Br. J. Pharmacol. (1988), **95**, 1125–1132

The dihydropyridine derivative 202-791: interpretation of the effects of the racemate considering inverse agonistic enantiomers

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1 The influence of the enantiomers and the racemate of the dihydropyridine derivative 202-791 [isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridinecarboxylate] on force of contraction and action potential was studied in guinea-pig isolated papillary muscles. The effects were investigated during regular stimulation (1 Hz) and after a period of rest (10 min).

2 The enantiomers of the dihydropyridine derivative 202-791 had opposite effects on the mechanical and bioelectrical parameters: the (+,S)-enantiomer enhanced contractility and prolonged action potential duration whereas the (−,R)-enantiomer reduced force and shortened action potential duration. Analogous to the effects during regular stimulation, the post-rest adaptation was modified adversely: in the presence of the (+,S)-enantiomer the pattern of adaptation was intensified while the (−,R)-enantiomer caused an attenuation. The term 'inverse agonism' seems more suitable than the commonly used comparison of agonist and antagonist, because each enantiomer possesses intrinsic activity, albeit in opposite directions.

3 The racemate of 202-791 acted like the (+,S)-enantiomer. In concentrations up to 1 μM, the racemate increased the force of contraction to the same extent as if the cardiodepressant (−,R)-enantiomer was not present. Only at the highest concentration (3 μM) did the counteracting effect of the (−,R)-enantiomer become evident. The racemate prolonged the action potential duration like the (+,S)-enantiomer although to a lesser extent. Moreover, the typical post-rest adaptation of contractile force and action potential duration was accentuated by the racemate as with the (+,S)-enantiomer.

4 The results demonstrate that in case of 202-791, the effects of the racemate do not reflect the opposite actions of the two enantiomers, but rather mimic that of the (+,S)-enantiomer. A prediction concerning the effects of the enantiomers which is based on findings obtained with the racemate is not possible.

Introduction

Slight modifications of the nifedipine molecule lead to a dihydropyridine derivative that, in contrast to the traditional calcium antagonists, enhanced contractility of the heart muscle and stimulated smooth muscle preparations. The positive inotropic and vasoconstrictor effect of this dihydropyridine derivative, Bay K 8644, were competitively antagonized by nifedipine (Schramm *et al.*, 1983).

In patch-clamp experiments, Bay K 8644 increased the mean calcium current amplitude mainly by prolonging the mean open time of calcium channels (Hess *et al.*, 1984). The vasoconstrictor and positive inotropic effect as well as the stimulation of the calcium inward current and the reversible binding to high-affinity binding sites in cultured myocardial cells (Bellemann, 1984) led to its classification as a 'calcium agonist'. Since then, several other dihydropyridine derivatives were reported to increase force of contraction in heart muscle (e.g. CGP 28392, Loutzenhiser *et al.*, 1984; YC-170, Rogg *et al.*, 1985; H 160/51, Beyer *et al.*, 1986).

The pronounced structural similarity between nifedipine and Bay K 8644 (Figure 1) raises the question as to how compounds of such chemical analogy

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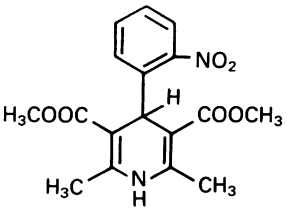
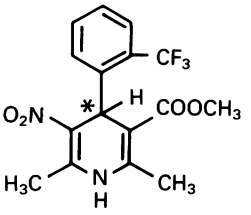
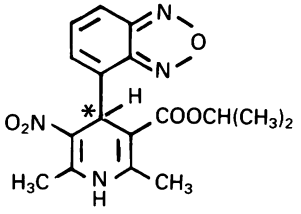


Nifedipine



Bay K 8644



202–791

Figure 1 Structural formulae of nifedipine, Bay K 8644, and 202-791. The asterisk marks the centre of asymmetry.

produce opposite effects. Even the optical isomers of Bay K 8644 which differ only in their sterical configuration act oppositely (Franckowiak *et al.*, 1985); however, in the racemate the positive inotropic effect prevails.

