

Calcium Binding and Tension Development in Detergent-Treated Muscle Fibers

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ABSTRACT The nonionic detergent Brij 58 eliminates irreversibly the capability of the sarcoplasmic reticulum (SR) of skinned crayfish muscle fibers to sequester Ca and to release it under appropriate stimulation. In contrast to deoxycholate (DOC) which causes an irreversible diminution of tension as well, Brij 58 does not affect the contractile proteins. Comparison of the time-course of tension development before and after Brij treatment demonstrates that Ca is accessible to the contractile proteins more rapidly after the SR is destroyed but, nevertheless, much more slowly than is predicted for free diffusion of Ca in the myoplasm. Slowing apparently results because of the presence of ca 1 mmol/kg fiber of myoplasmic Ca-binding sites that remain after Ca uptake of the SR is eliminated. A theoretical model is presented which allows for the effects of binding sites and of an unstirred layer in the vicinity of the fiber on Ca diffusion into the myoplasm.

INTRODUCTION

Mechanical removal (skinning) of the surface membrane of an isolated crayfish muscle fiber can be performed in an aqueous medium of appropriate ionic composition (Reuben et al., 1967, 1971). This procedure which removes the sarcolemma and mitochondria exposes the contractile system directly to the bathing medium. The fiber is now activated by changes in the bathing medium which are ineffective on intact fibers. The preparation therefore is useful in elucidating the regulation of tension in the muscle fibers by the substrate, MgATP, (Reuben et al., 1971) and by Ca (Brandt et al., 1972).

The present report describes further simplification of the skinned fiber by treatment with a nonionic detergent, Brij 58. Evidence is presented that this disrupts the internal membrane system of the sarcoplasmic reticulum (SR). The fiber no longer responds to agents that release Ca from the SR and it

loses the ability to accumulate Ca, but the contractile mechanism is not disrupted, since the fiber remains as responsive to activation by substrate and by Ca as before Brij treatment. Data on the kinetics of tension development confirm the finding by Hellam and Podolsky (1969) that the presence of the Ca-sequestering system of the SR contributes markedly to the slow rise of tension evoked by applied Ca. However, after disruption of the SR by Brij there is still appreciable Ca binding in the myoplasm. Preliminary reports were presented at the 1972 meetings of the Biophysical Society (Reuben and Brandt, 1972; Orentlicher et al., 1972).

METHODS

Chamber

To achieve more precise control of the experimental parameters several changes have been made in our procedures. The experimental chamber (Fig. 1) has been redesigned to increase circulation of the bathing fluid and temperature control. A stainless steel tube runs around the perimeter of the chamber and a thermistor controls a pump which circulates cold water through the tube whenever the temperature rises above a preset level, here 21°C. Two clamps, one fixed to the chamber and another fixed to a movable strain gage dip into the open channel of the chamber to hold the ends of the fiber.

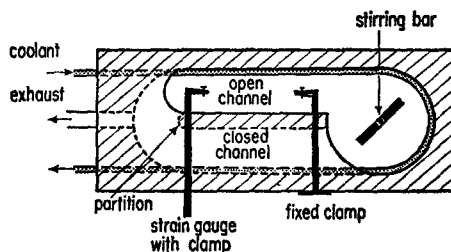


FIGURE 1

FIGURE 1. Experimental lucite chamber. The fiber is held between two clamps in the open channel. Saline, driven by a magnetic stirring bar, circulates about the partition which separates the open and closed channels. In most experiments the stirring bar (length of 1.8 cm) was driven at 300 rpm. The heavy double line represents a stainless steel tube which lies at the bath perimeter. Cold water circulated through this tube provides temperature regulation.

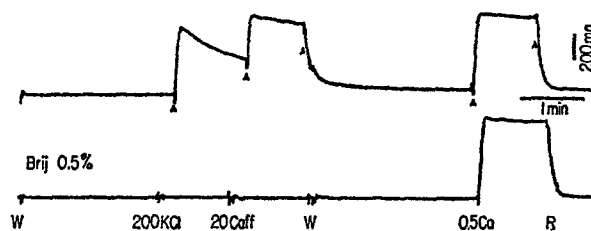


FIGURE 2

FIGURE 2. Effects of treatment with Brij 58. Upper row: At first arrow the bathing solution was changed from K propionate to KCl. The change evoked almost 0.6 gm of tension. At the second arrow KCl was washed out and replaced with the propionate saline containing 20 mM caffeine. A still larger tension was evoked. Caffeine was removed at third arrow. 0.5 mM Ca was applied near the end of the record. Lower row: The fiber was exposed to 0.5% Brij 58 for about 30 min. and the detergent was then washed out. The fiber no longer responded either to KCl or to caffeine, but it responded as before to 0.5 mM Ca.

Preparation

A fiber skinned over 80 to 90% of its length while bathed in the "skinning" solution (185 mM K propionate, 1 mM MgATP and 5 mM EGTA) is transferred to the experimental chamber. The ends of the fiber, still covered by sarcolemma, are inserted into the two clamps and the fiber is extended to a sarcomere length of about 8 μm which is in the optimal range for tension development of the length tension relation for crayfish fibers (April, 1973; Brandt et al., 1972).

Treatment with Detergent

The detergent solution is the skinning solution plus 0.1–0.5% Brij 58 (Ruger Chemical Company, Irvington on Hudson, N.Y.). The presence of the EGTA is essential to prevent a violent contraction of the fiber which is otherwise induced by the detergent. This solution was washed out of the chamber after 20–60 min and the fiber was allowed to equilibrate in the "rest" (or relaxing) solution which contained 185 mM K propionate, 5 mM ATP and buffered with EGTA to $\text{pCa} \geq 8$. Deoxycholate, DOC, (1 mM–5 mM) was applied in four experiments.

