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EFFECTS OF DIHYDROPYRIDINES ON CALCIUM RELEASE FROM THE ISOLATED MEMBRANE COMPLEX CONSISTING OF THE TRANSVERE TUBULE AND SARCOPLASMIC RETICULUM

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SUMMARY: We have investigated a) the effects of the dihydropyridines (DHPs) nifedipine and nimodipine on depolarization-induced (T-tubule-mediated) Ca^{2+} release in the vesicles consisting of the complex of the T-tubule and SR, and b) the binding of [^{3}H]nimodipine to these vesicles. These DHPs inhibited the slow but not the fast phase of depolarization-induced release, both of which are mediated via the T-tubule. The DHPs have no effect on caffeine-induced release in which T-tubules are not involved. There are two classes of DHP binding sites: one, with high affinity and small capacity, and another, exhibiting low affinity and a much larger capacity. The inhibition paralleled the low affinity binding of DHP with no correlation with the high affinity binding. These results suggest that the low affinity DHP binding sites located probably in the DHP receptor, rather than the high affinity DHP binding site, are responsible for the inhibition of e-c coupling.

The T-tubule membrane contains the specific receptor for DHP; the primary structure of its α_1 subunit was deduced from the cDNA sequence (1) and its high affinity DHP binding has been thoroughly investigated (review: ref. 2). Recent studies have led to unveiling of the critical role played by the DHP receptor in the initial step of e-c coupling in muscle. Thus, e-c coupling is missing in dysgenic mouse muscles where there is a specific genetic defect in the α_1 subunit of the DHP receptor (3), while expression of the α_1 subunit led to restoration of e-c coupling (4). Moreover, DHPs and phenylalkylamines inhibit a non-linear charge movement (5,6), Ca^{2+} transient (6), and contracture (7-10) in muscle fibers. However, such inhibition occurs at concentrations of the

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Abbreviations: DHP, dihydropyridine; e-c coupling, excitation-contraction coupling; MES, 2-(N-morpholino) ethane sulfonic acid; PMSF, phenyl methyl-sulfonyl-fluoride; SR, sarcoplasmic reticulum; T-tubule, transverse tubular system.

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reagents (e.g. 10-500 nM nifedipine, 1-100 μM D-600) that are much higher than the high affinity (or specific) DHP binding range.

The main purposes of this study were to investigate the effects of DHPs (nifedipine and nimodipine) on depolarization-induced Ca^{2+} release using the isolated microsomal vesicles consisting of the T-tubule-SR complex as an in vitro model of e-c coupling (cf. refs. 11-13), and to correlate these effects with DHP binding to the vesicles. As shown here, these DHPs inhibit depolarization-induced Ca^{2+} release, but not caffeine-induced release. Binding data suggest that the inhibition of Ca^{2+} release by DHPs is mediated by their binding to the low affinity site of the DHP receptor rather than to the receptor's high affinity site.

MATERIALS AND METHODS

Materials: Nimodipine (Bay E 9736) was the generous gift from Miles Laboratories, Inc., nifedipine was purchased from Sigma Co.

Preparation of microsomal fraction enriched in the triad vesicles: The triad-containing microsomal fraction was prepared from rabbit leg and back muscles by differential centrifugation as described previously (14). After the final centrifugation, the sedimented fraction was homogenized in a solution containing 0.3 M sucrose, 0.15 M K gluconate, proteolytic inhibitors (PEI: 0.1 mM PMSF, 10 μg/ml aprotinin, 0.8 μg/ml antipain, 2 μg/ml trypsin inhibitor) and 20 mM MES (pH 6.8) at a final protein concentration of 20-30 mg/ml. The preparation was quickly frozen in liquid nitrogen and stored at -70^{o}.

