Vol. 131, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1985 Pages 13-21

PROFILE OF THE OPPOSITELY ACTING ENANTIOMERS OF THE DIHYDROPYRIDINE 202-791 IN CARDIAC PREPARATIONS: RECEPTOR BINDING, ELECTROPHYSIOLOGICAL, AND PHARMACOLOGICAL STUDIES

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Received July 5, 1985

Receptor binding, electrophysiological, and inotropic effects of the pure dihydropyridine enantiomers (+)S202-791 and (-)R202-791 were studied in cardiac preparations. The K_I for (+)S202-791 binding correlated with the ED_{50}'s for an increase in contractile force and an increase in calcium current, the latter effect occurring at depolarized as well as resting holding potentials. The K_I for (-)R202-791 binding was much lower than the IC_{50}'s for inhibition of calcium current measured at holding potentials of -80 or -90 mV and a negative inotropic effect, but correlated closely with the IC_{50} for inhibition of calcium current measured at -30 mV. Thus, (+)S202-791, is a voltage independent calcium channel activator and (-)R202-791 is a voltage dependent calcium channel inhibitor. © 1985 Academic Press, Inc.

There are two major problems confronting the investigation of dihydropyridine (DHP) actions in cardiac muscle. First, is the approximate 1,000-fold discrepancy that exists between binding of calcium channel inhibitors, half-maximal inhibition of calcium influx through slow channels and contractility (1,2). Two explanations for this discrepancy have been proferred: 1) high and low affinity DHP "receptor sites" exist and these regulate calcium currents (3,4,5) and 2) only one high affinity site may be present, and alteration of the membrane potential can change the affinity of this site (6,7,8). Two recent studies, however, show that contrary to the expected change in affinity of DHPs with depolarization, a significant increase in only the number of DHP binding sites (B_{max}) occurs (9,10). A second major problem

0006-291X/85 $1.50
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is the biphasic effect of both calcium channel inhibitors and calcium channel activators (5,11-15). The dual stimulatory and inhibitory effects are consistent with the concept of multiple binding sites, but a single site explanation is also possible (8). However, in a recent intriguing study employing optically pure enantiomers of the DHP 202-791, Hof et al. (16) showed that in isolated rabbit aortic rings, biphasic effects of this DHP could be due to mixtures of oppositely acting enantiomers.

One approach to resolving both problems would be a detailed study of radioligand binding, electrophysiology and pharmacology using optically pure enantiomers, which have either calcium channel activating or inhibiting actions. These studies using (+)S202-791 and (-)R202-791 were carried out in cardiac preparations.

METHODS

Radioligand Binding Experiments

Canine cardiac sarcolemma was isolated and binding of [^{3}H]nitrendipine to high affinity sites was measured as previously described (3). Briefly, 25 µg of protein were incubated with [^{3}H]nitrendipine, either in the absence or presence of (+)S202-791 or (-)R202-791, in a final volume of 1 ml of 50 mM Tris-maleate buffer, pH 7.4, containing 150 mM NaCl and 1 mM $CaCl_2$. After 50 minutes of incubation at 37°C, the bound [^{3}H]nitrendipine was separated by rapid filtration and the radioactivity on the filters was measured with a Beckman LS 8100 liquid scintillation counter. Binding experiments with guinea pig microsomes were performed in 2 mls of 50 mM MOPS, pH 7.4, using 50 µg of protein. After 2 hrs of incubation at room temperature, bound [^{3}H]nitrendipine was separated by rapid filtration. The filter binding was measured by conducting experiments in the absence of protein.

Electrophysiological Experiments

Single ventricular cells were isolated from guinea pig hearts following the procedure described by Brown et al. (15). Whole-cell voltage clamp currents were recorded using the patch clamp method of Hamill et al. (17). The patch pipets had tip resistance of 1.5 MΩ or less, and the input resistance of the cell membrane used was between 10 and 100 GΩ. Evaluations were made on cells with stable calcium currents. Results from cells in which calcium currents were decreasing with time, or in which progressively more negative holding potentials were required to sustain the current produced by a given test potential, were discarded.

The experimental chamber (0.2 ml) was superfused at a rate of 2 ml/min by gravity. Ca currents were isolated by placing cells in an extracellular solution of (in mM): $CaCl_2$, 5; tetraethylammonium chloride 135; 4-aminopyridine, 5; $MgCl_2$, 1; glucose, 10; HEPES, 10 (pH 7.4). The patch pipet contained Cs-rich internal solution of (in mM): Cs Aspartate, 110; CsCl, 20; ATP, 2; EGTA, 5; HEPES, 5 (pH 7.3). All experiments were performed at 20-22°C.

