Modulation of Calcium Channels in Arterial Smooth Muscle Cells by Dihydropyridine Enantiomers

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ABSTRACT The actions of the optical enantiomers of BAY K 8644 and Sandoz 202 791 were studied on barium inward currents recorded using the whole-cell configuration of the patch clamp technique from enzymatically isolated smooth muscle cells from the rabbit ear artery. The enantiomers were applied by bath perfusion or rapidly by a concentration jump technique, which enabled the study of drug action under equilibrium and nonequilibrium conditions. A larger effect of agonists was seen on peak inward current in 110 mM Ba when small rather than large depolarizations were applied. The midpoint voltage of the steady-state inactivation curve of I_{Ba} was -12.8 ± 1.9 mV ($n = 4$) in the absence of drug, -16.4 ± 2.5 mV ($n = 4$) in 1 μM (+)202 791, and -31.4 ± 0.4 mV ($n = 4$) in 1 μM (−)202 791. The rate of onset of action of the agonist and antagonist enantiomers of BAY K 8644 and Sandoz 202 791 was studied by rapid application during 20-ms depolarizing steps from different holding potentials to +30 mV at 1 or 0.2 Hz. The drugs were applied as concentration jumps between two single pulses of a pulse train. The rates of onset of drug action on peak I_{Ba} during a 1-Hz pulse train were concentration dependent over the range of 100 nM–3 μM for both (+) and (−)202 791. The rate of onset of inhibition of peak current by antagonist enantiomers was not significantly influenced by the test pulse frequency. At a holding potential of −60 mV, the onset rate of the increase in peak I_{Ba} on application of 1 μM of agonist enantiomers (+)202 791 or (−)BAY K 8644 during a train of pulses occurred with mean time constants of 2.1 ± 0.7 s ($n = 7$) and 2.3 ± 0.2 s ($n = 4$), respectively. The onset of current increase on application of 1 μM (+)202 791 during a single voltage clamp step to 20 mV was faster, with a mean time constant of 380 ± 80 ms ($n = 3$).

INTRODUCTION

1,4-Dihydropyridine (DHP) compounds interact in a complex way with calcium channels in various tissues (Triggle, Skattebol, Rampe, Joslyn, and Gengo, 1986; Bechem, Hebisch, and Schramm, 1988; Glossmann and Striessnig, 1990). The opposing actions (agonist or antagonist) of different stereoisomers of several DHPs

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on calcium channels demonstrate a highly specific mechanism of drug action (Hof, Ruegg, Hof, and Vogel, 1985), which seems to reflect an interaction with the gating of L-type calcium channels (Hess, Lansman, and Tsien, 1984; Kokubun, Prod'hom, Becker, Porzig, and Reuter, 1986; Hering, Kleppisch, Timin, and Bodewei, 1989*b*; Lacerda and Brown, 1989; Marks and Jones, 1992). There is general agreement that agonist DHP compounds increase, whereas antagonist DHPs decrease the open probability of L-type calcium channels in heart (Hess et al., 1984; Ochi, Hino, and Niimi, 1984), smooth muscle (Yatani, Seidel, Allen, and Brown, 1987) and neuronal calcium channels (Nowycky, Fox, and Tsien, 1985). However, agonist drug effects have been reported for several different "calcium antagonists" in studies of single calcium channels (Hess et al., 1984) as well as whole-cell calcium channel currents (Brown, Kunze, and Yatani, 1986; Hering, Beech, and Bolton, 1987*b*; Kass, 1987; Terada, Nakao, Okabe, Kitamura, and Kuriyama, 1987; Aaronson, Bolton, Lang, and MacKenzie, 1988).

Agonist-DHP-induced gating of calcium channels was recently studied directly by means of a concentration jump technique. A rapidly occurring equilibration between the applied DHP and the calcium channel receptor in such experiments can be observed as a corresponding change in membrane conductance on application of the drugs during single voltage clamp steps (Hering, Beech, Bolton, and Lim 1988; Hering et al., 1989*b*; Imaizumi, Muraki, Takeda, and Watanabe, 1989).