Induction and assay of depolarization-induce Ca^{2+} release: The fraction (3.2 mg/ml) was incubated in 0.15 M K gluconate, 20 mM MES (pH 6.8) with or without addition of various concentrations of DHPs (nifedipine or nimodipine) for 30 min at 22^{o}. The control contained no DHP but the same volume of ethanol (the solvent of DHPs). The vesicles (1.6 mg/ml) were incubated in a solution containing 0.15 M K gluconate, 200 μM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5 mM Na_2-ATP, 5.0 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase, 9 μM arsenazo III, and 20 mM MES, pH 6.8 (Solution A_1) at 22^{o} to actively load the SR moiety with Ca^{2+} and polarize the T-tubule moiety due to the (Na^+ + K^+) ATPase reaction. At various times after the addition of ATP, the solution A_1 was mixed with an equal volume of Solution B_1 containing 0.15 M choline chloride, 9 μM arsenazo III, 20 mM MES (pH 6.8) to produce the depolarization of the T-tubule. The time course of induced Ca^{2+} release was recorded using a stopped-flow spectrophotometer system (14). The kinetic constants of Ca^{2+} release were calculated by fitting a double exponential function to the signal-averaged traces (see the legend to Fig. 1A).

Induction and assay of caffeine-induced Ca^{2+} release: The microsomal vesicles (3.2 mg/ml) were incubated in 0.15 M KCl with or without addition of inhibitors for 30 min at 22^{o}. For active Ca^{2+} loading of SR, the vesicles (1.0 mg/ml) were incubated in a solution of 0.15 M KCl, 0.5 mM Mg ATP, 5.0 mM phosphenolpyruvate, 10 units/ml pyruvate kinase, 9 μM arsenazo III, and 20 mM MES, pH 6.8 (Solution A_2). After incubation for 4-8 min, Ca^{2+} release was induced by mixing one part of Solution A_2 with one part of Solution B_2 containing 0.15 M KCl, 4 mM caffeine, 9 μM arsenazo III, 20 mM MES (pH 6.8). The time course of induced Ca^{2+} release was recorded using a stopped-flow spectrophotometer system, and the data were analyzed by computer fitting of a single exponential function.

Assays of [^{3}H]nimodipine binding: The microsomal vesicles were incubated with various concentrations of nimodipine containing [^{3}H]nimodipine (2.5 μC$_i$/ml) for 30 min, and incubated further in Solution A_1 in the same way as done for the induction of depolarization-induced Ca^{2+} release (see above). For the assay of low affinity binding, a 0.4 ml portion of Solution A_1 was placed on a 0.5 ml layer of 0.3 M sucrose, 20 mM MES (pH 6.8), and centrifuged for 15 min at 65k

rpm in a Beckman TLA-100.2 rotor. The sedimented fraction was homogenized in 0.2-0.3 ml of 0.15 M K gluconate, 20 mM MES (pH 6.8), and mixed with ScintiLene for scintillation counting. The background count obtained with 0.5 mM nimodipine was subtracted from each count. The high affinity binding was assayed after incubation of the vesicles as described above using the filtration technique (15,16).

RESULTS

As previously described (11,12), chemical depolarization of the T-tubule moiety of the Ca^{2+}-loaded T-tubule-SR complex leads to biphasic release of Ca^{2+} accumulated in the SR moiety (see inset to Fig. 1A). We investigated the effects of DHPs on depolarization-induced Ca^{2+} release. As seen in Figs. 1 (nifedipine) and 2 (nimodipine), the release rate in the slow phase was sharply reduced by DHP in the sub-μM to μM range. These DHPs had no appreciable effect on the fast phase of depolarization-induced Ca^{2+} release (Figs. 1A and 2A) or on caffeine-induced Ca^{2+} release (Figs. 1B and 2B).

There are clearly two distinguishable classes of [^{3}H]nimodipine binding sites in the T-tubule-SR complex: one, with high affinity and a small capacity ($K_D \simeq 2 \times 10^{-9}$ M, B_{max} = 1.2 pmol/mg), and another, exhibiting low affinity and a much larger capacity ($K_D \simeq 1.5 \times 10^{-6}$ M, B_{max} = 107 pmol/mg). The low affinity binding, but not the high affinity binding, appears to occur in a cooperative fashion; viz. Hill coefficient, 1.89.