Solutions

The major part of the work reported in this paper was done with a relaxing solution buffered to $\text{pCa} 8$ and an activating solution containing 10 μM unbuffered Ca (no EGTA) or one buffered to $\text{pCa} 5$. Experiments were also conducted with higher, unbuffered Ca concentrations. Test solutions were made by mixing appropriate amounts of stock solutions of similar ionic strength: 200 mM K propionate, 40 mM MgATP, 67 mM Mg (Acetate)₂, 67 mM K₂ EGTA, 67 mM K₂ (Ca EGTA). Buffered [Ca] was calculated with a value of $2 \times 10^6 \text{ M}^{-1}$ for the apparent association constant at pH 7 of Ca and EGTA. This is an intermediate value of an association constant for which no universally accepted number exists. An exact determination of the Ca-EGTA association constant may necessitate a change in our buffered [Ca], but this is of no importance to our conclusions. The MgATP⁼⁼ was prepared by dissolution of Na₂H₂ATP in Mg(Acetate)₂, followed by neutralization with KOH. All salines were titrated to pH 7.0 and contained a standard amount of pH buffer. The latter was 4 mM Tris maleate in one series of experiments and 5 mM imidazole buffer in another. No specific effects of the different pH buffers were detected.

Intracellular Ca

Fluorometric analysis (Turner model 110 Fluorometer, G. K. Turner Associates, Palo Alto, Calif.) for calcium was done on bundles (1–10 mg wet weight) from flexor and extensor muscles which were dissected out in a control saline (200 mM NaCl, 5 mM KCl, and 13.5 mM CaCl₂). Some of these muscles were weighed and analyzed for Ca after a brief wash with 200 K propionate to remove extracellular Ca. Other bundles were Brij treated for 1–2 h, and were then equilibrated for 0.5 h in saline buffered to either $\text{pCa} 5$ or $\text{pCa} 8$. Each bundle was then washed with 200 mM K propionate, gently blotted on plastic, and weighed wet. The muscle was

then placed in a plastic centrifuge tube for digestion overnight with 0.15 ml of 0.3 N acetic acid.

After the digested muscle was centrifuged down, 0.1 ml of the supernatant was added to 5 ml calcein solution (0.6 mg calcein in 100 ml of 0.4 N KOH) for the fluorometric measurement. Before each series of measurements the fluorometer was calibrated with standard Ca solutions (10, 20, 40 μ M CaCl₂) added to the calcein solution in the same manner as the experimental sample.

RESULTS

Elimination of the SR by Brij 58

Before Brij treatment skinned muscle fibers respond with large, rapidly developing but transient tension on exposure to anions of the Hofmeister series in the rank SCN > I > Br > Cl (Reuben et al., 1967). A nearly maximal tension was induced (Fig. 2, upper line) on replacing propionate with Cl. Maximal tension was evoked on application of 20 mM caffeine. The last response in the sequence was evoked on applying 0.5 mM Ca. The fiber was then treated with 0.5% Brij 58. After removing the detergent the fiber no longer responded to Cl nor to caffeine, but the response to Ca was not significantly changed.

Brij treatment also eliminates the large "tension spike" oscillations which occur in media unbuffered or weakly buffered for Ca. These oscillatory responses are a consequence of cyclic Ca accumulation and Ca release by the SR (Reuben and Brandt, 1972; Reuben et al., 1973; and to be published) and their elimination by Brij treatment therefore is additional evidence that SR function is disrupted.

Light and electron microscopy of the detergent-treated fibers confirm the conclusion that Brij eliminates the SR as a functional entity. Fig. 3 A is a control electron micrograph of a skinned fiber. Fig. 3 B illustrates the disruption and reduction of the intracellular membranes resulting from a 20-min exposure to 0.5% Brij 58. The disruption of intracellular membranes was seen in all fibers fixed after 20 min to 1 h in 0.5% Brij 58. However, the reduction of membrane remnants was variable. The SR vesicles remaining after detergent treatment varied from none in one fiber to a consistently high level for detergent-treated fibers not mechanically skinned.

Fig. 4 shows four light micrographs of a fiber before and during exposure to a subthreshold Ca-buffered solution (pCa 6.7) containing 3 mM oxalate. After 24 min the fiber darkened, and by 62 min it was nearly opaque. Brij 58 (0.5%) was then added to the solution and the fiber again became transparent after undergoing a massive contracture. After removal of Brij the fiber remained transparent for hours even though exposed to the Ca and oxalate solution. Thus, the Ca accumulated by the SR when oxalate was present (Hasselbach, 1964) was released by Brij treatment and further accumulation was eliminated.

