Volume 255, number 1, 196–200 FEBS 07599 September 1989

Rapid relaxation of single frog skeletal muscle fibres following laser flash photolysis of the caged calcium chelator, diazo-2

I.P. Mulligan and C.C. Ashley

University Laboratory of Physiology, Parks Road, Oxford OX2 3PT, England

Received 31 July 1989

Diazo-2 is a calcium chelator based on BAPTA [(1989) J. Biol. Chem., in press], whose electron withdrawing diazoacetyl group may be rapidly (2000 s^{-1}) converted photochemically to an electron donating carboxymethyl group by exposure to near ultraviolet light, producing an increase in its calcium affinity (K_d changes from 2.2 μM to 0.073 μM) without steric modification of the metal binding site. Photolysis of a 2 mM solution of this compound with a brief flash of light from a frequency-doubled ruby laser (347 nm) caused single skinned muscle fibres from the semitendinosus muscle of the frog *Rana temporaria* to relax with a mean half-time of 60.4 ± 5 ms (range 30–100 ms, $n = 15$) at 12°C, which is faster than the relaxation observed in intact muscles (half-time 133 ms at 14°C [(1986) J. Mol. Biol. 188, 325–342]) and similar to the rate of the fast phase of tension decay in intact single fibres (20 s^{-1} at 10°C [(1982) J. Physiol. 329, 1–20]).

Ca^{2+}; Muscle; Relaxation

1. INTRODUCTION

A rapid change in the intracellular free calcium forms an important step in stimulus response coupling in many biological preparations, e.g. excitation–contraction coupling in intact striated muscle [4]. The initiation of muscle contraction has been successfully studied in skinned muscle fibres following the rapid release of calcium from a photolabile chelator upon illumination with a brief pulse of near ultraviolet light [5–8] thus avoiding diffusion delays which hampered earlier methods [9]. In order to study muscle relaxation, which has not been feasible to examine in the same detail todate, a photolabile chelator is needed whose affinity for calcium increases on photolysis.

Recently Diazo-2, a photolabile derivative of BAPTA, has been described [1] which upon photolysis is rapidly (>2000 s^{-1}) converted from a chelator with a low affinity for Ca^{2+} ($K_d = 2.2$ μM) to one with a high affinity for Ca^{2+} ($K_d =$ 0.073 μM). We have used this technique in conjunction with laser flash photolysis of single-skinned muscle fibres [5,6] to study the time course of muscle relaxation when the sarcoplasmic free Ca^{2+} is rapidly reduced.

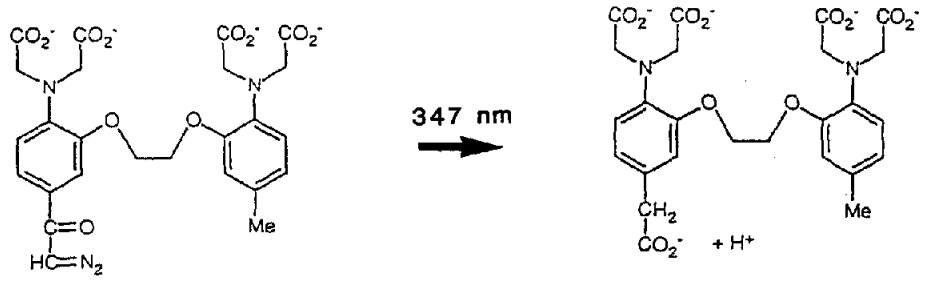
Correspondence address: I.P. Mulligan, University Laboratory of Physiology, Parks Road, Oxford OX2 3PT, England

2. MATERIALS AND METHODS

Semitendinosus muscle fibres were dissected from the frog *Rana temporaria* under mineral oil at 8°C and then skinned chemically by immersion in a relaxing solution containing 1% (v/v) Triton X-100 for 10 min at 12°C. Single skinned fibres were mounted on aluminium T-clips [10] and suspended between stainless-steel hooks attached to a tension transducer (Akers) at one end and to a fixed mount at the other. The apparatus, previously described by Ferenczi et al. [11] allows rapid changes of the solution which bathes the fibres. The laser photolysis technique and recording instrumentation was essentially the same as that described previously [11]. A frequency-doubled ruby laser (Lumonics, Rugby, England) produced 20 ns pulses at 347 nm. The beam energy was varied by altering the charging potential of the capacitors used to drive the xenon flash lamps of the laser. A cylindrical lens was used to focus the laser beam into an ellipse of 8 mm × 2 mm and shutters were used to prevent illumination of the T-clips.

