Dependence of Contractile Responses by Some Calcium Antagonists on External Calcium in the Skeletal Muscle

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Abstract Dependence of contractile potentiation by calcium antagonists on external calcium was investigated on the frog's twitch muscle. Low concentrations (10^{-6} to 10^{-5} M) of nicardipine and verapamil enhanced the peak tension of both twitch and electrically induced contracture in the presence of calcium. In the calcium-free media the drugs suppressed the contractile tension. Caffeine contracture was inhibited by the calcium antagonists at 20°C. This inhibition was caused by an early onset of relaxation, which was not observed at 7°C. The results suggest that some interaction between calcium ions and drug molecules at the voltage sensor on the transverse tubular membrane, which regulates E-C coupling but is not directly related to the functional calcium channel, may play an important role for the phenomena. The inhibitory action of calcium antagonists on the caffeine contracture in the presence of calcium is probably independent of the potentiating effect seen in the depolarization-induced contractions.

Key words: frog twitch muscles, E-C coupling, nicardipine, verapamil.

It has been shown that in skeletal muscle Ca channel blockers such as diltiazem, D600, and nicardipine, a dihydropyridine derivative, potentiate the twitch, tetanus, and potassium contracture (FRANK, 1982; GONZALEZ-SERRATOS *et al.*, 1982; HATAE, 1986; SATO and FUJINO, 1987), although these drugs inhibit the Ca current through the transverse tubular (T) membrane (SANCHEZ and STEFANI, 1978; ALMERS *et al.*, 1981; GONZALEZ-SERRATOS *et al.*, 1982; ILDEFONSE *et al.*, 1985). Based on these observations GONZALEZ-SERRATOS *et al.* (1982) concluded that the slow Ca channel plays no essential role in the excitation-contraction (E-C) coupling of the skeletal muscle. Furthermore, it is assumed that most of the receptors for dihydropyridines (DHP), have no relation to the functional Ca channel (SCHWARTZ *et al.*, 1985). However, the role of these Ca channel-unrelated receptors in the E-C coupling is still unclear, although some authors seek their localization in the voltage sensor at the junctional site between T membrane and sarcoplasmic reticulum (SR) (LAMB, 1986; RIOS and BRUM, 1987; WALSH *et al.*, 1987).

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In the present investigation we examined the dependence of the effects of Ca antagonists, such as nicardipine and verapamil, on the external Ca, and found that the twitch as well as the electrically induced contracture of the frog's fast twitch muscle were potentiated by the Ca antagonists in the presence of external Ca whereas they were inhibited in the absence of external Ca.

Similar results on the rat skeletal muscle were recently reported by DULHUNTY and GAGE (1988). A preliminary report has been published (KAWATA and HATAE, 1989).

MATERIALS AND METHODS

Mechanical recording. Small bundles containing ten to twenty fibers, about 10 mm in length and 0.4 mm in diameter, were dissected from semitendinosus muscle of *Rana japonica*. To inject stimulus current effectively and to get a spatial electrical homogeneity as much as possible, the muscle was partitioned into three parts by perfusing the middle compartment with isotonic sucrose solution as described previously (KAWATA, 1985). The proximal tendinous end in the test compartment was connected to the sensitive arm of a force transducer (Fuji-Keisoku, TDS-101). The length of muscle segment of this portion was between 1.5 and 2.0 mm. Both end-compartments were constantly superfused with normal Ringer's solution. The bath volume of the test compartment was 0.4 ml and the flow rate was about 4 ml/min.

Constant depolarizing current pulses delivered from an electric stimulator (Nihon Kohden, SEN 7103) were applied through a pair of platinum electrodes interposing both end-compartments at an interval of 30 s. The isometric tension (F) and its first derivative (dF/dt) were measured from the short muscle segment in the test compartment. For twitch experiments, suprathreshold pulses of 0.5 ms were given while for contracture experiments pulses of 1 s with variable intensities were applied under the condition where generation of the Na spike was completely blocked by tetrodotoxin (TTX). Basically the electrically induced contracture was kept below the maximum contraction level (70–90%). To achieve this we examined the tetanic tension for 50 to 100 Hz prior to application of TTX. In the measurements of caffeine contracture, 5 mM caffeine was applied for 30 s every 5 min without electrical stimuli. The values in the results represent mean $\pm$ S.E.

