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THE EFFECTS OF DIHYDROPYRIDINE DERIVATIVES ON FORCE AND CA^{2+} CURRENT IN FROG SKELETAL MUSCLE FIBRES

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SUMMARY

1. The effects of dihydropyridine (DHP) derivatives on current through the slow Ca^{2+} channel and on isometric force were investigated in short toe muscle fibres of the frog (*Rana temporaria*). The experiments were performed under voltage-clamp conditions with two flexible internal microelectrodes.

2. The non-chiral DHP derivative nifedipine was used mainly because it allowed control measurements after the inactivation of the drug with UV light.

3. In a TEA sulphate solution containing 4 mM-free Ca^{2+}, nifedipine (1 μM) caused no relevant alterations in the time course of successive contractures induced by depolarizing steps to 0 mV of 3·5 min duration followed by a restoration time at −90 mV of 1·5 min.

4. When external Ca^{2+} was replaced by Mg^{2+}, nifedipine caused a dose-dependent shortening of contractures. The effect reached saturation at about 50 % of shortening with 1–5 μM-nifedipine. In the absence of divalent cations and with Na^{+} being the only metallic cation in the solution, shortening became more pronounced and maximum force decreased.

5. The application of 2 μM-nifedipine to a Ca^{2+}-free, Mg^{2+}-containing solution shifted the voltage dependence of force inactivation by 5–10 mV to more negative potentials.

6. Force activation was facilitated by nifedipine. In the presence of 2 μM-nifedipine in a Ca^{2+}-containing solution, threshold potentials (rheobase) as negative as −75 mV were measured under microscopical observation. UV irradiation shifted the threshold potential back to the normal value of about −50 mV.

7. The slow Ca^{2+} inward current was blocked almost completely by ~5 μM-nifedipine, even when induced from negative holding potentials (−90 to −120 mV), i.e. under conditions where normal phasic contractures could still be observed.

8. Nifedipine (0·8 μM) caused a shift of the voltage dependence of current inactivation ($V_{0.5}$) by 4 mV from −26 to −30 mV and at negative holding potentials (−90 mV), a reduction of maximum current by 35 %.

9. The voltage dependence of current activation was not significantly altered by nifedipine (2 μM).

10. It is assumed that nifedipine binds with low affinity to the resting state of the DHP receptor (K_D 0·8 μM) and with high affinity (K_D 1 nM) to the inactivated *and*

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the active state (or to a precursor of this state). These assumptions could explain the relatively small shift of the inactivation curves (points 5 and 8) to more negative potentials and the facilitation of force activation (point 6).

INTRODUCTION

Calcium antagonists have been successfully used as tools for the analysis of events which take place at the T-tubular side of the triad in excitation–contraction coupling of skeletal muscle fibres. As a first step, dihydropyridines which bind with high affinity and specificity to Ca^{2+} channels were utilized to isolate (for review see Hofmann, Schneider, Röhrkasten, Nastainczyk, Sieber, Ruth & Flockerzi, 1989) and to sequence this integral protein, i.e. the dihydropyridine (DHP) receptor (Tanabe, Takeshima, Mikami, Flockerzi, Takahashi, Kangawa, Kojima, Matsuo, Hirose & Numa, 1987). Phenylalkylamines, another class of Ca^{2+} channel blockers, which also bind to the DHP receptor protein, were shown to block Ca^{2+} release from the sarcoplasmic reticulum (SR) and force development in a state-dependent manner (for literature see Feldmeyer, Melzer & Pohl, 1990). These drugs also suppressed intramembrane charge movements (Hui, Milton & Eisenberg, 1984; Feldmeyer *et al.* 1990), which are thought to reflect the T-tubular voltage control of Ca^{2+} release (Schneider & Chandler, 1973). Thus, phenylalkylamines helped to identify the DHP receptor protein as a voltage sensor for Ca^{2+} release and to strengthen the charge movement concept.

The effects of dihydropyridines on the DHP receptor are more complex and cannot yet be explained in a straightforward manner. Micromolar concentrations of these drugs, which blocked the Ca^{2+} current, had little or no effect on force development (McCleskey, 1985; Lüttgau, Gottschalk & Berwe, 1987; Dulhunty & Gage, 1988) and blocked intramembrane charge movements only partially (Lamb, 1986; Lamb & Walsh, 1987). Experiments with cut fibres performed at a more positive holding potential (−70 mV) revealed a stronger suppression of charge movements and Ca^{2+} release (Rios & Brum, 1987), suggesting a high-affinity binding to the inactivated state. This is in agreement with binding studies in which a dissociation constant for dihydropyridines of 10^{-9} M was measured (for review see Fosset & Lazdunski, 1987). Following the modulated receptor hypothesis, a high-affinity binding only to the inactivated state should result in a drastic shift of the steady-state force inactivation curve to more negative potentials (cf. Bean, 1984). This, however, was not observed (Caputo & Bolanos, 1987; present results) and suggests a more complicated mode of action.

With regard to the growing evidence that the DHP receptor acts as Ca^{2+} channel and controller of Ca^{2+} release (Lamb & Walsh, 1987; Tanabe, Beam, Povel & Numa, 1988), the differing actions of dihydropyridines on Ca^{2+} current and force (or Ca^{2+} release) are of particular interest. We therefore compared the effects of nifedipine (a photosensitive dihydropyridine derivative) on Ca^{2+} current and force, notably on their voltage dependence of inactivation. We conclude that, at least in intact frog skeletal muscle fibres, the nifedipine-induced effects can be explained by assuming a low-affinity binding of the drug to the resting state of the DHP receptor and a high-affinity binding to the inactivated *and* the active state.

Preliminary accounts of this work have been presented at a meeting of the Physiological Society (Neuhaus, 1986).

METHODS

Preparation

The m. lumbricalis digiti IV of the hindlimbs of the frog (*Rana temporaria*) was used. The animals were killed by decapitation and the muscles were dissected and stored in Ringer solution in a refrigerator until use. The experiments were performed with small bundles of fibres from this muscle. Isometric force of a single fibre (average length ~ 1·3 mm) was measured under voltage-clamp control using two flexible microelectrodes, as described by Gomolla, Gottschalk & Lüttgau (1983). A uniform depolarization under point voltage-clamp conditions was achieved in external solutions with TEA (tetraethylammonium) sulphate or propionate as main solutes (cf. Berwe & Feldmeyer, 1984).

General procedure

After the preparation had been transferred into the experimental chamber, Ringer solution (A) was replaced by solutions B and C in succession as described previously (Erdmann & Lüttgau, 1989). When nifedipine was applied we usually waited 5 min before we started the experiment. After a successful impalement, we clamped the fibre at a negative holding potential (−70 to −100 mV) for 5 min, before force or Ca^{2+} current was induced.

Measurement of the current through slow Ca^{2+} channels

These currents were measured in hypertonic solutions to prevent movement artifacts. We used Ca^{2+} or Ba^{2+} as current carriers at a concentration of 10 mM with propionate as an anion (solutions H and I). Barium was preferred because maximum current was greater, which allowed a better evaluation. We measured the slow Ca^{2+} (or Ba^{2+}) current, first described by Sanchez & Stefani (1978). It is detected as transient inward current (see Fig. 9) if Cl^- is absent and Na^+ and K^+ currents are mainly blocked by TTX and TEA. The difference between the negative peak of this inward current and the maximum of the subsequent outward current after inactivation of I_{Ca} was taken as a measure of the maximum Ca^{2+} inward current (see Fig. 9). Fibres with low Ca^{2+} currents or strong leakage currents were discarded. The fast Ca^{2+} inward current (Cota & Stefani, 1986) was only occasionally identified by its fast rising rate. It was neglected in the present work.

Threshold of force activation

These measurements were performed under optical control with a binocular (40 × magnification). In order to obtain reproduceable results, we always applied the same pulse programme. From a holding potential of −90 mV, we started with a first test pulse to −80 or −70 mV and increased the depolarization gradually in steps of 3 mV, until a clear movement became visible. Subsequently, the pulse was reduced in 2 mV steps, until movements were no longer detectable. This value plus 2 mV was taken as threshold potential.