Following equilibration of the fibres in solution containing Diazo-2, the trough was lowered pneumatically and the fibre suspended in air; after 600 ms the laser flashed and as soon as

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50



Diazo-2

Kd 2.2 μM

Substituted phenyl acetic acid

Kd 0.07μM

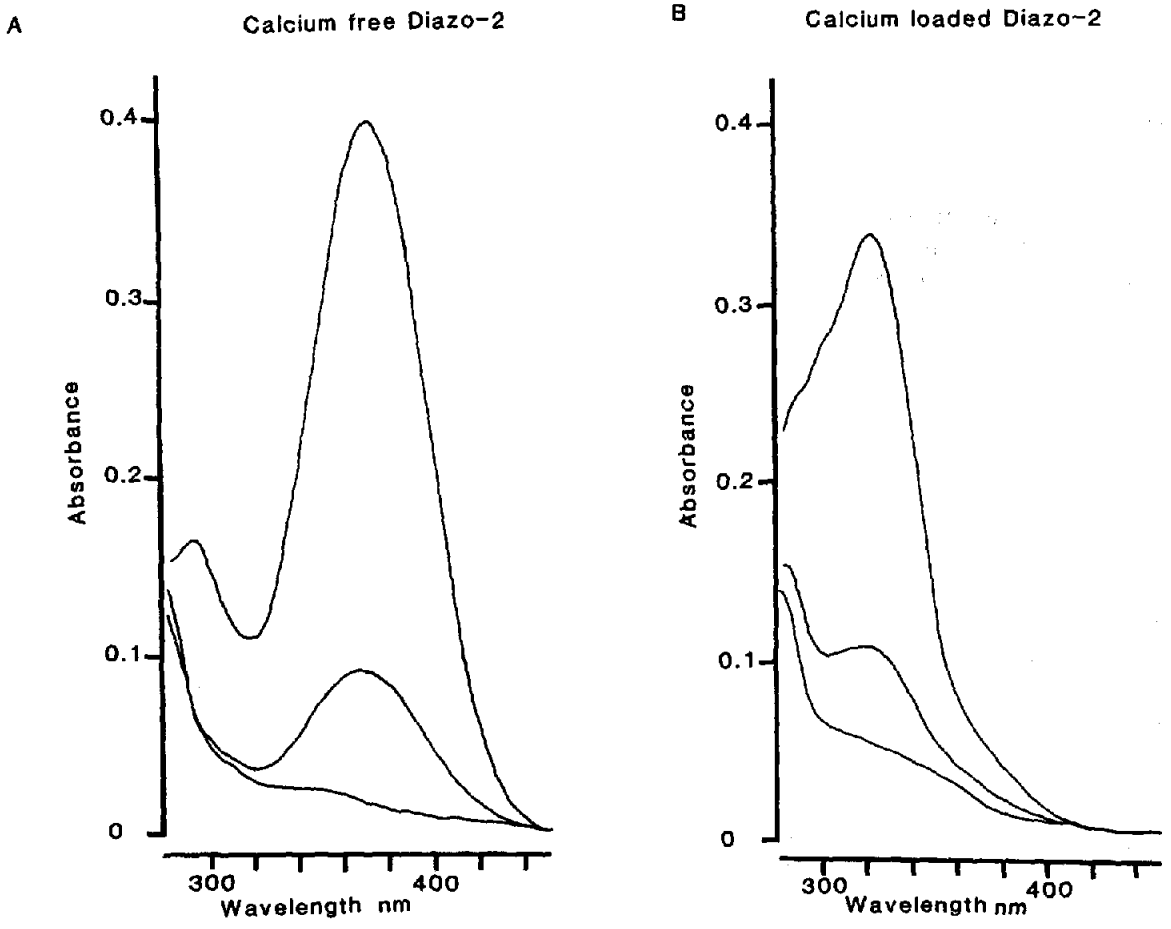


Fig. 1. Spectra obtained upon photolysis of 20 μM Diazo-2 in an acryl cuvette. (A) Calcium-free Diazo-2 in the presence of 1 mM EGTA. (B) Calcium-loaded Diazo-2 in the presence of 1 mM EGTA and 10 mM $CaCl_2$. In each panel the upper trace is the spectrum obtained prior to photolysis, the middle trace is the spectrum obtained after one laser flash and the lower trace is the spectrum obtained after ten further laser flashes.

the fibre had reached a new steady level it was returned to relaxing solution. The temperature of the solutions was kept at 12°C, this was the same as the dew point so that when the fibre was suspended in air its temperature should remain constant [12]. There was no evidence that the fibre temperature changed significantly following the laser flash, for if a fibre was immersed in a solution containing 2 mM Diazo-2 and saturating Ca^{2+} the tension did not change following photolysis as would be expected if the temperature of the fibre had risen significantly.

Diazo-2 was kindly supplied by S.R. Adams and R.Y. Tsien (University of California at Berkeley). This was added to a stock solution to give 2 mM Diazo-2 and 0.95 mM $CaCl_2$ was added to give a *p*Ca less than 5.7. The stock solution contained 60 mM Tes, 5.0 mM MgATP, 1.0 mM Mg^{2+}, 10 mM creatine phosphate and 15 U/ml creatine phosphokinase. The solution was made up to an ionic strength of 0.2 M by the addition of potassium propionate. The pH was adjusted to 7.00 ± 0.02. Relaxing and activating solutions were prepared by using 10 mM EGTA and 10 mM CaEGTA respectively in the basic stock solutions and adjusting with potassium propionate to give 0.2 M ionic strength (pH 7.00).

