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Effects of Ca^{2+} and Other Divalent Cations on K^+-Evoked Force Production of Slow Muscle Fibers from *Rana esculenta* and *Rana pipiens*

P. Krippeit-Drews* and H. Schmidt
Physiologisches Institut der Universität des Saarlandes, D-6650 Homburg/Saar, Federal Republic of Germany

Summary. Slow muscle fibers were dissected from *cruralis* muscles of *Rana esculenta* and *Rana pipiens*. Isometric contractures were evoked by application of K^+-rich Ringer's containing Ca^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+} or Mg^{2+}. High (7.2 mmol/liter) external Ca^{2+} concentration raised, 0 Ca^{2+} lowered the K^+ threshold. Replacing Ca^{2+} by Ni^{2+} or Co^{2+} had an effect similar to that of high Ca^{2+} Ringer's. In Mg^{2+} Ringer's the K^+ concentration-response curve was flattened. These effects were observed already after short exposure times in both species of slow fibers. When Ca^{2+} was removed for long periods of time the slow fibers of *R. esculenta* lost their contractile response to application of high K^+ concentrations much more quickly than those of *R. pipiens*, while the response to caffeine (20 mmol/liter) was maintained. Upon readmission of Ca^{2+} contractile ability was quickly restored in the slow fibers of both *R. esculenta* and *R. pipiens*, but the effects of Ni^{2+} (or Co^{2+}, Mn^{2+} and Mg^{2-}) were much larger in *R. esculenta* than in *R. pipiens* slow fibers. It is concluded that divalent cations have two different sites of action in slow muscle fibers. K^+ threshold seems to be affected through binding to sites at the membrane surface; these sites bind Ni^{2+} and Co^{2+} more firmly than Ca^{2+}. The second site is presumably the voltage sensor in the transverse tubular membrane, which controls force production, and where Ca^{2+} is the most effective species of the divalent cations examined.

Key Words slow muscle fibers · K^+ contractures · divalent cations

I. Introduction

There is general agreement that extracellular divalent cations play an important role in excitation-contraction coupling (ECC) of skeletal muscle fibers. Recent experiments on twitch fibers of the frog have shown, that the importance of divalent cations resides in their binding to a membrane site which is presumably located in the transverse tubular system and which senses the transmembrane voltage. This voltage sensor is coupled to the Ca^{2+}-release channel of the sarcoplasmic reticulum (Pizarro et al., 1988), and intramembrane charge movement (Schneider & Chandler, 1973; Adrian, Chandler & Rakowski, 1976) is regarded to be an essential step in this coupling process. Under normal conditions the voltage sensor is occupied by Ca^{2+}, but other divalent cations can replace Ca^{2+} in its function in ECC, while monovalent cations are much less effective (Brum et al., 1988; Pizarro et al., 1989).

ECC in frog slow muscle fibers exhibits many similarities to that of twitch fibers (Gilly & Hui, 1980). In both types of fibers, extracellular Ca^{2+} is important, since 10–15 min after omission of Ca^{2+} from the medium twitch and slow fibers were found unable to develop force upon application of K^+-rich solutions (Lüttgau, 1963; Frank, 1964; Nasledov, Zachar & Zacharová, 1966). Moreover, it was shown by Nasledov et al. (1966) and Lännergren (1967) that after reducing or increasing the Ca^{2+} concentration of the medium the K^+-threshold for slow fibers from *R. esculenta* and *R. temporaria* as well as for slow fibers of *Xenopus laevis* decreased or increased, respectively, just as had been shown for frog twitch fibers (Lüttgau, 1963; Frankenhaeuser & Lännergren, 1967).

In the present experiments the above-mentioned investigations were extended by studying K^+ contractures of slow fibers during prolonged exposure to media from which Ca^{2+} was omitted. In addition the capability of foreign divalent cations to replace Ca^{2+} in its effect on force production was examined. Some of the results were presented to the German Physiological Society (Krippeit-Drews & Schmidt, 1990).

II. Materials and Methods

The experiments were done on 81 single slow fibers dissected from *cruralis* muscles of *R. esculenta* (*R.e.*); these fibers had a

* *Present address:* Laboratoire de pharmacologie, UCL, Av. Mounier 73, B-1200 Brussels, Belgium

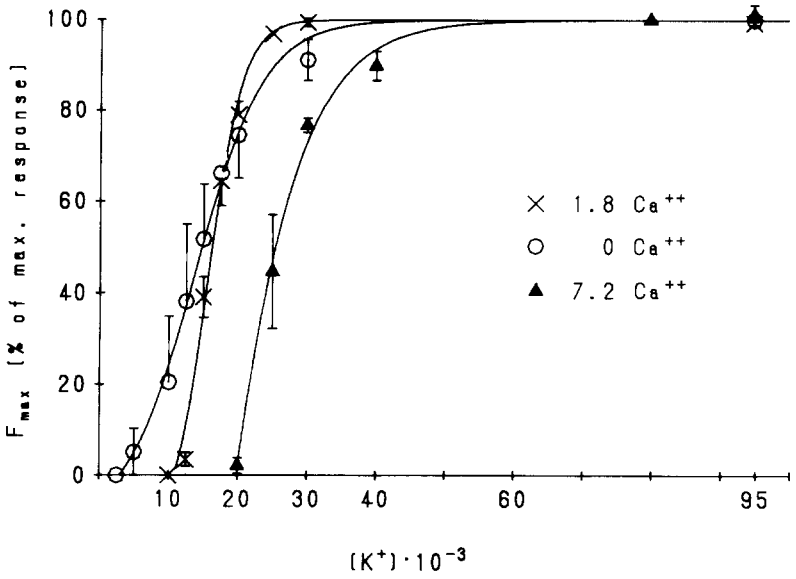

Fig. 1. Relationship between peak contracture tension (ordinate) and K^+ concentration of bath solution (abscissa) containing 0 (open circles), 1.8 (crosses) or 7.2 (filled triangles) mmol Ca^{2+}/liter. Fibers were bathed in the contracture solutions until peak tension was reached (between 20 sec in high, and 4 min in low K^+ concentrations). Contractures in 0 or 7.2 mmol Ca^{2+}/liter were evoked after presoaking periods of 60 sec each in Ringer's containing the corresponding Ca^{2+} concentration. Between contractures the fibers were returned to normal Ringer's. Mean values ± SEM (3–18 fibers). Note changes of K^+ threshold in 0 and 7.2 mmol Ca^{2+}/liter.

