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# Photolysis of caged calcium using a low-cost flash unit: efficacy analysis with a calcium selective electrode

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**Abstract — Photolysis of caged calcium (Nitr5®, Calbiochem) can be used to study calcium dependent processes such as excitation-contraction coupling and muscular mechanics. Expensive high energy light sources are routinely used for UV light exposure, but this study describes an alternative low cost xenon flash unit constructed in our laboratory. A 300 J short arc xenon flash lamp (Heimann) was mounted in an elliptical reflector and driven by a modified Metz 60 CT 4 photoflash unit up to 240 J input energy and 4 ms flash duration. A 20 µl cuvette containing a test solution was placed in a complementary elliptical reflector. An ion selective calcium electrode was used to measure the free calcium concentration [$Ca^{2+}$] before and after flash in test solutions containing 1.00 mM Nitr5 in combination with different added [$Ca^{2+}$]s. Using this technique we estimated that 1 flash on 1.00 mM Nitr5 increased the free [$Ca^{2+}$] from $10^{-7}$ to 1.1 x $10^{-5}$ M. When the added [$Ca^{2+}$] was less than 2.3 x $10^{-4}$ M, the used Nitr5 behaved as a strong calcium chelator because 23% of it was unloaded with calcium. It is concluded that a physiologically relevant change in free [$Ca^{2+}$] can be evoked by photolysis of Nitr5 using a low cost (approximately $1500) xenon flash unit, and that ion selective Ca electrodes can be adequately used to monitor the resulting changes in [$Ca^{2+}$].**

Photolabile calcium chelators have been designed to study intracellular calcium dependent processes [1,2]. These 'caged' calcium compounds are useful tools to bypass rate limiting steps in muscular excitation-contraction coupling [3–5]. When using the chelators intracellularly [6–8], it is possible to evoke a stepwise increase in the intracellular calcium concentration [$Ca^{2+}$] by exposing the cells to high intensity ultra-violet (UV) light. The minimum energy density required for photolysis of the caged calcium compound Nitr5 is approximately 250 mJ/cm$^2$ in a wavelength band from 320–370 nm [9,10]. The commonly used frequency doubled ruby laser [6,7] meets these specifications in a flash duration of 25 ns, but is very expensive. When a longer flash duration, ± 4 ms, is acceptable, the commercially available xenon flashlamp developed by Rapp and Güth [11] can be used. Even at one fourth of the price of the laser, this is still not a very economical alternative. We decided to develop a UV

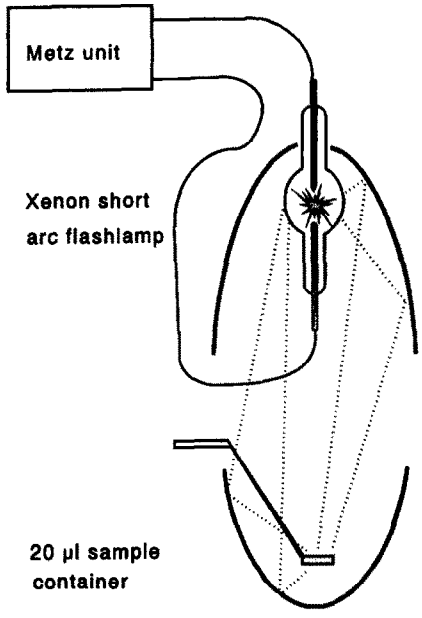


**Fig. 1** A schematic representation of the fluid sample illumination setup. A short arc xenon flash lamp was mounted in an elliptical reflector and driven by a modified Metz 60 CT 4 photo flash unit. A 20 µl cuvette was placed in a complementary second elliptical reflector.

light source for the release of caged Ca based on a standard photo flashlamp, costing one tenth of the price of the commercially available xenon flash unit.

The efficacy of the developed UV light source was quantified by measuring the increase in free calcium after exposure of a 20 µl sample of caged calcium with a calcium selective electrode.

