Detection of Ca^{2+}-transients elicited by flash photolysis of DM-nitrophen with a fast calcium indicator

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Abstract A confocal spot detection optical setup was used to record fluorescence signals in response to calcium pulses, elicited by flash photolysis of DM-nitrophen, with the calcium indicators CaOrange-5N and Fluo-3. Our results yield the following conclusions: $[Ca^{2+}]$ changes are almost perfect spikes at pCa 9 and broader transients followed by a step at pCa 7. The $[Ca^{2+}]$ spikes were used to measure the dissociation rate constant of the Ca^{2+} dyes. Experiments at pCa 7 were used to verify the kinetic rate constants of the dyes and to obtain those of DM-nitrophen. The association rate constant of this compound was found to be more than one order of magnitude faster than that suggested previously. CaOrange-5N was able to track changes in $[Ca^{2+}]$ more accurately than Fluo-3. This latter dye introduced severe distortions which preclude a quantitative deconvolution of the fluorescence transients into changes in the free $[Ca^{2+}]$.

Key words: Flash photolysis; Ca^{2+} dyes; Kinetic rate constant; Ca^{2+} transient

1. Introduction

The 'caged Ca^{2+}' chelators Nitr-5 and DM-nitrophen [1–3] have been increasingly used during the last five years as tools to investigate the role of Ca^{2+} in the regulation of various biological processes [4–7]. Their photolysis, elicited by illumination with UV light, results in rapid increases in the concentration of Ca^{2+} $[Ca^{2+}]$. DM-nitrophen is particularly effective in releasing massive amounts of Ca^{2+} ions upon UV flash photolysis and thus, in spite of Mg-binding limitations, has been the tool of preference for investigation of Ca^{2+} signaling with these techniques [5,6]. Unfortunately, attempts to provide a quantitative evaluation of the actual kinetics of the Ca^{2+}-transients elicited by flash photolysis of DM-nitrophen have been only moderately successful due to technical limitations in the Ca^{2+} detection methodology, and in the availability of dyes that are able to track these transients [8,9]. There is a consensus in the literature that release of Ca^{2+} by flash photolysis of DM-nitrophen leads to the creation of '$[Ca^{2+}]$-spikes' [8–13]. However, the characteristics of these spikes and the dependence of their kinetic properties on the initial conditions at which the flash was delivered, have not been well documented. A consequence of this lack of information has been the open controversy [10,11,14–16] about the interpretation of data in relation to the opening of muscle Ca^{2+}-release channels in response changes in $[Ca^{2+}]$ versus their steady-state properties [14–16]. More importantly, the usefulness of flash photolysis techniques to investigate the Ca^{2+} metabolism in living cells has been limited by the inability to measure the resulting $[Ca^{2+}]$-transient. The purpose of this paper is to demonstrate that by using the Ca^{2+} indicator, CaOrange-5N [12,13,17], in combination with novel features in the experimental device used to deliver a UV flash and to record fluorescence, we are able to measure the kinetic properties of the transients elicited by flash photolysis of DM-nitrophen. The comparison of the fluorescence data with theoretical predictions of a model that include both DM-nitrophen and a calcium dye provide new values for the kinetic parameters of DM-nitrophen which partially confirm and modify previous values in the literature [9].

2. Materials and methods

A confocal spot detection optical setup [18] was used to record fluorescence signals in response to calcium pulses, elicited by flash photolysis of DM-nitrophen, with the fluorescent calcium indicators CaOrange-5N and Fluo-3 (Molecular Probes, Eugene, OR). The source of light for excitation of the Ca^{2+} dyes was the 514 nm wavelength (about 1 W of power) of a multiline argon laser (Model 95, Lexel, Freemont, CA). A 20× objective (Fluo20, NA 0.75, Nikon, Japan) was used to focus the illuminating beam and to collect light from a small spot (6 μm in diameter) centered with respect to a fiber optic used to deliver UV pulses. The fluorescence was detected with a PIN photodiode (HRC008, United Detector Technologies, Culver City, CA) connected to an integrating head stage of a patch clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA; see [18] for a description of the spot detection methodology). Flashes of UV light (347 nm; 30 ns) were generated by a frequency-doubled ruby laser (Lumonics, UK) and guided through a cladded fused-silica fiber optic (diameter 200 μm) to 10 μl droplets of solutions placed on a coverslip that constituted the bottom of a experimental chamber on the stage of an inverted microscope (Model IM, Zeiss, Oberkochem, Germany). The energy of every UV flash was measured by a peak detection circuit and acquired simultaneously with the fluorescence traces.