mean diameter of 58.5 ± 1.4 μm, with only 12% of the fiber population being below 50 μm. Comparative experiments were also performed on 25 slow fibers from *R. pipiens* (*R.p.*). Although it was always attempted to select slow fibers with the largest possible diameter, none of these fibers measured more than 48 μm (mean 37.9 ± 1 μm). Contracture tensions obtained from these two species were much the same (30 to 40 N/cm²) as those developed by slow fibers from *R.t.* upon application of 95 mmol K^+/liter (Schmidt, Siebler & Krippeit-Drews, 1988).

Slow fibers were selected as previously described (Lüttgau, 1963; Lehmann & Schmidt, 1979), and contractures were recorded isometrically using an improved version (Schmidt et al., 1988) of the device originally described by Lehmann and Schmidt (1979). All solution changes were made automatically by operating electromagnetic valves. The resting tension was set to approximately 0.5 N/cm². Fiber diameters were usually determined before and after the experiments by taking several values along the fiber length.

A. Measurement of Resting Potential

A few isolated slow fibers from *R.e.* were impaled with conventional microelectrodes (dc resistance ≥ 50 MΩ) after prolonged exposure to Ca^{2+}-free Ringer's. Comparative measurements in different solutions were not done because of the high susceptibility of the slow fibers to damage produced by repeated impalements (Stefani & Steinbach, 1969; *unpublished results*).

B. Solutions

Ringer's solution had the following composition (in mmol/liter): NaCl 110.4; KCl 2.5; $CaCl_2$ 1.8; HEPES 5.0; pH 7.3. K^+-rich solutions were obtained by equimolar replacement of NaCl by KCl. Solutions named Ca^{2+}-free in this paper were obtained by omitting the 1.8 mmol Ca^{2+}/liter without adding Ca^{2+} chelators. In a few experiments on slow fibers from *R. pipiens* EGTA (1 mmol/liter) and Mg^{2+} (3 mmol/liter) were added to the Ca^{2+}-free solutions, and some of these solutions were buffered with imidazol. Results obtained under these experimental conditions will be dealt with separately in the text. Foreign divalent cations were added as chloride salts (Merck AG, Darmstadt, Germany). The temperature of the solutions was kept between 18 and 20.5°C.

III. Results

When the Ca^{2+} concentration of the bath solution was changed, it could be noted that some effects on contractile properties of the slow fibers were already fully developed shortly afterwards, while others were observed only after long-lasting exposures of the fibers to the test medium.

It must also be noted that some of the slow fibers dissected from *R.p.* showed a slowly developing transient increase in resting tension, when the Ca^{2+} concentration was reduced below 0.2 mmol/liter for prolonged periods of time. This effect was more pronounced in Ca^{2+}-free solutions containing EGTA (*see* section B.3.).

The origin of this increase in resting tension is unknown; it does not appear to be due to an increase in sodium permeability, because it could not be avoided by exchanging TEA^+ for Na^+. Further experiments are required to elucidate the mechanism of this observation, which was never made in slow fibers of *R.e.*

A. Rapid Effects

Figure 1 shows the relation between the K^+ concentration of the medium and the amplitude of the contracture evoked by the corresponding solutions. At normal Ca^{2+} concentration (1.8 mmol/liter) the K^+

threshold was near 12.5 mmol/liter, but with 30 mmol/liter maximum contracture amplitudes were already recorded. When Ca^{2+} was omitted 1 min prior to the application of the contracture solution, the K^+ threshold was lowered to about 3 mmol/liter and the concentration-response curve was slightly flattened. An increase of the Ca^{2+} concentration to four times normal had the opposite effect, namely an increase in the K^+ threshold value to about 20 mmol/liter. In all three Ca^{2+} concentrations examined the maximum contracture amplitude (evoked with 40 mmol K^+/liter or more) was unchanged.

Slow fibers of *R.p.* showed the same behavior as those of *R.e.*, and these results are therefore in good agreement with those obtained earlier in frog and toad slow fibers (Nasledov et al., 1966; Länner-gren, 1967).

1. Replacement of Ca^{2+} by Foreign Divalent Cations

When the external Ca^{2+} was replaced by an equivalent amount of Mg^{2+} for 1 to 10 min, the K^+ concentration-response curve was flattened, but a marked shift of the threshold K^+ concentration could not be noted (Fig. 2*A*). When Ni^{2+} or Co^{2+} replaced Ca^{2+} an effect similar to that of an increase in Ca^{2+} concentration was noted (Fig. 2*B*). In all these experiments maximum force as obtained with K^+ concentrations of 60–95 mmol/liter was virtually the same. Again, slow fibers of *R.p.* did not differ appreciably from those of *R.e.*