The purpose of this study is to investigate the interaction of DHPs with the calcium channels in different states (C, O, and I) in isolated arterial smooth muscle cells. The voltage-dependent effects of two pairs of DHP enantiomers with agonist and antagonist activity (Sandoz 202 791 and BAY K 8644) were compared under equilibrium and nonequilibrium conditions. Taking advantage of the less pronounced rundown of the calcium channel current in isolated ear artery cells compared with other preparations (see Benham, Hess, and Tsien, 1987; Hering, Bolton, Beech, and Lim, 1989*a*) and using the technique of rapid solution exchange (Hering et al., 1987*a*), we were able to study for the first time the rate of action of agonist and antagonist DHPs in smooth muscle at different membrane potentials.

METHODS

Single smooth muscle cells were freshly dispersed from rabbit ear artery using a modified procedure of Benham and Bolton (1986). Short segments (1–2 mm) of artery were incubated for 1 h in a modified physiological salt solution containing 10 μM calcium, 2 mg/ml bovine serum albumin, 1 mg/ml collagenase (130 U/mg; Worthington Biochemical Corp., Freehold, NJ), 0.5 mg/ml papain (15 U/mg; Sigma Immunochemicals, St. Louis, MO), and 5 mM dithiothreitol, respectively. Cells were dispersed by mild agitation in this low-calcium physiological salt solution. After centrifugation the cells were resuspended in normal physiological salt solution containing (mM): 130 NaCl, 6 KCl, 1.7 $CaCl_2$, 1.2 $MgCl_2$, 14 glucose, and 10.7 HEPES buffered to pH 7.4 with NaOH. The cells were stored on coverslips at 4°C and used within 6–8 h. The experiments were performed using the whole-cell configuration of the patch clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) by means of a patch clamp amplifier (EPC-7; List-Electronic, Darmstadt, FRG). Patch pipettes had resistances of 3–5 MΩ. The internal (pipette) solution contained (mM): 126 NaCl, 3.2 $MgSO_4$, 1 NaH_2PO_4, 2 EGTA, 11.5 glucose, 2 Mg_2 ATP, 10 TEA and 5 HEPES buffered to pH 7.2 with NaOH. The experiments were carried out in high-barium solution containing (mM): 110 $BaCl_2$ and 10 HEPES buffered to pH 7.4 with TEA-OH.

A BBC microcomputer which communicated with a 1401 CED-programmable interface provided the voltage clamp command pulses through a 12-bit digital-to-analogue converter. Data were recorded on FM tape and later analyzed off-line after analogue-to-digital conversion. The currents were low-pass filtered at 1 KHz with an 8-pole Butterworth filter (Barr and Stroud Inc., Glasgow, UK). Leak currents were subtracted either digitally using average values of steady leakage currents elicited by a 20-mV hyperpolarizing pulse or electronically by means of an analogue circuit. All recordings were made between 20 and 25°C. The current decay was fitted to an exponential function using the algorithm of Marquardt (1963). Curve fitting of dose–response data was performed by nonlinear regression using GraphPad Inplot 3.0

