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Competitive Action of Divalent Cations and D600 in Frog Slow Muscle Fibers

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Summary. Single, slow muscle fibers from *Rana temporaria* were equilibrated in normal Ringer's. 95 mmol/liter K^+-solution containing various concentrations of Ca^{2+}, Ni^{2+}, Mn^{2+} or Mg^{2+} was applied, and the ensuing contractures were recorded isometrically. While peak tension (F_{max}) was little affected, maintained tension (measured 1 min after onset of contracture) strongly depended on the concentration and species of divalent cations. Tension was maintained at its peak value in the presence of all species of divalent cations provided their concentrations were adequately increased. Dose-response curves were hyperbolic; Lineweaver-Burk plots revealed straight lines with different slopes intersecting near $1/F_{max}$, and indicating the following order of efficiency: $Ni^{2+} > Ca^{2+} > Mn^{2+} >> Mg^{2+}$. Hill plots for these cations resulted in straight lines with slopes near 1. Qualitatively similar relationships were obtained with contracture solutions containing D600 (3–12 μmol/liter). However, under these conditions higher concentrations of Ca^{2+} or Ni^{2+} were required in order to fully maintain tension. After a step concentration change in the medium during contracture, the effects of Ca^{2+} or D600 were detectable only after a delay of 9 and 18 sec, respectively. It is concluded that divalent cations and D600 compete for the same binding site according to a 1 : 1 reaction. This site is presumably located inside the transverse tubular system and controls inactivation of the contractile force.

Key Words slow fiber · K contracture · D600 · divalent cations · competitive binding

Introduction

In a previous paper (Schmidt, Siebler & Krippeit-Drews, 1988) it was shown that the organic Ca^{2+} channel blocker gallopamil (D600) strongly reduces the ability of slow muscle fibers to maintain contracture tension during prolonged exposure to K^+-rich Ringer's. This effect of D600 was counteracted not only by Ca^{2+}, but also by divalent cations known to block Ca^{2+} channels, such as Ni^{2+}, Co^{2+} or Mn^{2+}. It was concluded from those results that in slow fibers of *Rana temporaria* maintenance of tension is not related to a continuous influx of Ca^{2+}; instead, both D600 and divalent cations seem to bind to sites at the outer membrane surface and thereby regulate the degree of contractile inactivation during K^+ contractures.

In the present experiments the question was examined whether the antagonistic effects of divalent cations and D600 may be due to their binding to the same membrane site. This was done by comparing maintained tension measured at various concentrations of several species of divalent cations in the presence or absence of D600. The general result from these investigations is that divalent cations and D600 compete for the same binding site, presumably located inside the transverse tubular system. A preliminary report of some of these results has appeared (Krippeit-Drews & Schmidt, 1988).

Materials and Methods

Experimental procedures were essentially the same as described previously (Schmidt et al., 1988). Briefly, single slow fibers were dissected from iliofibularis and cruralis muscles of *Rana temporaria*[1]). The fiber under investigation was placed just over the ground of a narrow groove which was filled with Ringer's solution. Whenever the composition of the external medium was changed, the muscle chamber was perfused with the new solution at about 12 ml/min which was 30–50 times the volume of solution surrounding the fiber. Isometric contractures were evoked by application of Ringer's solution containing 95 mmol/liter K^+. Contractures generally lasted 1 min, and between contractures the fibers were bathed in normal Ringer's solution containing 1.8 mmol/liter Ca^{2+}. With the exception of the experiments described in paragraph C, changes of the Ca^{2+} concentration or replacement of Ca^{2+} by other divalent cations as well as addition of D600 to the external medium were always done simultaneously with the application of the high K^+ concentration used to evoke contractures. Intervals between successive contractures were 20 or 30 min according to whether D600 had been absent or present during the preceding contractures.

[1] With permission from the Government of the Saarland (§28 Abs. 3 No. 3 SNG).