The experimental solutions contained 100 mM KCl, 10 mM MOPS (pH 7), 7 mM DM-nitrophen (Calbiochem, La Jolla, CA) and 100 μM of a calcium indicator. Their pCa values were carefully adjusted to different values by successive titration with $CaCl_2$, monitoring the free $[Ca^{2+}]$ with Ca^{2+}-selective electrodes [19,20]. This titration procedure was used to generate Scatchard plots [21,22] that predicted a dissociation constant (K_d) for DM-nitrophen of 4.4 nM. The final pH of the solutions ranged between 7.2 and 7.5. The steady-state properties of Fluo-3 and CaOrange-5N were determined by fitting saturation curves to fluorescence data obtained in the same experimental setup where the flash transients were recorded. The K_d and F_{max}/F_{min} ratio values thus obtained were, respectively, 0.75 μM and 30 for Fluo-3, and 53 μM and 3.4 for CaOrange-5N [12]. It should be noted, however, that steady-state saturation curves for CaOrange-5N clearly suggested the existence of a lower affinity complex with a K_d of about 150–180 μM. The implications that the formation of multiple complexes have in the theoretical interpretation of the kinetic data presented here will be only briefly discussed later in this paper; it will be contained in detail in a manuscript currently in preparation.

The bandwidth and sampling rate of the data acquisition system were selected to optimize the signal-to-noise ratio of the fluorescence traces but they could be as fast as 30 kHz and 6 μs/sample, respectively. In order to prevent the saturation of the fluorescence recording system by

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the UV flash artifact, the gain of the patch clamp amplifier was reset for 50 μs to close to zero by short-circuiting the integrating capacitor of the patch clamp amplifier while the flash was delivered. As a consequence, the fluorescence records presented below show only the actual increase in fluorescence of the Ca^{2+} indicators in response to the release of Ca^{2+} ions induced by flash photolysis. The fluorescence transients are expressed in terms of normalized fluorescence changes ($\overline{\Delta F}$), defined as:

$$\overline{\Delta F} = \frac{(F - F_{min})}{(F_{max} - F_{min})}$$

in which F is the fluorescence acquired in every record as a function of time, F_{min} is the basal fluorescence recorded at pCa≥9, and F_{max} is the fluorescence recorded at saturating $[Ca^{2+}]$ (pCa<3). Traces expressed as $\overline{\Delta F}$ are normalized from 0 to 1. The advantage of this scaling method compared with the popular system of expressing fluorescence transients in terms of $\Delta F/F =: (F-F_{rest})/F_{rest}$ units is that results obtained with different dyes are readily comparable due to the normalization. Moreover, it can be readily demonstrated that for a fluorescent dye forming a single Ca-dye ([CaD]) complex

$$\overline{\Delta F} =: [CaD]/D_{tot}$$

where D_{tot} is the total dye concentration of the Ca^{2+} indicator. The experiments were performed at a room temperature of 18°C.

3. Results

Fig. 1 shows flash-induced Ca^{2+}-transients detected with the dyes CaOrange-5N (closed circles) and Fluo-3 (open circles) in a solution at pCa 9. Under these conditions both dyes report sudden fluorescence increases in response to UV flashes of identical energy (delivered at the instant indicated by the arrow) which are followed by exponential decays with very different kinetics. The Fluo-3 transient has a time constant of decay (τ) of about 5 ms and the CaOrange-5N transient decays with a τ of about 150 μs. From the dissociation constant (K_d) of DM-nitrophen (4.4 nM; see section 2) we can estimate that for this experiment at pCa 9, the concentration of Ca-DM-nitrophen complex before flash photolysis was 1.3 mM, the rest of the chelator (5.7 mM) being in its free form. As mentioned above, it has been suggested in several publications [8–13] that the existence of this very large concentration of free DM-nitrophen predicts the generation of $[Ca^{2+}]$-spikes (rather than '$[Ca^{2+}]$-steps') in response to flash photolysis. The sudden destruction of a fraction of the Ca-DM-nitrophen complex, which results in an equally rapid release of Ca^{2+} ions and increase in the free $[Ca^{2+}]$ of the solution, is followed by a subsequent re-binding of the Ca^{2+} by un-photolyzed free DM-nitrophen. Fig. 1A illustrates the severe limitation of Fluo-3, a commonly used Ca^{2+}-indicator, in reporting the existence of a fast $[Ca^{2+}]$-spike at an optimal experimental condition to generate it. CaOrange-5N, on the other hand, does a much better job in monitoring the presumably very fast time course of a $[Ca^{2+}]$-spike. The CaOrange-5N transient provides an experimental record with which the slowest estimation of the kinetics of the $[Ca^{2+}]$-spike has to be compared. For example, it may be argued that the τ of decay of the $[Ca^{2+}]$-spike at pCa 9 is accurately tracked by CaOrange-5N, a suggestion compatible with the relatively slow k_{on} reported for DM-nitrophen (e.g. 1.5×10^6 $M^{-1} \cdot s^{-1}$; [9]). In order to test the validity of this assumption, we recorded flash photolysis transients with both dyes at a pCa close to 7. At these conditions, the concentration of the Ca- DM-nitrophen complex is about 6.7 mM, and that of free DM-nitrophen is only 0.3 mM. Fig. 1B shows that for approximately the same energy of flash photolysis, the amplitude of the transients recorded by Fluo-3 and CaOrange-5N increased by a factor of about five, in agreement with the higher availability of photoreleasable complex. It can be observed in Fig. 1B that the Fluo-3 fluorescence trace reports a significant resting fluorescence at this pCa, also in agreement with the equilibrium dissociation constant measured for this dye (about 0.8 μM). In contrast, the CaOrange-5N fluorescence trace does not report a significant resting Ca^{2+} complexation at this pCa due to its very low affinity. The transient responses of both dyes to the release of Ca^{2+} by flash photolysis of DM-nitrophen are,