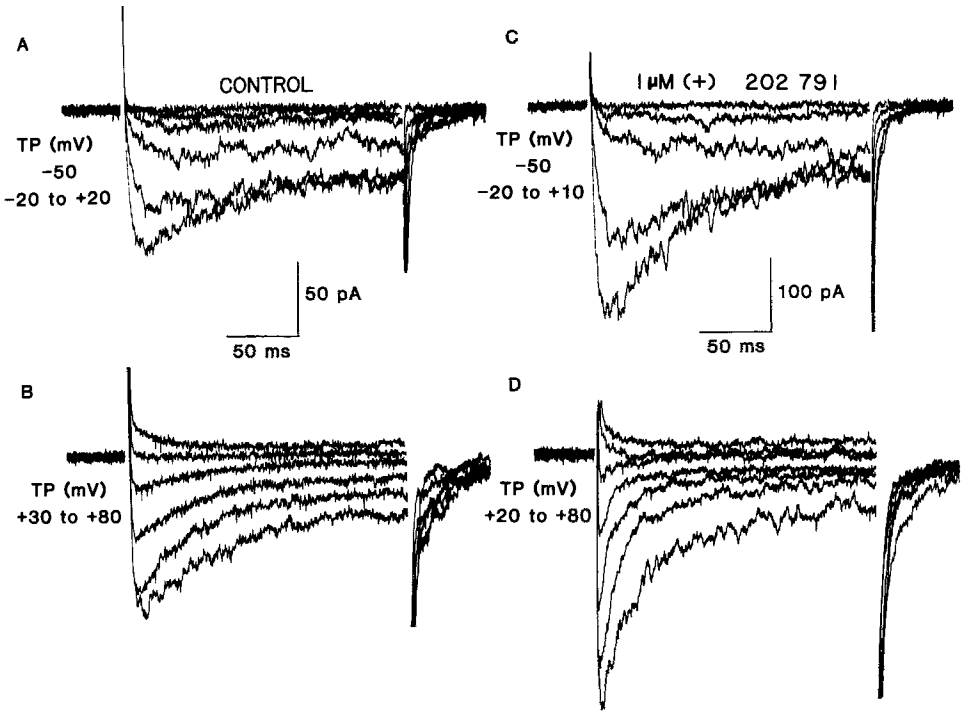

FIGURE 1. Effects of the agonist (+)202 791 on calcium channel currents in an arterial smooth muscle cell from the rabbit ear artery. (*A* and *B*) Inward currents were recorded in 110 mM extracellular $BaCl_2$, stepping from a holding potential of −60 mV to the indicated test potentials (*TP*) (10-mV steps). (*C* and *D*) Currents of the same cell at the same test potentials in the presence of 1 μM (+)202 791. Note the different current calibration in *A* and *B* vs. *C* and *D*.

(GraphPad Software, San Diego, CA). The enantiomeric DHPs (+)–(S) 202 791 and (−)–(R) 202 791 (isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridine-carboxylate) (gifts from Sandoz AG, Basel, Switzerland) and (+) BAY K 8644 and (−) BAY K 8644 (otherwise known as BAY R 4427 and BAY K 5417; gifts from Prof. H. Glossmann, Institut für Biochemische Pharmakologie, Innsbruck, Austria) were applied by bath perfusion or by a concentration jump technique previously described in detail (Hering et al., 1987*a*). This enabled the application of the drugs within < 10 ms (Hering et al., 1987*a*, 1989*a*, *b*). Stable whole-cell recordings were made from single cells in a micro-drop formed within an inner bath that was separated from an outer bath by a Sylgard polymer ring. The rapid application of new solution to the single cell from which recording was made took palce when solution (from the

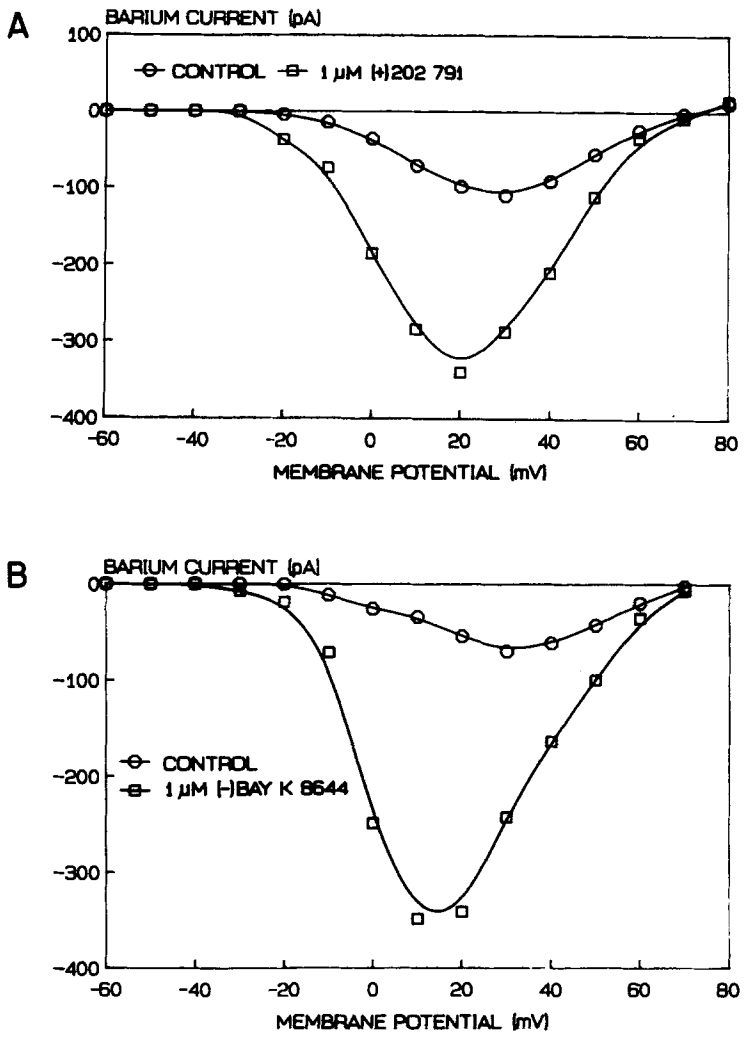

FIGURE 2. (*A*) The current–voltage relationship of peak inward currents in control 110 mM $BaCl_2$ (○) and after application (□) of 1 μM (+)202 791 (same experiment as Fig. 1). (*B*) The current–voltage relationship of peak inward currents in control 110 mM $BaCl_2$ (○) and after application (□) of 1 μM (−)BAY K 8644. Currents were recorded from a holding potential of −60 mV.