Solutions

The following solutions were used (all specifications in mM):
Solution A = standard Ringer solution, NaCl, 115; KCl, 2·5; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85; $CaCl_2$, 1·8.
Solution B = sulphate Ringer solution, Na_2SO_4, 38·75; K_2SO_4, 1·25; Na_2HPO_4, 1·08; NaH_2PO_4, 0·43; $CaSO_4$, 9·4; saccharose, 113.
Solution C = sulphate Ringer solution without divalent cations, Na_2SO_4, 38·75; K_2SO_4, 1·25; Na_2HPO_4, 1·08; NaH_2PO_4, 0·43; saccharose, 129.
Solution D = TEA solution, $(TEA)_2SO_4$, 40; K_2SO_4, 1·25; MOPS (3-(*N*-morpholino)propanesulphonic acid), 5; $CaSO_4$, 9·4; saccharose, 113.
Solution E = TEA solution in which Ca^{2+} was replaced by an equivalent amount of Mg^{2+}, $(TEA)_2SO_4$, 40; K_2SO_4, 1·25; MOPS, 5; $MgSO_4$, 17·4; saccharose, 101.

Solution F = solution E plus 1 mM-EGTA.
Solution G = solution E plus 1 mM-EGTA and 10 mM-perchlorate.
Solution H = hypertonic calcium propionate solution, (CH_3CH_2COO)TEA, 120; (CH_3CH_2COO)K, 2·5; $(CH_3CH_2COO)_2$Ca, 10; imidazol-Cl, 2; saccharose, 350.
Solution I = hypertonic barium propionate solution, (CH_3CH_2COO)TEA, 120; (CH_3CH_2COO)K, 2·5; $(CH_3CH_2COO)_2$Ba, 10; imidazol-Cl, 2; saccharose, 350.

Solutions B–I contained 10^{-7} g/ml TTX (tetrodotoxin). The pH of solutions A–H was 7·0 and that of solution I was 6·8. The concentration of free Ca^{2+} in solutions B and D was ~ 4 mM and that of free Mg^{2+} in solutions E, F, G was likewise ~ 4 mM. The dihydropyridine derivatives nifedipine, Bay K 8644 (Bayer, Leverkusen), (+)-(*S*)-202-791 and (−)-(*R*)-202-791 (Sandoz, Basel) were dissolved in DMSO (dimethylsulphoxide) stock solution at a concentration of 1 or 2 mM. Since the solutions used in the experiments contained up to 5 μM of the dihydropyridines, the DMSO concentration reached a maximum of 0·5% which, in control experiments, did not cause significant alterations of the time course of contractures.

Photoconversion of nifedipine into an ineffective compound

Nifedipine is a photosensitive drug which can be transferred into an ineffective nitroso-compound (Fig. 1) by irradiation with visible and UV light (Ebel, Schütz & Hornitschek, 1978).

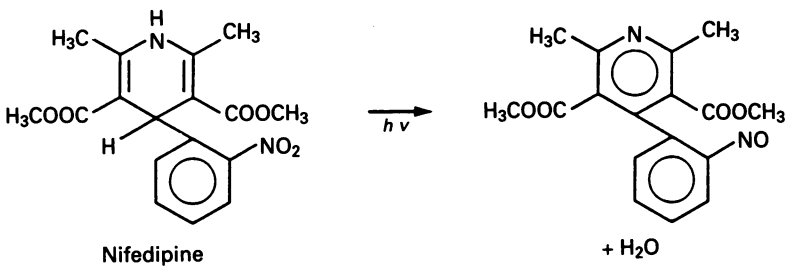


Fig. 1. Photoconversion of nifedipine.

The experiments were, therefore, performed at a dim yellow darkroom illumination and the stock solutions were stored in the dark. This photosensitivity was of great help in so far as it allowed to perform controls at the end of experiments or even in between. For this purpose, light from a mercury high-pressure lamp passed through a converging lens to illuminate the whole chamber. The light intensity of this elementary device was sufficient to restore the main part of the Ca^{2+} current blocked by nifedipine within about 15 s. To obtain a fairly complete inactivation of nifedipine we generally irradiated the whole chamber for 60 s. In control experiments we could show that irradiation for such a period of time and even longer periods did not alter Ca^{2+} or leakage currents and it was possible to reapply and irradiate nifedipine several times without damaging the preparation. After reapplication of the drug, the previous level of current inactivation was reached within a rather short time of 1–2 min.

Temperature

The muscles were dissected in a pre-cooled standard Ringer solution and the experiments were performed at temperatures between 6 and 15 °C, mostly at 10 or 15 °C.

Fitting procedure

For least-squares fitting we made use of the so-called MINPACK library (More, Garbow & Hillstrom, 1980). The FORTRAN source was provided by the Ruhr-University Computation Center and modified for use on our laboratory minicomputer (SMS 1000, Scientific Micro Systems Inc. with processor LSI 11/73 and RT 11 operating system, Digital Equipment Corporation). Data are expressed as means ± S.E.M. (n = number of experiments).

RESULTS

The effect of nifedipine on isometric force

Contractures in solutions containing different metal ions

Phenylalkylamines (D600, D888), which form another class of Ca^{2+} antagonists, are known to cause 'paralysis' by stabilizing the force-controlling system in the inactivated state (Eisenberg, McCarthy & Milton, 1983). As a consequence, force restoration is very much delayed and it takes minutes instead of seconds until the ability to develop force is fully restored (Berwe, Gottschalk & Lüttgau, 1987). It was therefore our first aim to find out if nifedipine also induces paralysis. We applied a pulse programme similar to that of Berwe *et al.* (1987) with a long-lasting depolarization (3·5 min) to induce paralysis and a restoration time of 1·5 min at −90 mV, which under normal conditions is sufficient to gain complete restoration.

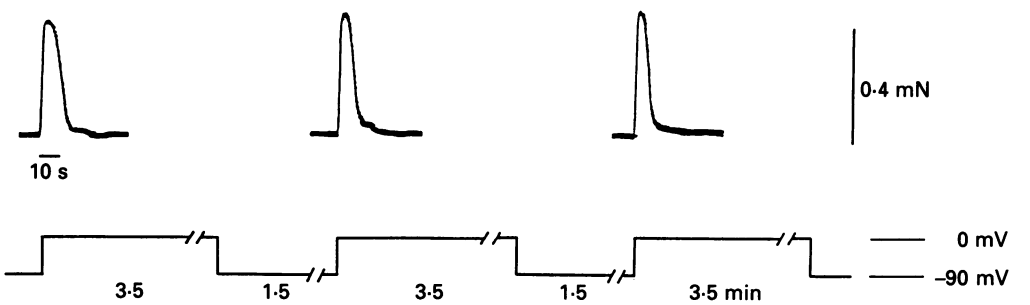


Fig. 2. 'Paralysis' experiment. The fibre was activated three times by long-lasting depolarizations as indicated by the pulse programme. Solution D, 1 μM-nifedipine, temperature = 6 °C.

The temperature was reduced to 6 °C, since it is known that in the presence of D600 lowering the temperature prolongs the restoration time considerably; although it does not *prevent* restoration (Siebler & Schmidt, 1987). Nifedipine (1 μM) was added to solution D before the first contracture was induced (Fig. 2). The results show that the restoration time was sufficient to restore maximum force, i.e. paralysis did not take place. The contractures became slightly shorter. However, this was sometimes also observed without nifedipine and is not regarded as an indication of a beginning paralysis. Using the same preparation and an identical pulse programme Lüttgau *et al.* (1987) obtained no paralysis either, when they applied nitrendipine, another dihydropyridine derivative, at the very high concentration of 100 μM.

Since nifedipine had no effect on the time course of contractures, we tried to sensitize the force-controlling system by replacing external Ca^{2+} with other metallic cations which can partially substitute Ca^{2+} in its role as a stabilizer of the resting and activatable state of the voltage sensor (Lüttgau & Spiecker, 1979; Pizarro, Fitts, Uribe & Rios, 1989). Representative results are shown in Fig. 3. The experiments were performed as follows. The fibre concerned was clamped at a holding potential of −80 mV for 5 min before a control contracture was induced by a depolarizing step

of 20 s (*A* and *B*) or 10 s (*C*) duration to 0 mV. Afterwards nifedipine (4 μM in *A* and *B*, 2 μM in *C*) was applied for 5 min before the test contracture was induced. Subsequently the fibre recovered at −80 mV for 5 min. It was then irradiated for 1 min before a second control contracture was induced. In the presence of Ca^{2+}, we observed no alteration in the time course of the contracture after nifedipine had been