Pharmacological Experiments

Myocardial contractility was measured as previously described (18). Briefly, left and right ventricular trabeculae of dog hearts and guinea pig papillary muscles were suspended in Krebs-Henseleit solution containing 2 mM Ca^{2+} and 5.9 mM K^+, at a pH of 7.4 and a temperature of 35°C. Stimulation

rate via point electrodes was 1 Hz. Equilibration time was 80-100 minutes. The contractile force and its derivative df/dt were recorded on a Grass P7 polygraph. The concentrations of (+)S202-791 and (-)R202-791, and the solvent ethanol, were added in a cumulative manner. Exposure time to each concentration was 20-25 minutes, a time sufficient for equilibration.

Materials

[^{3}H]nitrendipine, specific activity 78-79 Ci/mmol, was purchased from New England Nuclear. The stereoisomers of 202-791 were gifts from Sandoz Ltd., Basel, Switzerland, courtesy of Dr. Robert P. Hof. Stock solutions of the stereoisomers were made up to 10^{-2} M in 100% ethanol, and diluted with ethanol and water. The highest concentration of ethanol used in the experiments was 1%, which had no effect on the parameters measured in the binding and electrophysiological experiments. 0.1% ethanol produced a slight decrease in force in the pharmacological experiments. The drugs were used under sodium lamps.

Data Analysis

Radioligand binding data were analyzed using the nonlinear least squares Ligand program (19), converted to Applesoft by M.H. Teicher, MED-50 Ligand program (Biomedical Computing Technology Information Center) and modified by Dr. C.L. Johnson (University of Cincinnati).

The current and voltage in response to command pulses were monitored on a storage oscilloscope, digitized at 50 μs, and stored and analyzed on a PDP 11/70 computer. The concentration-response curves were fit to a single occupancy model using a modified Marquardt-Levenberg non-linear least squares method (20).

ED_{50}'s, i.e. the drug concentration that produced a 50% increase in contractility, and IC_{50}'s, the drug concentration producing a 50% decrease in contractility, were determined graphically.

RESULTS

Both (+)S and (-)R202-791 inhibited high affinity [^{3}H]nitrendipine binding to dog cardiac sarcolemma apparently in a competitive manner. This was demonstrated both by saturation and competition binding experiments (Fig. 1a and b, respectively). The calculated K_I for (-)R202-791 (1.60±0.08 nM) was much lower than the K_I for (+)S202-791 (230±12 nM) indicating that the binding is stereospecific. The inhibition of nitrendipine binding by these compounds to guinea pig membranes was also examined, and at room temperature, the K_I for (-)R202-791 was 0.9±0.3 nM and the K_I for (+)S202-791 was 90±30 nM (Table 1).

A few preliminary studies on the putative low affinity DHP site showed that these drugs may be weak inhibitors; however stereospecificity could not be demonstrated (data not shown).

(+)S202-791 produced only stimulatory effects on calcium currents at holding potentials from -20 to -100 mV. The effects were greatest at test potentials between -40 and zero mV and were smaller at positive test poten-