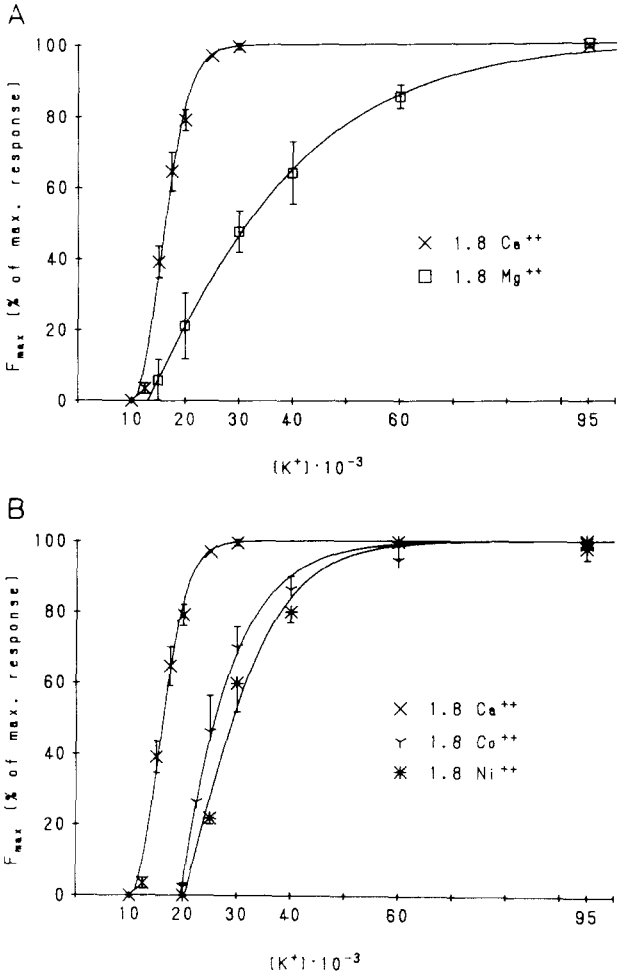

Fig. 2. Relationship between peak contracture tension and K^+ concentration of bath solution containing 1.8 mmol/liter of Ca^{2+} (same curve as in Fig. 1), Mg^{2+} (*A*), Ni^{2+} or Co^{2+} (*B*). Insets show symbols for the various divalent cations which replaced Ca^{2+} 10 min before evoking the contractures. Note unchanged K^+ threshold and flattening of curve in Mg^{2+}; in the presence of Ni^{2+} or Co^{2+} there is a marked increase of the K^+ threshold. After only 1-min replacement of Ca^{2+} by the foreign divalent cations the observed effects were virtually the same, flattening of the relationship in Mg^{2+} being somewhat less pronounced. Mean values ± SEM obtained from 6 (Mg^{2+}) and from 2–18 slow fibers (Ni^{2+} and Co^{2+}).

B. Effects Observed after Long Exposure Times

This series of experiments revealed rather large differences between slow fibers of *R.e.* and *R.p.* Therefore the results will be described separately, those from *R.e.* in sections B.1. and B.2., results from *R.p.* in section B.3.

1. Ca^{2+} Removal from the Medium (R.e.)

Figure 3 shows the effect of 10–60 min Ca^{2+} deprivation on the amplitude of the contractures evoked by 20-sec application of 95 mmol K^+ Ringer's. Contractures in normal Ca^{2+} (1.8 mmol/liter; *a*) and after presoaking the fibers for 10 (*b*), 20 (*c*), and 40 (*d*) min in Ca^{2+}-free Ringer's are shown in the inset of Fig. 3. After each test contracture the fibers were returned to normal Ringer's for at least 30 min and complete restoration of the contracture amplitude was verified. It can be noted that within 40–60 min of Ca^{2+} deprivation the slow fibers became unresponsive to high K^+ Ringer's, and as will be shown below, this effect of Ca^{2+} removal was completely reversible.

A much faster decrease of the K^+ contractures was observed when the K^+ applications were repeated at 5-min intervals in the continued absence of Ca^{2+} (Fig. 4*A*). In this case only about 15 min were required to completely block contractility. This accelerated loss of contractile force is presumably due to insufficient repriming between the contractures, because it has already been shown, that

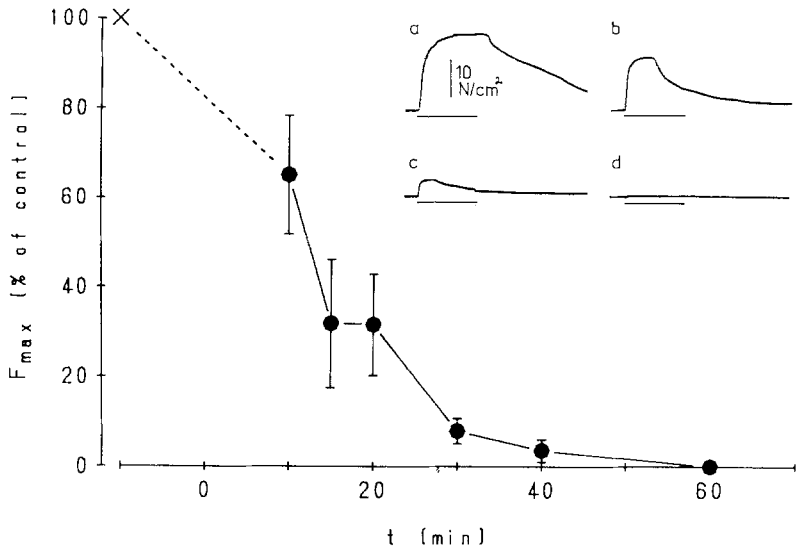

Fig. 3. Amplitude of contracture (in % of control value, cross) elicited by application of 95 mmol K^+/liter as obtained after soaking slow fibers in Ca^{2+}-free Ringer's for periods of time given on the abscissa. Between contractures the fibers were returned to normal Ringer's until contractile force was restored. Note, that contractures were abolished after 40–60 min. Inset shows original contractures recorded from one of the fibers in 1.8 mmol Ca^{2+}/liter (*a*), and 10 (*b*), 20 (*c*) and 40 (*d*) min after omission of Ca^{2+} from the bath solution. Horizontal lines below records indicate 20-sec periods of application of the contracture solution. Mean values ± SEM of 3–5 fibers.

reduction of external Ca^{2+} considerably slows recovery of contractile force following exposure to K^+-rich solutions (Schmidt et al., 1988). Figure 4*A* also illustrates that the fibers completely recovered within about 10 min after return into normal (1.8 mmol/liter) Ca^{2+}.

