

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

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The kinetics of  $\text{Ca}^{2+}$  dissociation from fura-2 and indo-1 were measured using a stopped-flow spectrofluorimeter. The dissociation rate constants were  $84 \text{ s}^{-1}$  and  $130 \text{ s}^{-1}$ , respectively, in  $0.1 \text{ M KCl}$  at  $20^\circ\text{C}$ . 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  $5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$  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  $\text{Ca}^{2+}$ .

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

## 1. INTRODUCTION

Changes in intracellular  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  under such circumstances, techniques which accurately measure both the amplitude and time-course of the  $\text{Ca}^{2+}$  concentration changes are required. Any reactions between  $\text{Ca}^{2+}$  and the sensor must therefore be faster than the transient changes in  $\text{Ca}^{2+}$  concentration to be measured.

The synthesis of the fluorescent  $\text{Ca}^{2+}$  indicators fura-2 and indo-1, which show 1:1  $\text{Ca}^{2+}$  binding stoichiometry, have a high quantum yield and are available as cell-permeable acetoxymethyl derivatives [1], have provided a major advancement as a technique for the detection of in-

tracellular  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  concentration changes, an application recently reviewed by Poenie and Tsien [5].

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

## 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  $0.1 \text{ M KCl}$ ,  $10 \text{ mM Tes}$  (pH 7.0 or 8.0). The final indicator concentration in the mixing chamber was  $10 \mu\text{M}$ . 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 Celoid 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.

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

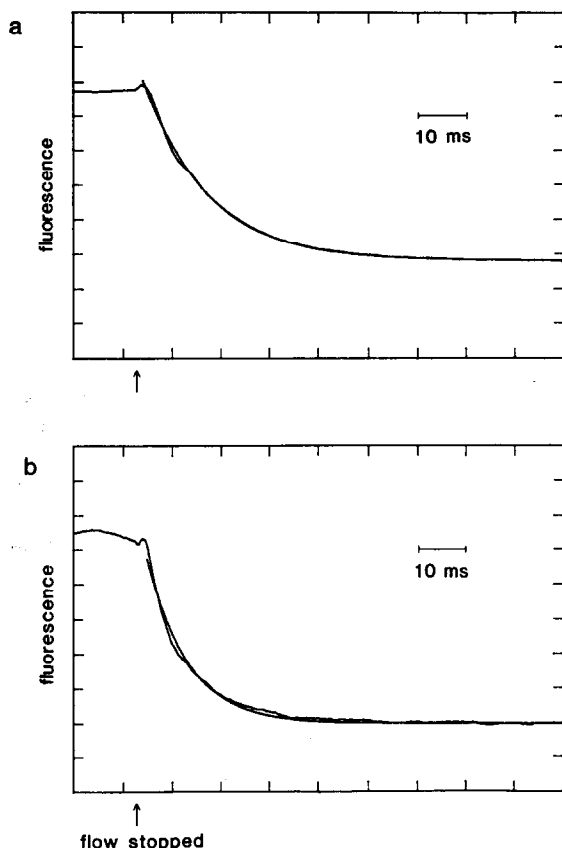


Fig.1. (a) Decrease in fura-2 fluorescence after mixing 20  $\mu\text{M}$   $\text{Ca}^{2+}$ -saturated fura-2 with 1.6 mM EDTA (syringe concentration) at pH 7.0 and the fit of this change by a single exponential ( $k = 75.7 \text{ s}^{-1}$ ), superimposed upon the recorded decrease. Horizontal axis: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

### 3. RESULTS

#### 3.1. $\text{Ca}^{2+}$ -fura-2 kinetics

The dissociation kinetics of the fura-2- $\text{Ca}^{2+}$  complex were measured by mixing 20  $\mu\text{M}$  fura-2 and 20  $\mu\text{M}$   $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  [6,7].