outer bath) flooded over the Sylgard ring and mixed with the micro-drop. Data are presented as mean values ± SEM.

RESULTS

Action of the Enantiomers of Sandoz 202 791 and BAY K 8644 under Equilibrium Conditions

The effects of the optical enantiomers of 202 791 and BAY K 8644 on the current–voltage relationship of the peak barium current were compared. The inward

TABLE I

A Comparison of the Effect of Agonist and Antagonist Drugs on Peak Currents (I_{Ba} Amplitude) Evoked by 200-ms Pulses to −10 or +40 mV from a Holding Potential of −60 mV and Steady-State Inactivation Parameters (I_{Ba} Availability)

	I_{Ba} amplitude		I_{Ba} availability		
	At −10 mV	At +40 mV	V_h	k	I_{ss}
	(*% control*)		(*mV*)	(*mV*)	(*% peak*)
Control	100	100	−12.8 ± 1.9	8.5 ± 3.4	10.7 ± 0.1
(+)202 791	433 ± 124	244 ± 19	−16.4 ± 2.5	6.7 ± 0.8	4.0 ± 3.0
(−)BAY K 8644	472 ± 187	238 ± 31	—	—	—
(−)202 791	82 ± 6	37 ± 6	−31.4 ± 0.4	5.1 ± 0.6	0
(+)BAY K 8644	99 ± 13	45 ± 5	—	—	—

The values shown are means ± SEM of three or four cells. 1 μM of each drug was applied.

currents were recorded before and after a 4-min equilibrium period with the drugs during the application of 200-ms pulses from a holding potential of −60 mV at 0.3 Hz (Fig. 1).

Both agonist compounds induced a concentration-dependent shift in the peak of the current–voltage relationship (Fig. 2, *A* and *B*). In the presence of 1 μM (+)202 791 the peak of the current–voltage relationship was shifted by 10 ± 3 mV ($n = 5$) and in the presence of (−)BAY K 8644 by 14 ± 2 mV ($n = 4$) in the negative direction. The drug-induced increase in peak current amplitude was much larger at