Abolition of the K^+ contracture by removal of extracellular Ca^{2+} was not accompanied by any appreciable decrease of the amplitude of contractures evoked by a high caffeine concentration (Fig. 4*B*); moreover, the decrease in K^+-evoked active tension following removal of Ca^{2+} was never accompanied by an increase in resting tension in the slow fibers of *R.e.*

An attempt was made to find out whether the loss of active tension was correlated with depolarization of the membrane. Six slow fibers from *R.e.* were therefore kept in 0 Ca^{2+} Ringer's for 30–60 min; microelectrode insertion revealed resting potentials between −46 and −72 mV (mean −59.8 ± 3.9 mV). These values are in the same range as those frequently observed in Ringer's containing 1.8 mmol Ca^{2+}/liter (Stefani & Steinbach, 1969; Forrester & Schmidt, 1970).

2. *Replacement of Ca^{2+} by Foreign Cations (R.e.)*

In the following experiments K^+ contractures were repeated at 10-min intervals in Ca^{2+}-free Ringer's until the contracture amplitude had fallen to values near 0%. Then Ni^{2+}, Co^{2+}, Mn^{2+} or Mg^{2+} (1.8 mmol/liter each) were added for the following 2 hr. As Fig. 5 shows, the contracture ability of the slow fibers recovered quickly and almost fully in media containing Ni^{2+}, Co^{2+} or Mn^{2+}; upon admission of Mg^{2+}, however, the contracture amplitude was restored to only about 40% of the control value.

These results were compared with experiments (on the same fibers) in which the foreign divalent cations replaced Ca^{2+} for the same period of time without prior abolition of the K^+ contracture by omission of Ca^{2+}. It can be seen, that—with the exception of Mg^{2+}—only between 5 and 20% of the initial tension was lost. Comparing the two sets of experiments it can be stated that the effects of the foreign divalent cations were largely independent of the experimental protocol used. In all cases, return to the normal Ca^{2+} concentration restored the contracture amplitude to its initial values.

Thus, although the fibers were kept in Ca^{2+}-free solutions for more than 2 hr, the ability to produce contracture tension was almost normal provided the external medium contained Ni^{2+}, Co^{2+}, or Mn^{2+} at the same concentration as Ca^{2+}.

3. *Effect of Ca^{2+} Removal and Other Divalent Cations on Amplitude and Maintenance of K^+ Contracture in Slow Fibers of R.p.*

Experiments were done in much the same way as in Figs. 4 and 5, contractures being repeated at intervals of 10 min. The amplitude of the 95-mmol K^+ contracture decreased rather slowly in the absence of Ca^{2+} (without addition of EGTA and Mg^{2+}), and after 60 min it was still 34, 81 and 85% of its initial value (three fibers), which is a considerably higher tension than that observed under the same condi-