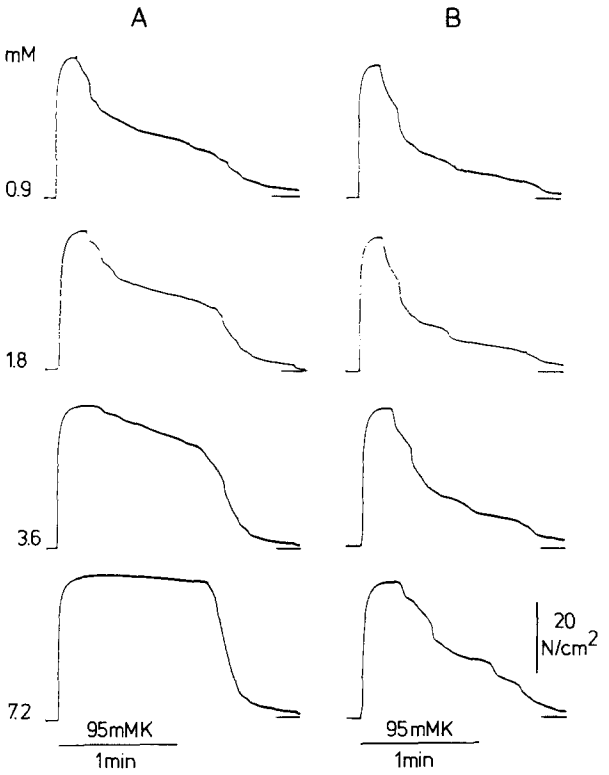

Fig. 1. Effect of Ca^{2+} (*A*) or Mg^{2+} (*B*) on contractures evoked by application of 95 mmol/liter K^+-Ringer's (horizontal lines below records) containing the concentrations of divalent cations given by the numbers on left edge of figure. Note that divalent cations had little effect on the peak contracture tension, while maintenance of tension improved as their concentration increased. All records are from the same slow fiber (diameter 55 μm)

Solutions

Normal Ringer's had the following composition (mmol/liter): NaCl 110.0, KCl 2.5, $CaCl_2$ 1.8, HEPES 5.0, pH 7.3. The contracture solution contained 95 mmol/liter K^+, its Na^+ concentration being reduced by 92.5 mmol/liter. D600 was kindly provided by Prof. Dr. R. Kretzschmar (Knoll AG, Ludwigshafen, FRG). All divalent cations were used as chloride salts (Merck AG, Darmstadt, FRG). Experiments were performed at 18–22°C.

Results

A) Maintenance of Tension at Different Concentrations of Divalent Cations

In agreement with previous results (Schmidt, 1987) maintenance of tension was clearly dependent on the Ca^{2+} concentration of the contracture solution (Fig. 1*A*). In this experiment tension decreased from its peak value of 39 to 21 N/cm^2 (54%) by the end of the 1 min exposure to 95 mmol/liter K^+-Ringer's containing the normal Ca^{2+} concentration (1.8 mmol/liter). Loss of tension (inactivation) was stronger when the contracture solutions contained less Ca^{2+}, while little or no tension was lost when the Ca^{2+} concentration was increased. In contrast to maintained tension there was only a small effect of the Ca^{2+} concentration on peak contracture tension. This is shown more clearly in Fig. 2*A* for a series of four experiments.

Results similar to those obtained with Ca^{2+} were obtained with Ca^{2+}-free contrature solutions containing different concentration of Ni^{2+} or Mn^{2+}. In general Ni^{2+} was somewhat more effective than Ca^{2+} and Mn^{2+}, but when Mg^{2+} replaced Ca^{2+} as the only divalent cation present in the contracture solution, tension decreased more quickly, and at the end of the contracture period it was markedly reduced as compared with Ca^{2+} (Fig. 1*B*). As with Ca^{2+}, peak tension was very little affected by the concentration of Mg^{2+} (Figs. 1*B* and 2*B*).

In Fig. 3 values of contracture tension as obtained after 1 min are plotted against the concentration of divalent cations. It can be seen that with all four cations examined contracture tension approached 100% of the peak value; thus, loss of tension during prolonged exposure to high potassium solution can be avoided, provided the external concentration of divalent cations is sufficiently high. For Ni^{2+}, Ca^{2+} and Mn^{2+} fully maintained contractures were obtained at 7.2–14.4 mmol/liter; in the case of Mg^{2+} more than 28.8 mmol/liter would have been required. Figure 3 also shows that the experimental points could be fitted with hyperbolic functions, only a small deviation being present for Mg^{2+} at the lowest concentrations used.