3. RESULTS

Fig.1 shows the spectrum obtained when 20 μM Diazo-2 (either calcium-free A or calcium-loaded B) was photolysed in an acryl cuvette (Sarstedt, FRG) with a 1 cm optical pathlength (absorbance 0.4 similar to that obtained with a 100 μm optical pathlength in the experiments with muscle fibres). After one flash of the frequency-doubled ruby laser (beam energy 250 mJ, beam height 1 cm (unfocused) as compared with a beam energy of 50–100 mJ, beam height focused to 2–3 mm, in the experiments with muscle fibres) calcium-free Diazo-2 was photolysed by 77% whilst calcium-loaded was photolysed by at least 69%. Exposure of these two samples to a further series of 10 flashes reduced the absorbance of calcium-free Diazo-2 at 370 nm to 4% of its initial value and the absorbance of calcium-loaded Diazo-2 at 330 nm to 15% of its initial value. The spectrum of photolysed calcium-loaded Diazo-2 was obtained when 10 mM $CaCl_2$ was added to the fully photolysed sample of calcium-free Diazo-2.

Fig.2 shows the effect of photolysis of 2 mm Diazo-2 on a skinned muscle fibre. The fibre had been dissected, mounted on the apparatus described and skinned with Trixon X-100. It was then transferred to a trough containing relaxing solution and the sarcomere length was adjusted to 2.3 μm. The fibre was then transferred to the stock solution with no EGTA and then to a solution containing 2 mM Diazo-2 (p*Ca* less than 5.7). Tension developed and was allowed to reach a steady level. Then the trough containing the solution was pneumatically lowered and the fibre left suspended in air; 600 ms later the frequency-doubled ruby laser was discharged. Tension declined rapidly with a $t_{1/2}$ of 88 ms (mean $t_{1/2}$ 60.4 ± 5 ms, $n = 15$). After a few seconds the fibre was transferred back to a relaxing solution. The fibre was then transferred via the solution with no EGTA to an activating solution (p*Ca* 4.5), which confirmed that the tension in the Diazo-2 solution prior to photolysis was indeed maximal.

