The Effects of Calcium Channel Modulators on Contractions of Tonic Frog Muscle Fibres*

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Abstract. Effects of adrenaline (ADR) and the dihydropyridine Ca channel agonist CGP-IOS were studied on twitch and tetanic contractions of isolated tonic muscle fibres or small muscle fibre bundles containing tonic fibres of the frog *Rana temporaria*. Tetanization caused a gradual increase of tension between 10 and 70 Hz. CGP-IOS produced an increase in twitch amplitude. After CGP-IOS administration ($2 \times 10^{-6} - 10^{-5}$ mmol/l), the twitch amplitude increased and the high frequency tetanus developed more rapidly. However, during the phase of high tension level a breakdown of tetanic tension appeared and this occurred earlier at higher stimulation frequencies. After the end of tetanization the contraction curve did not return to the initial level, so that the remaining contraction (contracture) lasted 2 to 5 min. Sometimes just after replacement of CGP-IOS by Ringer, a large enhancement of tetanus amplitude was observed followed by long lasting contractures. ADR (10^{-5} mmol/l) increased the tetanic tension without changing the shape of tetanus but did not affect twitch amplitude. Adrenergic modulation of tension requires extracellular Ca^{2+}. Combined administration of ADR and CGP-IOS had an integrative effect, so that independent action of each of them can be assumed. It is to suggest that direct and indirect Ca channel modulators, DHP derivatives and ADR, have different targets. The sites responsible for adrenergic modulation of the contraction may be Ca channels not identical to DHP-sensitive channels, i.e. Ca-releasing channels in SR or the DHP-insensitive Ca channels in muscle membrane.

* Dedicated to Prof. Dr. med. habil. G. Küchler on the occasion of his seventieth birthday.

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Key words: Contractions — Tonic fibres — Adrenaline — DHP agonist

Introduction

The adrenergic stimulation of mammalian or frog skeletal muscles produces an increase in tension output evoked by direct or indirect muscle activation (Oliver and Schaefer 1895; Orbeli 1923; Bowman and Ziamis 1958; Bowman and Nott 1969; Gonzales-Serratos et al. 1981; Jami et al. 1984). A key for the understanding of this phenomenon appeared when adrenoreceptor activation was disclosed. Activation may be evoked by β-agonists that act via G-protein signalling system on ionic channel protein complexes, including that in SR and T-membranes (Oota and Nagai 1977; Rosenthal et al. 1988; Yatani et al. 1988; Brown 1991). Considering the changes in excitation-contraction coupling (ECC) as a cause of adrenergic modulation of muscle activity, the understanding of the G-protein mediated processes can be expected to be important. The slow dihydropyridine (DHP) sensitive Ca channels in T-membrane may play a role of voltage sensors for contraction activation (Agnew 1987; Lamb and Walsh 1987; Rios and Brum 1987; Rios and Pizarro 1991).

The purpose of the present study was to examine both, the direct action on ECC by an L-type Ca channel agonist and the DHP derivative CGP-IOS, and the indirect action by adrenoreceptor stimulation resulting in modulation of muscle tension output. To minimize the influence of inactivation of contraction the experiments were performed on frog tonic muscle fibres which are known to lack the spontaneous inactivation of contraction during long term electrical stimulation or depolarization (Zhukov and Leushina 1950; Kuffler and Vaughan-Williams 1953; Lorkovic 1959; Lüttgau 1963; Nasledov et al. 1966; Lännergren 1967, 1975; Gilly and Hui 1980). Huerta et al. (1991) reported that in frog tonic fibres the adrenergic stimulation also generates a positive inotropic effect.

A preliminary report has already been published (Kössler et al. 1993).