Solutions. The normal Ringer's solution had the following composition (mM): NaCl, 111.2; KCl, 3.0; CaCl_2 , 2.0; HEPES, 5.0, and pH 7.2. Ca-free solution was prepared either by omitting CaCl_2 from and adding 1 mM EGTA to the normal Ringer's solution or by replacing Ca ions with Mg ions and further by adding 1 mM EGTA, where 3 Mg ions were assumed to roughly be equipotent to 2 Ca ions (DÖRRSCHEIDT-KÄFER, 1976). For measurements of electrically induced contracture, tetrodotoxin (5×10^{-6} M, Sankyo Co. Ltd.) was added. Nicardipine (gift from the Yamanouchi Pharmaceutical Co. Ltd.) and verapamil (gift from Eisai Co. Ltd.) were stocked as aqueous solutions at a concentration of 3×10^{-4} and 10^{-3} M,

respectively. The drugs were diluted to 10^{-6} to 10^{-5} M shortly before the application according to the experimental purpose. All solutions contained 10^{-6} g/ml *d*-tubocurarine. The temperature of the bath was kept at $20 \pm 1^\circ\text{C}$ by using a circulator pump device.

RESULTS

Effects of depletion of extracellular Ca

When external Ca ions were depleted by omitting Ca from and by adding 1 mM EGTA to the normal Ringer's solution, spontaneous activity appeared and the twitch tension was transiently augmented. Figure 1A shows an example in which Ca-free solution was perfused for 5 min. The enhanced activity probably results from a decrease of the resting membrane potential as well as from a lowering of the mechanical threshold.

Substitution of 3 mM Mg for Ca resulted in a rapid decrease of the twitch tension by about 50% followed by an extremely slow decline of the twitch tension (Fig. 1B). The mean value of the twitch height at 10-min perfusion of Ca-free solution was $43.5 \pm 1.2\%$ ($n=5$) of the control in normal Ringer. This would indicate that the twitch of the semitendinosus muscle consists of an external Ca-dependent component and -independent or less dependent component.

Dependence of twitch potentiation by Ca antagonists on external Ca

Figure 2A shows that the twitch tension was enhanced by about 22% by applying nicardipine at a concentration of $3 \mu\text{M}$ for 10 min. Since this concentration of the drug had no effect on both the resting potential and the action potential (HATAE, 1986), the observed enhancement of twitch force may reflect a facilitation of the E-C coupling process. However, the same concentration of the drug failed to show any twitch potentiation when it was applied after the twitch tension had been reduced by perfusing Ca-free, Mg-containing solution for 10 min, where only an external Ca-independent twitch component was manifest (Fig. 2B). Figure 3 summarizes data from three other muscles. In the figure, open circles indicate the control twitch height in the Ca-containing normal medium while filled circles indicate the twitch height in the Ca-free solution. The tension was expressed as percent of the twitch just before the application of nicardipine. Open triangles show the post-control in the Ca-containing solution. It can be seen that the effect of nicardipine on the contraction strongly depends upon external Ca.

Quite similar results were obtained by applying verapamil ($10 \mu\text{M}$). Figure 4 illustrates the results from one muscle preparation. The twitch potentiation in the normal Ringer's solution (Fig. 4A) was changed to an inhibitory effect in the Ca-free, Mg-containing medium (Fig. 4B). This potentiating effect was reversible (Fig. 4C).

Experiments on the electrically induced contracture

In such a short fiber preparation as used in the present experiment, a fairly

Fig. 1. Effects of Ca depletion on twitch. A: Ca-free, 1 mM EGTA solution was perfused for 5 min between arrows. B: Mg-replaced (3 mM), Ca-free solution was perfused for 10 min between arrows.

uniform and steady depolarization can be expected when a constant electrical pulse (1 s) is applied in the TTX-containing Ringer's solution (KAWATA, 1985). Figure 5 compares the effect of Ca-free perfusion on twitch (Fig. 5A) and that on electrically induced contracture (Fig. 5B) in the same preparation. In this example, contracture/twitch ratio was 2.85. In the record B, Ca depletion resulted in a reduction of the terminal tension to about 71% after 10 min of perfusion ($75.6 \pm 1.1\%$, $n=4$). Any contribution of changes in the action potential to this

Fig. 2. Effects of nicardipine ($3 \mu\text{M}$) on twitch tension were examined in the presence (A) and in the absence (B) of Ca. Nicardipine was given for 10 min in A while in B the drug was applied for 5 min after the twitch was decreased by perfusing Ca-free solution for 10 min. Experiments in different muscles. NR: normal Ringer.

change is unlikely because the contracture was elicited in the TTX-containing solution throughout the experiment.