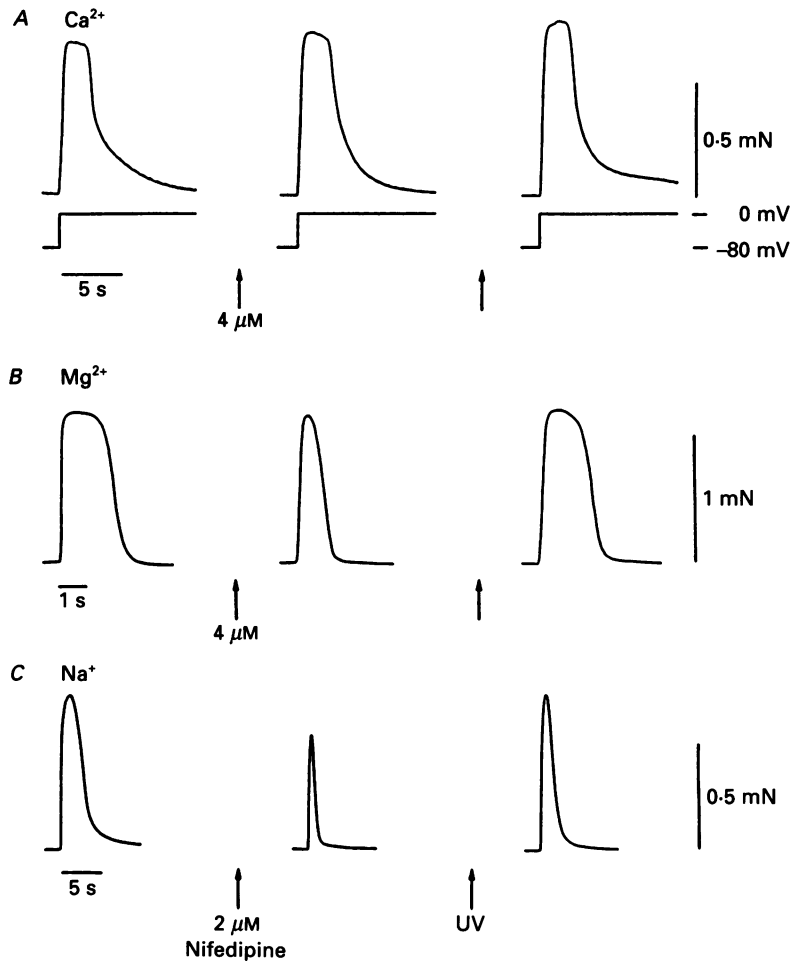


Fig. 3. Contractures induced by a depolarizing step (20 s in *A* and *B*, 10 s in *C*) from −80 to 0 mV in the presence of Ca^{2+} (*A*), Mg^{2+} (*B*) and Na^+ (*C*). In each row, a control contracture is followed by a contracture in the presence of nifedipine and one after irradiation with UV light. *A*, Solution D, 4 μM-nifedipine; *B*, solution E, 4 μM-nifedipine; *C*, solution C, 2 μM-nifedipine; three different fibres, temperature = 15 °C.

applied (*A*). When Ca^{2+} was replaced by an equivalent amount of Mg^{2+} (solution E), nifedipine caused a shortening of the plateau of maximum force which could be substantially reversed by irradiation (*B*). Similar effects of nifedipine in mammalian muscles have recently been described by Dulhunty & Gage (1988). In a solution without divalent cations and with Na^+ (solution C containing 80 mM-Na^+) being the only metal ion, we observed contractures without a plateau. The application of

nifedipine caused a distinct shortening and a decrease in maximum force. In most cases, this effect was only partially reversible, as shown in Fig. 3*C*, which is probably due to a general 'run-down' of fibres under this condition.

The reduction in the area of force activation (force time integral) in Ca^{2+}-free, Mg^{2+}-containing solutions was used as a characteristic, although not ideal, parameter for investigating the dose–response relation in the action of nifedipine. In Fig. 4 we plotted the reduction in force area in relative units *vs.* the concentration of nifedipine on a logarithmic scale. The mean value of areas before application of nifedipine and after irradiation served as a control ($F_{Control}$). It can be seen that the nifedipine effect approached saturation at a reduction in area to a value slightly below 50% with

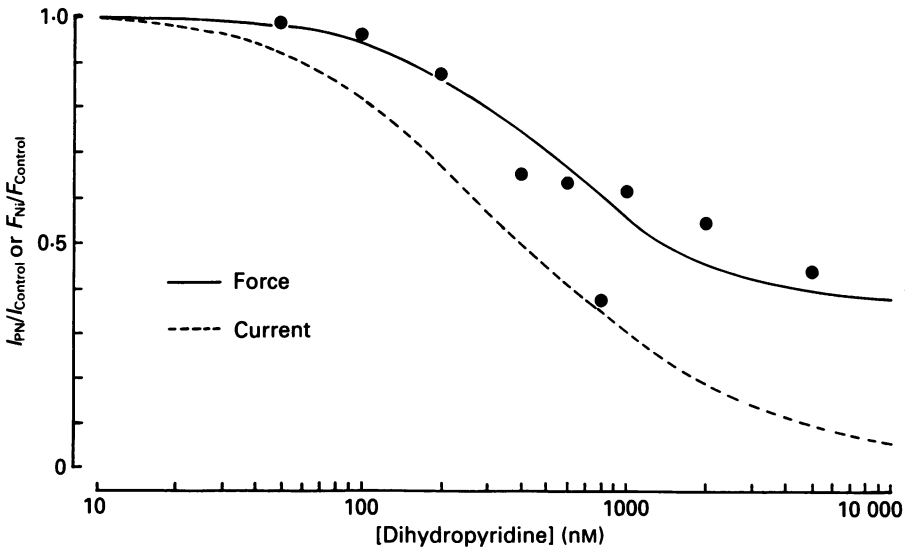


Fig. 4. Dependence of force on the concentration of nifedipine. The force time integral in the presence of the drug (F_{Ni}) divided by that in the control ($F_{Control}$) is plotted on the ordinate *vs.* the concentration of nifedipine on the abscissa (logarithmic scale). Each point of the curve which was fitted by hand corresponds to one fibre. Ca^{2+}-free solution containing Mg^{2+} (E), temperature = 15 °C. The dashed line shows peak Ca^{2+} current remaining 4–5 min after the application of PN-200/110 (another dihydropyridine derivative) as a fraction of that recorded immediately before (data (Fig. 3d) from a publication by Schwartz *et al.* 1985).

concentrations beyond 1000 nM. In this experiment, the fibres were kept at a holding potential of −70 mV. Hyperpolarizing fibres for several minutes to −120 or −150 mV could not reverse the shortening of the plateau of maximum force or the reduction in force development induced by nifedipine.

Potential dependence of force inactivation

By assuming a high-affinity binding of nifedipine only to the inactivated state of the DHP receptor, the modulated receptor hypothesis predicts a distinct shift of the steady-state inactivation curve towards more negative potentials (cf. Hille, 1977). We tested this prediction in the following experiment (Fig. 5*A*). In the presence of 2 μM-nifedipine, the fibre was clamped at a conditioning potential (given on the abscissa) for 240 s to establish a new distribution between resting and inactivated

DHP receptors, before the remaining resting receptors were activated by full depolarization. Afterwards, the preparation was irradiated and the pulse programme repeated to obtain the control value. A direct depolarization from −100 to 0 mV was finally induced to estimate the 100 % reference value to present force in relative units on the ordinate.

We used a Ca^{2+}-free solution containing Mg^{2+} and EGTA (F) because, as shown in Fig. 3, a nifedipine effect became detectable only in the absence of external Ca^{2+}. Fibres did not survive well in solution F. This, together with the rather long-lasting polarization at intermediate potentials, explains why usually only one pulse cycle could be accomplished. It may also account for the relatively large scattering of data