Materials and Methods

Single tonic muscle fibres were isolated mechanically from iliofibularis muscle of the frog *Rana temporaria*. Tonic fibres were distinguished by their ability to retain contracture in high K^+ concentration (Nasledov et al. 1966). In some experiments phasic fibres were also used for comparison. Fibres were mounted horizontally in a perspex chamber filled with Ringer solution that could be exchanged within some tens of seconds. Electrical stimulation was performed by single pulses (1 ms) or by trains of 5–6 s and 10, 20, 40, or 70 Hz via two parallel platinum plate electrodes. As the fibres were stimulated throughout their length, the term "twitch" used in the following text for tonic fibres should be understood just as a contractile response to

a single stimulus. ADR (Boehringer), the calcium channel blocker D600 (Sigma), or the DHP calcium channel agonist CGP-IOS (2-methylmethoxycarbonyl-4-(2-difluoromethoxyphenyl)-5-oxo-1,4,5,7-tetra-hydrofuro-(3,4-b)pyridine) was added to Ringer solution. CGP-IOS (Institute of Organic Synthesis, Riga, Latvia), differing from CGP-28392 by CH_3 in the carboxyl group being substituted by C_2H_5, was dissolved in dimethylsulphoxide (DMSO) giving a final concentration of 10^{-5} mmol/l CGP-IOS and 0.1% DMSO. The tension output was measured by a 6 MX 2B force transducer (Russia) and recorded oscillographically. Ringer solution contained (in mmol/l): NaCl 115; KCl 2.5; $CaCl_2$ 2; Tris-HCl 10; pH 7.3–7.4. In Ca-free solution $CaCl_2$ was replaced by equimolar amount of $MgCl_2$; no Ca buffering was made to get distinct micromolar concentration of Ca^{2+}. The frogs were caught in autumn and kept in tanks at 4–5 °C without feeding. Experiments were conducted during winter time at room temperature 20–22 °C.

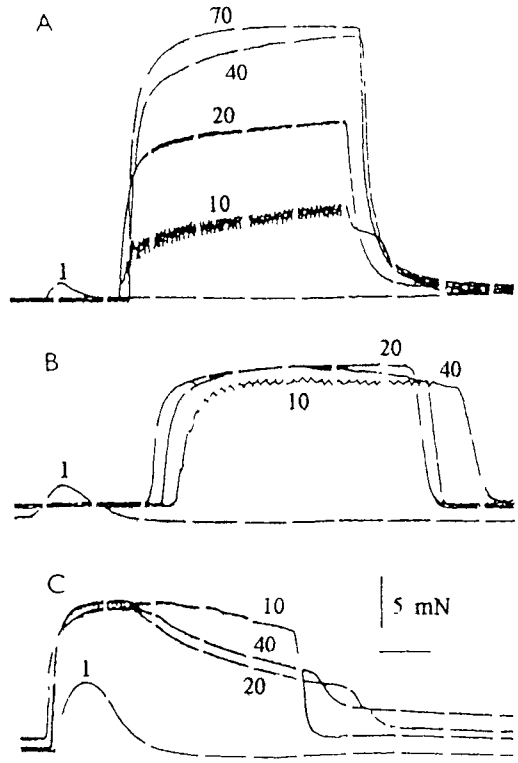

Figure 1. Effects of CGP-IOS (10^{-5} mmol/l) on twitches (1) and tetanic tension in tonic muscle fibres, stimulation frequency 10, 20, 40, and 70 Hz, respectively; *A* – Ringer, *B* – 5 min, and *C* – 8 min of drug action. Time bar: 0.2 s for twitches and 1 s for tetani.

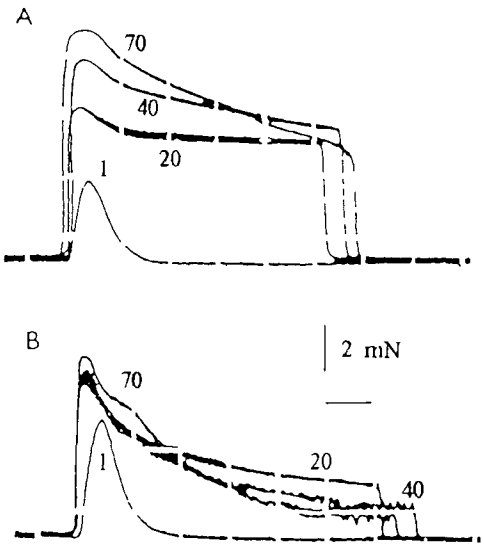

Figure 2. Effects of CGP-IOS (2×10^{-6} mmol/l) on twitches (1) and tetanic tension in phasic muscle fibres, stimulation frequency 10, 20, 40, and 70 Hz, respectively; *A* – Ringer, *B* – 10 min of drug action. Time bar: 0.2 s for twitches and 1 s for tetani.