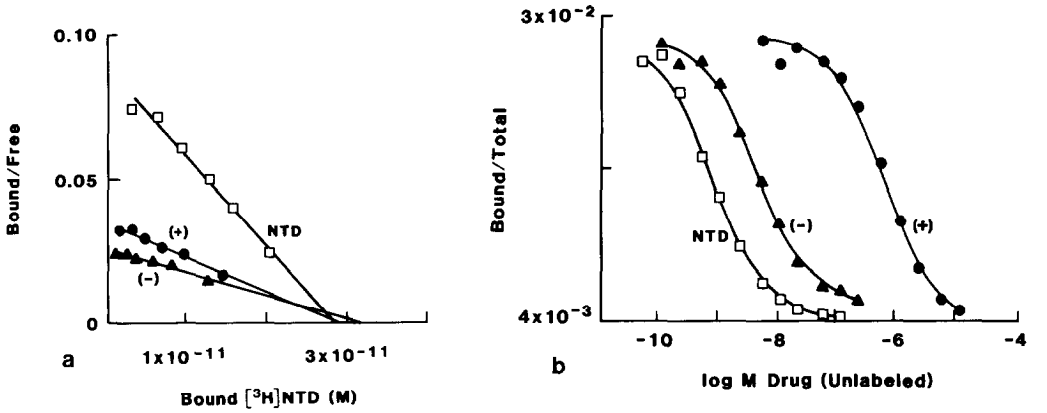


Figure 1. Effects of (-)R202-791 and (+)S202-791 on high affinity [^{3}H]nitrendipine binding to isolated canine cardiac sarcolemma. In panel a, computer fitted Scatchard plots of saturation [^{3}H]nitrendipine binding obtained in the absence (□) and in the presence of either 5 μM (-)R202-791 (▲) or 300 nM (+)S202-791 (●) are shown. Panel b illustrates computer fitted competition (displacement) binding curves. For these experiments, 0.5 nM [^{3}H]nitrendipine was used as labeled ligand and the concentrations of non-labeled nitrendipine (□), (-)R202-791 (▲) and (+)S202-791 (●) were varied as indicated on the abscissa. B, F and T indicate bound, total and free [^{3}H]nitrendipine concentrations, respectively.

TABLE 1

Binding	Electrophysiology	Pharmacology
	(+)S202-791	
GUINEA PIG	GUINEA PIG	GUINEA PIG
K_I = 90 ± 30 nM n = 3	ED_{50} = 80 ± 7 nM n = 6	ED_{50} = 315 ± 108 nM n = 4
DOG		DOG
K_I = 230 ± 12 nM n = 5		ED_{50} = 117 ± 22 nM n = 3
	(-)R202-791	
GUINEA PIG	GUINEA PIG	GUINEA PIG
	At - 90 mV IC_{50} = 200 ± 60nM n = 8	IC_{50} = 825 ± 48 nM n = 4
K_I = 0.9 ± 0.3 nM n = 3	At - 30 mV IC_{50} = 1 ± 0.95 nM n = 8	
DOG		DOG
K_I = 1.6 ± 0.08 nM n = 6		IC_{50} = 320 ± 93 nM n = 3

Values are mean ± S.E.M

tials. Fig. 2 shows calcium currents in the absence (a) and presence of (+)S202-791 at 100 nM (b), 200 nM (c) and 1 μM (d). The amplitude of the calcium currents increased without a significant change in the time course of activation and inactivation at concentrations between 1 nM and 100 nM. At higher concentrations, the inactivation rate was reduced. The onset of action was rapid (half time less than 10 seconds) and washing the cell resulted in recovery (data not shown).

The concentration dependence of the effects of (+)S202-791 on calcium currents for the response measured at 5 to 8 minutes after drug exposure is shown in panel (e) of Fig. 2. The relative changes in calcium current were plotted against the log of the drug concentration (abscissa). The continuous line was best fit by a one-to-one drug receptor interaction model with an ED_{50} of 80±7 nM (Table 1).

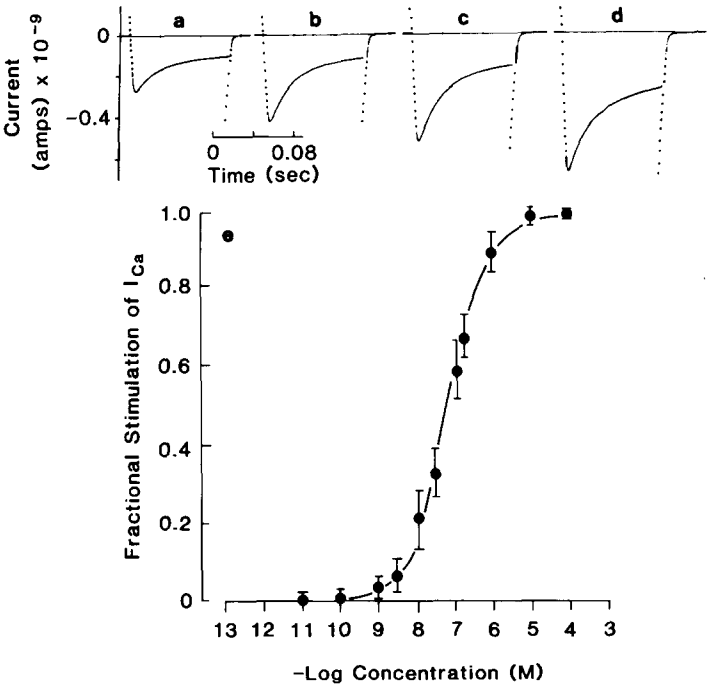
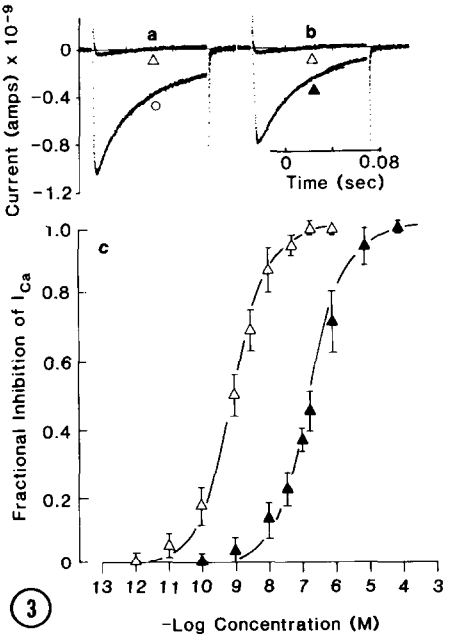