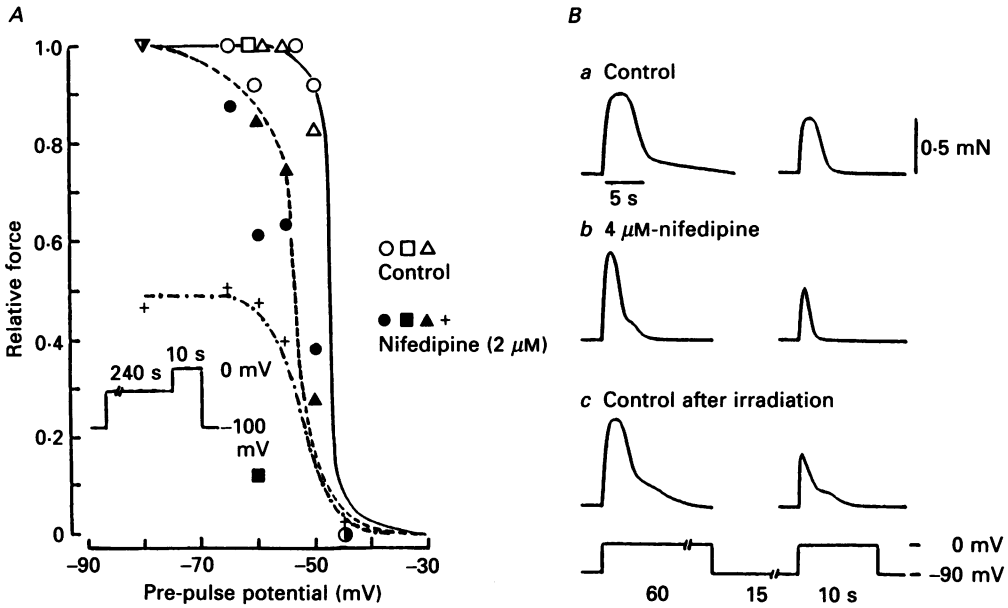


Fig. 5. *A*, the effect of 2 μM-nifedipine on the potential dependence of force inactivation. The pulse programme is shown in the inset. Between pulses the holding potential was −100 mV. Maximum force developed after the conditioning pulse (pre-pulse) in relation to maximum force induced by a reference depolarization step from −100 to 0 mV is plotted on the ordinate *vs.* the pre-pulse potential on the abscissa. Control, open symbols; 2 μM-nifedipine, filled symbols, maximum force; crosses, corresponding time integrals of contractures (the crosses at −55 and −60 mV are mean values). Solution F, nine fibres, temperature = 15 °C; the curves were fitted by hand. *B*, restoration of force during a short restoration time (15 s) at −90 mV: *a*, under control conditions; *b*, in the presence of 4 μM-nifedipine; *c*, after irradiation. The pulse programme is shown below. One fibre, solution F, temperature = 10 °C.

points. However, the results quite clearly show that nifedipine causes only a small shift of the inactivation curve towards more negative potentials in the order of about 10 mV. In addition, the curve is also likely to decrease in steepness. (Concerning the great steepness of normal force inactivation curves, see Erdmann & Lüttgau, 1989.) The evaluation of maximum force gives the impression of a complete cancellation of the nifedipine effect by hyperpolarization. That this is not the case became apparent when the area of force development was estimated. The lowest curve marked by crosses (Fig. 5*A*) shows that the force time integral in the presence of 2 μM-nifedipine was restored only by about 50 %.

These results show that, in a narrow potential range close to −50 mV, the difference between force development with and without nifedipine can become very pronounced. In a restoration experiment (not illustrated), which was again performed in a Ca^{2+}-free solution (F), we depolarized a fibre for 20 s and induced a contracture. Afterwards it was repolarized for 40 s to −50 mV, i.e. a potential where the nifedipine effect was most marked. A subsequent full depolarization revealed a restoration of maximum force by more than 50% in the first control and after irradiation (second control), but nearly no restoration in the presence of 4 μM-nifedipine.

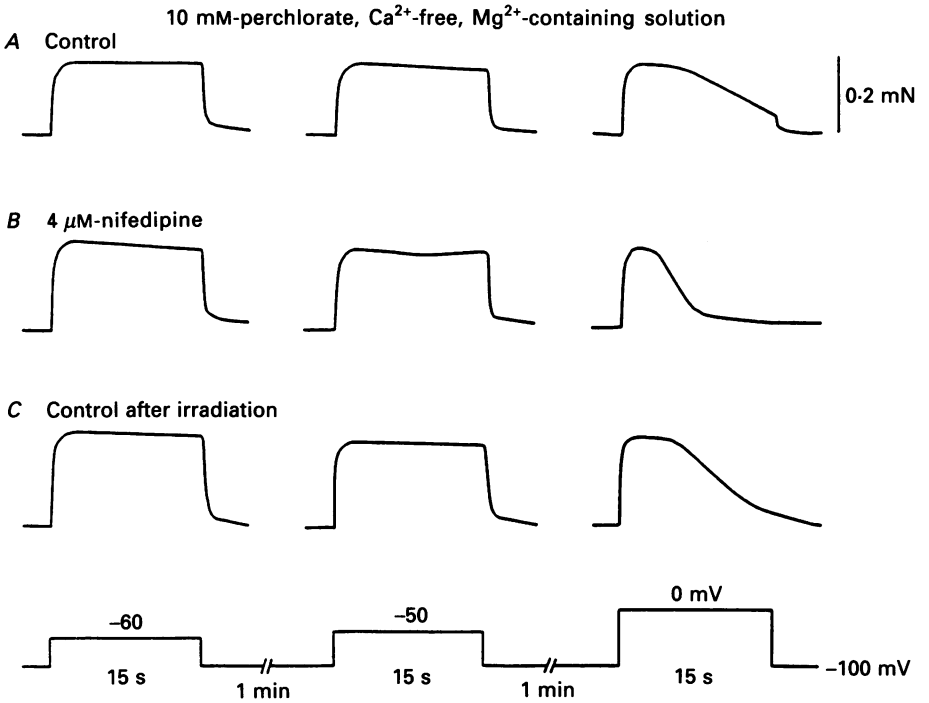


Fig. 6. The effect of nifedipine on long-lasting contractures induced by depolarization steps from −100 mV to −60 and −50 mV in the presence of 10 mM-perchlorate. *A*, control; *B*, after the application of 4 μM-nifedipine; *C*, second control after 1 min of irradiation with UV light. Ca^{2+}-free solution containing Mg^{2+} (G), temperature = 10 °C.

In an additional restoration experiment, shown in Fig. 5*B*, we applied 4 μM-nifedipine in a Ca^{2+}-free solution (F), depolarized for 60 s and reprimed at −90 mV for only 15 s. After this time, we induced a contracture which (in relation to the peak of the preceding contracture) reached 60% in the presence (*b*) and 64% (mean value of *a* and *c*) in the absence of the drug. This result was confirmed in further experiments, partly with different pulse programmes. It shows that restoration occurred unchanged in the presence of nifedipine apart from the component that was blocked in a voltage-independent way (see above). In Ca^{2+}-free solutions containing nifedipine, contractures were always shorter. This effect, described as an accelerated inactivation, could not be removed even by long-lasting periods of hyperpolarization (see Fig. 5*A*). With Na^+ as the only metallic ion in the external solution the potential dependence of inactivation shifted to more negative potentials (~ −70 mV). Since no

stable conditions could be obtained in this solution we did not measure complete inactivation curves. However, several tests allowed the conclusion that the shift in the potential dependence of inactivation to more negative potentials, induced by 2 μM-nifedipine, was likewise again in the order of only 10 mV.

The effect which a relatively high concentration of nifedipine (100 μM) has on the potential dependence of *restoration* in the presence of Ca^{2+} (1·8 mM) has already been measured by Caputo & Bolanos (1987). They observed a shift to negative values by about 12 mV (restoration time 30 s). From this finding and our experiments it appears that the effect of nifedipine on the potential dependence of inactivation tends to be independent of the metallic ion composition of the external solutions. These ions on the other hand had quite different effects on the duration of contractures (Fig. 3).

The chaotropic anion perchlorate is known to shift the potential dependence of intramembrane charge movement and force activation towards more negative potentials (Lüttgau, Gottschalk, Kovács & Fuxreiter, 1983), while the potential dependence of force inactivation remains unchanged (Gomolla *et al.* 1983). This results in a voltage range in which activation without inactivation of force takes place. We made use of this new possibility of force prolongation to find out if nifedipine affects longer lasting contractures (Fig. 6). In these experiments, 10 mM-ClO_4 were added to a Ca^{2+}-free, Mg^{2+}-containing solution (G), which caused a negative shift in threshold by 20 mV. The fibre in question was depolarized from -100 to -50 or -60 mV for 15 s. Figure 6 shows that no relaxation occurred at either potential in the absence and in the presence of 4 μM-nifedipine. Upon full depolarization, the fibre relaxed faster in the presence of nifedipine. This is in line with conclusions of the Mg^{2+} experiment illustrated in Fig. 3 and shows that perchlorate did not suppress the action of nifedipine.

One problem linked with the analysis of force measurements is the fact that force may reach saturation while the myoplasmic Ca^{2+} concentration (Ca_i^{2+}) still increases. Thus it could be argued that, in the experiment shown in Fig. 6, Ca_i^{2+} declined during the long-lasting pulses, but did not fall below the force saturation value. The following observations, however, contradict this interpretation. In some experiments of this type, force did not reach its maximum at -60 mV. However, a decline in force during the pulse did not take place.