Results

Twitch and tetanus in tonic fibres

In tonic fibres or fibre bundles the twitches, evoked by direct electrical stimulation were very small in relation to maximal tetanic tension. The mean tetanic twitch ratio measured at 70 Hz readed a value of 9.85 in tonic fibres ($n = 14$). In phasic fibres this ratio was 2.06 ($n = 8$).

The tetanic tension in tonic fibres increased during tetanization at 10, 20, 40 and 70 Hz gradually in parallel with the frequency rise (Fig. 1*A*). In tonic fibres 70 Hz stimulation resulted in a higher plateau of tension that was not reached during stimulation at 20 and 40 Hz. In phasic fibres a gradual increase of tension rise was seen in parallel with the frequency, but this increase was not so graduated as in tonic fibres (Fig. 2*A*). The second difference in tetanic responses between phasic and tonic fibres consisted in a slow rise of tension (during 5–6 s) for each stimulation frequency in tonic fibres, and a fast rise of tension to its maximal value in phasic fibres. In addition, during 5–6 s lasting tetanus, some decrease of tetanus amplitude appeared in phasic fibres in contrast to a slow continuation of rising force in tonic fibres. In phasic fibres, the highest frequency (70 Hz) caused a faster drop of tension during tetanization compared to 20 and 40 Hz (Fig. 2).

Effect of CGP-IOS

Application of CGP-IOS ($2 \times 10^{-6} - 10^{-5}$ mmol/l) for 5–20 min resulted in the following changes of the contraction slope (Figs. 1 and 2). First, an increase of

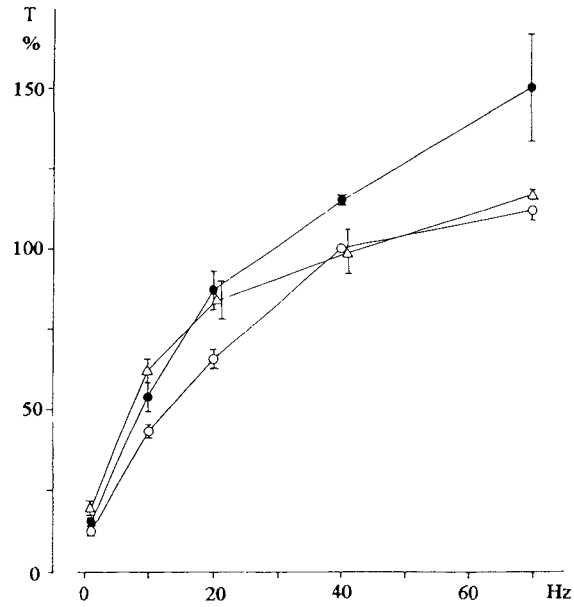

Figure 3. Dependence of isometric tension (T) on stimulation frequency of tonic fibres. Tetanus at 40 Hz in Ringer was taken as 100%, in Ringer (○, $n = 11$), in the presence of CGP-IOS, 10^{-5} mmol/l (△, $n = 6$), and in the presence of adrenaline, 10^{-5} mmol/l (●, $n = 6$).

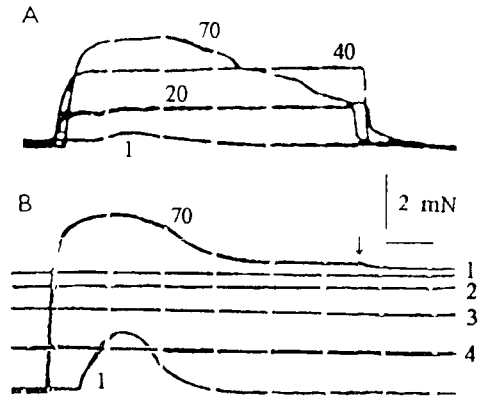

Figure 4. Time course of twitch and tetanic tension of tonic muscle fibres before (*A*) and 3 min after the end of CGP-IOS (10^{-5} mmol/l) treatment for 6 min (*B*), lowest lines (1), twitches; *A* – stimulation frequency 20, 40, and 70 Hz, *B* – stimulation frequency 70 Hz. The arrow indicates the end of tetanization, time after the end of tetanization 1, 2, 3, 4 min, respectively. Time bar: 0.2 s for twitches and 1 s for tetani.