Comparison of the inhibition of depolarization-induced Ca^{2+} release (Figs. 1A and 2A) with binding of [^{3}H]nimodipine (Fig. 3A) shows that the inhibition closely parallels the low affinity binding of [^{3}H]-nimodipine, without appreciable inhibition in the high affinity binding range ($\leq 3.0 \times 10^{-8}$ M).

DISCUSSION

The isolated microsomal fraction enriched in the T-tubule-SR complex serves as an *in vitro* model of e-c coupling (11-13). As shown here, some DHPs such as nifedipine and nimodipine inhibit depolarization-induced Ca^{2+} release. The important new aspect of this study is the finding that DHPs inhibit the slow phase of depolarization-induced Ca^{2+} release, whereas they have virtually no effect on the fast phase. This suggests that the slow phase is mediated by DHP-sensitive protein(s), while the fast phase is mediated by DHP-insensitive protein(s). According to our recent studies (17), the monoclonal antibody mAb #78 directed against the α_1 subunit of the DHP receptor (18) inhibits the slow phase of depolarization-induced Ca^{2+} release. On the other hand, antibodies directed against a ~28 kDa protein of the T-tubule inhibit the fast phase (refs. 19,20). Thus, it appears that the fast phase and the slow phase of depolarization-induced Ca^{2+} release are mediated by at least two different T-tubule proteins, *viz.* the 28 kDa protein and the DHP receptor, respectively.

