming that the second term of eqn 8 is negligible. Consequently, the EGTA concentration where $k_{obs} = k_{-2}/2$ corresponds to the condition where $k_1 \cdot [F] = k_2 \cdot [E]$. In fig.2 this occurs when [E]/[F] = 4.9. Assigning a value for k_2 of 5×10^7 $M^{-1} \cdot s^{-1}$ for EGTA at pH 8.0 [6] indicates that k_1 for fura-2 is 2.5×10^8 $M^{-1} \cdot s^{-1}$. The data obtained with EDTA as the competitor (fig.2) are consistent with these conclusions given that the EDTA association rate constant is $>10^7$ $M^{-1} \cdot s^{-1}$ at pH 7.0 [7].

The estimation of k_1 for fura-2 is dependent on the constants assigned to EGTA or EDTA which, in turn, are very pH dependent. It is clear however that k_1 is in excess of 10^8 $M^{-1} \cdot s^{-1}$ and probably approaches the diffusion-controlled limit. The pH independence of fura-2 thermodynamics [1] and its kinetics around neutrality (fig.2) contribute much to its usefulness as an accurate intracellular Ca^{2+} indicator.

3.2. *Mg^{2+}-fura-2 kinetics*

Experiments were also performed to check the kinetics of interaction of Mg^{2+} with fura-2. When 200 μM Ca^{2+} from one syringe was mixed with fura-2 and 100 μM EGTA from the other syringe more than 95% of the fluorescence change was complete within the dead-time of the instrument (1 ms), as expected from the above analysis. Ca^{2+} binding to fura-2 remained too fast to be measured, after inclusion of 10 mM Mg^{2+} in the fura-2-containing syringe. Fura-2 shows a weak affinity for Mg^{2+} with $K_d \sim 10$ mM [1], which we confirmed by titration using a conventional

Table 1

Kinetics of Ca^{2+} and Mg^{2+} binding to fura-2 and indo-1

	Fura-2		Indo-1
	Ca^{2+}	Mg^{2+}	Ca^{2+}
k_{-1} (s^{-1})	84	>500	130
k_1 ($M^{-1} \cdot s^{-1}$)	2.5×10^8–6.5×10^8		5×10^8–1×10^9

Rate constants invariant between pH 7.0 and 8.0, 0.1 M KCl, 20°C

fluorimeter. Thus, in the stopped-flow experiment above, the bound Mg^{2+} must dissociate rapidly (>500 s^{-1}).

3.3. *Ca^{2+}-indo-1 kinetics*

Similar methods were applied to study the kinetics of Ca^{2+} dissociation from the indo-1-Ca^{2+} complex (fig.1b). On increasing the EDTA concentration at pH 7.0 or EGTA concentration at pH 8.0, the observed rate reached a maximum of 130 s^{-1}. The association rate constant based on the known equilibrium constant of the indo-1-Ca^{2+} complex [1] or kinetic competition with EGTA was calculated to be in the region of 5×10^8 to 1×10^9 $M^{-1} \cdot s^{-1}$. Thus the kinetics of indo-1 interaction with Ca^{2+} are marginally faster than with fura-2.

4. DISCUSSION

Indicators useful for monitoring changes in intracellular free Ca^{2+} require reaction kinetics faster than the changes they are intended to detect. The dissociation rate of Ca^{2+} from fura-2 or indo-1 determined in vitro (table 1) indicate that the measurement of a transient decay in Ca^{2+} with a half-time as short as 15 ms should be possible in vivo.

The dissociation rate constants reported in this study are slightly faster than those found for quin2 [8,9] using stopped-flow methods, and consistent with the small difference in Ca^{2+} affinity between these indicators.

The association rate constants for both fura-2 and indo-1 of $>10^8$ $M^{-1} \cdot s^{-1}$ approach the diffusion-controlled limit (cf. [5]). Indo-1 has a slightly faster response than fura-2, although the choice of indicator for in vivo studies is likely to depend more on its optical properties as discussed in [1].

Hollingworth and Baylor [10] recently used fura-2 and antipyrylazo III to measure Ca^{2+} transients in intact frog muscle fibres and found a slower time-course for the fura-2 transient compared to antipyrylazo III. They suggested an explanation for this by a fura-2 Ca^{2+} 'off' rate constant of 25 s^{-1}, some 3.4-times slower than our value measured in vitro. It is possible that the kinetics of the Ca^{2+}-fura-2 interaction are altered in vivo, but further studies are required to clarify this point.

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