tension appeared in the single twitches likely at low frequency tetanic tension at 10 Hz stimulation. On the contrary, the high frequency tension decreased in its amplitude (Fig. 1), but sometimes did not change at all. The rise in tension at all tetanic frequencies was markedly enhanced, and the plateau was reached much faster than

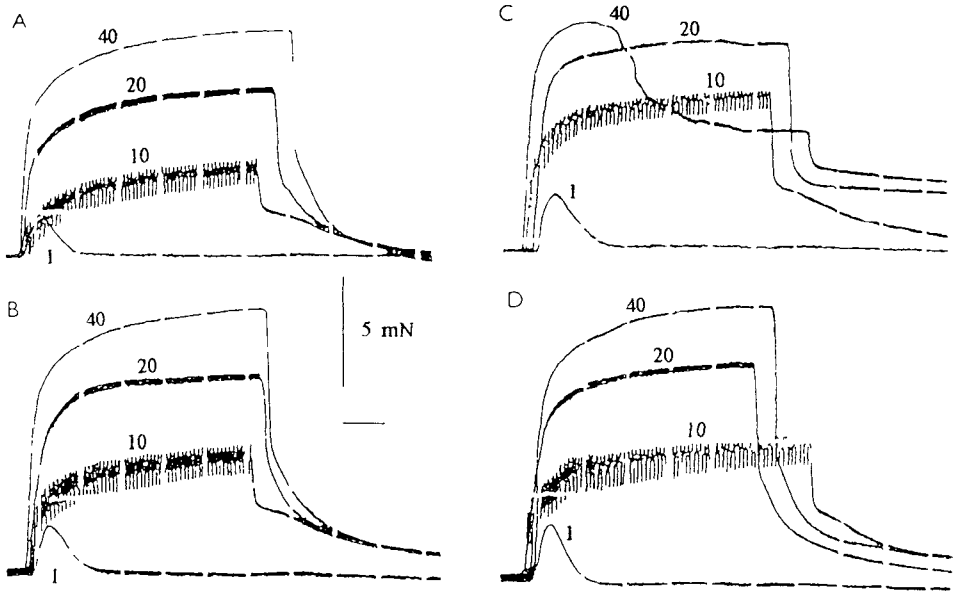

Figure 5. Effects of cooperative action of adrenaline and CGP-IOS on twitch (1) and tetanic contractions of single tonic muscle fibres, stimulation frequency 10, 20, and 40 Hz; *A* – Ringer, *B* – adrenaline, 10^{-5} mmol/l, *C* – adrenaline with addition of CGP-IOS, 10^{-5} mmol/l each, *D* – washout Ringer. Time bar: 0.2 s for twitches and 1 s for tetani.

in the control (Ringer, Fig. 1*B*). The mean percentual changes of the tetanic tension after CGP-IOS treatment are shown in Fig. 3. Second, the tetanus plateau in tonic fibres lost its stability and a decay of contractile tension appeared already during the stimulation. The higher the tetanus frequency the earlier the decrease in tension (Figs. 1*C* and 6*B*). Third, after the end of stimulation in tonic fibres the tension did not return to its initial level like in Ringer solution. In contrast, a residual contraction (contracture) occurred which lasted for 1–3 min. This increase of resting tension depended on the duration of CGP-IOS exposure (Figs. 1*C* and 6*B*). Fourth, after the cessation of CGP-IOS application (usually lasting for 10–15 min), a large increase of tetanic tension was observed during the washout process. This phenomenon was followed by a very slow relaxation lasting for 5 min or longer. The twitch contraction in these cases was also much extended (Fig. 4).