The enantiomers of other dihydropyridine derivatives, e.g. 202-791 [isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridinecarboxylate], were also reported to have opposite effects in blood vessels and other tissues (Hof *et al.*, 1985; Kongsamut *et al.*, 1985; Uematsu *et al.*, 1986; Wei *et al.*, 1986). In the present study we have investigated how the steric configuration of compound 202-791 influences the electrical and mechanical responses of cardiac muscle to this agent. The effects were measured during regular stimulation and after a period of rest, since we have shown previously for the racemates of calcium agonists that their effects were enhanced in a characteristic manner after a pause (Beyer *et al.*, 1986). We chose compound 202-791 because little is known about its effects in multicellular heart preparations, in spite of the many published details about its action at the level of single channel conductance (Kokubun *et al.*, 1986).

Some of these results were communicated at the Spring Meeting of the German Pharmacological Society, 1987 (Damarowsky, 1987).

Methods

Papillary muscles of less than 1 mm diameter were excised from right ventricles of guinea-pig hearts. For recording of action potentials and contractions, the papillary muscles were mounted horizontally in a 3 ml muscle chamber that was continuously perfused with prewarmed, oxygenated Tyrode solution at a rate of 10–12 ml min^{-1}. The experiments were performed at a temperature of 35.0 ± 0.3°C. The preparations were stimulated electrically via two platinum electrodes located in the floor of the bath at the nontendineous end of the muscle (frequency of stimulation 1 Hz; impulse duration 2 ms; voltage 2 × threshold voltage).

Tension (preload 5 mN) was registered with an isometric force transducer (Statham UC-2 cell) and recorded continuously on a penwriter (Helco-Scriptor, HE 16, Hellige). The contractions were also displayed on one channel of a dual beam oscilloscope (Tektronix 502 A).

Transmembrane action potentials were recorded with conventional glass microelectrodes of 10–20 MΩ tip resistance when filled with 3 M KCl. For documentation the action potentials were displayed on the second beam of the oscilloscope using a capacitance compensated preamplifier of high impedance (10^{11} Ω). Mechanical as well as bioelectrical signals were photographed at regular intervals. The following parameters were analysed: force of contraction, time-to-peak tension, duration of contraction, amplitude of action potential, resting membrane potential, maximum upstroke velocity of the action potential (dV/dt_{max}) and duration of the action potential at various stages of repolarization.

An initial equilibration period of 90 min was required until all parameters became constant. Then the perfusion medium was changed from normal bathing solution to solution containing the drug. The preparations were re-exposed to normal bathing solution in order to test the reversibility of the effects. For investigation of post-rest adaptation, regular stimulation was interrupted for 10 min both during the equilibration and the drug period. Only

one drug concentration was tested in any single preparation.

The composition of the normal bathing solution (in mmol l^{-1}) was as follows: NaCl 137.0, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 12.0, NaH_2PO_4 0.2 and glucose 5.5. The pH of the solution in the reservoir was maintained at 7.4 by gassing with a mixture of 97% O_2 and 3% CO_2.

Racemate and enantiomers of the dihydropyridine derivative 202-791 which were a gift from Sandoz Ltd., Basel, Switzerland, were dissolved in a mixture of water and 94% ethanol (6:4) to yield a stock solution with the concentration of 1 mM. The final concentration of solvent in the muscle chamber never exceeded 0.5% (volume/volume). Under these conditions the parameters studied were not influenced by the solvent. Although 202-791 is less sensitive to destruction by daylight than for example nifedipine all experiments were carried out by the light of a sodium vapour lamp.

The optical method of 'circular dichroism' was used to demonstrate not only the purity of the enantiomers but also the existence of a 1:1 racemic mixture (Figure 2; H. Walkenhorst, unpublished observation). The measurement was made by means of a Jasco J 40-A spectropolarimeter.