4. DISCUSSION

We have demonstrated that a single 20 ns flash of laser light at 347 nm is able to photolyse at least 77% of calcium-free Diazo-2 and 68% of calcium-loaded Diazo-2. Using these values for percentage photolysis and the rate constants for photolysis and calcium binding described by Adams et al. [1] we have simulated the likely time course of the change in free calcium within the muscle fibres in these experiments (see fig.3). This simulation indicates that the free calcium falls very rapidly from 2 μM to 0.25 μM (within 3 ms) and that presence of 200 μM TnC calcium-specific regulatory sites on the thin filaments of the muscle fibre produces only a small deviation in the predicted calcium transient.

The speeed of relaxation of frog muscle fibres in vivo has been investigated and found to vary with the length of the preceding tetanus [13,14]. Kress et al. [2] found a half-time of 133 ms at 14°C for whole muscle while for intact single frog fibres at 10°C, Edman and Flitney [3] report a rate of relaxation of 2 s^{-1} for the 'slow' phase followed by a rate of 20 s^{-1} for the 'fast' phase during which sarcomeric inhomogeneity develops [3,15].

In addition the relation between the fall of free Ca^{2+} and rate of relaxation has been studied in intact muscle fibres [13,16–18] but these studies on relaxation are limited by the rate at which free calcium can be removed from the sarcoplasm, presumably mainly by the activity of the sarcoplasmic reticulum calcium pump (14 s^{-1} at 10°C [14] or 25 s^{-1} following a single twitch decreasing to 10 s^{-1} after a tetanus of at least 2 s duration [13]). There is also an elevation or 'tail' to the free