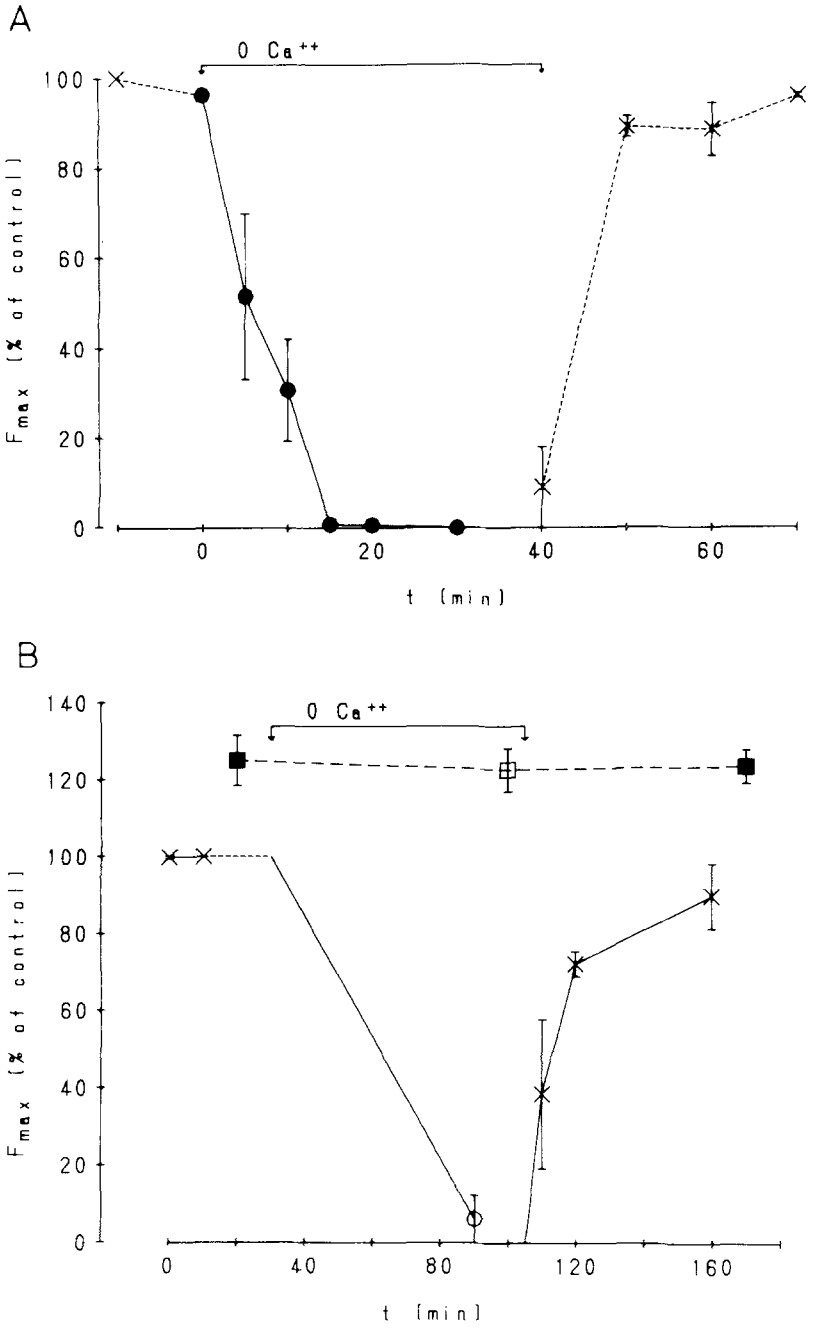

Fig. 4. Amplitude of K^+ contractures measured in the continued absence of Ca^{2+} from the medium (horizontal lines). In (*A*) contractures were evoked by 20-sec application of 95 mmol K^+/liter at 5-min intervals during the early phase after omission of Ca^{2+}. Note that contractile force was lost within 15 min (*A*), but it was restored quickly and completely following reapplication of Ca^{2+}. In (*B*) only one K^+ contracture was evoked 60 min following removal of Ca^{2+}; nevertheless, its amplitude was very small, while that evoked by 20 mmol caffeine/liter was practically unchanged 10 min later. Mean values ± SEM obtained from 3–7 slow fibers in *A* and from 3 fibers in *B*

tions in slow fibers of *R.e.* In Fig. 6 an experiment is shown in which exposure to Ca^{2+}-free Ringer's was continued for 315 min. The slow fiber (similar to others) had transiently increased its resting tension at normal K^+ concentration (small dots), but this decreased steeply together with the active tension developed upon application of high K^+ solution. After more than 6 hr in Ca^{2+}-free medium the fiber was unresponsive to K^+. Addition of 1.8 mmol Mg^{2+}/liter restored contractile force to only 4% (within 70 min), and the following replacement of Mg^{2+} by Ni^{2+} increased it to only 7% (within 60 min). In two more slow fibers 1.8 mmol Ni^{2+}/liter restored the contracture amplitude to 30 and 50% within 40 and 50 min, respectively. In large contrast to these observations, return to 1.8 mmol Ca^{2+}/liter