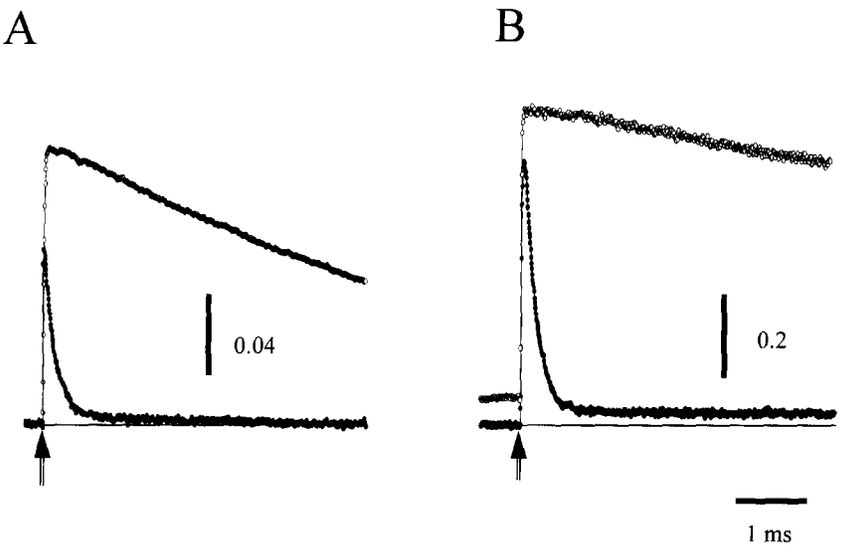

Fig. 1. Flash-induced Ca^{2+}-transients recorded with Fluo-3 and CaOrange-5N at two different resting Ca^{2+} levels. (A) UV flashes of 820 μJ were delivered to droplets of solution containing DM-nitrophen and either 100 μM Fluo-3 (open circles) or 100 μM CaOrange-5N (solid circles). The traces obtained with each dye correspond to an average of 4 sweeps. Fluo-3 and CaOrange-5N traces were superimposed for comparison of their time courses. The free $[Ca^{2+}]$ of the solution was adjusted to pCa 9. The vertical bar indicates the same normalized fluorescence value ($\overline{\Delta F}$; see section 2) for both dyes. (B) Same as in panel (A) but with the $[Ca^{2+}]$ adjusted at 80 nM (pCa 7.1) and energy 860 μJ. The traces obtained with each dye correspond to single sweeps. Also note that in this case the vertical calibration bar is 5 times larger than in (A).