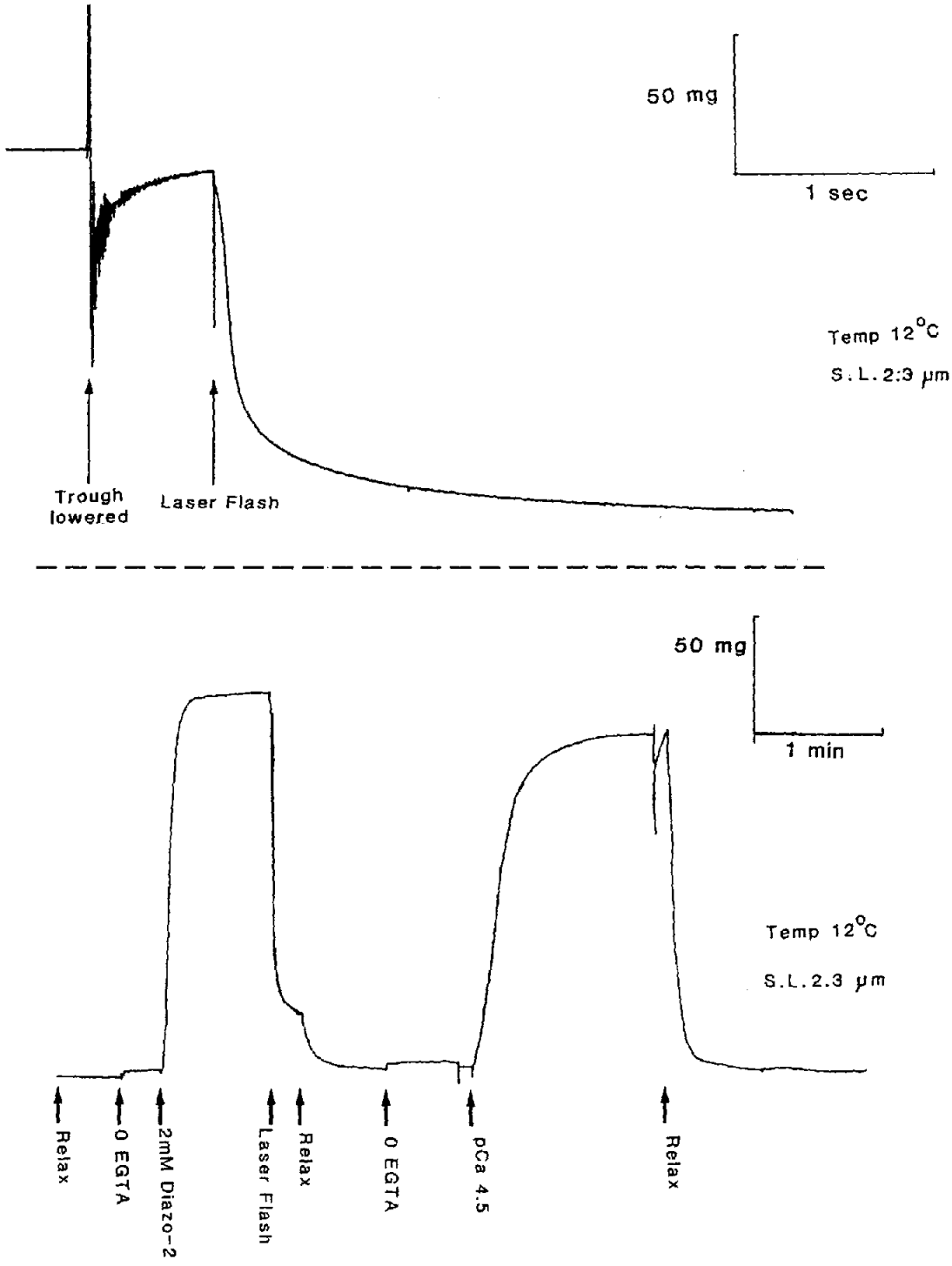


Fig.2. The upper panel shows the tension record of a single skinned muscle fibre which has been equilibrated in 2 mM Diazo-2 and exposed to a brief flash of light from a frequency-doubled ruby laser (347 nm). The lower panel shows the same tension recordin a slower time scale allowing the development of tension in Diazo-2 to be seen and compared with the tension developed in an activ solution, p*Ca* 4.5.

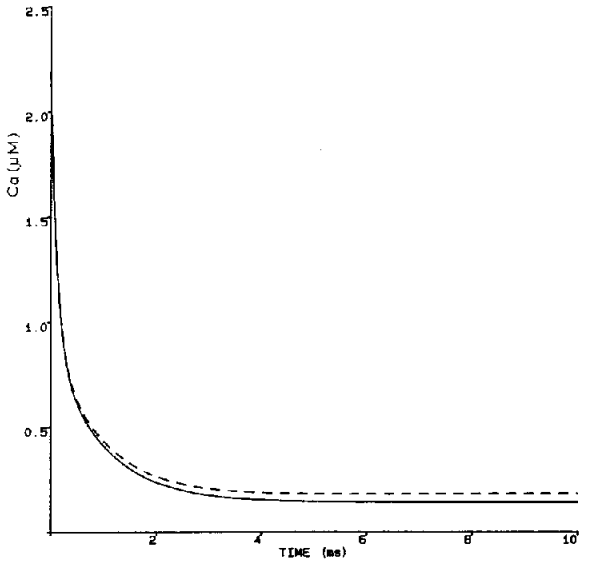


Fig.3. A computer simulation of the time course of the calcium transient following photolysis of 2 mM Diazo-2 in the absence of troponin (solid line) and in the presence of 200 μM calcium-specific regulatory sites (dotted line). Rate constants: k_{phot} for calcium-free Diazo-2 2300 s^{-1}, k_{phot} for calcium-loaded Diazo-2 7500 s^{-1}, k_{on}^{Ca} Diazo-2 and its photolysis product a substituted phenyl acetic acid 8.0×10^8 $M^{-1} \cdot s^{-1}$, k_{off}^{Ca} Diazo-2 1780 s^{-1}, k_{off}^{Ca} photolysed Diazo-2 58 s^{-1} [1], k_{on}^{Ca} calcium-specific sites on troponin C 10^8 $M^{-1} \cdot s^{-1}$ and k_{off}^{Ca} 100 s^{-1} [21].