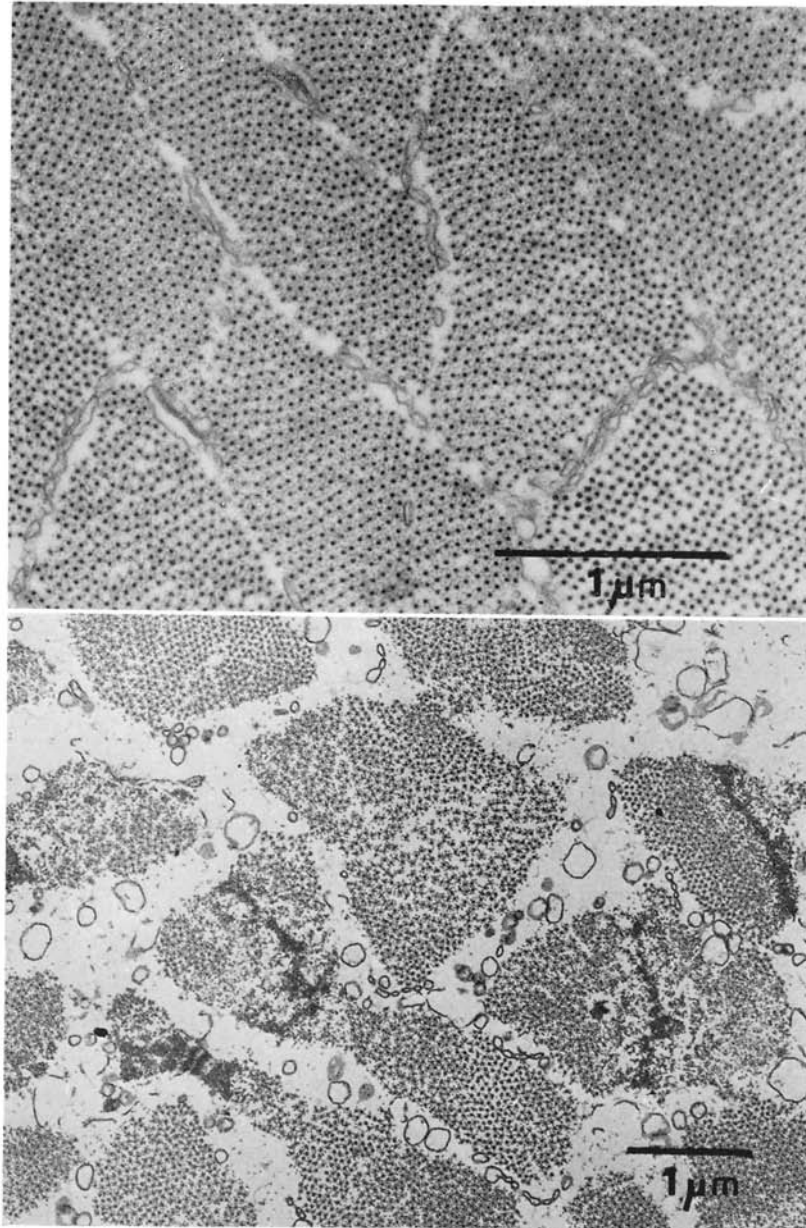


FIGURE 3. Electronmicrographs of skinned fibers. (A) The extensive SR of crayfish muscle is not destroyed by skinning. (B) Total disruption of the SR produced by a 20-min exposure to a 0.5% solution of Brij 58. Although resolution is too low to provide information regarding the myofilaments, it is clear that there is more space between myofilament bundles and that only vesicles remain of the extensive membrane system shown in A. Fibers were fixed with 0.2% glutaraldehyde in the experimental chamber prior to transfer to the osmium fixative.

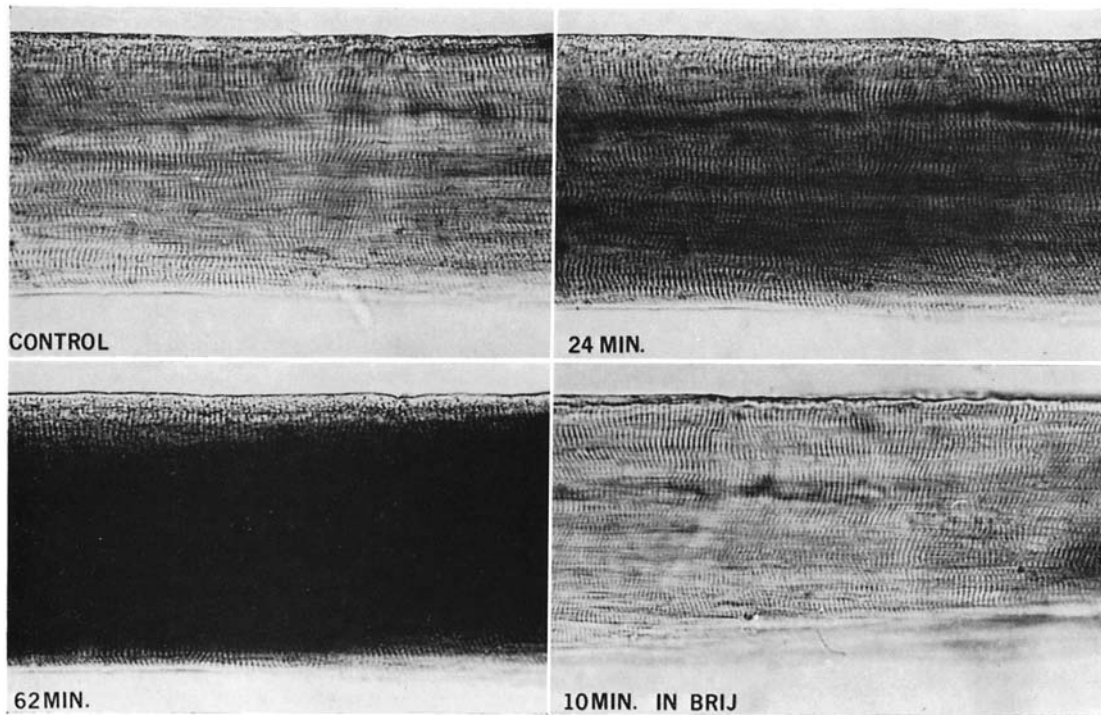


FIGURE 4. Effect of Brij on Ca accumulation by the skinned fiber in the presence of 3 mM oxalate. Upper left: The fiber before the addition of subthreshold buffered Ca and oxalate. Upper right: Darkening after 24 min. The fiber became nearly black (lower left) after 62 min. At this time Brij 58 was added to the bath and the fiber reverted to a transparent state within minutes (lower right). It remained so thereafter even though the fiber was still in the buffered Ca-oxalate bath without Brij.

Absence of Effects on Contractile System

Used as described, Brij 58 produces no marked effects on the response of the actomyosin system to substrate and Ca. The absence of an effect on the tension response to saturating Ca levels is demonstrated in Fig. 2. Two other properties of the actomyosin system have been measured before and after Brij treatment. These are variation of tension with substrate, $pS = -\log [MgATP]$ at $pCa > 8$; and the variation of tension with calcium (pCa) at $[MgATP] = 1$ mM. Detailed descriptions of the experimental procedures will be found in Reuben et al. (1971) and Brandt et al. (1972). The tension- pS curve (Fig. 5 A) was obtained with $[ATP] = 5$ mM and $[Mg]$ varied with EDTA as the buffer. After Brij treatment this relation was virtually unchanged. The small decline in tension apparent at all values of pS is due to the gradual decline of fiber tension with time. The companion experiment is shown in Fig. 5 B. In this $[MgATP]$ was fixed at 1 mM and tension was

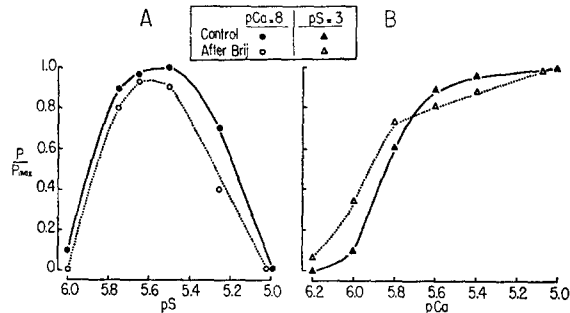


FIGURE 5. Characterization of the contractile elements before and after Brij treatment. (A) The response to increasing [Mg ATP] by increasing [Mg] with [ATP] = 5 mM. The [Mg] is buffered with EDTA. The upper curve is the control. (B) [Mg] = 5 mM, [ATP] = 1 mM, and [Ca] is varied with EGTA as the buffer. The maximum tension (pCa = 5) was 270 mg both before and after Brij treatment.

measured as a function of pCa before and after Brij treatment. The slight broadening of the tension-pCa relation is of the same order as that observed upon repetition of control curves after intervals of about 1 h.