Figure 2. The effects of (+)S202-791 on whole cell calcium currents of isolated guinea pig ventricular myocytes. Original current traces are shown before (a) and after a 5 minute superfusion with (+)S202-791 at each of 100 nM (b), 200 nM (c), and 1 μM (d). The holding potential was -50 mV and depolarizing pulses to zero mV for 100 msec were applied at 0.1 Hz. Panel (e) shows the concentration-response curve of (+)S202-791. Each point represents the mean and S.D. for 5 to 8 cells. As results were the same regardless of the holding potential, data obtained at a range of holding potentials (-20 to -80 mV) were combined.

In contrast, (-)R202-791 had a purely inhibitory effect on calcium current. Panel (a) of Fig. 3 shows that 500 nM of the drug strongly inhibited the calcium current, and this inhibition could be considerably reduced by shifting the holding potential from -30 mV to -80 mV (b). Since these results suggest that the effects of (-)R202-791 are dependent upon the holding potential, more comparisons between the effects of holding potentials of -80 or -90 mV and -30 mV were made. The concentration-dependence of the drug effects at the two holding potentials are shown in panel (c) of Fig. 3. The concentration-response curves were best fit by a single binding site model. The IC_{50} at -30 mV was 1±0.95 nM, and the IC_{50} at -90 mV was 200±60 nM (Table 1).



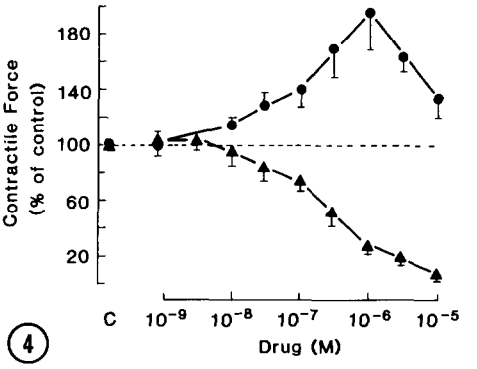


Figure 3. Panel (a) shows the calcium current response to a +10 mV test potential from a holding potential of -30 mV, in the absence (O) and presence (Δ) of the (-)R202-791. Panel (b) compares the current response to a +10 mV test potential from a resting potential of -30 mV (Δ) with the response to the same test potential when the cell had been held at -80 mV for 1 minute (▲) in the presence of 500 nM (-)R202-791. Panel (c) shows the concentration-response curves for (-)R202-791 at -30 mV (Δ) and at either -80 or -90 mV (▲). Each point represents the mean and S.D. for 5 to 8 cells.

Figure 4. Concentration-dependent effects of (+)S202-791 (●) and (-)R202-791 (▲) on contractile force of canine ventricular trabeculae. The drugs were added in a cumulative manner, and each concentration was allowed to act for at least 20 minutes to ensure its maximal effect. Points are mean ± S.E.M. for 3 tissues.

When 10 μM (-)R202-791 was added as a single dose and test pulses were applied from -30 mV, a maximal inhibition of calcium current was observed. If (+)S202-791 was then added cumulatively, an increase in calcium current was not observed until a concentration of 1 μM had been reached (data not shown).

In experiments using isolated dog heart ventricular trabeculae and guinea pig papillary muscles, (+)S202-791 produced a marked increase in contractile force to 197% and 252% of control, respectively, with ED_{50}'s of 117±22 nM (Fig. 4 and Table 1) and 315±108 nM. At concentrations higher than 1 μM in the dog and 10 μM in the guinea pig, the contractile force began to decline significantly. At 10 μM (+)S202-791, the solvent alcohol (0.1%) contributed less than 5% to the decline in contractile force. Calcium applied during the decline in force reversed this effect (data not shown). In concentrations higher than 10 nM, (-)R202-791 produced a negative inotropic effect with IC_{50}'s of 320±93 nM in the dog and 825±48 nM in the guinea pig. In both species, (-)R202-791 produced a very small (less than 13%) but consistent increase in force at concentrations between 0.1 and 10 nM. The positive inotropic effect of (+)S202-791 and the negative inotropic effect of (-)R202-791 could not be washed out in 15 minutes.