Nicardipine ($3 \mu\text{M}$) showed a potentiating effect on the contracture (Fig. 6A, $6.26 \pm 1.17\%$, at 5-min perfusion). Then the effects of nicardipine on the electrically induced contracture were examined in TTX-containing, Ca-free solution. The peak tension did not increase by applying the same concentration of the drug but rather decreased (Fig. 6B), as seen with twitches.

A low concentration of Ca antagonists such as nicardipine or verapamil thus has a potentiating effect on tension in the presence of Ca ions in the external medium, while in the absence of Ca the effect is reversed.

Caffeine contractures

This dual effect of Ca antagonists raised the possibility that the drugs have some intracellular effects, either on SR or contractile elements. To explore this possibility the experiments shown in Fig. 7 were performed. As described in METHODS, 5 mM caffeine was applied for 30 s every 5 min. When nicardipine ($3 \mu\text{M}$) was applied 3 min prior to the application of caffeine, the next caffeine

Fig. 3. Summary of the effects of nicardipine on twitch. Responses in the normal Ringer (open circles), in the Ca-free solution (filled circles), and recovery (open triangles) were plotted as relative values to the peak tension immediately before the drug application. Nicardipine ($3 \mu\text{M}$) was applied for 10 min in normal Ringer while it was given for 5 min in Ca-free solution. Mean of three different muscles.

contracture was consistently suppressed. The application of the drug was limited to 5 min. The change was fairly reversible (Fig. 7A). A quite similar inhibition was observed when applying verapamil ($10 \mu\text{M}$) (Fig. 7). Interestingly, the drug-induced inhibition was exclusively due to an early onset of the relaxation while the rate of tension rise was essentially unchanged (Fig. 7C, D). In order to examine the possibility of any contribution of the energy-consuming process, the experiments were repeated at 7°C . Figure 8 summarizes and compares the results obtained from 6 and 4 muscles at 20 and 7°C , respectively. In the figure, open circles show the peak tension and filled circles show the contraction time measured at 50% level of the peak tension. The inhibitory effect of the Ca antagonists seen at 20°C (Fig. 8A) almost disappeared at 7°C (Fig. 8B).

Fig. 4. Effects of verapamil ($10 \mu\text{M}$) on twitch. A: pre-control. B: effect in the Ca-free medium. C: recovery after re-perfusion of Ca. Experimental procedure is the same as in Figs. 2 and 3, except that Ca-free solution was perfused for 15 min instead of 10 min in the case of nifedipine (B). NR: normal Ringer.

DISCUSSION

The results of this study indicate that the contractile tension, irrespective of twitch or contracture, depends on the external Ca concentration. There are many controversial results on the effects of Ca depletion on the contractility of skeletal

Fig. 5. Inhibition of electrically induced contracture by a superfusion of Ca-free, Mg solution. A: effect of 10-min perfusion of Ca-free solution on twitch. B: the same maneuver on the 1-s-depolarization-induced contracture. Results from the same muscle.

Fig. 6. Experiments of contracture on dependence of the effects of nicardipine ($3 \mu\text{M}$) upon external calcium. A: experiment in Ca-containing solution. B: effects of Ca-free solution. In both cases tetrodotoxin (TTX) (10^{-6} g/ml) was given at the downward arrow. Panels A and B are the separate experiments on different muscles. NR: normal Ringer.

muscle (FRANK, 1960; CAPUTO and GIMENEZ, 1967; LÜTTGAU and SPIECKER, 1979; COTA and STEFANI, 1981; GRAF and SCHATZMANN, 1984; TAKAUJI *et al.*, 1984; CAILLÉ *et al.*, 1985). We found that the contraction of the semitendinosus muscle of *Rana japonica* consisted of a Ca-dependent component and a Ca-independent one. In our Ca-free solution, Ca ions were replaced with Mg ions. Measuring the mechanical threshold, DÖRRSCHEIDT-KÄFER (1976) found that the ratio of effectiveness Ca^{2+} : Mg^{2+} is about 1:0.55 to 1:0.6. We used a ratio of 1:0.67 (2 Ca:3 Mg) for the replacement of divalent cations. The fact that, even under this condition, depletion of external Ca resulted in a fall of tension by about 55% for twitch and 25% for contracture would indicate the possibility of either a participation of inward Ca current or a shift of the inactivation curve for tension generation to more negative membrane potential range. It is believed that slow Ca current is not essential for twitch generation (AVILA-SAKAR *et al.*, 1986). On the other hand, a shift of the inactivation curve of contracture to the left by Ca depletion was observed both in amphibian and mammalian muscles (LÜTTGAU and SPIECKER, 1979; CAPUTO 1981; COTA and STEFANI, 1981; GRAF and SCHATZMANN, 1984). Thus the latter seems to play a major role in the reduction of tension observed in the present study.