Change in the Kinetics of Ca-Induced Tensions

Of particular relevance to the present work are the changes induced by various agents in the time-course of the rise of tension in response to a supra-threshold concentration of Ca (Fig. 6). Before treatment with Brij, addition of 0.03 mM Ca to the control saline induced a rise of the tension with a half-time to the peak level ($t_{\frac{1}{2}}$) of 106 s (record 1). When 10 mM caffeine was present (record 2) $t_{\frac{1}{2}}$ was reduced to 50 s. The change is consistent with the assumption that the reduction of $t_{\frac{1}{2}}$ reflects the inhibition of the SR Ca-uptake system (Hellam and Podolsky, 1969). The third record was obtained with 5 mM procaine present in the bathing solution. On removing the procaine (record 4) $t_{\frac{1}{2}}$ returned to the initial value. Thus, caffeine and procaine altered $t_{\frac{1}{2}}$ in a reciprocal manner, consistent with their respective physiological actions (Chiarandini et al., 1970). Treatment of the skinned fiber with 0.5% Brij also decreased $t_{\frac{1}{2}}$ and this value was not changed in the presence of caffeine or procaine (records 5-7). Thus, treatment with Brij 58 eliminated permanently a delay that is reversibly eliminated by caffeine and is prolonged reversibly in procaine. All these effects appear to be related to the state of the SR, the effect of Brij treatment being correlated with the permanent elimination of the SR as a system regulating Ca in the fiber. The tension induced by 30 μ M Ca was not decreased but rather was slightly increased after the Brij treatment, in this experiment, but usually no increase was seen.

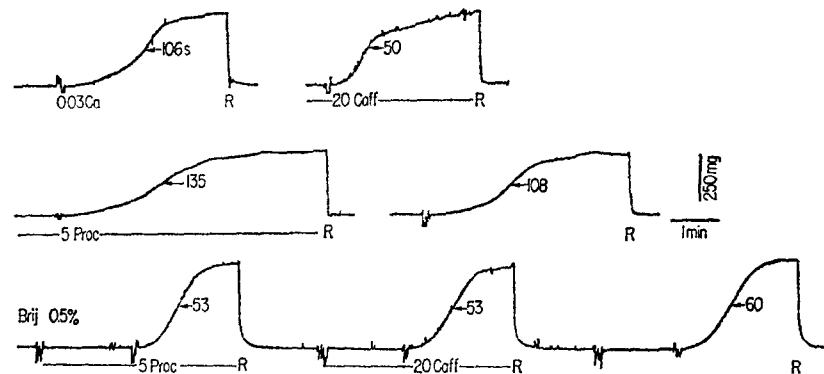


FIGURE 6. Time-course of tension development in a single fiber under different conditions. Tension was evoked by applying $30 \mu\text{M}$ Ca in each case. Upper left: Control medium. Artifacts on the traces indicate application of Ca. Upper right: The tension evoked by 20 mM caffeine had subsided at the time the record was made. The tension in response to Ca rose more rapidly than in the control condition. Middle left: With 5 mM procaine present the rise of the Ca-induced tension was slowed. Middle right: Another response in the control medium. Brij 58 (0.5%) was then applied for about 30 min. The lowest trace is a continuous registration of three responses to $30 \mu\text{M}$ Ca after the detergent was washed out. Tension developed with a half-time of 53–60 s (control in last record), independently of the presence of procaine or caffeine. Note that introduction of caffeine did not induce tension.

Effects of Deoxycholate

This surfactant, which eliminates uptake of Ca by SR fragments (Briggs and Fuchs, 1963), was used by Hellam and Podolsky (1969) to eliminate Ca uptake by the SR in skinned frog muscle fibers. Like Brij 58, DOC induces a vigorous contraction of skinned crayfish fibers when the bathing solution is not buffered with EGTA. This response is presumably caused (as with Brij) by release of Ca from the SR. However, fibers exposed to 1–5 mM DOC show a rapid and irreversible time- and concentration-dependent diminution of contractile activity in response to added Ca. With a brief exposure of the fiber (<2 min) to 5 mM DOC tension was virtually abolished in marked contrast to the absence of such an action by Brij (Figs. 2 and 5). It is, however, consistent with the finding that DOC causes irreversible diminution of ATPase activity (Briggs and Fuchs, 1963).

Tension Kinetics of Detergent-Treated Fibers

All the tension data presented in the remainder of this report relate to Brij-treated fibers in the standard, propionate saline with 5 mM Mg and 1 mM ATP, unless specifically noted. The excess Mg was used to minimize the concentration of CaATP and its possible effect on Ca diffusion. Tension was elicited with either unbuffered Ca at $[\text{Ca}] \geq 10 \mu\text{M}$ or by Ca buffered with

EGTA to 10 μM free Ca ($\text{pCa} = 5$). Although the initial rate of tension development can be modified by changing the concentrations of ATP, MgATP, and Mg as well as Ca the major limitation in all conditions is clearly the inward diffusion of ionized Ca.

In free diffusion of Ca from a bath into a cylindrical volume of radius, $a = 100 \mu\text{m}$ (the dimension of a typical crayfish muscle fiber) the quantity of Ca in the cylinder would be expected to reach nearly maximum value within a diffusion time $a^2/D \approx 10 \text{ s}$; since D_{Ca} is of the order $10^{-5} \text{ cm}^2/\text{s}$. In fact, in no case did tension of the Brij-treated fiber in response to 10 μM Ca reach 10% of maximum at 10 s. The time-course of these tensions was variable, but the time to steady tension was always extended over several minutes. Figs. 6 and 7 illustrate the slowness of the response and the effect of increasing the total Ca concentration. Fig. 7 A shows the time-course of tension elicited by unbuffered Ca in the concentrations of 10 μM and 20 μM , respectively. The steady-state tension with 20 μM Ca is about 15% higher than with 10 μM and the tension developed in about one half the time.

Fig. 7 B shows data on the same fiber when it was activated with Ca buffered to $\text{pCa} 5$ (10 μM free Ca) but with the total Ca varied by varying the total concentration of EGTA (L_T), from 0.06 mM to 6 mM. As the calcium buffering capacity of the solution increased, thereby increasing the Ca available for diffusion to the contractile machinery, the tension developed more rapidly. The steady-state tension did not change appreciably, however, since the free Ca in the activating solution did not change.