If reciprocal values of contracture tension are plotted *vs.* reciprocal values of divalent cation concentrations (Lineweaver-Burk plots) it becomes obvious that the experimental values are well fitted by straight lines which have different slopes, but intersect in a common point near $1/F_{max}$ (Fig. 3, inset). Further analysis (Hanes-Woolf plots, *not shown*) resulted in apparent K_D values of 0.9, 1.2, 4.1 and 11.6 mmol/liter for Ni^{2+}, Ca^{2+}, Mn^{2+} and Mg^{2+} respectively. Moreover, as Fig. 4 shows, the relationship between log F/F_{max}-F and log concentration (Hill plots) is linear for all divalent cations examined; the slopes of the straight lines are near 1.

B) Maintenance of Tension in the Presence of D600

It has been described previously that in the presence of 30 μmol/liter D600 maintenance of tension is considerably impaired, while peak tension is only slightly reduced (Schmidt et al., 1988); in addition, the effect of D600 could be counteracted by increas-

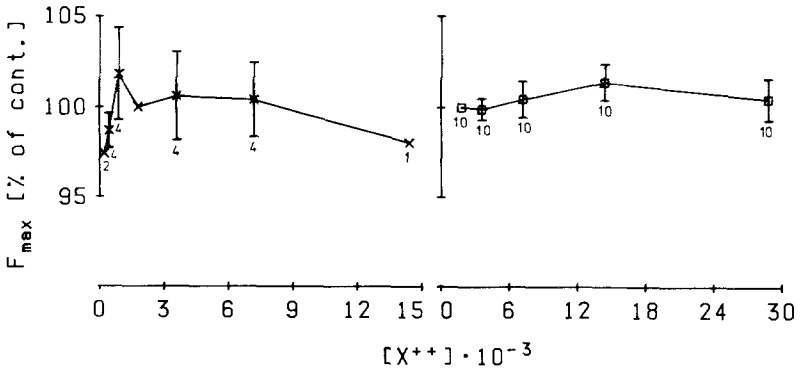

Fig. 2. Relation between peak tension (F_{max}) and concentration of Ca^{2+} (left) or Mg^{2+} (right) in the contracture solution (95 mmol/liter K^+). Peak tension at 1.8 mmol/liter of the divalent cations was taken as 100%. Small numbers near experimental values indicate number of corresponding experiments. Note that peak tension varied unsystematically by not more than 5% when the concentration of Ca^{2+} or Mg^{2+} was changed