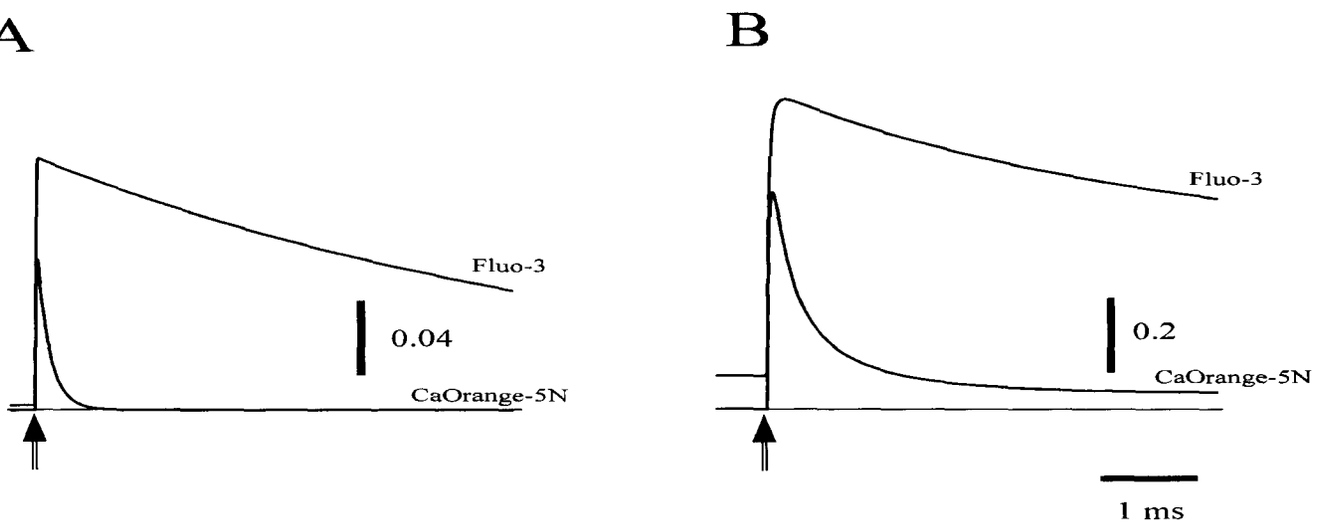

Fig. 2. Predictions of a kinetic model of flash photolysis of DM-nitrophen and detection by one Ca^{2+} indicator. The UV flash (arrow) photolyses a fraction (α) of DM-nitrophen with a time constant of 20 μs, changing its affinity from 4.4 nM to 3.3 mM [3]. The photoreaction has 2.5 higher quantum efficiency for Ca-DM-nitrophen than for free DM-nitrophen. Panels (A) and (B) were simulated for the same resting Ca^{2+} concentrations than the experiments shown in Fig. 1A and B, respectively. α was set at 5.2% and 5% for simulations in panels (A) and (B), respectively. The kinetic parameters for DM-nitrophen and the Ca^{2+} dyes are given in the text.

also at this pCa, very different from each other. The CaOrange-5N signal shows an initial transient that decays with a τ of about 185 μs followed by a small sustained step of about 5% of the amplitude. The Fluo-3 transient is similar to that recorded at pCa 9, but with an even slower τ of decay of approximately 20 ms. The surprising result in the comparison of the transients shown in Fig. 1A and 1B is that the 100-fold change in resting $[Ca^{2+}]$ of the solutions at which flash photolysis was performed, had a relatively small effect in the kinetics of the fluorescence transients. This is remarkable since, as said above, the concentration of free DM-nitrophen was reduced from 5.7 mM to 0.3 mM by this manipulation, yet there is still a clear indication of a significant, fast transient component in the CaOrange-5N record. In terms of the time course of the $[Ca^{2+}]$ induced by the photorelease of DM-nitrophen, the early transient component in the CaOrange-5N fluorescence signals strongly suggests that 0.3 mM free DM-nitrophen is still able to chelate very rapidly the relatively large amounts of Ca^{2+} released by the flash, an observation incompatible with the possibility that the k_{on} of DM-nitrophen is relatively slow, as previously suggested.

Fig. 2 illustrates the results of computer simulations solving a system of kinetic equations that represent the physico-chemical conditions of the experiments shown in Fig. 1. The model is quite similar to that described by Zucker [9]. Fig. 2A shows the simulated normalized fluorescence transients at pCa 9, and Fig. 2B at pCa 7.1. It can be observed that the model quantitatively predicts the main features of the flash-induced transients with two vastly different Ca^{2+} indicators at two distinct pCa conditions. The agreement between the model simulations and the data is remarkable considering that both the magnitudes and kinetics of the Fluo-3 and CaOrange-5N transients were predicted without changes in the model parameters other than the free $[Ca^{2+}]$ of the solutions (for comparison of traces in Fig. 2A and B) and the kinetic rate constants of the dyes (for comparison between Fluo-3 and CaOrange-5N transients at each pCa). There are several features of the model that should be highlighted: (a) The association rate constant (k_{on}) of DM-nitrophen was 8×10^7 $M^{-1} \cdot s^{-1}$, significantly faster than that reported by Zucker [9]. Simulations with slower values for this rate constant fail to predict the main features of the fluorescence transients, mostly those of CaOrange-5N at the higher $[Ca^{2+}]$; (b) the time constants of decay (τ) of the fluorescence transients at pCa 9 is almost exclusively determined by the dissociation rate constants (k_{off}) of the Ca^{2+} dyes, approximately 7×10^3 s^{-1} for CaOrange-5N and about 170 s^{-1} for Fluo-3; (c) the amplitude of the fluorescence transients is mainly determined, at equal dye concentrations, by the association rate constants (k_{on}) of the dyes, approximately 1.3×10^8 $M^{-1} \cdot s^{-1}$ and 2.3×10^8 $M^{-1} \cdot s^{-1}$ for CaOrange-5N and Fluo-3, respectively.