Results

Effects of the stereoisomers and the racemate in papillary muscle

In isolated papillary muscles of guinea-pig heart, (+,S)-202-791 (0.3 μM) prolonged the action potential duration, increased force of contraction, and retarded relaxation (Figure 3). The same concentration of the (−,R)-enantiomer showed opposite actions. The racemic mixture (0.3 μM) had similar effects as the (+,S)-enantiomer, but they were smaller in size. Resting membrane potential did not change significantly; the maximum velocity of depolarization, dV/dt_{max}, also remained constant.

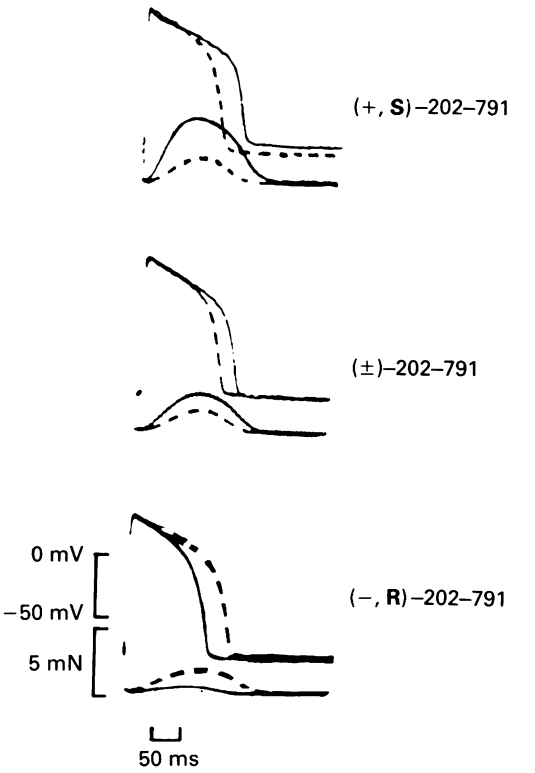


Figure 3 Effects of (+,S)-202-791, (−,R)-202-791, and (±)-202-791 (0,3 μM) on transmembrane action potentials and force of contraction of guinea-pig papillary muscles. The control records (dashed lines) were superimposed on the records obtained after 60 min of exposure (continuous lines) to the drugs. Calibrations are valid for all frames.

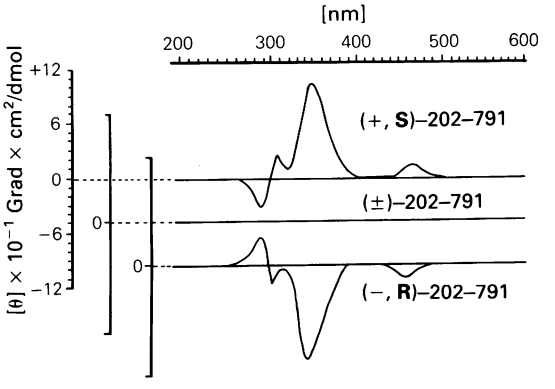


Figure 2 Circular dichroism spectrum of (+,S)-202-791, (−,R)-202-791, and the racemate. Abscissa scale: wave length in nm; ordinate scale: molar ellipticity. Note: the mirror-like spectrums of the enantiomers and the absence of any signal in the case of the racemate.