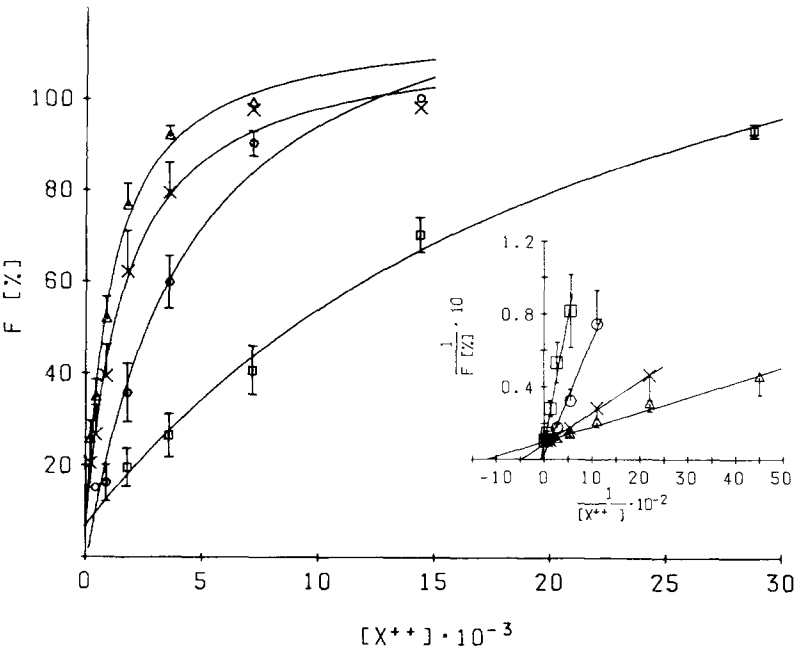

Fig. 3. Effect of molar concentrations of divalent cations (abscissa) on contracture tension measured 1 min after application of 95 mmol/liter K^+-Ringer's (ordinate). The contracture solution contained only one species of divalent cations: Ni^{2+} (△), Ca^{2+} (×), Mn^{2+} (○) or Mg^{2+} (□). Note that with all species of divalent cations contracture tension approached 100% peak tension. Ca^{2+} and Ni^{2+} were the most effective divalent cations, while Mg^{2+} was least effective. Experimental values were fitted with a hyperbolic function; values are means ± SEM of 4–10 experiments, expressed in % of corresponding peak tension. *Inset:* Relation between the reciprocal values of maintained tension (ordinate) and concentration of divalent cations (abscissa; Lineweaver-Burk plot). Note that measured values can be connected by straight lines which have different slopes and intersect at a common point near $1/F_{max}$

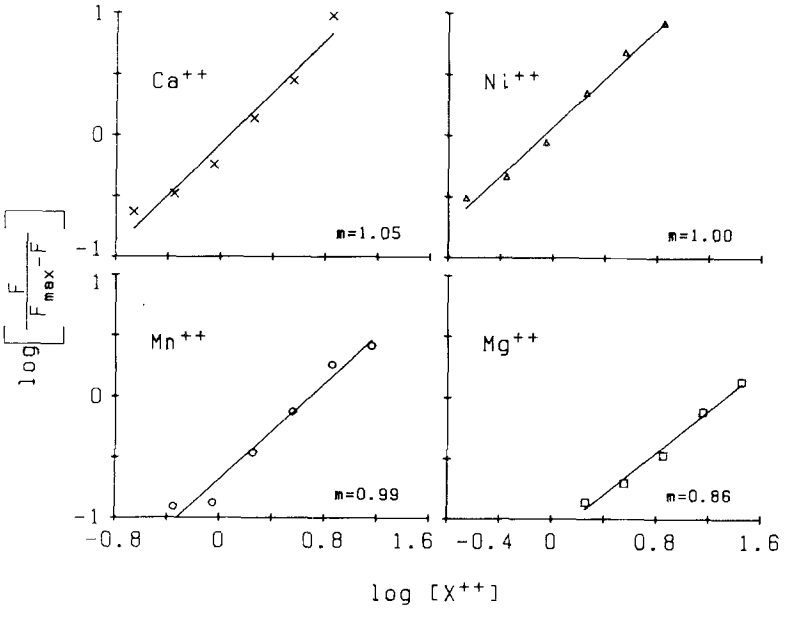

Fig. 4. Hill-plots of contracture tension (log F/F_{max}-F; ordinate) against log concentration of divalent cations (abscissa); same experiments as in Fig. 3. Note that the straight lines connecting experimental values all have slopes (m) near 1.0

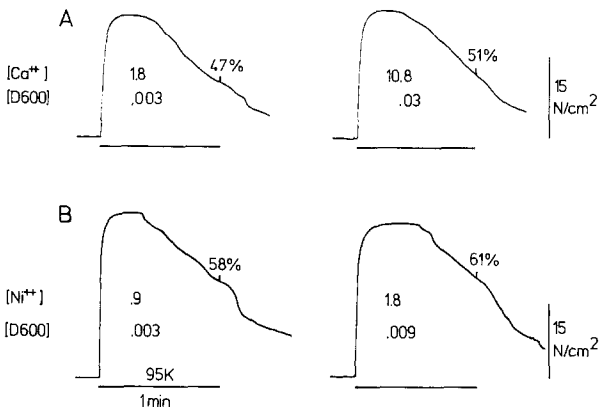

Fig. 5. Effect of different concentrations of Ca^{2+} (*A*) or Ni^{2+} (*B*) and D600 on contracture tension. Contractures evoked by application of 95 mmol/liter K^+-Ringer's containing the concentrations of divalent cations and D600 indicated near records. Note that the effect of a higher concentration of D600 can be balanced by increasing the concentration of divalent cations; diameters of fibers were 68 μm (*A*) and 65 μm (*B*)

ing the concentration of divalent cations. This antagonism was investigated in more detail in the present experiments by systematically varying the concentrations of D600 and divalent cations of the contracture solution. Figure 5*A* shows contractures which were evoked with 95 mmol/liter K^+-Ringer's containing different concentrations of D600 and Ca^{2+}; these records were selected from a longer series of records in order to illustrate that similar contracture tensions (1-min values) could be obtained with low concentrations of both D600 and Ca^{2+}, or with a higher D600-concentration combined with an appropriately increased concentration of Ca^{2+}. Mean values obtained in several experiments of the same type are plotted in Fig. 6A. It clearly shows that, as the concentration of D600 increases, more Ca^{2+} is required to maintain tension at the same level. The figure also shows that the experimental values can be fitted reasonably well with hyperbolic functions.