Effect of adrenaline

ADR (10^{-6} – 10^{-5} mmol/l) enhanced the tetanic tension. This increase could be seen at each tetanic frequency (10–70 Hz). Unlike with CGP-IOS, the shape of the tetanic curve remained without alterations. No stable effect of ADR on the

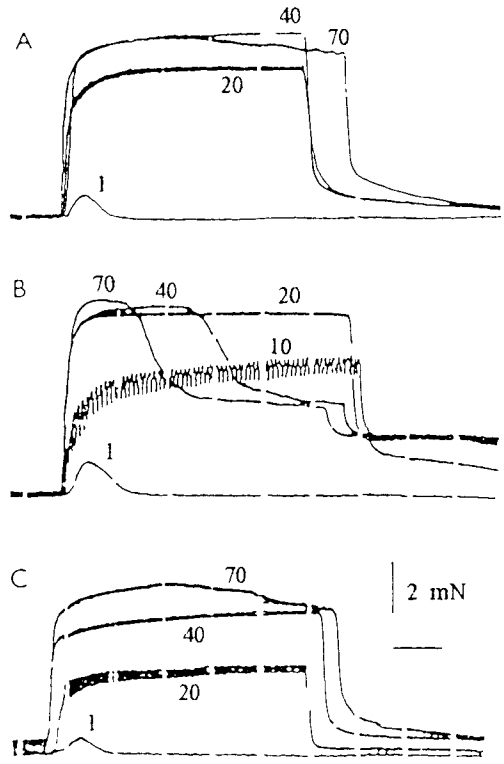

Figure 6. Effects of adrenaline (10^{-5} mmol/l) on twitch (1) and tetanic contraction of single tonic muscle fibre preincubated in CGP-IOS (10^{-5} mmol/l) for 10 min, stimulation frequency 10, 20, 40, and 70 Hz; *A* – Ringer, *B* – 10 min of action of both drugs, ADR added after 10 min CGP-IOS alone, *C* – washout Ringer, 10 min. Time bar: 0.2 s for twitches and 1 s for tetani.

amplitude of twitch tension could be observed (Fig. 5). The mean changes of peak amplitudes of twitch and tetanic tension of tonic fibres following ADR treatment are shown in Fig. 3. The starting points of the curves correspond to twitch responses.

Effect of cooperative action of Ca agonist CGP-IOS and adrenaline

We deemed it important to elucidate how Ca channel agonist and ADR act when applied in combination. The combined administration of CGP-IOS and ADR induced changes of contractile responses which can be considered as a sum of both actions (Fig. 5*C*). ADR evoked an additional increase in twitch tension as compared with CGP-IOS acting alone. An additional increase in the rate of rise of tetanus also appeared. The changes, typical for CGP-IOS action remained in the presence of ADR as well (Fig. 6): (1) an increase of twitch tension; (2) interruption of the plateau and partial relaxation during stimulation at high frequency; (3) incomplete relaxation and long lasting "tail" contracture following tetanization.

All these features have been described above as typically of CGP-IOS action. On the other hand, the increase of tetanic tension, especially under high frequency tetanization, is attributed to specific ADR action described above. Fig. 6 shows that

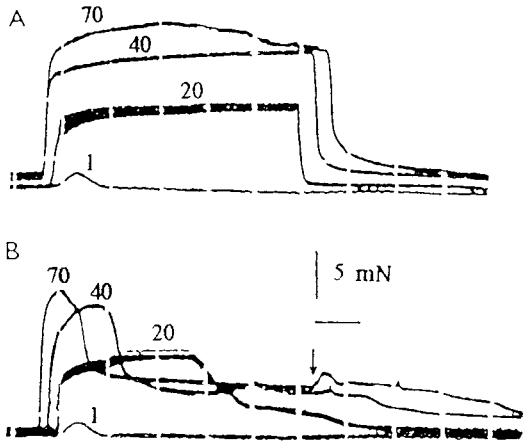

Figure 7. Effects of D600 (10^{-5} mmol/l) on twitch (1) and tetanic tension in tonic muscle fibre, stimulation frequency 20, 40, and 70 Hz. *A* – Ringer, *B* – 5 min of drug exposure. Time bar: 0.2 s for twitches and 1 s for tetani.The arrow indicates washout of the drug after 70 Hz stimulation.