The results of this section show that, even in the absence of Ca^{2+}, nifedipine caused the force inactivation curve to shift only slightly towards more negative potentials. This small shift and the corresponding minor shift of the Ca^{2+} current inactivation curve of only 4 mV (0·8 μM-nifedipine, see Fig. 11) are difficult to reconcile with a high-affinity binding only to the inactivated state. If one assumes a high affinity to the inactivated state with a dissociation constant of $\sim$ 1 nM (cf. Fosset & Lazdunski, 1987) and a low affinity of $\sim$ 500 nM to the resting state (compare Bean, 1984; Gurney, Nerbonne & Lester, 1985), the modulated receptor hypothesis (cf. Hille, 1977; Bean, 1984) would predict a shift of the mid-point voltage to more negative potentials by more than 40 mV. This is certainly not the case. An alternative explanation is given in the Discussion.

Facilitation of force development

Several investigators (McCleskey, 1985; Dulhunty & Gage, 1988) have shown that, under certain conditions, nifedipine facilitates activation. We were able to confirm their findings by using several experimental procedures. In the experiment shown in Fig. 7, we measured strength–duration curves in the presence of 2 μM-nifedipine, and after irradiation. The threshold of force development was estimated by optical inspection under the binocular, as described in detail under Methods. In the presence of the drug, we estimated a rheobase of −75 mV. After a first irradiation of 60 s, it shifted to −65 mV and after a second irradiation, the curve with open symbols was

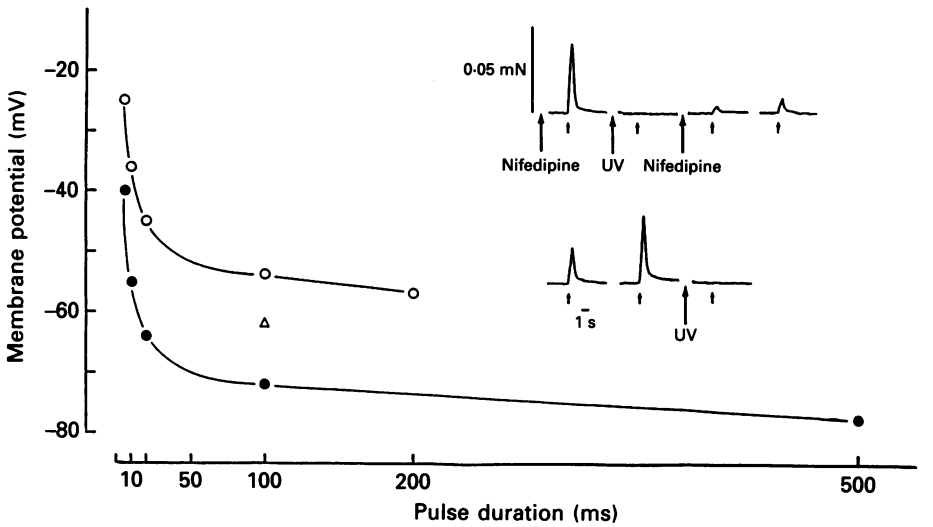


Fig. 7. Facilitation of force development. Strength–duration curves in the presence of 2 μM-nifedipine (●) and after two successive periods of irradiation, each lasting one min (○). The triangle point was measured after the first irradiation. Abscissa, duration of the rectangular depolarizing pulse. Ordinate, threshold potential of force activation. Holding potentials −90 mV. One fibre, solution D, temperature = 6 °C. Inset: the initiation of force by means of voltage steps to normal threshold potential (500 ms from −90 to −50 mV) after applying 2 μM-nifedipine and its abolition by UV light (twice, as indicated). Time between stimuli 10 s, solution D, temperature = 6 °C.

measured. The new rheobase near −50 mV corresponds to the normal value observed under the present conditions. In further experiments of this type, nifedipine mostly caused a negative shift of the rheobase. However, we observed great variations. Applying the same concentration of nifedipine we observed shifts in the rheobase of between 30 and only a few millivolts. The induction of minor contractures prior to the threshold tests appeared to augment this facilitating action of nifedipine.

The inset of Fig. 7 shows the effect of 500 ms pulses from −90 to −50 mV in the presence of 2 μM-nifedipine, and after irradiation. Following the first irradiation, the fibre developed no force in response to a stimulus. However, force reappeared after reapplying nifedipine with increasing strength during successive stimuli.

In a further experiment (Fig. 8), which also revealed facilitation, the fibre was stimulated every 2 s by short pulses (200 ms) from a relatively positive holding

potential of −70 mV to 0 mV. The upper (*A*) row shows the control contractures. As in further experiments of this type, the second stimulus induced a prolonged response, for which a substantiated explanation cannot be given. This prolongation decreased with the 2nd, 3rd and 4th response. When nifedipine (2 μM) was applied (Fig. 8*B*), the responses nearly fused, i.e. nifedipine caused a slowing of deactivation. This effect of nifedipine could be reversed either by irradiation, as illustrated in

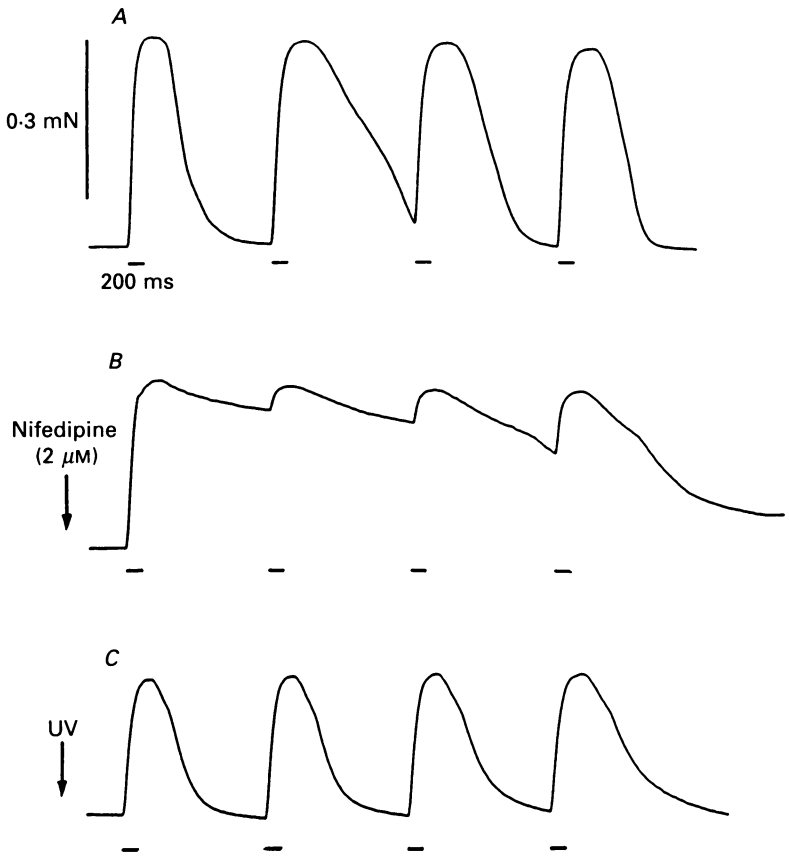


Fig. 8. The effect of nifedipine on successive contractures induced by four short stimuli (200 ms) from a holding potential of −70 to 0 mV. Interval between pulses, 2 s. *A*, control; *B*, after the application of nifedipine; *C*, after irradiation. Solution D, temperature = 10 °C.

Fig. 8*C*, or by hyperpolarizing the fibre to −100 mV (not shown). The latter finding shows that Ca^{2+} release is still under the control of the voltage sensor. In Fig. 8*B*, the effect became less pronounced during subsequent responses. This was always observed in similar experiments. It reveals that the nifedipine-induced activation of force negative to the normal threshold of about −50 mV cannot be maintained. This is a potential range in which inactivation is normally absent. Thus, long-lasting contractures could be obtained in the presence of perchlorate, which likewise causes a threshold shift to negative potentials (see Fig. 6). The transient character of the

nifedipine-induced force development could possibly be explained by a slow return of the threshold to the normal value, probably caused by intracellular processes in connection with Ca^{2+} release (see Discussion). In our opinion, a transformation into the inactivated state is not involved, because the time course of contractures upon full depolarization remains unchanged in Ca^{2+}-containing solutions. The transient character of the phenomenon explains the difficulties of getting reproducible results and the impossibility of quantitatively describing the dependence of threshold on nifedipine concentration.

In further experiments we compared the effect of the dihydropyridine (+)-*S*-202-791, which is known to be an activator of myocardiac Ca^{2+} channels ('agonist'), with its enantiomer (−)-*R*-202-791, a blocker of Ca^{2+} channels ('antagonist'), on the threshold of force activation. The threshold was estimated before and 5 min after the application of the drug. We used concentrations of between 0·5 and 2·5 μM and, in ten successful attempts, we observed no (two fibres) or a small shift between 5 and 9 mV to more negative potentials, with no distinct difference occurring between the two enantiomers. The results suggest a minor facilitation of force development. However, since the exchange of the solution alone often caused a minor threshold shift and exact threshold measurements were difficult to obtain, we did not pursue these investigations further.