Qualitatively similar results were obtained with D600 and Ni^{2+} (Figs. 5*B* and 6*B*). Again, experimental values of contracture tension could be fitted by hyperbolic functions, and the corresponding Lineweaver-Burk plots resulted in straight lines intersecting at the same point near $1/F_{max}$ (Fig. 6*B*). The slopes of the straight lines as obtained in Fig. 6*A* and *B* were plotted against the concentration of D600 and fitted with straight lines. From their intercepts with the abscissa apparent K_D values of 2.2 and 4.2 mmol/liter were determined for D600 binding in the presence of Ca^{2+} or Ni^{2+}, respectively. The higher binding affinity of Ni^{2+} as compared with Ca^{2+} is evident also from this type of analysis.

C) Site of Action of D600 and Divalent Cations

In the course of previous (Schmidt et al., 1988) as well as in the present experiments it became apparent that slow fibers need not be equilibrated in D600 for 10 min (as described for twitch fibers by Eisenberg et al., 1983) in order to observe its full effect. Instead, the effect of D600 on the K^+ contracture is virtually the same when it is applied together with the high K^+ concentration used to evoke the contracture. Thus, it seemed obvious that D600 reaches its site of action rather quickly. In order to find out whether the presumed binding site might be located on the free membrane surface, we applied D600 and divalent cations during the K^+ contracture when tension had passed its peak value. One of these experiments is shown in Fig. 7. First, a contracture was evoked with 95 mmol/liter K^+-Ringer's containing normal Ca^{2+} concentration. After a sufficiently long wash interval the same contracture solution was reapplied, but 18 sec later (arrow) the external Ca^{2+} concentration was increased from 1.8 to 14.4 mmol/liter. Figure 7 shows that the contracture continued unchanged for about 6 sec and then started to deviate from the control record. Thus the latency after which Ca^{2+} exerts its effect may be taken to be 6–9 sec. A third contracture was evoked again with the usual contracture solution, but by 18 sec D600 was added to the medium. In this case it took 12–18 sec until the contracture tension began to decrease more quickly than under control conditions. It should be noted that these latencies cannot be due to delayed concentration changes in the bath because the muscle chamber was perfused at high speed (*see* Materials and Methods). Experiments of the same type were done with a total of six slow fibers; the latencies for the action of D600 varied between 9 and 30 sec (mean 17.8 ± 4.4 sec; $n = 4$), while those for Ca^{2+} were 6–16 sec (mean 8.6 ± 1.3 sec; $n = 6$). In four of these fibers latencies for D600 and Ca^{2+} were compared, and they were always twice as long for D600 as for Ca^{2+}. It thus seems obvious that divalent cations as well as D600 bind to a site which is not immediately available, and therefore is not located at the external free surface of the slow fiber membrane.

Discussion

In the present experiments the effect of increasing concentrations of Ni^{2+}, Mn^{2+} or Mg^{2+} on maintained contracture tension of slow muscle fibers was compared with that of Ca^{2+}. It could be shown that all divalent cations improve maintenance of