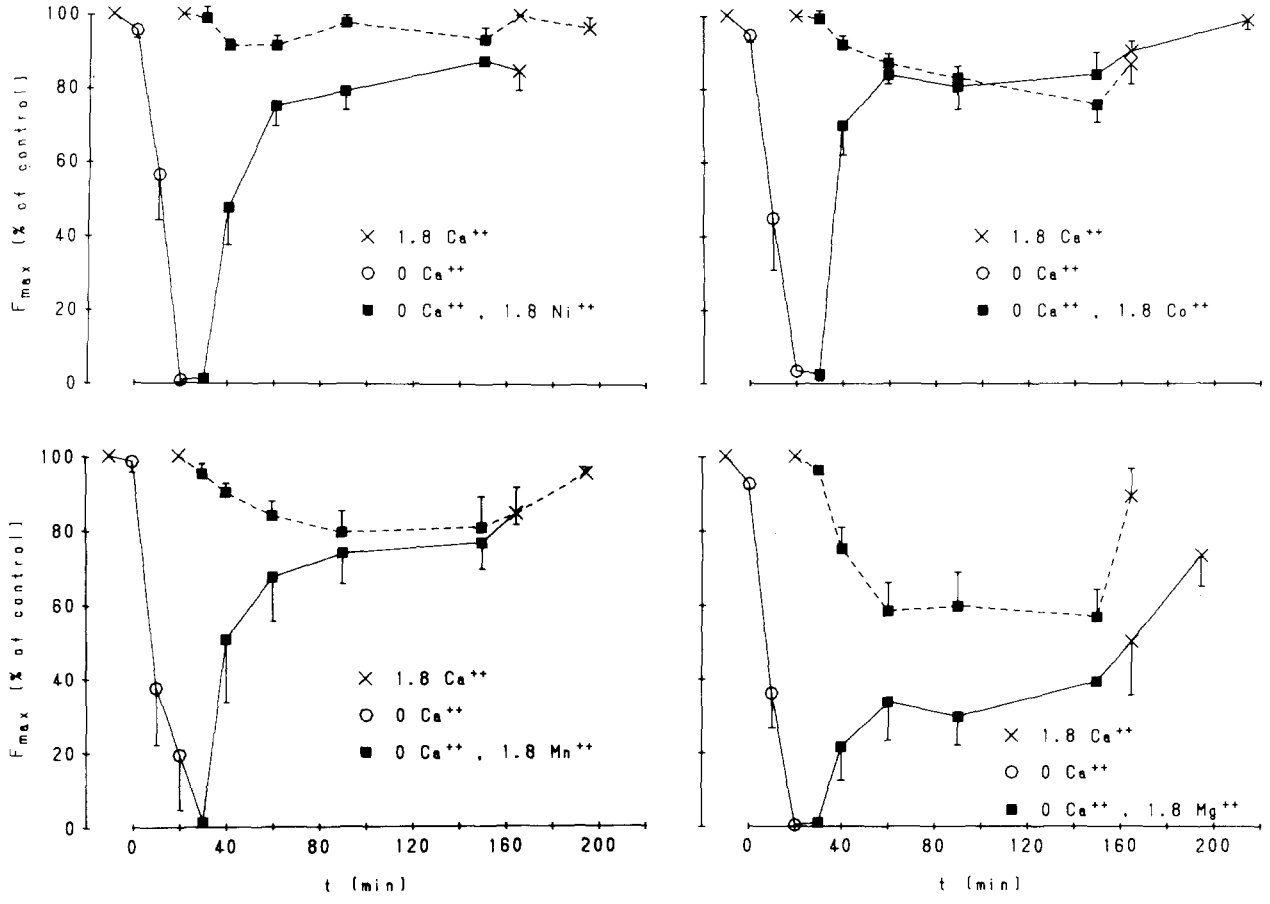

Fig. 5. Effects of substituting Ni^{2+}, Co^{2+}, Mn^{2+} or Mg^{2+} for Ca^{2+} (1.8 mmol/liter each) on amplitude of K^+ contracture. Ringer's solution containing 95 mmol K^+/liter was applied for 20 sec at regular intervals of 10 min each. Two experimental protocols were followed with each of the slow fibers examined. First, Ca^{2+} was omitted from the bath for 30 min which practically abolished the contractures in all cases; at the end of the last 10-min interval in 0 Ca^{2+} one of the four foreign divalent cations was added to the bath solution for a period of 2 hr (solid lines). Fibers were then returned to normal Ringer's (with 1.8 mmol Ca^{2+}), and after approximately 1 hr the second run was started (dashed lines). The same divalent cations replaced Ca^{2+} for 2 hr without passing through a Ca^{2+}-free period. Note that similar contracture amplitudes were observed with three of the foreign divalent cations independent of the protocol used. The effectiveness of Mg^{2+} to replace Ca^{2+} was clearly less than that of the other cations. Mean values ± SEM from 3–5 slow fibers.

after 7.5 hr in Ca^{2+}-free media restored the contracture ability to 90% within 10 (Fig. 6) to 20 min. Thus, Mg^{2+} and Ni^{2+} are considerably less effective in slow fibers of *R.p.* than in those of *R.e.* (Fig. 5).

The decrease of active tension observed after immersion in Ca^{2+}-free Ringer's was not dependent on the presence or on the amplitude of a change in resting tension. For instance, in a similar experiment on another slow fiber from *R.p.* active tension decreased to 3% of its control within 100 min but resting tension remained unchanged.

In view of the rather important differences between the slow fibers of *R.e.* and *R.p.* we made additional experiments to find out whether the slow fibers of *R.p.* also behaved differently as to the effect of Ca^{2+} on maintenance of contracture tension. These experiments seemed necessary because several years ago Huerta, Muñiz and Stefani (1986), using "bundles of tonic fibres" from *R.p.*, reported that Ca^{2+} restores contracture tension which had been lost during exposure to K^+-rich Ringer's containing EGTA and Mg^{2+} in the place of Ca^{2+}; Ni^{2+} did not exert this effect. We repeated the above-mentioned experiments of Huerta et al. (1986) under their conditions (solutions buffered with imidazol and containing 1 mmol EGTA/liter plus 3 mmol Mg^{2+}/liter) as well as with solutions buffered with HEPES. Single slow fibers as well as bundles consisting of several identified slow and twitch fibers from *R.p.* were used. The preparations were bathed for 5 min in 0 Ca^{2+} Ringer's; then contractures were evoked by increasing the K^+ concentration from 2.5