The slowness of the response to 10 μM Ca is illustrated further in the record of Fig. 8. Note that the $\text{pCa} 8$ buffer was removed by two washes with un-

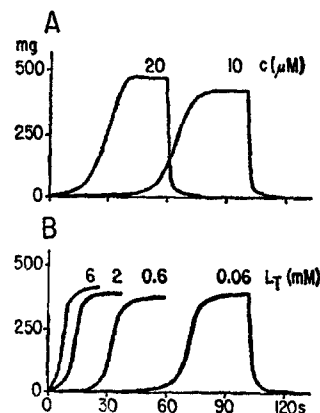


FIGURE 7. Change in kinetics of tension development with increasing concentration of Ca. (A) Response to unbuffered Ca solution containing 10 μM and 20 μM Ca, respectively. (B) pCa of the activating solution was maintained constant ($\text{pCa} 5$) but the amount of total Ca was increased by increasing the total concentration of EGTA (L_T) from 0.06 mM to 6 mM.

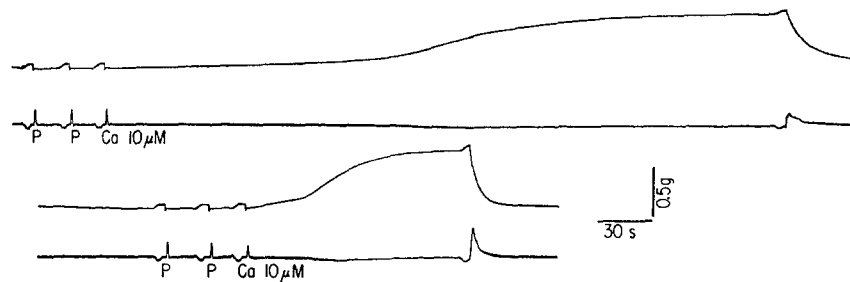


FIGURE 8. Kinetics of tension development in a Brij-treated fiber. Continuous recording. Upper line: The fiber had been equilibrated in a solution buffered for pCa 8. It was then washed twice with propionate saline at 20-s intervals and about 20 s later with an unbuffered saline containing 10 μ M Ca. Note the slow onset of tension and the slow rise to a steady value. The fiber was then relaxed by washing with a solution buffered for Ca at pCa 6.2. Lower line: After 2 min in this solution the fiber was washed twice with K propionate and then 10 μ M Ca was added. Tension began to rise almost immediately and the same steady-state level was attained much more rapidly. The fiber was then relaxed with pCa 8 solution.

buffered saline before the addition of Ca. It is essential that all EGTA be removed from the fiber before tension is elicited with low levels of (total) Ca.

Fig. 8 illustrates another way in which the kinetics of tension development can be varied experimentally. The tension developed in Fig. 8 (upper trace) was relaxed by washing the fiber with a solution buffered to pCa 6.2. After 2 min this solution was replaced twice with K propionate wash before applying the activating solution containing 10 μ M Ca. The onset of tension (lower trace) and its rise were much more rapid. The quickened development of tension achieved by presoaking the fiber in a subthreshold concentration of Ca before exposure to 10 μ M Ca (Fig. 8 B) was also observed when the tension was induced by buffered Ca (pCa 5). This is shown in Fig. 9 where the shortening of the time-course of tension, measured by the time to 10% P_{max} , t , was observed at values of L_T , ranging up to 12 mM, in fibers presoaked in pCa 6.2 solutions.

The data of Fig. 10 emphasize the slow equilibration between solutions containing subthreshold levels of Ca and the detergent-treated skinned fiber. In (A) the fiber was first exposed to pCa 6.0 solution ($L_T = 3$ mM) for approximately 2 min before decreasing pCa to 5.0. The tension increased immediately and attained a maximum value in 20 s. The fiber relaxed upon increasing pCa to 8.0 and after a 2-min soak in this solution pCa was again reduced to 5.0 (B). A delay of about 15 s preceded the tension which rose slowly, reaching P_0 after 1.0 min in the pCa 5 solution. The effect of equilibration in pCa 6 can be reduced by a brief wash with the pCa 8 saline before exposure to the activating solution (Fig. 10 C). Likewise, after the fiber had been equilibrated with pCa 8 saline, a brief exposure to pCa 6 en-

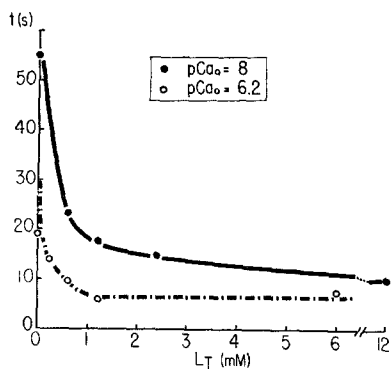


FIGURE 9

FIGURE 9. Variation of time to 10% P_{max} (t) with buffer concentration, L_T . Note that conditioning the fiber at pCa 6.2 reduces t . The stimulus for tension was 10 μ M unbuffered Ca.

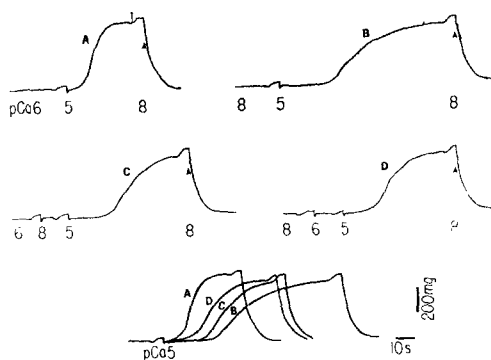


FIGURE 10

FIGURE 10. Influence of conditioning with subthreshold levels of Ca upon the rise of Ca induced tension. (A) The fiber had been kept in pCa 6 for 2 min before a change to pCa 5 (denoted by artifact). (B) The fiber was then bathed in pCa 8 medium for 2 min. Note the slower onset and rise of tension in response to pCa 5. (C) After bathing in pCa 6 the fiber was exposed for 10 s to pCa 8 before stimulation with pCa 5. (D) The procedure was reversed. The fiber was first bathed in pCa 8 then exposed to pCa 6 for about 10 s before applying pCa 5. Tracings of the four records are superimposed below, with the wash artifact as the reference on the time axis. Further discussion in text.

hances the response to pCa 5 (Fig. 10 D). Thus reversal of the effects of equilibrating salines required at least 15 s for this fiber. The tensions as a function of the described presoaking conditions are superimposed at the bottom of Fig. 10. These findings indicate that even in the absence of a functional SR some "barrier" hinders the diffusion of Ca to activator sites in the muscle fiber.