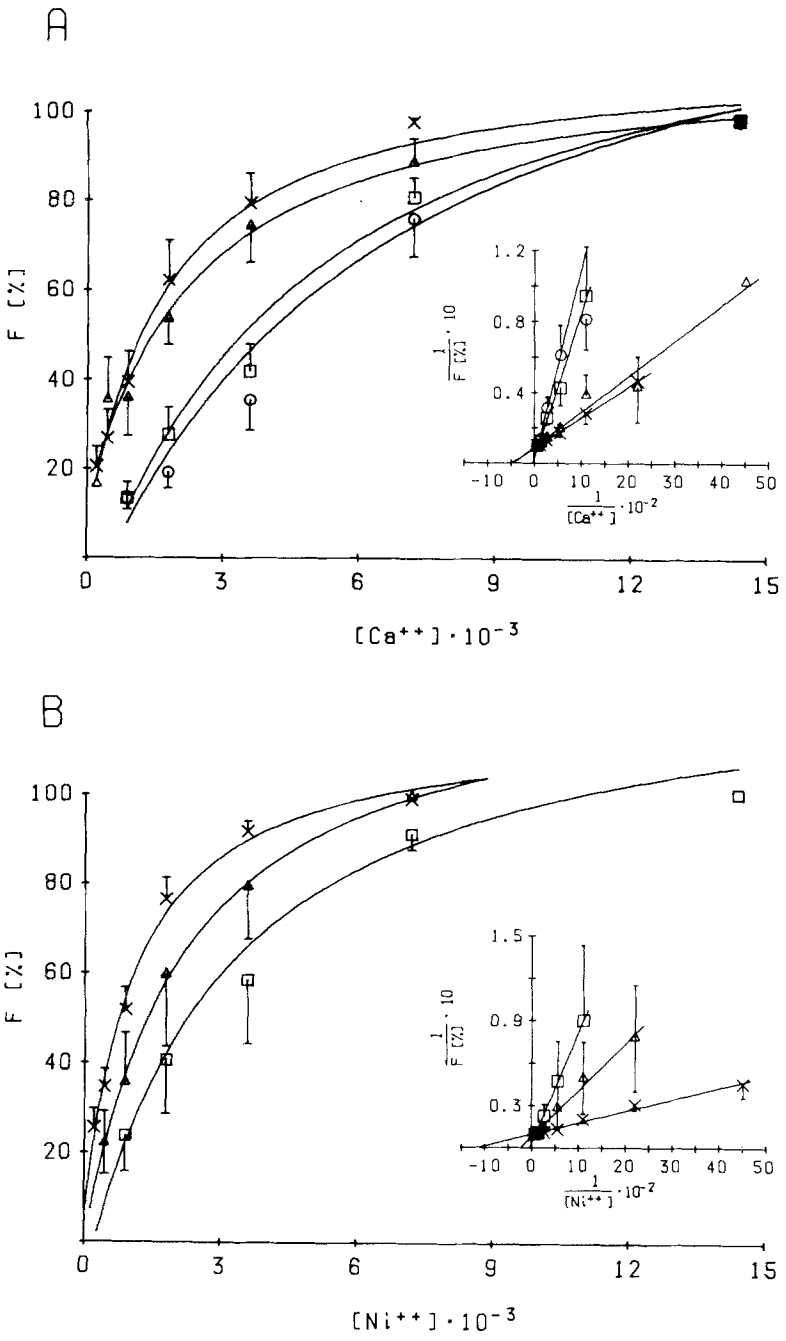

Fig. 6. Effect of different concentrations of D600 on maintained tension (1 min values) recorded in 95 mmol/liter K^+-Ringer's containing the concentrations of Ca^{2+} (*A*) or Ni^{2+} (*B*) indicated on the abscissae. Concentrations of D600 were 0 (×), 3 (△), 9 (□) or 12 (○) μmol/liter. Mean values ± SEM from 4–10 experiments fitted with a hyperbolic function. Note that depressing effect of D600 on maintained tension is counteracted by the divalent cations. *Insets:* Relation between reciprocal values of contracture tension and concentration of Ca^{2+} (*A*) or Ni^{2+} (*B*) as shown in the diagrams. Experimental values were fitted with straight lines which have different slopes but intersect near $1/F_{max}$. Note that Ni^{2+} acts in the same way as Ca^{2+}, but with a higher degree of efficiency

tension in a dose-dependent manner, all dose-response curves being hyperbolic. With Ni^{2+}, Ca^{2+} and Mn^{2+} fully maintained tension was observed in the range between 7.2 and 14.4 mmol/liter, while a much higher concentration (>28.8 mmol/liter) of Mg^{2+} was required. Hence the binding capacity of Mg^{2+} is clearly much less than that of the other divalent cations examined. These conclusions are strengthened by the results of corresponding Lineweaver-Burk plots. The straight lines have different slopes for the divalent cations studied, but they intersect at the same point near $1/F_{max}$. Moreover, the Hill plots revealed straight lines with slopes around 1. It can thus be concluded that all divalent cations examined bind in a 1 : 1 reaction to the same site, which is intimately related to the mechanism of inactivation of contractile force in the slow muscle fiber.