## Materials and methods

### *Ultra-violet light source*

A modified Metz 60 CT 4 flash unit was used as an ultra-violet (UV) light source for the photolysis experiments. When this unit is used for photographic applications, the UV light from the flashtube is absorbed by a filter in the front window.

This front window was removed and the 70 mm long flashtube was replaced by a 300 J/flash, 120 W, type DG 8907 ST II xenon flashtube manufactured by Heimann, Wiesbaden, Germany. This short arc lamp (arc length 7 mm), was placed in the focus of a Melles and Griot, type 02 REM 001 elliptical mirror.

In order to compensate for the lower impedance of this lamp (20 mohm) as opposed to the original 70 mm tube (1.5 ohm), an extra choking coil of 22 µH was placed in the high power circuit of the Metz unit in series with the short arc tube. The trigger circuit of the driver unit was not altered. The focus size, determined by video tape recording of flashes on a graduated grey plastic screen, was approximately 5 mm in diameter. Flash duration was 4 ms measured by means of a photo detector with amplifier connected to a storage oscilloscope and the input energy was calculated to be 240 J [12,13].

### *Fluid sample illumination setup*

A 20 µl fluid sample was exposed in a 7 mm long quartz tubular container of 1.8 mm inner diameter and 200 µm wall thickness that was placed in the second focus of the elliptical reflector, as shown in Figure 1. In order to gain a more intense and even exposure the container was surrounded by a custom made second elliptical reflector complementary to the 02 REM 001. A cedar wooden mould was made for this reflector using a computerized milling machine. The glass for the reflector was blown inside this mould to a wall thickness of 2 mm. The inside surface of the elliptically shaped glass was coated with aluminium.

### *Ion-selective calcium electrode setup*

A calcium-selective electrode, Orion type 9320, was used in combination with a double barrel reference electrode, Orion type 900200 (inner compartment filled with saturated AgCl solution and outer compartment with 4 M KCl), for the measurement of free $[Ca^{2+}]$ in the 20 µl sample. Initially, a setup was tested in which a continuous flow of fluid was pumped via the Ca selective electrode past the reference electrode with a syringe pump. The following (Fig. 2) simpler assembly, however, showed far more stable potentials. In this setup (Fig. 2) the Ca electrode was positioned upside down and a teflon spacer ring with a thickness of 0.3 mm (inner

diameter 6 mm and outer diameter 12 mm) was placed around the 6 mm diameter Ca sensitive membrane. The 20 µl fluid sample was applied centrally in the ring on the membrane. The reference electrode was gently lowered onto the membrane and loaded with a mass of 1.5 kg, so that a fluid film over the telfon ring made contact with the peripheral junction of the reference electrode. The potential between both electrodes was measured using a Consort pH-mV meter and recorded on a W+W chart recorder. Values were read after allowing 20 s for stabilization. The calcium-sensitive membrane, the ring and the reference electrode were rinsed extensively with de-ionised water and dried with a clean tissue between measurements.

*Solutions*

For calibration, standard buffer solutions were made containing saturated Ca-EGTA and EGTA in proportions calculated so that free $[Ca^{2+}]$ of $10^{-8}$, $10^{-7}$, $10^{-6}$ and $10^{-5}$ M were established at pH 6.8; total EGTA concentration was 3 mM.

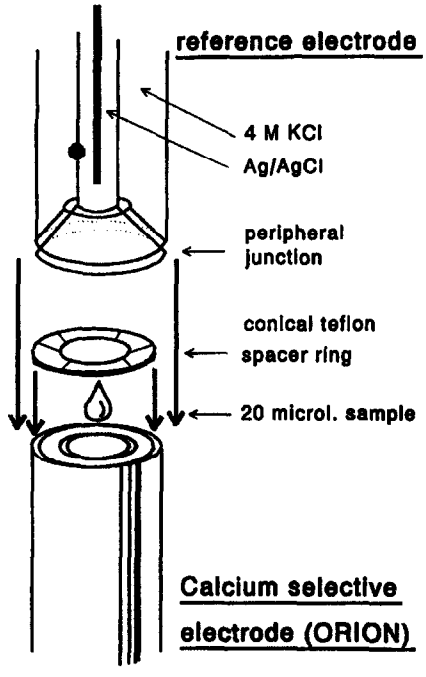


Fig. 2 A schematic representation of the ion selective calcium electrode setup. The calcium selective electrode was positioned upside down, the 20 µl sample was applied centrally on the membrane and the reference electrode was lowered so that a fluid film over the ring made contact with the peripheral junction of the reference electrode.