The time courses of force of contraction and action potential duration after exposure to various concentrations of the stereoisomers and the racemate of 202-791 are depicted in Figure 4a and b. For comparative purposes, the parameters were expressed as a multiple or fraction, respectively, of the control values measured at the end of the equilibration period. The (+,S)-enantiomer and the racemate had biphasic positive inotropic effects, the maximum values of which were reached earlier with higher concentrations (Figure 4a). Similar time courses were observed for the accompanying prolongation in

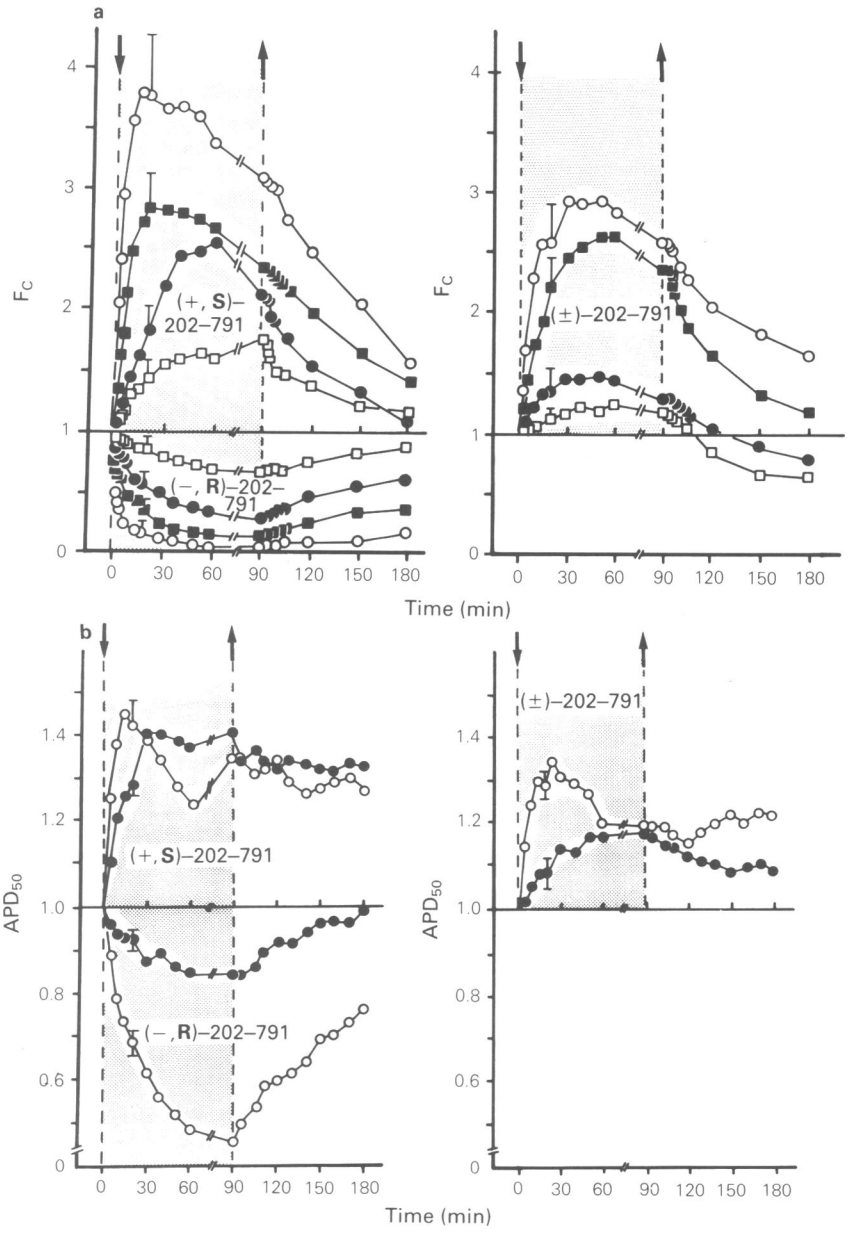


Figure 4 (a) Time courses of the effects of the enantiomers and the racemate of 202-791 on force of contraction (F_c). The concentrations investigated were: 0,1 μM (□), 0,3 μM (●), 1 μM (■), 3 μM (○). Ordinate scale: force of contraction as a multiple or fraction of the values measured at the end of the equilibration period. Standard errors of the mean (3–7 experiments) were determined for each value; however, only representative s.e.means are illustrated for the sake of clarity. The hatched area marks the period of exposure (downward arrow: start of exposure, upward arrow: end of exposure by washing with drug-free bathing solution). After 60 min of exposure, regular stimulation was interrupted for 10 min and post-rest adaptation was recorded subsequently (see Figure 6); the interruptions in the lines of the figure mark the pauses. (b) Time courses of the effects of the enantiomers and the racemate of 202-791 on the action potential duration at 50% of repolarization. For the sake of clarity, the time courses of only two concentrations are presented: 0,3 μM (●) and 3 μM (○). Ordinate scale: action potential duration at 50% of repolarization (APD_{50}) as a multiple or fraction of the values measured at the end of the equilibration period. For further explanations see (a).

action potential duration (Figure 4b): at higher concentrations a maximum prolongation was followed by a decrease in the induced changes which declined even below the effects of low concentrations. The time course of action potential duration, which is less liable to spontaneous decline than the amplitude of contraction, suggests a dualism in action rather than a time-dependent decline in muscle function.