Ca-Binding Sites in the Detergent-Treated Muscle Fiber

Calcium bound to muscle bundles after they were separated from chitin and tendon was chemically determined. A 20–30-min detergent treatment before equilibration in detergent-free pCa = 8 solution reduced the Ca from 10 mmol/kg to 0.46 ± 0.26 mmol/kg wet weight. Equilibration at pCa = 5 increased the bound Ca to 1.47 ± 0.66 mmol/kg. The difference between these values is significant at $P < 0.001$. All values of bound Ca were the same for bundles in 5 mM Mg, 1 mM ATP, and 1 mM Mg, 5 mM ATP. These findings, therefore, indicate that detergent-treated fibers, in which the SR can no longer accumulate Ca contain about 1 mmol/kg wet weight of binding sites for Ca. Presumably the presence of these binding sites is a factor in slowing the equilibration at the contractile machinery with Ca.

DISCUSSION

Brij Affects SR Functioning but not Contractile Activity

Destruction of the functional capacity and structure of the SR with Brij (Figs. 2–6) does not significantly affect the performance of the contractile machinery as measured by the maximum tension output of the fiber in response to Ca (Figs. 2, 5, and 6). Nor are the relations between tension and substrate concentration (pS) with $pCa \geq 8$, or between tension and pCa with $pS = 3$ significantly altered (Fig. 5). These findings make the Brij-treated muscle fiber a particularly useful preparation for the study of the properties of the contractile system. In contrast DOC not only destroys the SR but has an effect on the contractile proteins as originally described by Briggs and Fuchs (1963)¹.

Ca-Binding by the Brij-Treated Fibers

When the SR is intact the response of the skinned muscle fiber to a high level of Ca (buffered or unbuffered) is slow (Fig. 6). It is also slow in frog muscle fibers skinned mechanically (Hellam and Podolsky, 1969) or chemically (Julian, 1971). The slow development of tension has been ascribed by Hellam and Podolsky (1969) to the rapid uptake of Ca by the powerful Ca-binding system of the SR. Disruption of the SR by Brij reduces but does not eliminate the delay before the onset of tension. The half-time ($t_{1/2}$) for the rise of tension is still large and is of the order of 1 min when the Ca bathing the fiber is increased from about pCa 8 to pCa 5 (Figs. 7 and 8). This is too slow to be accounted for by free diffusion of Ca. Both the delay and the slow rise of tension are diminished (Figs. 8–10) by presoaking the fiber in solutions buffered for pCa at levels which are subthreshold for inducing tension ($pCa > 6$). Our data demonstrate that in addition to the uptake system of the SR the muscle fibers possess other binding sites at a concentration of about 1 mmol/kg cell.

Nature of the Binding Sites

Some fraction of the binding sites which have been disclosed in the present work may have nothing to do with the contractile proteins. For example,

¹ Our finding confirms Briggs and Fuchs (1963). In order to avoid irreversible effects Hellam and Podolsky (1969) exposed frog fibers to 1 mM DOC for 5 min or less and under these conditions reported an increased sensitivity of the fibers to buffered Ca (their Figs. 2 and 3). This effect may have been due to the increase in myoplasmic Ca upon its release from the SR by DOC since we also observed an apparent transient increase in sensitivity to Ca which was eliminated by higher concentrations of free EGTA, i.e., ≥ 5 mM in conjunction with our standard vigorous stirring.

some might be associated with the SR fragments shown in Fig. 3 B. It has been reported (Fiehn and Hasselbach, 1970; Carvalho, 1972) that SR vesicles after lipid extraction may bind Ca, but lack the capacity to actively accumulate Ca. On the other hand, biochemical data have demonstrated the existence of Ca-binding sites in the regulatory protein, troponin (Ebashi et al., 1968; Fuchs, 1971; Bremel and Weber, 1972). Identification of these with some fraction of the sites demonstrated in the present work requires their further characterization. The skinned, Brij-treated muscle fiber is a preparation that is amenable to experiments on this matter and we withhold further discussion of this problem for a future report.

Kinetic model of Tension Development

The Brij-treated skinned muscle fiber may be idealized as a cylinder containing binding sites for Ca. On exposure of the cylinder to a solution containing a given concentration of Ca the latter will diffuse inward but the rate at which Ca reaches the core is slowed from that predicted for free diffusion by the binding capacity of the sites. Contractile activity will occur only as the concentration of Ca rises above that level required to fill critical binding sites. In the experiments of the present work the steady-state level of tension for a given pCa is not markedly affected by the different experimental conditions and the final concentration of Ca in the fiber is adequate to achieve maximal tension. Thus the significant characteristics are the delay in onset of tension and the rate of rise of the tension.

Both the onset and the rate of rise are accelerated in high concentrations of Ca (Fig. 7 A) and with an increased Ca capacity of the buffer system (Fig. 7 B). In the case of buffered Ca either CaEGTA dissociation or CaEGTA diffusion might in principle be rate limiting. The relative importance of the two can be estimated as follows: if the ratio of diffusion time to reaction time $t_d/t_r = k^+a^2/D$ is calculated with fiber radius $a = 0.01$ cm, diffusion coefficient, $D = 3 \times 10^{-6}$ cm²/s, and the dissociation rate constant, $k^+ = 0.4$ s⁻¹ (Hellam and Podolsky, 1969), we have $t_d/t_r = 13$. Note that if $a = 50$ μm, as is typical for frog fibers $t_d/t_r = 3$. If diffusion and reaction times are comparable, the system approximates an infinitely rapid reaction (Crank, 1957, p. 145). Therefore while both release of Ca and diffusion of CaEGTA may be significant for frog (Ford and Podolsky, 1972), diffusion should completely dominate in the crayfish fiber. For diffusion to be slowed in muscle, there must be Ca-binding sites in the fiber to hinder the movement of Ca. It is important that this conclusion is insensitive to the values of k^+ and D . For example, it would be necessary to decrease k^+ by 100 (or increase D by 100) to modify the conclusion that diffusion is the limiting process in crayfish muscle. The delay in onset of tension decreased and the rate of tension de-

velopment increased when the fibers were presoaked in subthreshold concentrations of Ca (Fig. 10). These findings indicate that reduction in the concentration of available Ca-binding sites also promotes diffusion of Ca to the sites responsible for tension generation.