Since the work of Frank (1962) on whole frog muscle it is known that divalent cations are able to replace Ca^{2+} in its effect on contractile force (for literature *see also* Caputo, 1983). In a more recent

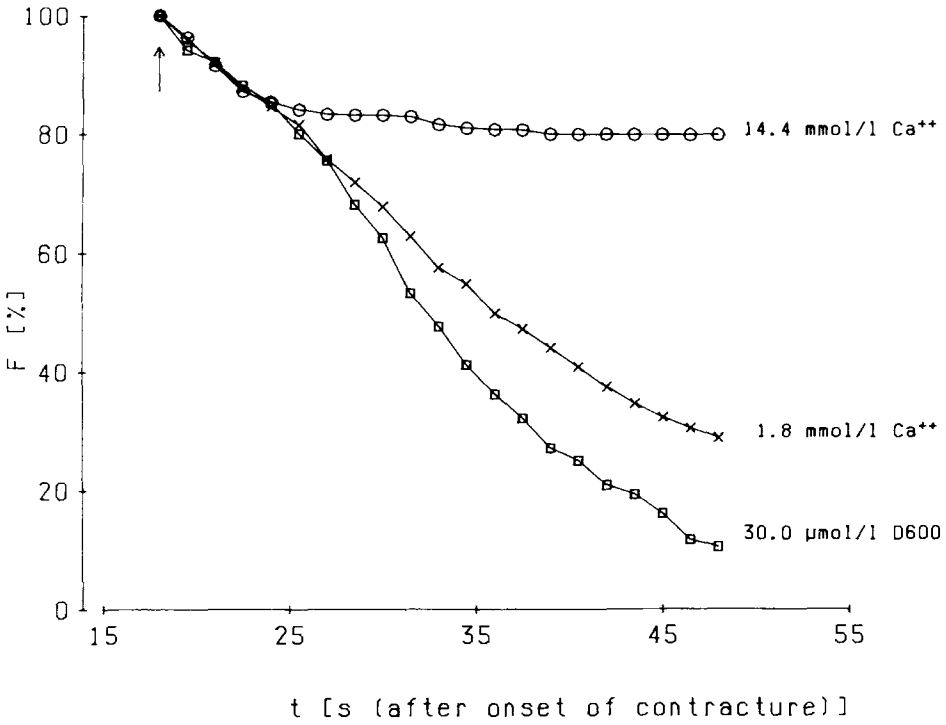

Fig. 7. Time course of contractures evoked with 95 mmol/liter K^+-Ringer's. Three contractures were elicited successively at intervals of 20–30 min. The control contracture (×) was performed with constant Ca^{2+} concentration (1.8 mmol/liter). 18 sec after beginning of the second contracture (arrow) the Ca^{2+} concentration was increased from 1.8 to 14.4 mmol/liter (○). During the third contracture (□) the Ca^{2+} concentration remained constant at 1.8 mmol/liter, but 18 sec after beginning (arrow) D600 (30 µmol/liter) was added to the medium. Note that contracture curves after addition of Ca^{2+} or D600 start to deviate from control curve with different latencies. Contracture tension at t = 18 sec was taken as 100%. Diameter of slow fiber was 56 µm; F_{max} = 39 N/cm²

paper Lorković and Rüdel (1983) found evidence "for interaction of Ca^{2+} and Co^{2+} (or Ni^{2+}) at some site on the muscle membrane" responsible for regulation of contracture duration in frog twitch fibers. Thus, the slow muscle fiber does not seem to be unique as regards (competitive) binding of divalent cations.

Binding of Divalent Cations in the Presence of D600

When D600 was added to the contracture solution the dose-response curves for Ca^{2+} and Ni^{2+} remained hyperbolic, but with increasing concentrations of D600 more Ca^{2+} or Ni^{2+} was required to fully maintain contracture tension. Lineweaver-Burk plots still showed straight lines intersecting near $1/F_{max}$, thus indicating that Ca^{2+}, Ni^{2+} and D600 compete for the same binding site.