4. Discussion

We present a new methodology that allows for the detection of very rapid fluorescence transients in response to flash photolysis of DM-nitrophen. The UV light used for flash photolysis of this photolabile chelator was an extremely short pulse (30 ns in duration) delivered, at a precise instant, through a fiber optic to droplets of solution on the stage of an epifluorescence microscope. The brief duration of the UV flash, combined with electronic 'blanking' of the UV illumination artifact and with the use of a fast fluorescence detection system, allowed us to record accurately, not only the decaying phase of the fluorescence transients, but also their rising phases. Failure to resolve the entire time course of the fluorescence transients would preclude the comparison between the data presented in Fig. 1 and the model simulations shown in Fig. 2, thus considerably weakening the determination of kinetic rate constants.

We present data with the novel fluorescent Ca^{2+} indicator CaOrange-5N and demonstrate that it is well suited to track the very fast $[Ca^{2+}]$-spikes elicited by flash photolysis of DM-nitro-

phen. Model simulations predict the time course of the CaOrange-5N fluorescence transients using values for K_d, k_{on} and k_{off} of 53 μM, 1.3×10^8 $M^{-1} \cdot s^{-1}$ and 6,800 s^{-1}, respectively. Close inspection of the equilibrium binding properties of the dye, and the relatively fast decay time constant of the transient component at pCa 7, however, suggest the possible formation of two Ca^{2+}-dye complexes. One of them would have very low affinity ($K_d > 100$ μM) and very fast dissociation rate constant ($k_{off} > 10{,}000$ s^{-1}) and the other moderate affinity (10 $\mu M > K_d > 100$ μM) and dissociation rate ($1{,}000 < k_{off} < 10{,}000$ s^{-1}). The simulations shown in Fig. 2 are greatly improved with these new assumptions. Nevertheless, the kinetic properties of both Fluo-3 and CaOrange-5N fluorescence transients are only compatible with the selection of k_{on} values for DM-nitrophen at least 30 times faster than those reported by Zucker [9]. The discussion about the possible origin of this discrepancy goes beyond the scope of this report, but we believe that the use of a fast indicator dye and our improved methodology allow us to better discriminate between different values of this parameter.

The experimental records and theoretical simulations strongly suggest that the $[Ca^{2+}]$-spikes at pCa 9 are extremely fast transients that can be mathematically approximated to Dirac delta functions. Under these conditions the kinetic responses of one-complex Ca^{2+} indicators can be demonstrated to behave as a single exponential decays with $\tau = 1/k_{off}$ of the indicator. We used this approximation to calculate the dissociation rate constants for Fluo-3 and CaOrange-5N given in section 3. In the future, this methodology may prove to be optimal to evaluate the kinetic responses of Ca^{2+} indicators in vitro and in vivo. Based on the model we conclude further that for flashes at pCa 9, the heights of the normalized fluorescence transients are determined by the Ca^{2+} indicators' association rate constants (k_{on}). At pCa 7.1, on the other hand, the $[Ca^{2+}]$ waveform is significantly slower than at pCa 9 and CaOrange-5N is able to approximately track its time course. For example, in the simulation shown Fig. 2B, the peak $[Ca^{2+}]$ was 220 μM and the peak of the CaD/Ca_T transient was about 0.6, indicating a complex formation of 60 μM. If the usual procedure of dye calibration is applied to estimate the free $[Ca^{2+}]$ by assuming that the dye is at equilibrium, one would estimate that the peak $[Ca^{2+}]$ (for that particular waveform) was 80 μM, about 1/3 of the true value. In contrast, using Fluo-3 one would estimate a peak free $[Ca^{2+}]$ of about 4 μM, which is 1/50 of the true value. These experimental comparisons illustrate the advantage of a faster dye (e.g. CaOrange-5N) over a slower dye (e.g. Fluo-3) in detecting rate limited processes. Moreover, since the kinetic rate constants can be measured with the flash photolysis methodology, it is possible to make the necessary corrections for the rate limitation of a dye, provided that the distortion introduced is not as severe as it is the case of Fluo-3 for fast $[Ca^{2+}]$ transients.

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