Colquhoun et al. (1972) have presented a theoretical analysis of a somewhat similar condition, the kinetics of TTX binding and the penetration of the neurotoxin into nerve. The programs devised by these workers have been used to calculate the time-course of penetration of Ca into the muscle fiber with the different values of the parameters of binding constants and concentration of binding sites. From these computations we were able to determine that for sufficiently high binding constants, the influx of Ca is independent of the actual value of the binding constant, and Ca binding proceeds with a well defined front through the fiber. Although these are "empirical" conclusions from observation of the computer simulations of the diffusion process, they conform to intuition. If binding is very strong, wherever there is any Ca all binding sites must be occupied. This implies that Ca diffusion must proceed with a sharp front between filled sites and those not yet reached by Ca. It is shown in the Appendix that the penetration of this front is a function of Dc_0t/a^2M , where c_0 is the bath concentration of Ca and M is the concentration of binding sites. When the calculations also take into account the effect of an unstirred layer outside the fiber and the tension threshold, the forms of the observed time-course of tension are approximated. An analysis of the diffusion problem is given in the Appendix.

The results of these calculations may be summarized as follows. In the absence of an unstirred layer, tension should rise with an (ideally) infinite slope upon exposure of a fiber to saturation [Ca]. However, the unstirred layer reduces the initial rate of rise to a term inversely proportional to the unstirred layer thickness. The existence of a tension threshold may reduce the initial rate of rise to zero. Thus the combined effects of an unstirred layer and a finite tension threshold may give rise to the observed delay preceding the onset of tension. The absence of stirring effectively increases the unstirred layer thickness by an order of magnitude (Haydon and Hladky, 1972, pp. 144-145) and thereby further slows the rate of tension development. Another implication of these calculations is that the time required to reach a given tension is proportional to the number of Ca-binding sites in the fiber. Therefore, as was observed (Figs. 8-10), the most rapid tensions will be attained by pre-equilibration of a fiber to subthreshold [Ca] in order to saturate the high affinity Ca-binding sites. These are primarily responsible for the slow rise of tension of detergent-treated fibers. Whether these sites function in tension regulation is immaterial to the kinetics of the model, although a significant problem in itself.

APPENDIX

Diffusion with Binding in a Cylinder

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A program developed for calculating TTX diffusion into nerve bundles (Colquhoun et al., 1972) has been employed to compute several cases relevant to the present study, of which three are of special interest. These are calculations of the changes in bound Ca and free Ca in a muscle fiber suddenly exposed to a bath with $c_o = 10 \mu\text{M}$ unbuffered Ca. The first two computations are for $M = 1 \text{ mM}$ binding sites with binding constants: $K = 2 \mu\text{M}^{-1}$, or $K = 10 \mu\text{M}^{-1}$. As Fig. 11 demonstrates, these two conditions lead to very similar binding kinetics. Analysis of the concentration profiles (see below) indicates that for the case, $Kc_o \gg 1$, diffusion takes the form of a wave of binding, i.e., there is a very small region of transition between saturated binding sites and empty sites. The third condition in Fig. 11 gives the binding kinetics for a lower density of sites, $M = 0.3 \text{ mM}$, with $K = 1 \mu\text{M}^{-1}$. The shift of tension vs. time to shorter times caused by conditioning fibers at subthreshold Ca levels (Figs. 8–10) is explained by this analysis as due to a saturation of high affinity binding sites during conditioning, leading to a decrease in the binding capacity, M .

In order to facilitate further analysis, an algebraic approximation to the exact computer calculation was sought. The dotted curves in Fig. 12 represent profiles of

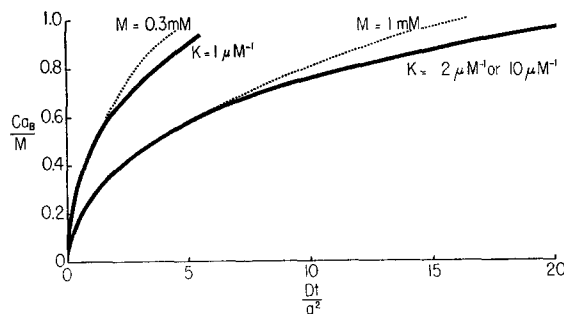


FIGURE 11. Computed kinetics of Ca uptake by a cylinder with Ca-binding sites at two concentrations: 0.3 mM, 1 mM. Solid lines are computer solutions for the diffusion equation with boundary condition $c_o = 10 \mu\text{M}$. At 1 mM, the calculated values at $K = 2 \mu\text{M}^{-1}$ and $K = 10 \mu\text{M}^{-1}$ fell on the same curve. The curve at 0.3 mM was calculated with $K = 1 \mu\text{M}^{-1}$. Dashed lines represent the algebraic approximation described in the text.

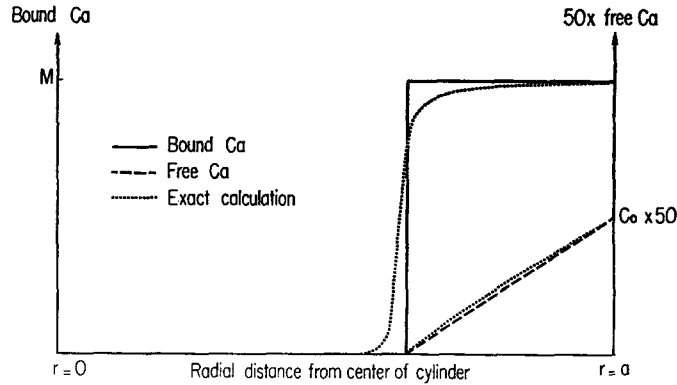


FIGURE 12. Concentration profiles of bound (Ca_B) and free (c) calcium for diffusion in a cylinder; $M = 1.0$ mM, $K = 10 \mu M^{-1}$, at 60% of saturation. The dark lines represent the idealized profiles used to derive an approximate equation for Ca binding, while the dashed lines and points are the exact diffusion-equation computation.