ADR increases the tetanic tension also when applied after the addition of the DHP agonist. An increased amount of maximum tension was registered although signs of DHP derivative action such as increase of twitches, run-down of high frequency tetanus, and delay in relaxation occurred (Fig. 5*C*). Fig. 7 shows the result of simultaneous administration of both CGP-IOS and ADR. The additive effects of the Ca agonist and adrenostimulation are easy to observe. It must be noted that ADR did not alter the characteristic shape of tetanic contraction elicited by the Ca channel blocker.

Effect of Ca channel blocker D600

The calcium channel blocker D600 ($5 \times 10^{-6} - 5 \times 10^{-5}$ mmol/l) did not affect the twitch tension in tonic fibres but considerably decreased the level of tetanic plateau (Fig. 7). At the begin of tetanic stimulation an almost normal rise of tension occurred but the tension dropped after one or two seconds and a fast decrease of tetanus appeared. The higher the frequency of tetanization the sooner the relaxation, as seen for CGP-IOS. In contrast to CGP-IOS action, relaxation appeared much earlier at each frequency in the presence of D600. Another similarity of the action of D600 and the DHP agonist was a contracture which continued in tonic fibres after the end of tetanic stimulation. The level of this contracture corresponds to the tetanization frequency (Fig. 7); the higher the frequency the higher the contracture.

Effect of Ca-free solution

Immersion of tonic muscle fibres into Ca^{2+}-free Ringer solution caused a significant drop in maximum tension output (Fig. 8). This drop concerned both single twitches and tetanic contractions. The tension drops progressed during the exposure to Ca-free solution. No initial rise of contraction could be seen (Fig. 8*B*). It is important

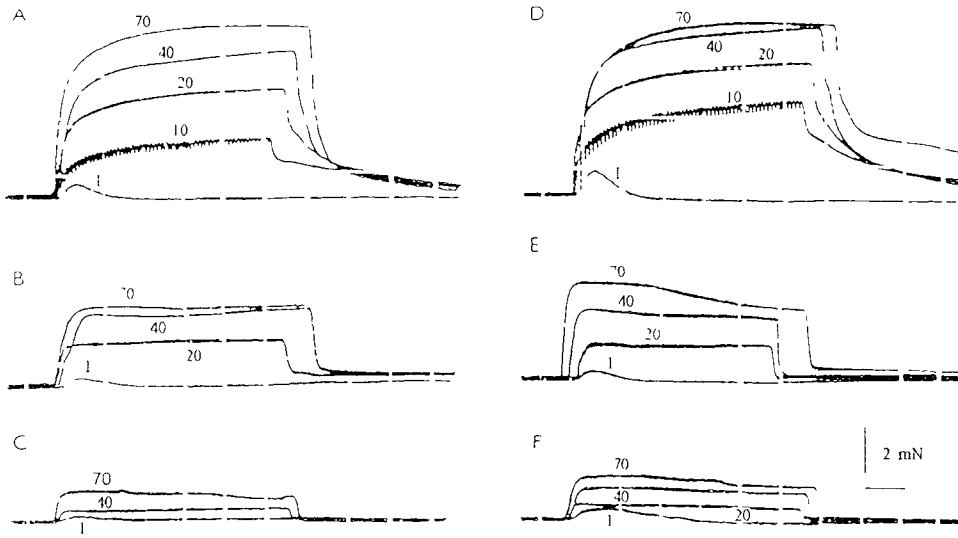

Figure 8. Twitch (1) and tetanic contractions of tonic muscle bundle, stimulation frequency 10, 20, 40, and 70 Hz; *A* – Ringer, *B* – 8 min Ca-free solution, *C* – addition of adrenaline (10^{-5} mmol/l) to Ca-free solution, 5 min, *D* - 20 min after washout in Ringer and then 7 min in CGP-IOS (10^{-6} mmol/l) in Ringer, *E* – CGP-IOS in Ca-free solution, 10 min, *F* – exposure to adrenaline (2×10^{-5} mmol/l) in Ca-free solution for 12 min. Time bar: 0.2 s for twitches and 1 s for tetani.

to note that ADR did not have any positive effect on tension output which was diminished in Ca-free solution. In contrast the tension continued to drop with time (Fig. 8*C*). When Ca channel agonist CGP-IOS was applied at a low concentration (10^{-6} mmol/l) prior to Ca-free solution, the tension output was suppressed less than in Ringer solution. It seems that the DHP agonist protected the muscle fibre from the tension depression elicited by external Ca^{2+} deprivation (Figs. 8*D* and *E*). In this case also ADR did not display any positive inotropic action in Ca-free solution (Fig. 8*F*).