In a number of muscle preparations organic and inorganic Ca^{2+} channel blockers have been compared as to their effects on contractile properties; yet, competition for the same binding site has not been reported so far (Huerta, Muñiz & Stefani, 1986; Kotsias, Muchnik & Obejero Paz, 1986; Léoty & Noireaud, 1987). However, in Ca^{2+} channels of mammalian skeletal muscle microsomes binding of verapamil (a close analogue of D600) and other Ca^{2+} channel blockers was inhibited by divalent cations (Goll, Ferry & Glossmann, 1984). These authors found a series of efficiency (Mn^{2+} > Ca^{2+} > Mg^{2+}) which is similar to that observed in the present and earlier (Schmidt et al., 1988) experiments on maintained tension.

Nature and Localization of Binding Sites

There is no indication that in slow fibers of *R. temporaria* maintenance of contracture tension is related to Ca^{2+} influx (Miledi, Parker & Schalow, 1981; Schmidt, 1987; Schmidt et al., 1988), although small Ca^{2+} currents have been found in some of the fibers investigated by Zacharovà et al. (1985). Nevertheless, organic and inorganic Ca^{2+} channel blockers strongly affect maintenance of tension in the same concentration range as has been shown for their effect on Ca^{2+} channels by Palade and Almers (1985). There is, however, a substantial difference:

with respect to maintenance of tension the two classes of substances exert antagonistic effects with only small quantitative differences between the favorable effects of Ca^{2+}, Ni^{2+} and Mn^{2+}. Even Mg^{2+} supports maintenance of tension, although much less than the other cations examined. In addition, we were able to show (Fig. 7) that the site binding divalent cations and D600 is not immediately accessible from outside, because both species of substances start to act only after a delay. It has been calculated that changes of the Ca^{2+} concentration inside the transverse tubular system of twitch fibers occur within several sec (Almers, Fink & Palade, 1981; Lorković & Rüdel, 1983). Since the slow fibers exhibit a much less developed tubular system (Flitney, 1971; Franzini-Armstrong, 1973) with sparsely occurring dyadic and triadic junctions, a latency of about 9 sec for the action of Ca^{2+} is conceivable. D600 is likely to diffuse more slowly than Ca^{2+}, and its latency of action should therefore be longer. In fact, we measured a mean value of about 18 sec. Thus, we come to the conclusion that the site of action of both inorganic and organic Ca^{2+} channel blockers is located inside the transverse tubular system, presumably at the junctions with the sarcoplasmic reticulum. Corresponding conclusions were already drawn for the force controlling system of twitch fibers (Berwe, Gottschalk & Lüttgau, 1987; Caputo & Bolaños, 1987).

Taken together, these results suggest that we are dealing with a binding site that could be a modified Ca^{2+} channel. Its predominant function may no longer be that of a conducting ion channel, but it has preserved its binding properties towards various types of Ca^{2+} channel blockers; in addition, it has acquired the property of being able to control maintenance of tension from outside the cell by a mechanism still not fully understood. Recently, it was shown in twitch fibers that the dihydropyridine receptor is involved in the control of intracellular Ca^{2+} concentration during excitation-contraction coupling (Rios & Brum, 1987). This receptor is highly concentrated in transverse tubular membrane preparations of rabbit and frog muscle, and dihydropyridine binding is noncompetitively reduced by verapamil and by Ni^{2+}, Co^{2+}, $Mn^{2+}$and Ca^{2+}. The potency sequence of these divalent cations (Fosset et al., 1983) is similar to that found in our experiments on frog slow muscle fibers. D600, on the other hand, has been shown to "paralyze" twitch fibers by blocking intramembrane charge movement (Hui, Milton & Eisenberg, 1984), which is regarded an essential step in excitation-contraction coupling (Schneider & Chandler, 1973; Adrian, Chandler & Rakowski, 1976). Since immobilization of charge movement leads to contractile inactivation, not only in twitch fibers (Chandler, Rakowski & Schneider, 1976) but also in frog slow fibers (Gilly & Hui, 1980), it is tempting to speculate that most of the Ca^{2+} channels originally existing in the transverse tubular system of slow muscle fibers may have been transformed into nonconducting molecules which control contractile inactivation by using the voltage-sensing part of the original channel. Prolonged depolarization (e.g., during K^+-contracture) presumably reduces the affinity for Ca^{2+} and increases the affinity of the receptor molecule for Ca^{2+} channel blockers. Binding of these substances thus effectively stabilizes the inactivated state, as has been proposed to be the mechanism of "paralysis" in twitch fibers (Berwe et al., 1987; Siebler & Schmidt, 1987; Brum et al., 1988).

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