Fig. 7. Effects of nicardipine on caffeine contracture. Five mM caffeine was given for 30 s at an interval of 5 min. Ca antagonist was perfused for 5 min between arrows (A, B). A, C: experiment on nicardipine (N) ($3 \mu\text{M}$); B, D: experiment on verapamil (V) ($10 \mu\text{M}$). Records C and D show superimposed tension traces of control contracture (Cont.) and contracture during Ca antagonist (N, V) with faster speed. Caffeine was applied for 30 s between arrowheads.

Our finding that low concentration of Ca antagonists such as nicardipine or verapamil augmented the contractile tension of the skeletal muscle is consistent with previous reports (GONZALEZ-SERRATOS *et al.*, 1982; FRANK, 1984; HATAE, 1986; SATO and FUJINO, 1987; DULHUNTY and GAGE, 1988). This potentiation occurred only in the presence of Ca ions. It is unlikely that the potentiation results from an increase in the Ca current since the drugs in the same range of concentration are known to inhibit the slow Ca current (SANCHEZ and STEFANI, 1978; GONZALEZ-SERRATOS, 1982; ILDEFONSE *et al.*, 1985; PALADE and ALMERS, 1985). Therefore the seeming agonistic action of the drugs on the contractile tension may not be related to the functional Ca channels.

On the other hand, an inhibition of the tension was consistently observed when extracellular Ca was depleted. DULHUNTY and GAGE (1988) have recently observed that the effect of nifedipine, a dihydropyridine, on twitch, tetanus, and potassium contracture of rat soleus muscle was also dependent on the external Ca concentration. They found that nifedipine ($50 \mu\text{M}$) potentiated both twitch and tetanus in 2.5 mM Ca solution whereas the drug suppressed them in low Ca media. Interestingly enough, Bay K8644 ($50 \mu\text{M}$) also caused contractile inhibition in a low Ca solution. Although they used higher concentration of drugs than we used here, the results present by DULHUNTY and GAGE (1988) and by the present authors are

Fig. 8. Inhibition of caffeine contracture by nicardipine at 20°C (A) was lost at 7°C (B). Relative peak tension (open circles) and contraction time measured at 50% of peak tension (filled circles) were plotted against time. Nicardipine (3 μ M) was applied for 5 min between arrows. Caffeine contracture was induced every 5 min. Results of 6 muscles for 20°C and 4 muscles for 7°C.

quite similar. The causal mechanism for the contractile inhibition by Ca antagonists in low Ca and/or Ca-free solution is unclear. It may be due to a Ca binding and dissociation reaction at the voltage sensor on the T membrane which somehow mediates the E-C coupling process as proposed by DULHUNTY and GAGE (1988) for rat muscle. An alternative possibility would be an involvement of the intracellular process rather than the T-SR junction. Our experiments on caffeine contracture

revealed that tension development was inhibited by applying of nicardipine or verapamil at the same concentration range as used in the experiments on twitch or electrically induced contracture. The inhibition is attributed to an early onset of the relaxation as described before. The fact that the initial rate of the rising phase remained unchanged would suggest that the contractile proteins were probably not modified. An accelerated dissociation of Ca ions from troponin is expected either by stimulation of Ca pump of the SR membrane or by activation of parvalbumins in the sarcoplasm. The former possibility is more likely because the inhibition of contracture disappeared at low temperature, which clearly indicates the process is metabolic energy dependent. WANG *et al.* (1984) investigated the effects of various Ca antagonists on SR microsomes from dog ventricle and rabbit back muscle and found that the Ca ATPase of SR from skeletal muscle was increased by verapamil, diltiazem, felodipine, and nisoldipine. In contrast, ISHIZUKA and ENDO (1983) reported a suppressing effect of diltiazem on the Ca uptake of SR measured on the skinned muscle of toad. The latter authors found, in addition, an increase of the Ca sensitivity of contractile protein system. The relationship between the inhibition of caffeine contracture by Ca antagonists and that of depolarization-induced contraction is not clear. Although recent experiments suggest the close relation or even identity of Ca channel of the SR to T-SR junctional proteins (INUI *et al.*, 1987; KUNDSON *et al.*, 1988; WAGENKNECHT *et al.*, 1989), the relationship between this potential-operated Ca channel and the caffeine-activated Ca channel is still undefined. Therefore, we cannot decide whether the inhibitory action of Ca antagonists on SR Ca pump could somehow be related to the inhibition of depolarization-induced contraction under Ca-free condition. This point remains to be clarified.

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