Discussion

In experiments described above ADR caused a positive inotropic effect, well established by many authors for skeletal muscles of mammalians and frogs (e.g. Gonzales-Serratos et al. 1981; Jami et al. 1984). The effect was more obvious at tetanic repetitive stimulation than in twitch contractions. This confirms the classical data of Orbeli (1923) who showed that sympathetic stimulation enhances the

contractions mostly in muscles that had been fatigued by repetitive stimulation. Alternatively, a positive effect of catecholamines on twitches was described (Arreola et al. 1987). The inconsistency can be explained by the large variety of experimental conditions in different experiments. For instance, in the present study the first twitches were evoked 5–12 min after ADR had been added to the resting fibres, whereas in the experiments of Arreola et al. (1987) the twitches were evoked all the time during exposure.

The mode of action of catecholamines causing positive inotropic effects on skeletal muscles has been questioned (Williams and Barnes 1989). It has been established that the effect of ADR and other adrenomimetics is achieved via membrane β-adrenoreceptors, which activate G-proteins and enhance the myoplasmic concentration of cAMP, in turn stimulating the phosphorylation of Ca channels by proteinkinase A (Yatani et al. 1988; Brown 1991). The existence of β-adrenoreceptors in skeletal muscles was described by Bowman and Nott (1969). Later enhancement of calcium current (I_{Ca}) in response β-adrenoreceptor activation was shown in muscle cells (Arreola et al. 1987; Garcia et al. 1990). Although no external Ca entry is required for ECC in skeletal muscles (Rios and Pizzaro 1991), an increase of I_{Ca} can be suggested as a main factor for force potentiation:

1. Slow Ca channels in muscle T-membrane have been shown to be DHP sensitive complexes including voltage sensors. The regulation of channels can induce changes in voltage sensor operation (Agnew 1987; Rios and Pizzaro 1991);

2. Ca^{2+} entering the muscle fibre may increase the intracellular Ca^{2+} concentration both, directly and by "Ca-induced Ca release" from intracellular stores and thus modulate the myoplasmic Ca^{2+} concentration during contraction (Ildefonce et al. 1985).

The dihydropyridine derivative agonist CGP-IOS is known to stimulate the current through slow (L-type) DHP-sensitive Ca channels in T-membrane as other widely used DHP agonists, e.g. Bay K 8644 or CPG-28392 (Shvinka et al. 1990). In our experiments CGP-IOS in concentrations of $10^{-6} - 10^{-5}$ mmol/l evoked several very prominent changes in tetanic contraction. These are much better seen on tonic than on phasic fibres, because of the failing of spontaneous inactivation of mechanical output in this type of fibres. The changes evoked by DHP agonist in the form of (1) suddenly occurring run-down of tetanic tension during stimulation; (2) long lasting tail contracture after cessation of tetanic stimulation; and (3) largely enhanced tetanic contraction immediately after washout of the DHP agonist have been described here for the first time.

It was not expected that the DHP agonist, which exhibits a stimulatory effect on Ca current (Shvinka et al. 1990), would have a negative effect on tetanic contraction. Indeed, DHP agonists of Ca channels are known to potentiate skeletal muscle contraction (Ildefonce et al. 1985). A paper published by Dulhunty and Gage (1988) mentioned a negative inotropic effect of Ca agonist Bay K 8644 on

peak tetanic and twitch tensions of mammalian muscle, but the mechanism of this phenomenon was not explained.