(+,S)-202-791 increased contractile force maximally 4 fold while the racemate was somewhat less effective: force of contraction was elevated 3 fold at the highest concentration of 3 μM. In contrast, the (−,R)-enantiomer produced at the highest concentration a complete inhibition of developed tension. Additionally, the two enantiomers influenced action potential duration oppositely, but the extent of both effects was of the same order, of approximately 50%.

After wash-out of the compounds, contractile force approached the pre-drug values. Similarly, the shortening of action potential duration induced by the (−,R)-enantiomer was reversible. Only the prolongation of action potential duration caused by the (+,S)-enantiomer and by the racemate seemed not to be reversed by simple wash-out.

Concentration-response curves

Referring to the effects of the racemate we tried to compute the concentration-response curve. We extrapolated the effects of (+,S)-202-791 and (−,R)-202,791 at half the concentration used and constructed for the racemate a concentration-response curve to be expected from simple additive effects.

Two different ways of construction were discussed: (1) Setting the maximum positive and negative inotropic effect as 100% we obtained for the enantiomers mirror-like concentration-response curves (Figure 5a). The constructed concentration-response curve of the racemate differs markedly from the experimental curve. Considering the enantiomers acting with similar activity in opposite directions, a neutralization of the opposite effects could be expected but was not present.

(2) By using the original values (Figure 5b), in the lower concentration range the experimental concentration-response curve shows fair agreement with the calculated one; however, in the high concentration range, larger effects are measured than expected from simple additive actions of the enantiomers.

The effects of the enantiomers on action potential duration (Figure 6) were not normalized, since the absolute amount of both prolongation and shortening was nearly identical. The computed curve corresponds to the measured values with the exception of the higher concentrations where again the influence of (+,S)-202-791 seemed to predominate.