The effect of nifedipine on the slow inward current

With regard to the dual function of the DHP receptor (see Introduction) it was of interest to compare the effects of nifedipine on force development, as described in the previous section, with those on the slow inward current. We concentrated our main effort on investigating what effect nifedipine had on the voltage dependence of inactivation, since the shift in mid-point voltage of inactivation is a central parameter for analysing a possible state-dependent binding of the drug to the DHP receptor. A few experiments also deal with current activation. We used Ba^{2+} and Ca^{2+} as current carriers and suppressed force development by applying hypertonic solutions. Further technical details have been described under Methods.

Voltage dependence of inactivation

The pulse programme for measuring the voltage dependence of inactivation as concerns the slow Ca^{2+} (or Ba^{2+}-) current corresponds to the classical procedure and was as follows. The fibre was first clamped at a holding potential of −90 mV for 5 min. From this potential, currents were induced by depolarizing to −20 (Ba^{2+}) or 0 mV (Ca^{2+}). After this first test, the fibre was clamped from −90 mV to a conditioning (pre-pulse) potential between −70 and −10 mV and in a second step, after 10 s, depolarized to −20 (Ba^{2+}) or 0 mV (Ca^{2+}). The currents induced by the second step were evaluated, as described under Methods, to estimate the extent of inactivation established during the preceding conditioning pre-pulse. Between these and all other pulse sequences, fibres were repolarized for 1 min to −90 mV. After the conditioning pulses at different potentials had been tested, the control pulses from −60 mV (10 s) to 0 mV with Ca^{2+} (or −90 mV to −20 mV with Ba^{2+}) were repeated. Only those experiments were further evaluated in which maximum current in the controls at the beginning and at the end differed by less than 10%.

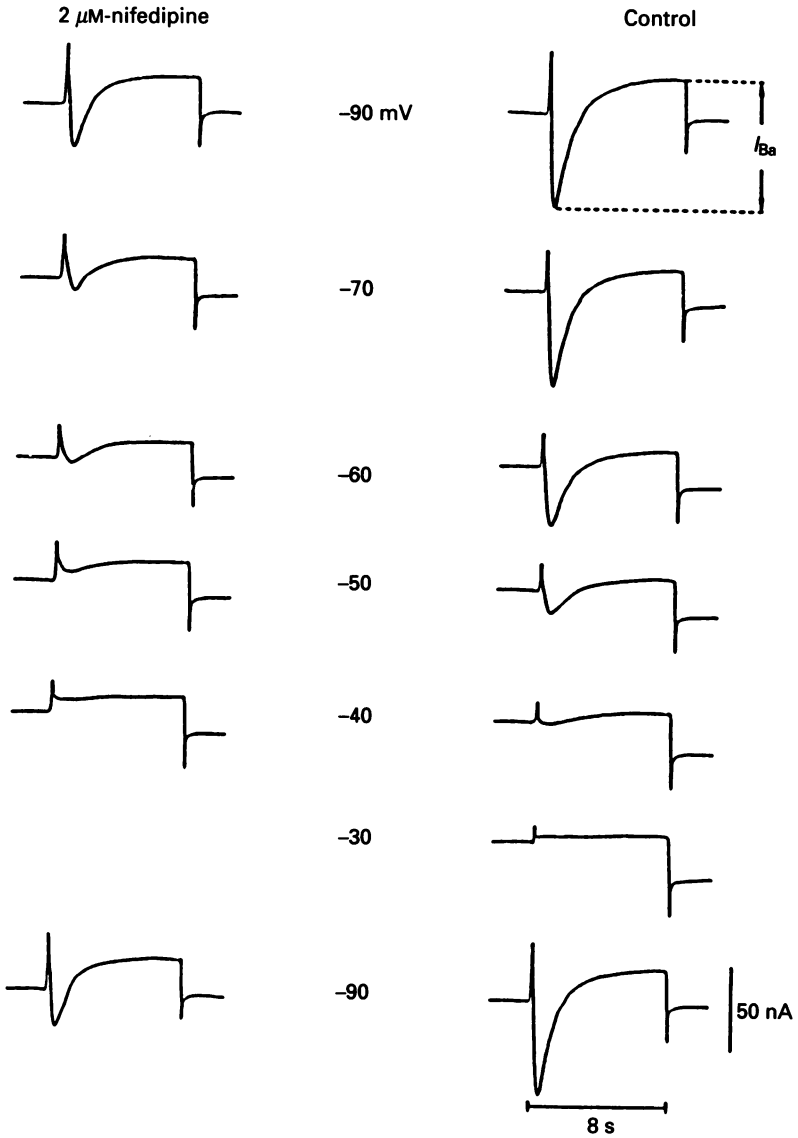


Fig. 9. Voltage dependence of current inactivation. Ba^{2+} currents through the slow Ca^{2+} channel in the presence of nifedipine (left) and subsequently after UV irradiation. The currents were induced by a depolarizing voltage step to −20 mV after a conditioning period of 10 s at the potentials indicated in the middle. Solution I (barium propionate) with 2 μM-nifedipine, temperature = 15 °C.

In the presence of Ca^{2+}, the current amplitude was often larger when induced from a pre-pulse holding potential of −60 mV than from −90 mV because, after a depolarization from −60 mV, the Ca^{2+} current rose with some delay and could be more easily distinguished from the early outward current. Therefore, with Ca^{2+} as current carrier the −60 mV value was taken as 100%. In experiments with nifedipine, the pulse programme was extended as follows. Prior to the application of nifedipine, the control pulses from −90 mV (Ba^{2+}) and −60 mV (Ca^{2+}) were

performed. Subsequently, the propionate solution (H or I) was replaced by a solution of the same composition plus nifedipine. After 5 min of incubation, we carried out the same conditioning pulse programme as the one without nifedipine. Afterwards, the preparation was irradiated for 1 min and control pulses from −90 mV (Ba^{2+}) and −60 mV (Ca^{2+}) were induced. The fibre was then irradiated a second time and the control pulse programme was repeated. Usually we observed only minor differences (plus and minus) between the current amplitudes after the first and second irradiation. The mean of the amplitude at −60 mV (Ca^{2+}) or −90 mV (Ba^{2+}) was taken as 100%. We evaluated only those experiments in which the current

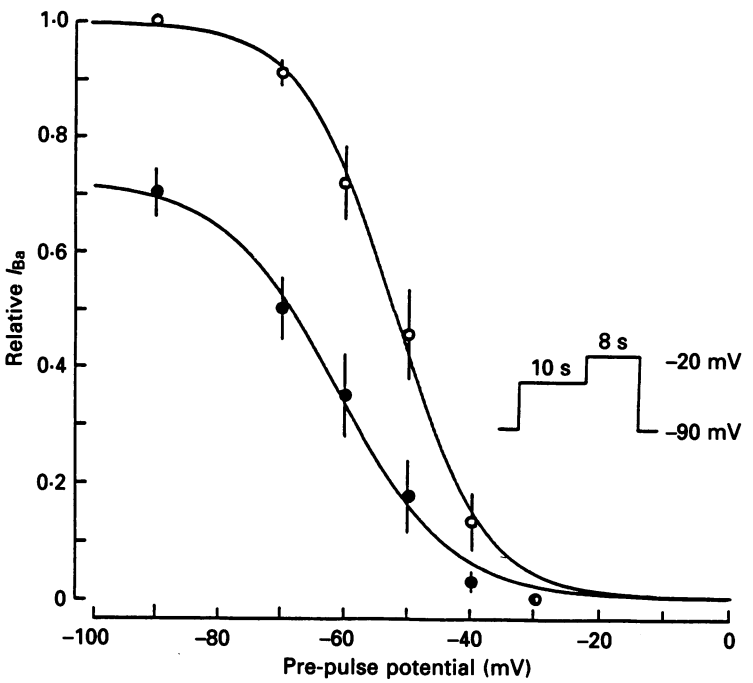


Fig. 10. Voltage dependence of Ba^{2+} current inactivation with (●) and without (○) nifedipine. The pulse programme is shown in the inset. For further details see text. Ordinate, Ba^{2+} current in relative units. Reference potential, −90 mV. For further details see text. Abscissa, conditioning potential. Curves were best fit of function $(1+\exp[(V_{0.5}-V)/k])^{-1}$ to data. Control (○): $V_{0.5} = -52$ mV, $k = -7{\cdot}0$ mV; 2 μM-nifedipine (●): $V_{0.5} = -61$ mV, $k = -9{\cdot}0$ mV. Means ± S.E.M. ($n = 6$). Solution I, temperature = 15 °C.

amplitude between the first (before application of nifedipine) and the final control differed by less than 20%.