Standards with $10^{-4}$ and $10^{-3}$ M free $[Ca^{2+}]$ were made by diluting a 2 x $10^{-3}$ M $CaCl_2$ (Merck) standard solution with de-ionised water ($[Ca^{2+}]$ 2 x $10^{-6}$ M). The solutions containing Nitr5 (Calbiochem) were composed of 1.00 or 3.00 mM Nitr5 and 10 mM KCl, the KCl was added to adjust the ionic strength (according to Orion $Ca^{2+}$ selective electrode manual). $CaCl_2$ was added in order to obtain solutions with 11 different added $[Ca^{2+}]$s: $10^{-7}$, $10^{-6}$, $10^{-5}$, 5 x $10^{-5}$, $10^{-4}$, 1.5 x $10^{-4}$, 2 x $10^{-4}$, 2.5 x $10^{-4}$, 3 x $10^{-4}$, 5 x $10^{-4}$, $10^{-3}$ M.

*Measurement protocol*

Ion-selective electrode (ISE) potentials were measured 5 times in the 6 different free Ca standard solutions in ascending order. Before and after a series of measurements using Nitr5, the electrode response was checked with 3 different standards: $10^{-6}$, $10^{-5}$, and $10^{-4}$ M Ca. The free $[Ca^{2+}]$s of the solutions containing 1.00 mM Nitr5 were measured 5 times in both flashed and unflashed condition for each of the solutions with different added $[Ca^{2+}]$s. The $10^{-7}$, $10^{-6}$, $10^{-5}$ and $10^{-4}$ M added $[Ca^{2+}]$ solutions, the $10^{-4}$, 1.5 x $10^{-4}$, 2 x $10^{-4}$, 2.5 x $10^{-4}$, 3 x $10^{-4}$ M $[Ca^{2+}]$ solutions and the 3 x $10^{-4}$ and $10^{-3}$ M $[Ca^{2+}]$ solutions were measured in separate sessions with intermediate reloading of the Ca electrode in high $[Ca^{2+}]$. Solutions containing 3 mM Nitr5 were analyzed 3 times, in both flashed and unflashed condition in 3 different solutions with added $[Ca^{2+}]$ $10^{-4}$, 5 x $10^{-4}$ and $10^{-3}$ M. The unflashed Nitr5 solutions were incubated in the 20 µl container in front of the flash lamp for the same period of time as the flashed solutions. Both flashed and unflashed solutions were transferred to the $Ca^{2+}$ measurement site with a 20 µl Hamilton syringe approximately 1 min after the flashes. Between measurements both syringe and container were cleaned thoroughly by flushing several times with de-ionized water. All solutions were flashed 5 times in order to obtain a better readability of the responses. In a separate series (n = 3) responses to 1 and 5 flashes were compared using a Nitr5 solution with an added $[Ca^{2+}]$ of 2.3 x $10^{-4}$ M. This solution was also used in a series (n = 3) to test Ca release with and with-