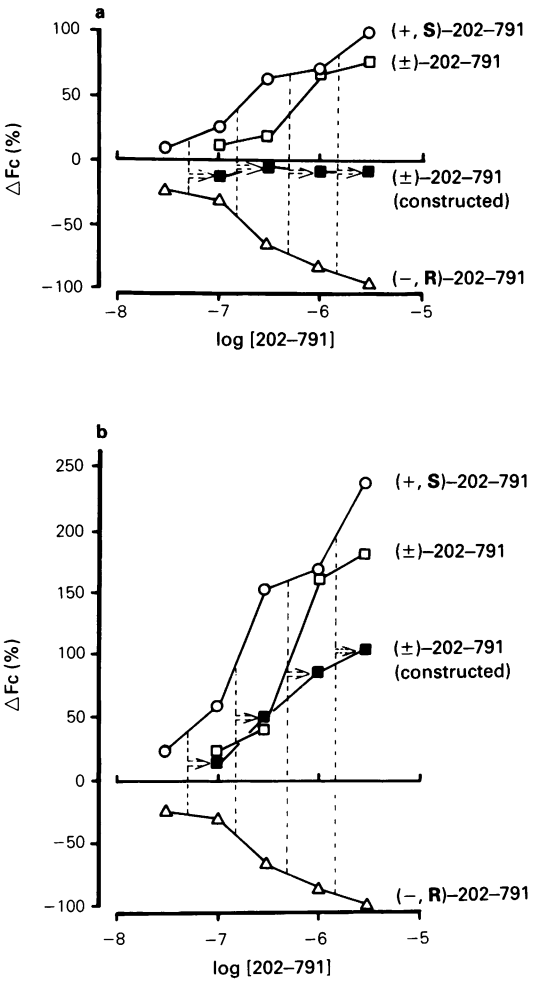


Figure 5 Concentration-response curves for the effects of (+,S)-202-791 (○), (−,R)-202-791 (△), and the racemate (□) on the force of contraction. The normalized (a) as well as the original Δ%-values (b) were obtained 60 min after the beginning of exposure. Mean values were taken from 3–7 experiments. The dashed lines serve to construct the concentration-response curves for the racemate (■) from the values obtained for the individual enantiomers on the assumption of simple addition of effects (see text for further explanation).

Post-rest adaptation

In a previous paper we have demonstrated that the effects of the calcium agonist Bay K 8644 became more prominent after a period of rest (Beyer *et al.*, 1986). If the differences in responses to the racemate

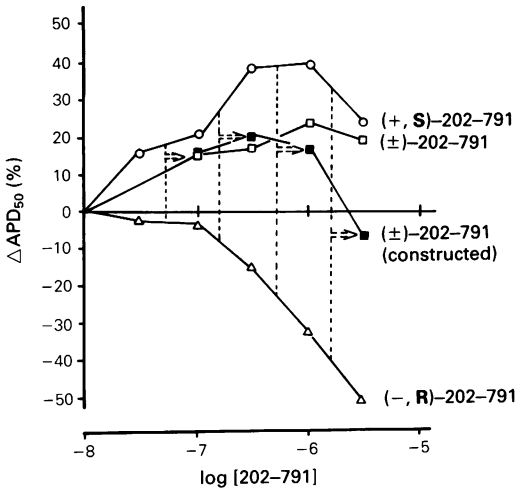


Figure 6 Concentration-response curves for the racemate of 202-791 (■) constructed from the respective curves of the effects of the enantiomers on the action potential duration at 50% repolarization (APD_{50}). The effects are expressed as original, non-normalized Δ%-values obtained 60 min after the beginning of exposure. Mean values were taken from 3–7 experiments. See text for further explanation.

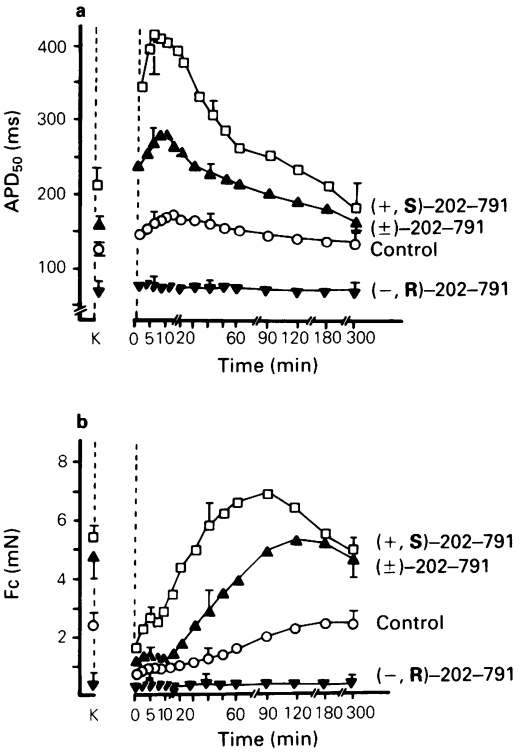


Figure 7 Effects of the 202-791 compounds (1 μM) on the time courses of adaptation of action potential duration (a) and force of contraction (b) after 10 min of rest. Stimulation was interrupted as marked by the vertical dashed lines. Mean values from 5–6 experiments with s.e.mean shown by vertical bars.

and the (+,S)-enantiomer are not due to experimental scatter but characterize the individual drug's action, they should persist during post-rest adaptation.