Figure 9 shows original records from an experiment with Ba^{2+} as a current carrier. The measurements, which were all performed with one fibre, started with a series of different pre-potentials in the presence of the drug. Subsequently, the whole programme was repeated after an irradiation of the fibre. This experiment was exceptional in so far as the fibre survived the pulse programme in the presence of nifedipine and after irradiation with only a minor 'run-down' and little alteration in leakage current. Normally, only half of the programme, i.e. either with or without nifedipine, could be carried out as described. Figure 9 reveals that nifedipine

decreased maximum current even at negative holding potentials. It also shifted the potential dependence of inactivation to more negative potentials. This can be seen more clearly in Fig. 10, in which the amount of inactivation is related to the conditioning pre-potential. In this figure the results obtained from six fibres in the presence of 2 μM-nifedipine are compared to those from six control fibres. Maximum Ba^{2+} current at -90 mV was reduced by about 30%. In addition, the mid-point voltage ($V_{0.5}$) shifted from -52 to -61 mV while the steepness factor k decreased from -7 to -9 mV.

Figure 11 illustrates related results with Ca^{2+} as a current carrier. At a concentration of 0·8 μM, nifedipine reduced maximum Ca^{2+} current at negative

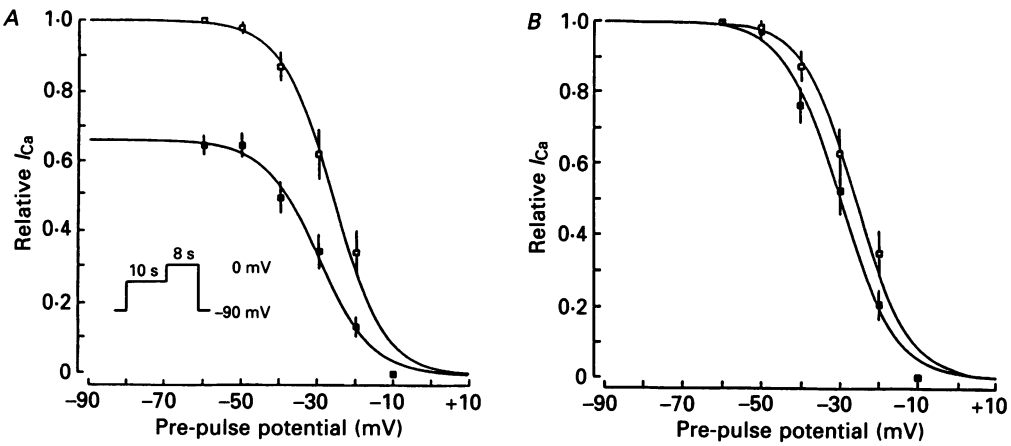


Fig. 11. *A*, the effect of nifedipine on the voltage dependence of channel inactivation in the presence of Ca^{2+}. Same procedure as in Fig. 10 with the following deviations: the fibres were depolarized to 0 instead of -20 mV and the reference potential was -60 instead of -90 mV. Ordinate, Ca^{2+} current related to the control current from -60 mV (reference potential). Abscissa, conditioning potential. Curves were best fit of function $(1+\exp[(V_{0.5}-V)/k])^{-1}$ to data. Control (□): $V_{0.5} = -26$ mV, $k = -6.7$ mV; 0·8 μM-nifedipine (■): $V_{0.5} = -30$ mV, $k = -7.1$ mV. Means ± S.E.M., $n = 10$ (control) and $n = 7$ (nifedipine), solution H, temperature = 15 °C. *B*, same experiment as in *A* with the maximum current in the presence of nifedipine scaled up to 1·0.

potentials by about 35%, while k changed from -6.7 to -7.1 mV. A comparison of the control inactivation curves in the presence of Ba^{2+} and Ca^{2+} shows that the mid-point voltage in the presence of Ca^{2+} was to be found at a value which is 26 mV more positive than that obtained with Ba^{2+}. Comparing the action of nifedipine in the presence of Ca^{2+} or Ba^{2+} it appears that, with Ca^{2+}, the decrease in I_{max} at negative potentials was comparatively more pronounced than the shift in $V_{0.5}$. For a better comparison of shift and steepness, the maximum Ca^{2+} current in the presence of nifedipine was scaled up to 100% in Fig. 11*B*.

The question of how much time is needed to reach a steady state in the binding of dihydropyridines during the conditioning pulse is still a matter of controversial discussion. In the experiments by Gurney *et al.* (1985), the conditioning pulse lasted for 250 ms and the authors observed a shift in the inactivation curve by more than 20 mV to negative potentials, which results in a calculated K_D value of 39 nM if a

binding of the drug to the inactivated state is assumed. Since this K_D value is still larger than the value obtained in binding studies the authors assumed that the steady-state inactivation had not been completely reached yet. However, with regard to the relatively large shift of the inactivation curve observed after only 250 ms we feel that 10 s, i.e. a 40 times longer pulse duration, as used in our experiments, should at least be sufficient to approach the steady state quite closely. This should in particular be expected in the case of high concentrations, which were up to 3 orders of magnitude higher than the dissociation constant of the high-affinity drug sites. Longer-lasting pulses in the order of minutes will probably shift the inactivation curve still further. However, this effect is most certainly related to secondary reactions. It is known that longer-lasting depolarizations, for example, transform the inactivated voltage sensor and probably also the Ca^{2+} channel into a secondary inactivated state, from which it recovers only slowly after hyperpolarization (Lüttgau, Gottschalk & Berwe, 1986). These slow alterations were accelerated in Ca^{2+}-free solutions containing Mg^{2+} and may also be promoted by Ca^{2+} antagonists.

Voltage dependence of activation

Since nifedipine facilitated the activation of force as described in the preceding section, a few experiments were performed to find out if the drug has a similar effect on the slow Ca^{2+} channel. To detect a possible shift in current activation we depolarized fibres for 8 s from a holding potential of −90 mV to potentials between −60 and +20 mV in 10 mV steps. Ba^{2+} was used as a current carrier. Nifedipine at a concentration of 2 μM was added and the results in the presence of nifedipine were compared with controls after inactivating the drug by UV light. The Ba^{2+} currents became detectable at potentials positive to −50 mV and reached a maximum between −30 and −20 mV. Nifedipine reduced the current but did not alter the current–voltage relation nor did it change the threshold potential, which corresponds to related findings in heart muscle fibres (Gurney *et al.* 1985). This, however, does not exclude entirely a threshold shift as observed in force measurements (Fig. 7) since tiny Ca^{2+} currents escape detection under the present experimental conditions.

In a few experiments, we applied the Ca^{2+} agonist Bay K 8644 (2 μM) (see Bechem, Hebisch & Schramm (1988) concerning the action of Ca^{2+} agonists) and observed an increase in Ca^{2+} current, a shortening in the time to peak and a shift in the potential dependence of activation by about 10 mV to more negative potentials. These results are comparable to those obtained by Ildefonse, Jacquemond, Rougier, Renaud, Fosset & Lazdunski (1985).

Dose–response relation. Only a few experiments were done with nifedipine concentrations above and below 0·8 μM (in the presence of Ca^{2+}). Due to a shortage in the supply of *R. temporaria*, we were not in a position to complete a dose–response curve as intended. For this reason, we included a dihydropyridine dose–response curve of Schwartz, McCleskey & Almers (1985) in Fig. 4 (dotted line), which roughly corresponded to our data (compare Fig. 11). At high concentrations, i.e. 5 and 10 μM, we obtained a slightly less pronounced inhibitory action. In addition, we had difficulties in restoring the original current strength, even with several irradiation periods of 1 min.

DISCUSSION

The action of nifedipine on Ca^{2+} current and force

The dihydropyridine receptor is an integral protein in the T-tubular membrane. Recently, it has been suggested that it is both a Ca^{2+} channel and a voltage-sensing device which in some way controls the release of Ca^{2+} from the sarcoplasmic reticulum (see Introduction). Ca^{2+} antagonists bind to the DHP receptor protein and interfere in a still unknown way with the gating mechanism, i.e. the voltage-sensitive domains of the integral protein. It is, therefore, not surprising that nifedipine affected both Ca^{2+} current and force development, although to a different extent. At a concentration of a few micromolar, nifedipine blocked Ca^{2+} current, while normal phasic contractures could still be elicited. This could imply the existence of two separate gating mechanisms or that one gating arrangement controls both systems, whereby a binding of nifedipine may have different effects on each process. We think that the second alternative is the case and that the obviously different effects on the two systems may finally prove helpful to gain a deeper insight into the function of the DHP receptor and the mode of action of the dihydropyridine class of Ca^{2+} antagonists.