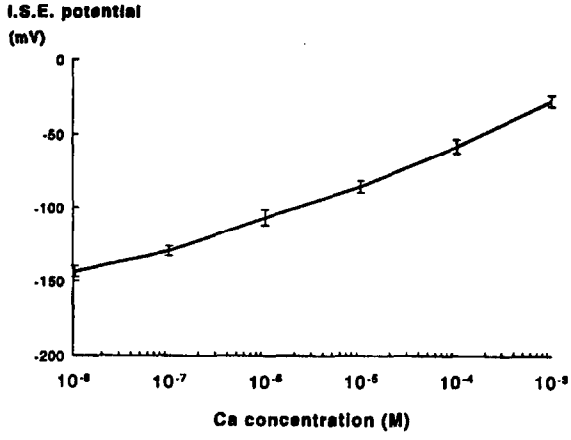


Fig. 3 Calcium electrode calibration curve showing the mean ion selective electrode (ISE) potential ± standard error of the mean (SEM) for 6 different calcium standard solutions with free [$Ca^{2+}$] of $10^{-8}$ to $10^{-3}$ M.

out the complementary second elliptical mirror and with and without a 3 mm thick UG11 (Schott) glass filter (80% transmission peak between 320–370 nm wavelength).

## Results

The ISE potential as a function of the free [$Ca^{2+}$], measured in 6 different calcium standard solutions is shown in Figure 3. Figure 4 shows on a double logarithmic scale the free [$Ca^{2+}$] in the Nitr5 buffer solution as a function of the total [$Ca^{2+}$], which is the sum of added [$Ca^{2+}$] and the calcium initially bound to the 1.00 mM Nitr5. Mean ± standard error of the mean (SEM) for 1.00 mM Nitr5, unphotolysed (solid line) and photolysed (dashed line) are plotted.

Figure 4 also shows a theoretical curve (0% dotted line) representing the relation between total

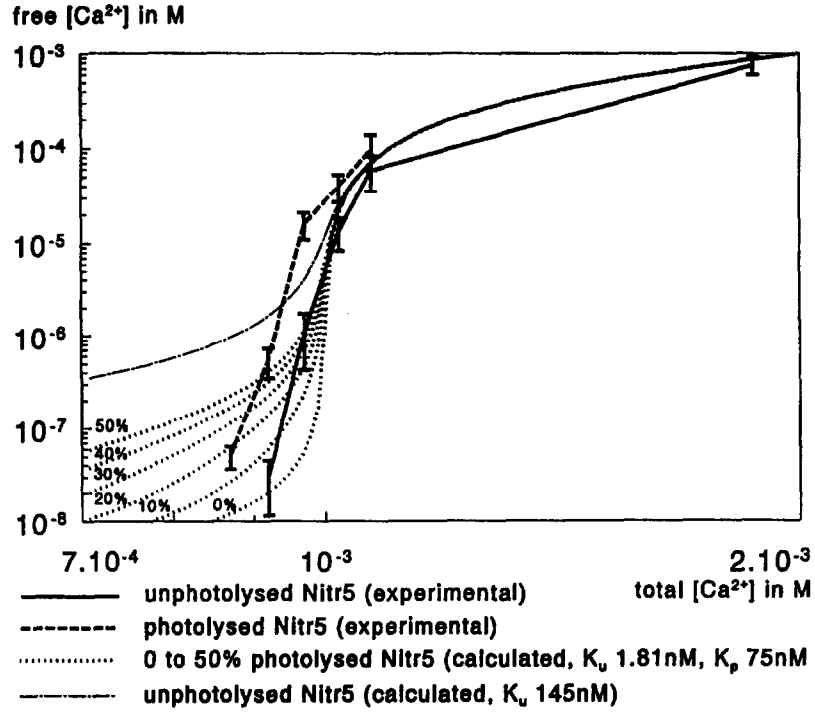


Fig. 4 Free [$Ca^{2+}$] as a function of total [$Ca^{2+}$] i.e. added [$Ca^{2+}$] plus the calcium bound initially to the $10^{-3}$ M Nitr5. The solid line represents average values ± SEM for a solution containing 1.00 mM Nitr5. The thick dashed line shows the values for 1.00 mM Nitr5 after exposure to 5 UV light flashes. The 6 dotted lines show calculated data for 6 different degrees of photolysis (from 0% to 50% in steps of 10%) of a theoretical 2-buffer system, containing unflashed and flashed Nitr5 with dissociation constants for calcium of 1.81 nM ($K_U$) and 75 nM ($K_P$). The dot-dashed line represents a calculated line for unflashed Nitr5 using a $K_U$ of 145 nM (published dissociation constant at 100 mM ionic strength [1]).