In present experiments a frequency-dependent decay of tetanic tension was observed during prolonged tetanization after exposure to CGP-IOS. It is suggested that the increase of Ca channel function, due to DHP agonist action and consequently enhanced Ca^{2+} entrance, leads to decay of tetanic contraction. This conclusion agrees with the finding that the decay starts the sooner the higher the applied tetanic frequency. The blocking effect of increased Ca^{2+} concentration on SR calcium release has recently been described (Shirokova et al. 1994; Györke and Pallade 1994). The tail contracture after the end of tetanization observed in these experiments on tonic but not phasic fibres can also be explained by high Ca^{2+} concentration in the myoplasm. The SR in tonic fibres is less developed (Franzini-Armstrong 1973) and myoplasmic Ca^{2+}, if enhanced, may not be reaccumulated by SR fast enough. The absence of inactivation of the contractile system makes it possible to indicate excess of Ca^{2+} in the myoplasm. It may be more difficult to explain the large increase of tetanic tension just after the start of washout of the DHP agonist. This phenomenon requires further experiments. After exposure to Ca blocker D600 (Fig. 7) the tetanic tension initially developed almost normally but dropped later during the stimulation. This corresponds to results showing that the block of contraction caused by D600 is dose-dependent (Neuhaus et al. 1990). The events seem to be similar to that elicited by CGP-IOS action but can be expected to have opposite underlying causes: a deficiency rather than excess of Ca ions. The experiments performed with Ca-free solution showed a significant drop of twitch and tetanic contractions (Fig. 8). It was expected that Ca-free solution may have a similar effect as does Ca blocker D600, namely cessation of Ca^{2+} entry into the myoplasm. Indeed, Ca-free solution caused a smooth decline of the amplitude already from the very beginning of tetanic stimulation. These differences can be explained by suggesting the specific mode of D600 action. Actually, it was proposed that D600 may bind to a receptor at the force controlling system and lead to stabilization of the inactive state (Neuhaus et al. 1990). Siebler and Schmidt (1987) have found that D600 prolongs the inactivation state of the contractile system in frog twitch muscle fibres. In present experiments a decline occurred only after an initial rise of contraction, whereas the lack of Ca ions caused contraction activation to decline from the very beginning.

Cooperative action of both the Ca channel agonist CGP-IOS and ADR results in a summation of reactions to each of them. This could be seen as the appearance of two different kinds of changes in tetanic contraction: a smooth increase in amplitude with ADR and the run-down of the tetanus plateau with a tail contracture with CGP-IOS. It seems that these changes occur independently suggesting that each modulator has its own target. The target for DHP agonists is the DHP-sensitive slow Ca channel in T-membrane. The target for adrenergic substances has been

discussed by many authors Phosphorylation of the DHP sensitive channels has been described in cardiac cells (Tsien et al 1986) as a result of adrenomimetics and cAMP action which in turn enhance the activity of Ca channels, apparently by increasing the channel opening probability Such mechanisms have been considered in recent years also for skeletal muscle cells (Oota and Nagai 1977, Arreola et al 1987, Huerta et al 1991)

If the positive inotropic effects were due to an increased Ca^{2+} entry through DHP-sensitive channels, the activation of these channels by direct DHP agonist action would lead to the same contractile effect as adrenergic stimulation Because of the difference between the effects of these two kinds of modulators obtained in present experiments it should be concluded that the adrenergic effect on contraction may be connected with targets other than DHP-sensitive Ca channels The potentiation of tension due to the adrenergic modulation of the SR pump via cAMP was suggested by Gonzales-Serratos et al (1981) and by Huerta et al (1991) An important role may play the SR Ca releasing channels DHP blocker nifedipine did not abolish the adrenergic effect (Arreola et al 1987), and the increase of myoplasmic Ca^{2+} after adrenomimetics action is not associated with intramembrane charge movement connected with DHP channel activity (Cairns et al 1993)

Alternatively, the positive inotropic effect of ADR could not be observed in the Ca-free external solution as is seen from experiments illustrated in Fig 8 and from experiments by Arreola et al (1987), who proposed that the main role may be played by the fast DHP-insensitive muscle membrane Ca current

In conclusion, it may state that the Ca channels, responsible for enhancing the Ca^{2+} concentration in the myoplasm, play a key role in the achievement of adrenergic positive inotropic effect in frog tonic skeletal muscles These channels are not identical to DHP-sensitive Ca channels in muscle T-membrane

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