Figure 7 shows the experimental pre- and post-rest values of force of contraction and action potential duration. In untreated papillary muscles, the first action potential after 10 min of rest was of a longer duration than the pre-rest action potential. The following time course of adaptation at regular stimulation showed a biphasic pattern: beginning with a further increase for 10 or 15 beats, action potential duration declined gradually within 3 to 5 min to reach finally the pre-rest control value. In the presence of both (+,S)-202-791 and the racemate the biphasic time course of adaptation was accentuated whereas (−,R)-202-791 caused an attenuation (Figure 7a). Moreover, the rate of adaptation was modified: in the presence of (+,S)-202-791 and the racemate the initial increase of action potential duration proceeded faster.

During the first beat after rest, tension development in control preparations was small. An initial phase lasting 6 to 8 beats of rapid recovery was separated from a later phase of slowly developing recovery (Figure 7b). The triphasic pattern of adaptation, especially the later phase, was intensified by both (+,S)-202-791 and the racemate while (−,R)-202-791 again suppressed the mechanical response.

Discussion

In isolated papillary muscles of guinea-pig hearts the enantiomers of 202-791 have opposite effects on force of contraction and action potential duration. In the current concept of action of the dihydropyridine derivatives, these drugs may modulate calcium channel activity leading either to inhibition or promotion of Ca^{2+}-influx (see Janis & Triggle, 1984, for recent review). The direction of response depends on several factors, e.g. chemical structure (with significant effects of the stereoisomeric configuration) and concentration of the compound (Schwartz *et al.*, 1984; Thomas *et al.*, 1984; Williams *et al.*, 1985).

Opposite effects of extremely low or high concentrations of dihydropyridines have been reported, e.g. nitrendipine and nifedipine were found to enhance force of contraction in the nanomolar concentration range, whereas Bay K 8644 decreased force again at concentrations $\geqslant 10\,\mu$M (Thomas *et al.*, 1984; Schwartz *et al.*, 1984). These effects cannot be due to differences in affinity of the oppositely acting enantiomers because nifedipine possesses only one steric configuration. Furthermore, a small consistent increase in force was also reported for low concentrations of a single stereoisomer, i.e. (−,**R**)-202-791 (Williams *et al.*, 1985). In the present study we were unable to detect a dual action of (−,**R**)-202-791 in the concentration-range studied. For concentrations of (+,**S**)-202-791 and the racemate above $1\,\mu$M, the observed changes in action potential duration and force of contraction were not stable but declined again.

Another important experimental variable that determines the direction of changes induced by dihydropyridines is the membrane potential (Bean, 1984; Sanguinetti & Kass, 1984a,b). A potential-dependent modification of the drug effects should not play any role in the present study because all interventions did not alter the normal resting potential which stayed at $-85{,}0 \pm 1{,}1$ mV ($\bar{x} \pm$ s.e.mean).

The presented results raise two problems, namely (a) the choice of appropriate terms to characterize the mode of action of the investigated compounds and (b) the interpretation of the effects of the racemate on the basis of the effects of the enantiomers.

These two points will be discussed separately.

If the function of a biological system can be modified pharmacologically in two directions, the choice of the appropriate term for the drug under investigation may become difficult. The action of atropine upon the frequency of heart beat may serve to illustrate this point. Both atropine and catecholamines increase the frequency. Not knowing the mode of action of atropine, the action had to be called agonistic like that of the catecholamines. However, since it is well known that atropine blocks the continuous action of acetylcholine the action of atropine is designated to be antagonistic, e.g. it merely blocks the action of acetylcholine without changing cellular properties.