calcium fall which extends over 5 s in frog at 10°C [13] or 1–2 s in arthropod fibres at 20°C [18]. In intact frog fibres the soluble sarcoplasmic parvalbumins may contribute to this prolonged elevation of sarcoplasmic free calcium [19,20].

In this study we show that we can produce rates of relaxation in single skinned frog fibres, with a caged chelator, which are at least as fast as those seen in vivo whilst the fall in free calcium is likely to be much faster ($t_{1/2} < 1$ ms) than even the fast phase of the calcium fall in vivo ($t_{1/2}$ = 70–80 ms). It is now possible to use this method of reducing free calcium to study how rapidly the TnC-bound calcium falls using fluorescently labelled derivates such as TnC_{Danz} [21] and to determine which steps are likely to limit the rate of relaxation of muscle.

Acknowledgements: This study was supported by grants from the BHF, MDA and NIH The help and advice of Dr Stephen Adams and Dr Roger Tsien in providing samples of Diazo-2 is gratefully acknowledged.

REFERENCES

[1] Adams, S.R., Kao, J.P.Y. and Tsien, R.Y. (1989) J. Biol. Chem., in press.
[2] Kress, M., Huxley, H.E., Faruqui, A.R. and Hendrix, J. (1986) J. Mol. Biol. 188, 325–342.
[3] Edman, K.A.P. and Flitney, F.W. (1982) J. Physiol. 329, 1–20.
[4] Ashley, C.C. and Ridgway, E.B. (1970) J. Physiol. 209, 105–130.
[5] Ashley, C.C., Barsotti, R.J., Ferenczi, M.A., Lea, T.J. and Mulligan, I.P. (1987) J. Physiol. 394, 24P.
[6] Ashley, C.C., Barsotti, R.J., Ferenczi, M.A., Lea, T.J. and Mulligan, I.P. (1988) Biophys. J. 53, 564a.
[7] Goldman, Y.E. and Kaplan, J.H. (1988) Biophys. J. 53, 25a.
[8] Kaplan, J.H. and Ellis-Davies, G.C.R. (1988) Proc. Natl. Acad. Sci. USA 84, 3496–3500.
[9] Moisescu, D.G. (1976) Nature 262, 610–613.
[10] Goldman, Y.E. and Simmons, R.B. (1984) J. Physiol. 350, 497–518.
[11] Ferenczi, M.A., Homsher, E. and Trentham, D.R. (1984) J. Physiol. 352, 575–599.
[12] Ferenczi, M.A. (1986) Biophys. J. 50, 471–477.
[13] Cannell, M.B. (1986) J. Physiol. 376, 203–218.
[14] Miledi, R., Parker, I. and Zhu, P.H. (1982) J. Physiol. 33, 655–680.
[15] Huxley, A.F. and Simmons, R.M. (1973) Cold Spring Harbor Symp. Quant. Biol. 37, 669–680.
[16] Gordon, A.M. and Ridgway, E.B. (1978) Eur. J. Cardiol. 7, 27–34.
[17] Blinks, J.R., Rudel, R. and Taylor, S.R. (1978) J. Physiol. 277, 291–323.
[18] Ashley, C.C. and Lignon, J. (1981) J. Physiol. 318, 10P.
[19] Robertson, S.P., Johnson, J.D. and Potter, J.D. (1981) Biophys. J. 34, 559–569.
[20] Gillis, J.M. (1985) Biochim. Biophys. Acta 811, 97–145.
[21] Griffiths, P.J., Potter, J.D., Coles, B., Strang, P. and Ashley, C.C. (1984) FEBS Lett. 176, 144–150.