DISCUSSION

Both (+)S202-791 and (-)R202-791 competed with [^{3}H]nitrendipine for the high affinity DHP binding site present in canine cardiac sarcolemmal and guinea pig microsomal preparations. The obvious difference between the stereoisomers is their affinities, with (-)R202-791 having a 140-fold higher affinity than (+)S202-791. In the case of (+)S202-791, high affinity binding appears to correlate quite well with the activation of calcium channels and the positive inotropic effect. This correlation is supported by the fact that the activation of calcium channel current by (+)S202-791 occurred at all holding membrane potentials that were tested (-30 to -90 mV). A similar correlation exists between high affinity binding, calcium channel activation and the positive inotropic effect of the DHP Bay k 8644 (3,15). Even the

magnitude of the positive inotropic effect of (+)S202-791 was similar to that produced by Bay k 8644, though (+)S202-791 was less potent (12).

The negative inotropic effect of (+)S202-791 at concentrations higher than 1 μM was less pronounced than with Bay k 8644. Since calcium applied during the decrease in contractility could restore the force, the role of calcium overload in the negative inotropic effect could be ruled out, as has been shown for Bay k 8644 (5,12). Therefore, pharmacologically, except for a small difference in potency, (+)S202-791 and Bay k 8644 appear to be similar. When biphasic inotropic effects of stereoisomers are observed, the possibility of cross contamination with the opposite isomer has to be considered. Alternatively, a site of action of these drugs unrelated to the calcium channel may exist, as biphasic actions of (+)S202-791 were not seen in the electrophysiological studies, except when a mixture of equal parts of the (-) and (+) drugs was applied (data not shown).

The K_I of (-)R202-791 for binding was more than two orders of magnitude lower than the concentrations that produced a 50% inhibition of calcium current (measured at -80 to -90 mV) and a 50% reduction in contractile force. Inhibition of calcium current by (-)R202-791 showed a marked membrane potential dependence. At a holding potential of -30 mV, where most of the channels are in an inactivated state, a 50% inhibition of calcium current was seen with 1 nM (-)R202-791. This concentration correlates with the K_I for binding of this drug to the high affinity sites. In intact cardiac tissue where there is a large (-80 to -90 mV) negative membrane potential, the concentrations of (-)R202-791 required to produce a 50% inhibition of contraction can be correlated with the concentration required to inhibit calcium current by 50% when measured at -80 to -90 mV. Thus, a good correlation between binding, pharmacological and electrophysiological data exists when the effects of membrane potential are considered. When all of the DHP-sensitive calcium channels were held initially in the "rested state," the two stereoisomers had opposite effects in response to a test pulse. The voltage dependence of (-)R202-791 was clearly established whereas there was no voltage dependence with respect

to holding potential for the (+)S202-791. This result is consistent with a two-site model for the action of DHPs, although other interpretations cannot be excluded (7,8).

The correlation between the binding, electrophysiology, and pharmacology of (+)S202-791 indicates that binding of this drug to a high affinity site may directly mediate its pharmacological effect. No such correlation exists for (-)R202-791, unless membrane potential is taken into consideration. The most interesting demonstration, however, is the opposite pharmacological effects of the two DHP stereoisomers, and this provides a basis for further study.

ACKNOWLEDGEMENTS

These studies were supported by PO1HL22619 (Ic, A.S.), RO1HL25145 (A.M.B.), RO1HL32935 (S.H.), RO1HL33662 (A.M.B.), American Heart Association, Texas and Southwestern Ohio Affiliates. Dr. L. Dumont is a visiting scientist from the Department of Cardiac Surgery, Sainte Justine Hospital, Montreal. Special appreciation is extended to Dr. Robert P. Hof of Sandoz, Ltd. (Preclinical Research Pharmaceutical Division, Basel) for the generous supply of (-)R and (+)S202-791 and for communicating his data prior to publication. We also thank Mrs. Liz Wendelmoot and Mrs. Robin Wright for their help in preparing the manuscript and Ms. Gwen Kraft for drawing the figures.

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