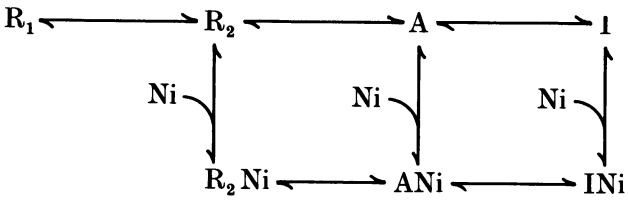
As to force development, it appears that nifedipine accelerates the inactivation of force. This effect, however, became detectable only after Ca^{2+} had been replaced by other metal ions. This dependence on the cation species in the external solution is to our mind related to recent observations by Pizarro *et al.* (1989). These authors showed that metallic ions in the external solution are needed for the normal functioning of the voltage sensor with an efficiency sequence of the order $Ca^{2+} > Sr^{2+} > Mg^{2+} > Ba^{2+} > Na^{+} > K^{+} > Cs^{+}$. They assume that this sequence corresponds to the affinity of metal ions to a specific site at the voltage sensor and that a dissociation of the cation upon depolarization is the cause of inactivation. Following this line, we assume that nifedipine binds to the voltage sensor. If Ca^{2+} is present, this has no visible effect on force inactivation. However, if metallic ions with lesser affinity are present, the bound nifedipine appears to accelerate the normal release of the cation, resulting in a shortening of contractures. The dose–response curve shown in Fig. 4 suggests that nifedipine is bound to a low-affinity binding site ($K_D \sim 0{\cdot}8\ \mu M$). This effect could not be reversed by long-lasting periods of hyperpolarization and the characteristic action of nifedipine on the duration of contractures could also be traced during the process of restoration after a depolarization-induced inactivation of force development (Fig. 5*B*). This indicates a rather stable binding to the resting state.

The effect of nifedipine on Ca^{2+} current is principally comparable to that on force in the absence of Ca^{2+}. It can likewise not be reversed by hyperpolarization and the dose–response curve reveals a similar K_D value (Fig. 4). However, the effect is more pronounced and nifedipine can suppress the Ca^{2+} current completely. This could, for example, be related to the slower time course in the activation of the Ca^{2+} current, compared to that of the Ca^{2+} release mechanism.

The state-dependent binding of nifedipine

Binding studies revealed a high-affinity binding site for nifedipine ($K_D \sim 1$ nM, see below). Since nanomolar concentrations of the drug were without effect on Ca^{2+}

current or force it is reasonable to assume that the measured high-affinity binding is restricted to the inactivated DHP receptor (cf. Bean, 1984). This should lead to a drastic shift of the steady-state inactivation curve to more negative potentials (modulated receptor hypothesis, cf. Hille, 1977). However, this was not the case; only a minor shift in the potential dependence of force and current inactivation was found. We feel that these and other findings could be reconciled by assuming a high-affinity binding ($K_D \sim 1$ nM) to both the inactivated *and* the active state (and probably a precursor of the active state, R_2) of the voltage sensor controlling the Ca^{2+} channel in the T-tubular membrane *and* Ca^{2+} release from the SR, as described in the following state diagram.



Here R_1 and R_2 mean different resting (closed) states, whereby the transition is associated with charge movements (compare Melzer, Schneider, Simon & Szücs, 1986), with A being the active and I the inactivated state. (The above-described low-affinity binding is not included in this state diagram.) With the assumption of a high-affinity binding to states R_2, A and I nifedipine should cause only a minor shift of the inactivation curve to more negative potentials, as was observed.

The state diagram could also explain the facilitating action of nifedipine since a high-affinity binding to A would increase the probability of finding the system in an active state. Unfortunately, nifedipine-induced activation of force was only transient. Therefore it was not possible to quantitatively investigate in how far the shift in threshold of force depends on the concentration of nifedipine. Since relaxation of nifedipine-induced force was apparently not accompanied by inactivation we assume that the system turns into a resting, but not into an inactivated state. A facilitating action of nifedipine on the Ca^{2+} current was not observed. However, a possible transient activation may have escaped detection (see p. 203).

In binding studies, Schwartz *et al.* (1985) observed high-affinity sites ($K_D \sim 1$ nM) in depolarized *and* polarized (−80 mV) fibres. However, in the latter case, B_{max} reached only $\sim \frac{1}{3}$ of the corresponding value found in depolarized fibres. These findings are difficult to interpret. Agreement with the present model could be achieved if the assumption is made that $\frac{1}{3}$ of the polarized fibres were actually in a partially depolarized state (~ -50 mV), which allowed a binding of nifedipine to R_2 or I without a further recruitment of binding sites from fibres with normal resting potentials.

Comparison with former results

High-affinity binding sites for the binding of nifedipine and other dihydropyridine derivatives to fractions enriched with T-tubular membranes were found by different authors (for reference see Fosset & Lazdunski, 1987). The dissociation constants

estimated (0·7–4·9 nM) were slightly higher than those measured in heart preparations (for reference see Glossmann & Striessnig, 1988).

With regard to the effect of nifedipine on force development, the present results are in good agreement with those found by McCleskey (1985) and Dulhunty & Gage (1988), who used intact frog and mammalian skeletal muscle fibres, respectively. These authors have already described the facilitating action of this drug and they likewise found only a minor depression of force development at even much higher nifedipine concentrations than those used in the present experiments. Dulhunty & Gage (1988) also showed that nifedipine became more effective when Ca^{2+} was replaced by Mg^{2+}. They interpreted their results by means of a reaction scheme in which both the transformation from a precursor state into the active state and subsequently from the active into the inactivated state involves a dissociation of Ca^{2+}. Dihydropyridines are hypothesized to act by reducing the affinity of the active and the inactive state to Ca^{2+}. When we compare this result with the present modulated receptor model, several points of agreement become obvious. In both models, the binding of nifedipine to the active state or a precursor is assumed and inactivation is associated with a dissociation of Ca^{2+} from a specific binding site.

Completely deviating results were obtained by two other groups. Cognard, Romey, Galizzi, Fosset & Lazdunski (1986) used myoballs of skeletal muscles from newborn rats and measured Ca^{2+} currents using the whole-cell recording mode of patch clamp. With 13 and 0·15 nM of PN-200-110, another dihydropyridine derivative, these authors obtained a 50% current block when they induced the current from a holding potential of −90 and −65 mV, respectively. Pizarro, Brum, Fill, Fitts, Rodriguez, Uribe & Rios (1988; see also Rios & Brum, 1987) measured intramembrane charge movements and Ca^{2+} transients in cut fibres. In a Ca^{2+}-free, Mg^{2+}-containing solution, the Ca^{2+} transient disappeared almost completely after the application of nifedipine (1 μM) while charge movements changed their polarity, indicating a transformation of the gating system into the inactivated state ($Q_1 \rightarrow Q_2$). This was measured with a holding potential of −110 mV. Hyperpolarizing the membrane to −130 mV caused a substantial recovery of the Ca^{2+} transient and a less pronounced restoration of charge movement, i.e. nifedipine must have caused a considerable shift of the Ca^{2+} release (force) inactivation curve. These results, in particular those of Cognard *et al.* (1986), can be quantitatively interpreted by assuming a high-affinity binding only to the inactivated state. In the experiments of the two groups, the intracellular solution was at least partially replaced by an artificial one. Since it is known that, in the absence of 'cytosolic regulatory control', Ca^{2+} channels may reveal a lower affinity to dihydropyridines (see Porzig & Becker, 1988), we argue that in artificial solutions the states R_2 and A may lose their high affinity to dihydropyridines. A variable affinity of dihydropyridines to secondary resting states and the active state, depending on the composition of external and internal solutions and other factors as holding potential, temperature, etc. may to our mind explain the different effects of the drug and the apparently controversial results described in the literature.

The present model does not exclude alternative concepts about the action of dihydropyridines, which were developed to describe the action of the drug in heart muscle cells. Of particular interest is the model of Lacerda & Brown (1989) who

assume a co-operative binding of DHPs at two sites. We preferred the above-mentioned extension of the modulated receptor concept (e.g. Bean, 1984) mainly because it provides an explanation for the diverging results obtained with perfused and intact preparations by altering only one binding constant.

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