$[Ca^{2+}]$ and free $[Ca^{2+}]$ in a 2 buffer system (*see* Appendix). This curve is characterized by a steeply increasing free $[Ca^{2+}]$ around a total $[Ca^{2+}]$ equal to the [Nitr5] as a result of the 1:1 $Ca^{2+}$ binding to Nitr5 at saturation. Such a steep part in the curve was also seen in the experimental data measured at different added $[Ca^{2+}]$s. In these data, total $[Ca^{2+}]$ in the solutions equalled the added $[Ca^{2+}]$ plus the $[Ca^{2+}]$ bound initially to the chelator. The amount of $Ca^{2+}$ bound initially to the chelator was estimated from the horizontal shift between the steep part of the experimental data curve and the comparable part of the theoretical curve. A best fit of both curves was obtained at a value of 0.77 mM $[Ca^{2+}]$ being bound to the 1.00 mM Nitr5. Total $[Ca^{2+}]$ values in the Nitr5 solution were therefore calculated by adding 0.77 mM $[Ca^{2+}]$ to the added $[Ca^{2+}]$. The 77% $Ca^{2+}$ loading of Nitr5 was verified by measuring free $[Ca^{2+}]$ in 3 mM Nitr5 with $10^{-4}$, $5 \times 10^{-4}$ and $1 \times 10^{-3}$ M added $[Ca^{2+}]$s. In this 3-fold higher [Nitr5] the steep rise in free $[Ca^{2+}]$ was expected at a 3-fold higher total $[Ca^{2+}]$. Consequently a 3-fold higher amount of added $[Ca^{2+}]$ was expected to be necessary to saturate the Nitr5, i.e. 0.69 mM. In a limited series of measurements (n = 3) the steep free $[Ca^{2+}]$ increase was indeed shifted to a value between 0.50–1.00 mM added $[Ca^{2+}]$.

Figure 4 shows that after exposing samples with a total $[Ca^{2+}]$ between 0.92–1.07 mM to 5 flashes from the flash unit, a significant increase in free $[Ca^{2+}]$ was detected. The free $[Ca^{2+}]$ was increased by a maximum of approximately $2.6 \times 10^{-5}$ M in response to the flashes in a total $[Ca^{2+}]$ of 1.02 mM.

The effects of 5 flashes, 1 flash, omission of the complementary elliptical reflector and addition of a UG11 filter in a solution containing 1.00 mM Nitr5 in 1.00 mM total $[Ca^{2+}]$ are compared in Figure 5. One flash released 67% of the amount of $Ca^{2+}$ compared to the amount that was released by 5 flashes. Without a complementary second elliptical reflector 5 flashes released 55% of the amount released with this reflector. 5 flashes with a UG11 filter released 97% of the amount released without this filter.

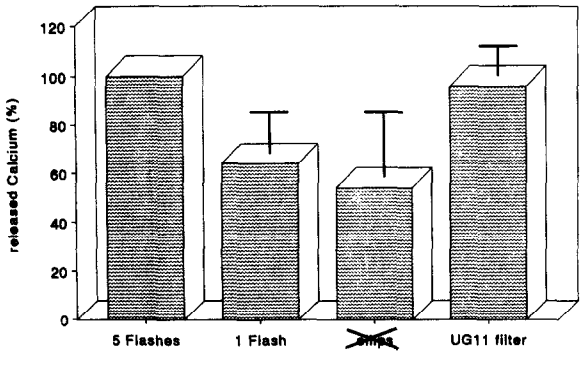


**Fig. 5** The relative increase in free $[Ca^{2+}]$ evoked by 1 flash, omission of the second complementary elliptical mirror and addition of a Schott UG11 filter expressed as a percentage ± SEM of the effect of 5 flashes.