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

Fig.2 shows the time constants of fitted exponentials to the fura-2- $\text{Ca}^{2+}$  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 ( $84.5 \text{ s}^{-1}$  and  $84.3 \text{ s}^{-1}$ , respectively) indicating a value for the  $\text{Ca}^{2+}$  dissociation rate from fura-2 of  $84 \text{ s}^{-1}$ , which is pH independent over the range 7.0 to 8.0.

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

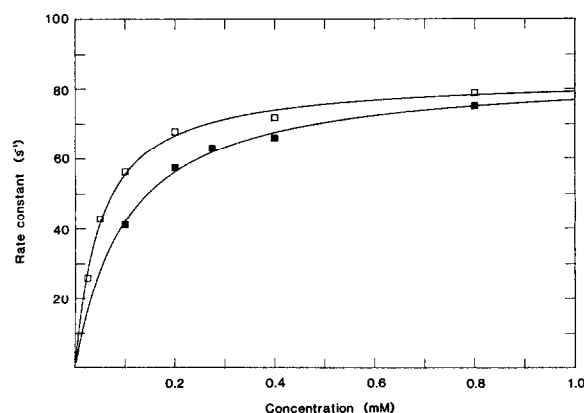


Fig.2. Rate constants of the fitted exponentials to the  $\text{Ca}^{2+}$ -fura-2 fluorescence changes (see e.g. fig.1) on mixing with different EDTA (pH 7.0; ■) and EGTA (pH 8.0; □) reaction chamber concentrations. The curves are hyperbolic least-square fits to the data.

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

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



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

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

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

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

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

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

Substituting into eqn 3:

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

$$[\text{Ca}_0] = [\text{CaF}] + [\text{CaE}]$$

$$\begin{aligned} \frac{d[\text{CaE}]}{dt} = & \frac{k_2 \cdot k_{-1} \cdot [\text{E}] \cdot [\text{Ca}_0]}{k_1 \cdot [\text{F}] + k_2 \cdot [\text{E}]} - \\ & \left\{ \frac{k_2 \cdot k_1 \cdot [\text{E}] + k_{-2} \cdot k_1 \cdot [\text{F}]}{k_1 \cdot [\text{F}] + k_2 \cdot [\text{E}]} \right\} \cdot [\text{CaE}] \end{aligned} \quad (7)$$

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

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

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

### 3.2. $\text{Mg}^{2+}$ -fura-2 kinetics

Experiments were also performed to check the kinetics of interaction of  $\text{Mg}^{2+}$  with fura-2. When  $200 \mu\text{M}$   $\text{Ca}^{2+}$  from one syringe was mixed with fura-2 and  $100 \mu\text{M}$  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.  $\text{Ca}^{2+}$  binding to fura-2 remained too fast to be measured, after inclusion of 10 mM  $\text{Mg}^{2+}$  in the fura-2-containing syringe. Fura-2 shows a weak affinity for  $\text{Mg}^{2+}$  with  $K_d \sim 10 \text{ mM}$  [1], which we confirmed by titration using a conventional

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

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

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  $\text{Mg}^{2+}$  must dissociate rapidly ( $>500 \text{ s}^{-1}$ ).

### 3.3. $\text{Ca}^{2+}$ -indo-1 kinetics

Similar methods were applied to study the kinetics of  $\text{Ca}^{2+}$  dissociation from the indo-1- $\text{Ca}^{2+}$  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  $130 \text{ s}^{-1}$ . The association rate constant based on the known equilibrium constant of the indo-1- $\text{Ca}^{2+}$  complex [1] or kinetic competition with EGTA was calculated to be in the region of  $5 \times 10^8$  to  $1 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ . Thus the kinetics of indo-1 interaction with  $\text{Ca}^{2+}$  are marginally faster than with fura-2.

## 4. DISCUSSION

Indicators useful for monitoring changes in intracellular free  $\text{Ca}^{2+}$  require reaction kinetics faster than the changes they are intended to detect. The dissociation rate of  $\text{Ca}^{2+}$  from fura-2 or indo-1 determined in vitro (table 1) indicate that the measurement of a transient decay in  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  affinity between these indicators.

The association rate constants for both fura-2 and indo-1 of  $>10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$  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].

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

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