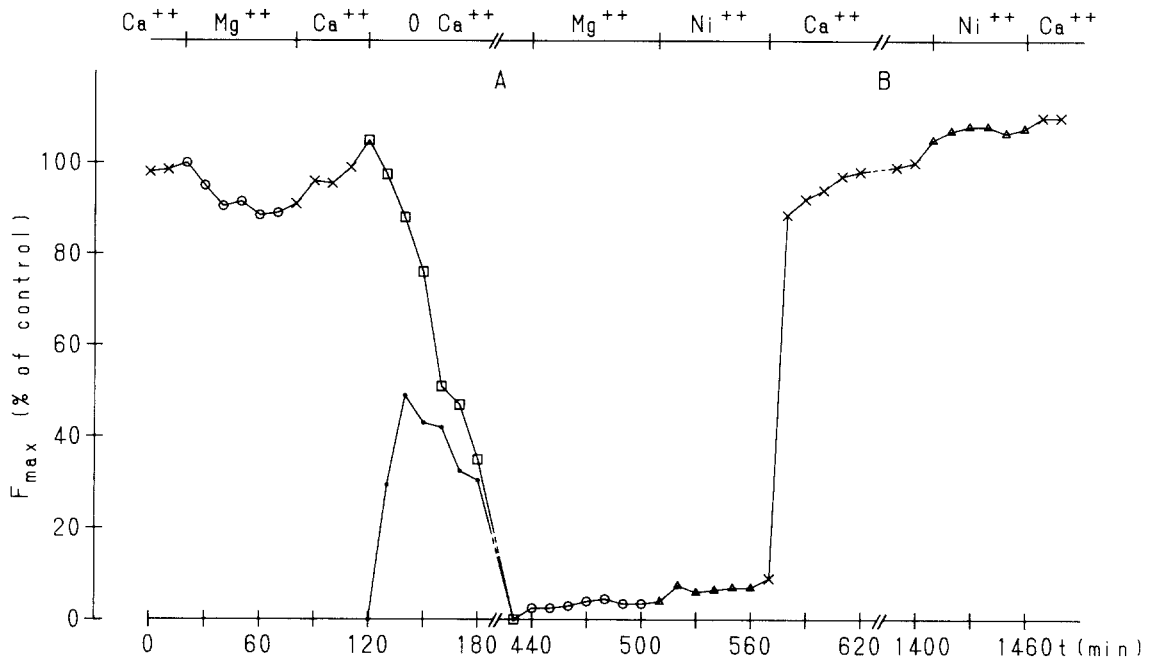

Fig. 6. Effects of equimolar substitution of Mg^{2+} (circles) or Ni^{2+} (triangles) for Ca^{2+} (1.8 mmol/liter; crosses) on amplitude of K^+ contracture. Contractures were repeated at 10-min intervals by applying 95 mmol K^+/liter for 20 sec each. Horizontal line indicates which of the three species of divalent cations was present in the bath. At points *A* and *B* the sequence of contractures was interrupted for about 4 and 13 hr, respectively. Note that the contracture amplitude decreased slightly when Mg^{2+} replaced Ca^{2+} (at t = 20 min) and that it hardly changed when Ca^{2+} was replaced by Ni^{2+} (at t = 1380 min). However, when Ca^{2+} was removed from the medium (0 Ca^{2+}) the contracture amplitude decreased to 35 and 0% within 60 min and 5 hr, respectively, and it was restored by a few percent only upon addition of Mg^{2+} (at t = 430 min) or Ni^{2+} (at t = 520 min). In contrast, readmission of Ca^{2+} after a total Ca^{2+}-free period of almost 8 hr restored the contracture amplitude from 7 to 89% within 10 min. Note also that in the absence of divalent cations (0 Ca^{2+}) the fiber developed resting tension (small dots) which disappeared by the end of the Ca^{2+}-free period (at t = 430 min). All results from one *R.p.* slow fiber, diameter 45 μm.

to 95 mmol/liter. In six experiments on pure slow fiber preparations (four single fibers and two bundles of two or three slow fibers) tension increased to a maximum and then decreased to 72.3 and 54.9% within 2 and 4 min, respectively. Addition of 3.6 mmol Ca^{2+} or Ni^{2+}/liter (with simultaneous omission of EGTA and Mg^{2+}) in none of these experiments restored contracture tension (Fig. 7). Instead, contracture tension was smaller by 9.2% (Ca^{2+}; six experiments), 8.9% (Ni^{2+}; four experiments) and 13.8% (Co^{2+}; one experiment) 2 min after adding the divalent cations. In three additional experiments on single slow fibers contractures were evoked with 40 (instead of 95) mmol K^+/liter; 3.6 mmol Ca^{2+}/liter were added 4 min later, and contracture tension again decreased by 2.3 to 8.6% within the following 4 min.

Similar results were obtained in five experiments on fiber bundles consisting of 5–10 slow and twitch fibers (maximum isometric tension 140–320 mg). The results are thus identical to those obtained earlier in slow fibers of *R.t.*and *R.e.*

We therefore conclude that maintained tension, which is lost during K^+ contractures, cannot be restored by addition of Ca^{2+} or Ni^{2+} to the medium; in this respect the slow fibers of *R. temporaria, esculenta* and *pipiens* exhibit identical behavior.

IV. Discussion

In the present series of experiments two main results were obtained. Following alterations of the external Ca^{2+} concentration force production of frog slow muscle fibers is affected in two different ways: (i) Changes of the K^+ threshold are observed rapidly after removal or addition of Ca^{2+} (or replacement of Ca^{2+} by other divalent cations). (ii) In contrast, maximum force (elicited with high K^+ concentrations) changes slowly after removal of Ca^{2+}. Time course of development and the efficiency sequence of the divalent cations examined are indicative of two different sites of action of the divalent cations.

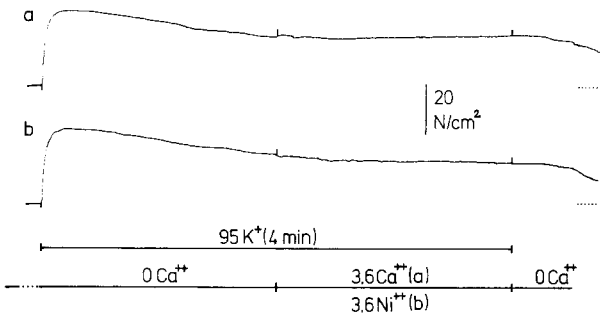

Figure 7. Effect of Ca^{2+} or Ni^{2+} on maintained contracture tension. Contractures were evoked by increasing the K^+ concentration of the medium from 2.5 to 95 mmol/liter 5 min after application of 0 Ca^{2+} Ringer containing EGTA (1 mmol/liter) and Mg^{2+} (3 mmol/liter). Two min later Ca^{2+} (*a*) or Ni^{2+} (*b*) (3.6 mmol/liter) were added, EGTA and Mg^{2+} being simultaneously omitted (horizontal lines). It can be seen, that both divalent cation species exert similar effects; tension is stabilized but not restored. Short vertical dashes indicate change of solutions. Preparation consisting of two identified slow fibers with 35 and 40 μm diameter (*R.p.*).

A. Rapid Effects

Removal or addition of Ca^{2+} shifts the K^+ threshold within less than a minute to lower or higher K^+ concentrations, respectively, without affecting the amplitude of contractures evoked with high K^+ concentrations in slow fibers from both *R.e.* and *R.p.* Insofar we have confirmed earlier results obtained in slow fibers of *R.t.* (Nasledov et al., 1966), slow fibers of *X. laevis* (Lännergren, 1967) or in frog twitch fibers (Lüttgau, 1963; Frankenhaeuser & Lännergren, 1967). There are minor deviations between the results reported by the various authors, but these seem to result from differences in exposure time and methods rather than from species differences.