In the case of the enantiomers of the dihydropyridine derivative, the situation is comparable. Both the contraction amplitude and the duration of the action potential can be altered in opposite directions. The enantiomers of the dihydropyridine derivative 202-791 produced opposite effects due to contrary intrinsic activities. Since an identical site of action can be assumed a competitive displacement of [^{3}H]-nitrendipine by the (+,**S**)- as well as the (−,**R**)-enantiomer has been demonstrated (Williams *et al.*, 1985, Wei *et al.*, 1986), both enantiomers display agonistic effects but in opposite directions.

The term 'inverse agonism' coined by Martin (1984) seems, therefore, more appropriate to characterize the action of (−,**R**)-202-791 whereas (+,**S**)-202-791 acts like an agonist increasing the Ca-effect on the contraction amplitude. In contrast, the effects of the two enantiomers upon the action potential duration are different from those expected after corresponding changes in $[Ca^{2+}]_o$: the Ca-agonistic enantiomer (+,**S**)-202-791 prolonged action potential duration whereas an increase of $[Ca^{2+}]_o$ shortens it. The (−,**R**)-enantiomer shortened action potential duration mimicking an increase of $[Ca^{2+}]_o$. This underlines the fact that both enantiomers are indeed 'agonists'.

In view of the inverse agonism of the enantiomers the interpretation of the racemate effects becomes complicated. The racemate influenced force of contraction and action potential duration essentially like the (+,**S**)-enantiomer in spite of the presence of 50% of the oppositely acting enantiomer.

Knowing the concentration-response curves of the enantiomers an attempt can be made to predict the concentration-response curve for the racemate. As can be seen from Figures 5 and 6, the concentration-response curve constructed for the racemate did not match the curve which was actually recorded. Two different ways of construction lead to the same result: when applied as a racemic mixture, the two enantiomers did not affect the electrical and mechanical parameters according to the sum of their individual action. The curve of the racemate was shifted towards the concentration-response curve of the (+,**S**)-enantiomer. This finding suggests that the effect of the (−,**R**)-enantiomer could not develop to the same extent as in the absence of the (+,**S**)-enantiomer. At the racemic concentration of $1\,\mu$M, the positive inotropic effect of (+,**S**)-202-791 even seemed to be enhanced by (−,**R**)-202-791.

The reason for the unexpected action of the racemate is unknown. We were able to exclude a 'wrong composition' of the racemic mixture by means of circular dichroism. Since the enantiomers produced completely opposite optical signals and the racemate failed to elicit any signal at all (Figure 2), it can be concluded that the racemate consisted of an exact 1:1 mixture of the enantiomers.

Patch-clamp studies in intact cardiac cells with normal resting potential (Kokubun *et al.*, 1986) demonstrated a further enhancement of the open state probability of the voltage-dependent Ca^{2+}-channel when (−,**R**)-202-791 was added to (+,**S**)-202-791. Based on supplementary binding studies the authors suggested a positive cooperative interaction, i.e. a possible mutual increase in binding affinity. The

increased effect of the (+,S)-enantiomer in the presence of (−,R)-202,791 agrees with our findings.

The effect of (±)-H 160/51 was also found to resemble the action of the stimulatory (−)-enantiomer; this observation was explained by a model postulating a hyperbolic relationship between force of contraction and calcium available for the excitation-contraction coupling (Gjörstrup *et al.*, 1986). This interpretation is restricted to events depending on the availability of calcium, for example the force of contraction. The prolongation of action potential duration in the presence of (±)-202-791 does not result from an increase of intracellular calcium. Therefore, this model does not seem appropriate to explain the effects of (±)-202-791.

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(Received April 26, 1988
Revised July 15, 1988
Accepted July 26, 1988)