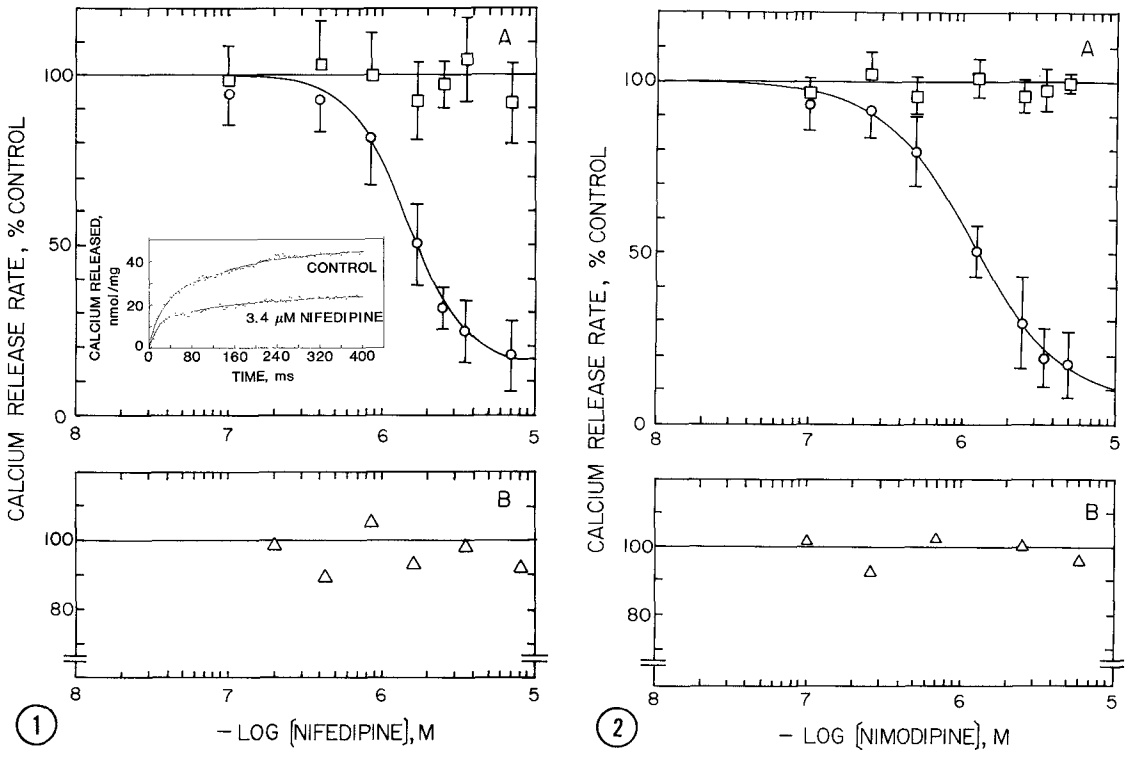

Fig. 1. A Effects of nifedipine on the fast phase (○) and slow phase (□) of depolarization-induced Ca^{2+} release in the microsomal vesicles enriched in the T-tubule-SR complex, as a function of the concentration of nifedipine. Ca^{2+} release was induced by replacement of K gluconate with choline Cl after incubating the triad-containing microsomes with various concentrations of nifedipine. The time course of Ca^{2+} release (see inset) was fitted by double-exponential: $A_f\{1-\exp(-k_f t)\}+A_s\{1-\exp(-k_s t)\}$, where A is the amount of Ca^{2+} released, and k is the rate constant of release in the fast (f) and slow (s) phases. The release activity (A·k, or the initial rate) was calculated for each concentration of nifedipine, and expressed as % activity relative to the value at [nifedipine]=0. Datum point: average ± standard deviation. n=2-10. B Lack of effect of nifedipine on caffeine-induced Ca^{2+} release. Ca^{2+} release was induced by caffeine after incubating the vesicles with various concentrations of nifedipine as above, and the time course of release was fitted by single exponential. The relative release activity was calculated as described above. Datum point: average of two experiments.

Fig. 2. A Effects of nimodipine on the fast phase (○) and slow phase (□) of depolarization-induced Ca^{2+} release as a function of the concentration of nimodipine. Relative activity of Ca^{2+} release was calculated as described in the legend to Fig. 1A. n = 2-4. B Lack of effect of nimodipine on caffeine-induced Ca^{2+} release. Induction of release and calculation of the data were performed in the same way as in the nifedipine experiments. Datum point: average of two experiments.

As shown here, the inhibition of the slow phase of depolarization-induced Ca^{2+} release occurs at high DHP concentrations at which the DHP binding with low affinity and high capacity takes place, while there is no inhibition in the high affinity (specific) binding range. Since a large portion of the low affinity binding described here may be ascribable to non-specific binding to heterogeneous components, it is likely that only a small portion of the low

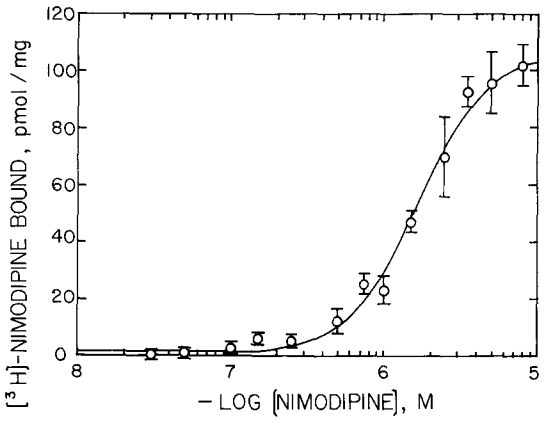

Fig. 3. Binding of [^{3}H]nimodipine to the microsomal vesicles. Binding assays were performed as described in Materials and Methods. Datum point: average ± standard deviation. n = 2-7.

affinity binding sites is critical for the inhibition. These critical sites are likely to be in the T-tubule, since DHPs have no effect on caffeine-induced Ca^{2+} release which is produced by direct stimulation of the SR moiety (13,21). Moreover, they appear to be associated with the DHP receptor for the following reasons. Firstly, the slow phase that is inhibited by DHPs is in fact mediated by the DHP receptor as described above. Secondly, in the intact muscle fiber system, nifedipine (10 nM - 0.5 μM) blocks charge movement, which is the event presumably occurring within the DHP receptor (e.g. ref. 6). However, the possibility that low affinity DHP binding proteins other than the DHP receptor (e.g. ref. 22) might also be involved in the inhibition cannot be excluded.

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