Replacement of Ca^{2+} by foreign divalent cations also changes the K^+ concentration-force relation within 1 min, and there is little or no additional change afterwards. The effect of an equimolar replacement of Ca^{2+} by Ni^{2+} or Co^{2+} is similar to that of increasing the Ca^{2+} concentration, while replacement by Mg^{2+} acts as if Ca^{2+} had been removed from the medium. The rapidity of these effects suggests that the divalent cations act at the outer membrane surface by adsorbing to negative charges and thereby changing the electrical field within the membrane (as first suggested by Huxley, *see* Frankenhaeuser & Hodgkin, 1957). Since 1.8 mmol Ni^{2+}/liter (or Co^{2+}) act in much the same way as 7.2 mmol Ca^{2+}/liter the additional assumption is required that these foreign cations bind much stronger than Ca^{2+}; Mg^{2+} must do so less firmly because its effect resembles that of a reduction of the Ca^{2+} concentration, as has already been shown for frog twitch fibers (Dörrscheidt-Käfer, 1976). Thus, our results indicate that the efficiency sequence for the effect of divalent cations on surface charges of slow muscle fibers is $Ni^{2+} = Co^{2+} > Ca^{2+} > Mg^{2+}$.

B. Effects Observed after Long Exposure Times

While short time removal of Ca^{2+} from the medium does not appreciably affect force production by high K^+ concentrations, there is a strong effect when the exposure to Ca^{2+}-free media exceeds 10 min. In slow fibers of *R.e.* the ability to produce force upon application of 95 mmol K^+/liter is lost after 40–60 min. Contractile force can, however, be restored quickly to 80–90% of the control value by adding Ni^{2+}, Co^{2+}, or Mn^{2+} (1.8 mmol/liter) to the Ca^{2+}-free medium, while Mg^{2+} is much less effective. Even 2 hr later the fibers' ability to respond to high K^+ is preserved. These results clearly demonstrate, that force production upon membrane depolarization does not require Ca^{2+} as long as other divalent cations are present in the external medium.

As regards both the effect of Ca^{2+} removal on contractile force as well as the restorative effect of foreign divalent cations there was a large difference between the slow fibers of *R.e.* and those of *R.p.* The latter lost their ability to produce force (in response to high K^+ concentrations) much more slowly, and force restoration (after complete loss in Ca^{2+}-free solution) following addition of Mg^{2+} or Ni^{2+} (1.8 mmol/liter) was almost absent, while return to normal Ca^{2+} concentration quickly restored contractile ability. This behavior contrasts strongly with the observation of an almost unchanged contractile force when Ca^{2+} is removed and simultaneously replaced by Ni^{2+} or Mg^{2+}. We suggest, that in the continued absence of external Ca^{2+} the site binding divalent cations undergoes a change of its functional state which cannot be reversed by divalent cations, except Ca^{2+}. This effect must be much stronger in slow fibers of *R.p.* than in those of *R.e.*

There seems to be agreement amongst many authors that in twitch fibers the coupling process between membrane depolarization and initiation of contraction is controlled by a voltage sensor located in the transverse tubular membrane (*see* for instance Pizarro et al., 1988). Proper functioning of this voltage sensor requires binding of metal ions, the most effective ones being Ca^{2+}, while Mg^{2+} is less powerful (Brum et al., 1988; Pizarro et al., 1989). In the absence of metal ions the voltage sensor becomes inactivated, i.e., undergoes changes usually associated with membrane depolarization.

It is likely that a similar situation exists in frog slow fibers. However, in contrast to the results discussed in section IV.A., our observations demonstrate that Ni^{2+}, Co^{2+} and Mn^{2+} cannot fully replace Ca^{2+}; therefore their efficiency sequence is different from that for surface charges. Moreover, slow fibers from different animal species seem to differ slightly as regards the binding capacity of their voltage sensors for divalent cations but in all of them the efficiency sequence is $Ca^{2+} > Ni^{2+} = Co^{2+} = Mn^{2+} \gg Mg^{2+}$.

It has been mentioned above, that the voltage sensor is inactivated not only by lack of metal ions in the external solution but also by depolarization. Complete loss of active tension in 0 Ca^{2+} could have therefore resulted from a marked depolarization of the slow fiber membrane. However, 30–60 min after removal of Ca^{2+} resting potentials did not differ from values measured in normal Ringer's. In addition, marked depolarization should have evoked an increase in resting tension, which was never observed in slow fibers from *R.e.;* on the other hand loss of active tension in the slow fibers of *R.p.* occurred irrespective of an increase in resting tension.

Finally, Gilly and Hui (1980) showed that the quantity of moveable charge decreased only slightly in slow fibers held at 0-mV membrane potential for 30 min. Thus, even if we concede that our membrane potential measurements may not be completely reliable, we must conclude that depolarization (if it occurred at all) must have been small and could therefore have contributed only negligibly to the complete loss of contractile force observed in the absence of external Ca^{2+}.

An interesting and so far unexplained observation is that upon removal of Ca^{2+} some slow fibers from *R.t.* (Schmidt, 1987) and *R.p.* (present paper) respond with an increase in resting tension; this was never observed in the slow fibers from *R.e.,* and more experiments are needed to clarify the mechanism of this effect.

No difference between the slow fibers from *R.t.* (and *R.e.*) and those from *R.p.* was found as to the effect of divalent cations added during prolonged K^+ contractures, i.e., we never found re-establishment of lost contracture tension, neither did we observe a difference between Ca^{2+} and Ni^{2+} in this respect. On the contrary, both ion species tended to stabilize contracture tension near the value reached at the moment of application of the divalent cations. This result is clearly at variance with that of Huerta et al. (1986), although we tried to mimick the experimental conditions of these authors as closely as possible (buffer system, K^+ concentrations, exposure times). We can see only one major difference between the two sets of experiments: Huerta et al. (1986) examined fiber bundles presumably containing a substantial proportion of twitch fibers (as suggested by the time course of the K^+ contractures), while our experiments were done on single slow fibers and, for comparison, on a few fiber bundles, which were obviously smaller than those used by Huerta et al. (1986). Whether the size of the mixed fiber preparations or other factors (seasonal variations?) may be responsible for the observed discrepancies we are unable to decide.

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