bound and free Ca in a fiber when 60% of its binding sites have been saturated by inward diffusing Ca ions. This computation was for the case $M = 1$ mM, $K = 10 \mu M^{-1}$. The heavy lines represent approximate profiles based on the assumptions that all Ca is confined to an annulus of saturated sites ($Ca_B =$ bound concentration) and that the free Ca ($c =$ free concentration) profile is linear. If the penetration of this Ca front is x , the approximate profiles (see Fig. 12) can be represented as:

$$\begin{aligned}
 r > a - x & \quad Ca_B = M \\
 & \quad c = c_0 \left(1 - \frac{a - r}{x} \right) \\
 r < a - x & \quad Ca_B = c = 0
 \end{aligned}$$

A derivation of the binding equations for the approximation is given below. Since this is an “all-or-none” model $Ca_B/M = P/P_{max}$. This property will facilitate use of the model as a predictor of tension kinetics. A measure of the accuracy of this approximation for Ca binding is given in Fig. 11. At both $M = 0.3$ and $M = 1.0$, the approximate curves (dotted lines) begin to deviate from the exact calculations at 60% of capacity and proceed to saturation more rapidly than the exact curves. The approximate relation seems a useful one, because, as mentioned above, the region of transition between saturated binding sites and empty ones is negligibly small when $Kc_0 \gg 1$.

Comparison of Fig. 11 with the experimental curves of tension kinetics in Fig. 7 shows that the experimental curves start much more slowly than the computed curves. In fact, the computed curves start with infinite slope, while the experimental curves begin with a small slope and then go through an inflection point. Any real interface presents a resistance to diffusion, frequently represented by an unstirred layer of fluid, with a thickness, δ . At an aqueous interface δ is generally 10–100 μm (Helfferich, 1962, p. 253). Due to this barrier the surface concentration, c_s , increases

only gradually from its initial value, rising towards the bulk concentration, c_o . Since there is an apparent tension threshold, $c^* \approx 1 \mu\text{M}$ free Ca, this interfacial resistance will cause an initial delay and slow the overall development of tension. The mathematical treatment of these effects is given below. The effect on tension kinetics of an unstirred layer of $20 \mu\text{m}$ and a tension threshold is shown in Fig. 13. This simple model accounts for the qualitative features of the Ca-induced tensions: delayed rise of tension, slow time course, shift of curve to shorter times with pre-equilibration at subthreshold Ca.

An Approximate Equation for Occupancy of Binding Sites in a Cylinder

Assume: (a) $M \gg c_o$, effectively all Ca in cylinder is bound. (b) Free Ca moves as a linear wave through cylinder: $c = c_s$ at $r = a$, $c = 0$ at $r = a - x$. Define the following distances from the cylinder surface: x' distance from surface of cylinder; x

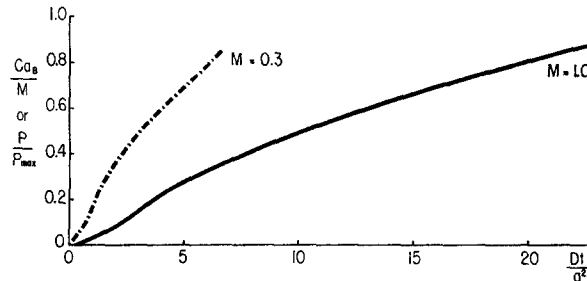


FIGURE 13. Effect of an unstirred layer, $\delta = 20 \mu\text{m}$, and a tension threshold, $c^* = 1 \mu\text{M}$ unbuffered Ca. Note that an inflection point occurs at both high and low binding sites. This is in sharp contrast to the curves of Fig. 11, calculated for the same values of M .

distance of Ca penetration ($c = 0$ at $x' = x$); x^* distance of activation-penetration ($c = c^*$ at $x' = x^*$).

NO UNSTIRRED LAYER, $c_s = c_o$. By assumption (a) the rate of change of the amount of Ca in the cylinder $= 2\pi M(a - x) dx/dt$. Also, rate of change of the amount of Ca in the cylinder $= -2\pi aD(\partial c/\partial r)_{r=a}$ which, by assumption (b), can be written as $2\pi Dc_o(a/x)$. Equating these expressions gives

$$M(a - x) \frac{dx}{dt} = Dc_o \frac{a}{x}, \quad (1)$$

integration of which gives

$$\frac{1}{2} (x/a)^2 - \frac{1}{3} (x/a)^3 = \frac{Dc_o t}{a^2 M}.$$

EFFECT OF AN UNSTIRRED LAYER Continuity of flux at interface implies that $\alpha(c_o - c_s) = -D(\partial c/\partial r)_{r=a}$ where α is a constant.

By assumption (b): $-(\partial c/\partial r)_{r=a} = c_s/x$. Replacing α by D/δ (i.e. taking $(\partial c/\partial r) \simeq (c_o - c_s)/\delta$) we obtain

$$\frac{c_s}{c_o} = 1 + \frac{\delta}{x}. \quad (2)$$

Using this result we obtain, in place of Eq. 1,

$$M(a-x) \frac{dx}{dt} = Dc_s \frac{a}{x} = \frac{Dc_o a}{(x+\delta)}.$$

Integration of this gives

$$-\frac{1}{3} (x/a)^3 + \frac{1-\delta/a}{2} (x/a)^2 + (\delta/a)(x/a) = \frac{Dtc_o}{a^2M}. \quad (3)$$

EFFECT OF A TENSION THRESHOLD, c^* , ON TENSION KINETICS For a linear concentration profile, $c = c_s(1 - x'/x)$, so $x^*/x = 1 - c^*/c_s$ as long as $c_s > c^*$. Substituting for c_s from Eq. 2 gives

$$\begin{aligned} x^* &= x - \frac{c^*}{c_o} (x + \delta), & c_s &\geq c^* \\ x^* &= 0, & c_s &\leq c^*. \end{aligned} \quad (4)$$

The fraction of the fiber volume that is fully activated (the annulus outside x^*), and hence the relative tension is

$$\frac{P}{P_{\max}} = 2 \left(\frac{x^*}{a} - \frac{1}{2} \left(\frac{x^*}{a} \right)^2 \right). \quad (5)$$

Combination of Eqs. 3, 4, and 5 gives P/P_{\max} as a function of time. These equations predict a delay phase in tension development corresponding to the time period $c_s < c^*$.

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