## Discussion

A low cost xenon flash unit was constructed as an alternative for more expensive light sources such as an UV laser or a commercially available xenon flashlamp to photolyse caged calcium. The driver and trigger circuit of a Metz flash unit, which is widely employed in professional photography, was used after slight modification. An additional advantage of this system is that it is battery powered, thus avoiding interference on the power lines. The external trigger system of the photoflash unit could easily be used for synchronization. The original flashtube was replaced by a short-arc xenon flashlamp, which is a more concentrated light source. The UV light emitted by the flashlamp was concentrated using a dual elliptical mirror arrangement, which provided a high efficacy and an even illumination of the preparation. The light spot in the focus was larger in diameter than one would expect from a lamp with 7 mm arc length. This effect was due to the fact that the discharge was not only limited to the arc between the anode and cathode but more or less extended through the whole 15 mm diameter bulb of the xenon lamp. A frame by frame analysis of images recorded on videotape of the flash on a graduated grey screen in the focus revealed that an intense spot in the focus with a diameter of 5 mm was surrounded by a larger less intense spot with a total diameter of 30 mm. This halo was reflected back to the preparation with the complementary elliptical reflector.

The efficacy of the light source was determined by measuring the free $[Ca^{2+}]$ before and after flash

in a 1.00 mM Nitr5 solution using a Ca selective electrode. This method had the advantage of multidirectional sensitivity as opposed to most electronic detectors, which are sensitive in one direction only. A disadvantage of the arrangement could be a gradient in Ca release from the surface towards the middle of the container, which was earlier described by Lando and Zucker [9]. However this would imply that the measured calcium release is a conservative estimate as the diameter of a muscle preparation would be at least a factor 10 less.

Using a calcium selective electrode in combination with a peripheral junction reference electrode and a spacer ring (Fig. 2) enabled the analysis of a very small sample while preventing the efflux of the reference solution from directly influencing the contents of the sample.

It was found that the performance of the electrode depended on the order in which measurements were done if free [$Ca^{2+}$]s were far apart. Therefore the low free [$Ca^{2+}$] range, the high free [$Ca^{2+}$] range and the steep part in the curve (Fig. 4) where saturation of Nitr5 occurred were measured in separate sessions to allow for intermediate $Ca^{2+}$ reloading and stabilization of the electrode. In solutions containing 1.00 mM Nitr5 and added [$Ca^{2+}$]s below $10^{-4}$ M the free [$Ca^{2+}$] was reduced dramatically. The differences between the potentials measured in standard Ca solutions and those obtained in solutions with a low added [$Ca^{2+}$] can be explained by the strong Ca buffering capacity of Nitr5. This implies that in muscle experiments cells should be loaded slowly with Nitr5 in order to prevent the intracellular [$Ca^{2+}$] from dropping too low. The buffering effect also explains why in lower added [$Ca^{2+}$]s the response to a flash was decreased — all released $Ca^{2+}$ is directly reabsorbed by empty 'cages'. The steep rise in free [$Ca^{2+}$] shown in the unphotolysed Nitr5 curve in Figure 4 between 0.87 and 1.07 mM total [$Ca^{2+}$] can be explained by $Ca^{2+}$ saturation of the empty cages of Nitr5 in this region. Therefore a flash evokes the largest increase in free [$Ca^{2+}$] in this region. As each Nitr5 molecule can contain 1 $Ca^{2+}$ molecule, the horizontal position of this saturation region in the free [$Ca^{2+}$]–total [$Ca^{2+}$] plot indicates the point where total [$Ca^{2+}$] equals [Nitr5]. This was used as described in the Results section to estimate that 1.00 mM of Nitr5 initially contained 0.77 mM $Ca^{2+}$.

Figure 4 shows that 5 flashes increased the free [$Ca^{2+}$] markedly in the range between 0.92–1.07 mM total [$Ca^{2+}$]. The amplitude of the calcium jump, which can be read from the y axis, was variable along this range. By interpolation we can estimate that at a free [$Ca^{2+}$] of $10^{-7}$ M, which corresponds approximately to the intracellular [$Ca^{2+}$] of a muscle cell in the relaxed state, 5 UV flashes would evoke an increase in free [$Ca^{2+}$] to $1.7 \times 10^{-5}$ M. Such an increase in free [$Ca^{2+}$] in the 20 µl sample is similar to or greater than the physiological calcium jump that has been measured intracellularly upon stimulation [14]. At 0.97 mM total [$Ca^{2+}$] which corresponds to $10^{-6}$ M free [$Ca^{2+}$] a maximum response to 5 flashes of $2.6 \times 10^{-5}$ M free [$Ca^{2+}$] was found. The fact that the free [$Ca^{2+}$] increase in response to UV flashes was variable along the range of added [$Ca^{2+}$] values can partly be understood by describing the tested solution as a system of two competing buffers, i.e. unflashed Nitr5 and flashed Nitr5. It was attempted to estimate the amount of Nitr5 that was photolysed to give the effect shown in Figure 4 by using the equations for this system described in the Appendix. The 6 dotted lines show simulated data based on a total [Nitr5] of 1.00 mM and depict different ratios of unphotolysed Nitr5 to photolysed Nitr5. The degree of photolysis increases from 0 to 50% in steps of 10%. To realize a closer resemblance of the experimental and the calculated data, it was necessary to decrease the dissociation constants Kd of both flashed and unflashed Nitr5 used in other studies [1]. It can be seen in Figure 4 that experimental data from unflashed controls resembled closely a line calculated using a Kd of 1.81 nM, which is a factor 80 less than the dot-dashed line calculated using a Kd of 145 nM [1]. Similarly, the dissociation constant for flashed Nitr5 was decreased to 75 nM. The lower Kd values can be explained by the factor 10 lower ionic strength that was used in the present experiments as compared to the ionic strength (100 mM) for which the Kd values were determined in other studies [1]. The present experiments were performed at this low ionic strength (10 mM) in order to be able to measure lower [$Ca^{2+}$]s, according to the Orion $Ca^{2+}$ selective electrode manual. For EGTA [15] it has been shown that the Kd

value decreases with decreasing ionic strength. By using the formula described by Thomas [15], we calculated that the Kd for Nitr5 would decrease 2-fold with a 10-fold decrease in ionic strength. It can thus be questioned whether the formula (2.11 in [15]) properly describes the relationship between Kd and ionic strength, considering the very low Kd value in the present experimental data. When comparing the calculated dotted lines in Figure 4 with the lines representing the experimental data, a more or less similar shape is seen. In the lowest range of the theoretical curve experimental data could not be collected because free $[Ca^{2+}]$s lower than $10^{-8}$ M could not be measured with the used electrode and total $[Ca^{2+}]$s lower than 0.77 mM could not be attained as this amount was bound to the 1.00 mM Nitr5. When comparing photolysed to unphotolysed curves, it seems (other than in the theoretical curves) that the shape of both curves is similar but that the experimental photolysed curve is more shifted to the left. This effect can be observed most clearly around 1.00 mM total $[Ca^{2+}]$ in Figure 4 and is more likely due to a reduction of overall buffer capacity than to an altered Kd of the same buffer. This could be the case when the UV light destroys the cages rather than changes the buffering properties. In the light of these uncertainties it is not possible to calculate a precise ratio of photolysed to unphotolysed Nitr5 in this manner.

It can be concluded that the effect of photolysis on free $[Ca^{2+}]$ is critically dependent upon the total $[Ca^{2+}]$, as can be seen in both experimental and calculated data in Figure 4. The figure also shows that there is a very small range of total $[Ca^{2+}]$ values in which free $[Ca^{2+}]$ values similar to intracellular $[Ca^{2+}]$ values can be attained. This indicates that the strong buffering capacity of Nitr5 intracellularly, i.e. when loaded as the Nitr5-acetoxymethyl ester, makes high demands on the calcium homeostasis mechanism of the cell, and might even interfere to a large extent with this mechanism.

In order to obtain a more distinct effect of the UV exposure initially 5 flashes were applied instead of 1 flash. In later experiments, it was shown that the effect of 5 flashes measured in 1.00 mM Nitr5 with a 2.3 x $10^{-4}$ M added $[Ca^{2+}]$ was not 5-times but only 1.5-times higher than the effect of 1 flash (*see* Fig.5). So as opposed to a 1.7 x $10^{-5}$ M $Ca^{2+}$ jump in response to 5 flashes a 1.1 x $10^{-5}$ M $Ca^{2+}$ jump would be attainable in response to 1 flash. Also the effect of the complementary second elliptical mirror was investigated. With this second mirror the response was 2-fold enhanced, which can be exactly explained by the illumination of the preparation from both sides. A 3 mm thick Schott UG11 filter decreased the increase in free $[Ca^{2+}]$ by 3%, which indicates that the effect measured is mainly evoked by UV light with a wavelength between 320–370 nm.

This study indicates that it is possible using one flash from a low-cost UV flash unit ($1500) to evoke a physiological $[Ca^{2+}]$ jump, from $10^{-7}$ to 1.1 x $10^{-5}$ M, in a 20 µl sample containing 1.00 mM Nitr5. It is most likely that the effect in a smaller sized (smooth) muscle preparation will be more distinct. Measurement of the free $[Ca^{2+}]$ increase using an ion selective electrode appears to be a useful tool to analyze both the behaviour of Nitr5 and the efficacy of the flash for photolysis.

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## APPENDIX

The relation of total $[Ca^{2+}]$ to free $[Ca^{2+}]$ in a solution containing 2 competing buffers, in this case photolysed and unphotolysed Nitr5, can be described according to Zucker and Steinhardt [16].

In such a solution the total calcium concentration ($[Ca_T]$) consists of free calcium ($[Ca_F]$) and calcium bound to both buffers:

$$[Ca_T] = [Ca_F] + [Ca^{2+}] \text{ bound to unphotolysed Nitr5} + [Ca^{2+}] \text{ bound to photolysed Nitr5} \quad \text{Eq. 1}$$

by introducing a dissociation constant $K_U$ for calcium binding to unphotolysed Nitr5 with a concentration of $[N_5U]$:

$$[Ca^{2+}] \text{ bound to unphotolysed Nitr5} = \frac{[Ca_F] \cdot [N_5U]}{Kd + [Ca_F]} \quad \text{Eq. 2}$$

and a similar dissociation constant $K_P$ for calcium binding to photolysed Nitr5 with concentration $[N_5P]$, Equation 1 can be rewritten as follows:

$$[Ca_T] = [Ca_F]\left(1 + \frac{[N_5U]}{K_U + [Ca_F]} + \frac{[N_5P]}{K_P + [Ca_F]}\right) \quad \text{Eq. 3}$$

Reduction to one denominator gives:

$$[Ca_F]^3 + (K_U + K_P - [Ca_T] + [N_5U] + [N_5P])[Ca_F]^2 + \{K_P [N_5U] + K_U [N_5P] - [Ca_T] (K_U + K_P) + K_U K_P\} [Ca_F] - K_U K_P [Ca_T] = 0 \quad \text{Eq. 4}$$

Using the computer program MATLAB® total $[Ca^{2+}]$s were calculated for 100 equally spaced free $[Ca^{2+}]$ values between $10^{-8}$ and $10^{-3}$ M and 6 different ratios of $[N_5U]$ and $[N_5P]$. Kd values (for 10mM ionic strength) of 1.81 nM and 75 nM were used. For comparison an extra curve was calculated using the dissociation constant for unphotolysed Nitr5 at 100 mM ionic strength [1]. The curves were plotted on logarithmic axes, as shown in Figure 4. The steeply increasing part of the experimental data curve was shifted to coincide with the calculated data curve by addition of $7.7 \times 10^{-4}$ to the added $[Ca^{2+}]$ values which represents the $[Ca^{2+}]$ bound to 1.00 mM Nitr5.