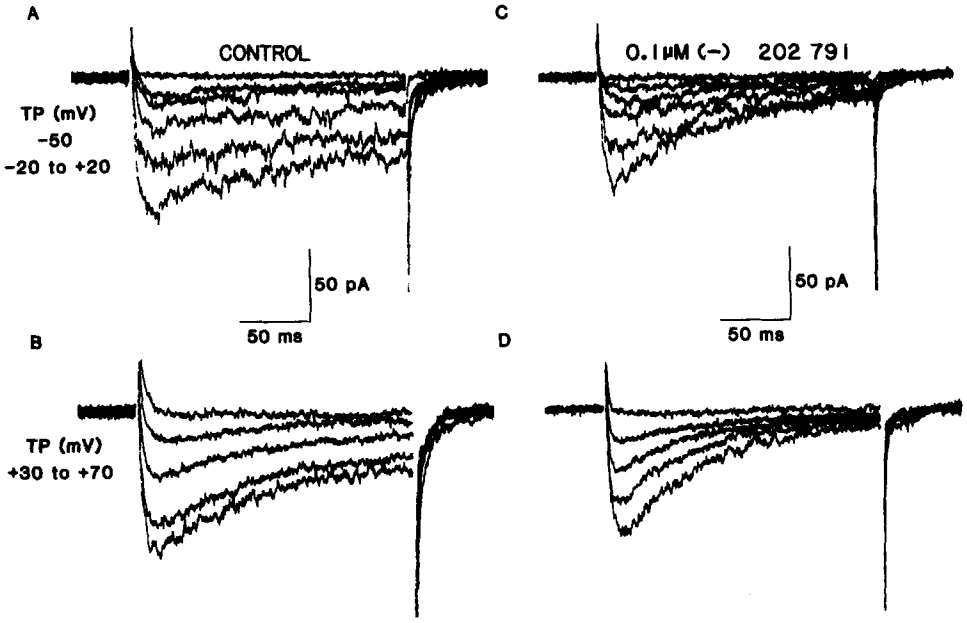

FIGURE 3. Effect of antagonist DHP enantiomers on barium currents of rabbit ear artery cells. (*A* and *B*) Inward currents were recorded in 110 mM extracellular $BaCl_2$ stepping from a holding potential of −60 mV to the indicated TP (10-mV steps). (*C* and *D*) Inward currents of the same cell in the presence of 0.1 μM (−)202 791.

small depolarizations compared with the increase in current at voltages near the reversal of the current. A comparison of the increase in peak current values at test potentials of −10 and +40 mV is shown in Table I.

Agonist enantiomers induced an increase in the transient component of the current. On application of the antagonist (−)202 791 (0.1 μM), a decrease of the peak current was observed which was accompanied by an acceleration in the current decay (Fig. 3). In the presence of 1 μM (−)202 791 the currents now inactivated almost completely during a 200-ms depolarizing pulse. The inhibitory effects of

TABLE II

Equilibrium data			Data from on/off rates			
HP	$-\log EC_{50}$	EC_{50}	$-\log K_D$	K_D	K_{off}	K_{-1}
mV	*M*	*nM*	*M*	*nM*	s^{-1}	s^{-1}
			(+) 202 791			
−40	7.21 ± 0.33	62	7.07 ± 0.11	85	0.078 ± 0.007 (n = 6)	0.1
−60	6.89 ± 0.10	128	7.13 ± 0.19	74	0.076 ± 0.005 (n = 4)	—
−80	6.95 ± 0.42	112	7.06 ± 0.19	87	0.080 ± 0.008 (n = 6)	—
			(−) 202 791			
−40	6.97 ± 0.03	110	6.60 ± 0.16	250	0.022 ± 0.005 (n = 3)	0.03
−60	6.28 ± 0.08	524	6.34 ± 0.05	457	0.016 ± 0.004 (n = 6)	0.017
−80	5.62 ± 0.38	2,400	5.87 ± 0.11	1,350	0.024 ± 0.005 (n = 3)	0.017

Stimulation or inhibition of peak I_{Ba} by (+) or (−) 202 791, respectively, were studied during 20-ms test pulse trains. The equilibrium data $-\log EC_{50}$ (molar) were obtained from dose–response studies (pulses applied at a frequency 0.3 Hz to minimize interaction with open or inactivated channels). The peak I_{Ba} inhibition or increase at steady state (i.e., after more than three onset time constants when the degree of change in peak I_{Ba} appeared to be stable) was fit to a logistic expression: $y = a + (b - a)/(1 + (10^x/10^c))$, where a and b are the minimum and maximum of the curve, respectively (maximum constrained to 1 for no inhibition in the absence of drug), $c = \log EC_{50}$, and x = drug concentration assuming a pseudo Hill slope of −1. The apparent K_D values from on and off rates were calcualted from the kinetics of peak current changes on application of (+) or (−)202 791 during a 1-Hz pulse train according to $K_{on} = (1/\tau_{on} - K_{off})/[x]$ with $K_D = K_{off}/K_{on}$. In the calculation the association rate constant (K_{on}), τ_{on} was taken to be the mean of τ_{on} measured at three concentrations of agonist and antagonist used (see Fig. 8, *A* and *B*). It should be noted, however, that τ_{on} for the agonist (+) 202 791 appeared not to be well described by a model based on a simple bimolecular interaction which could introduce some error in our estimation of K_D. The off rate of drug action (K_{off}) was estimated from washout of (+) or (−) 202 791 during a 20-ms pulse (1 Hz) train to 30 mV by concentration jump application of control solution between two pulses. Alternatively, the dissociation rate constant K_{-1} was estimated from the intercept of the $1/\tau$ plot against drug concentration as described in Fig. 8.

antagonist enantiomers were, in contrast to the agonist action, less pronounced during small depolarizing test pulses (see Table II; cf. Figs. 2, *A* and *B* and 4, *A* and *B*) and no obvious shift in the peak of the current–voltage relationship or activation curve was observed.

Agonist and antagonist enantiomers of the studied DHPs affected the steady-state inactivation of the calcium channel to different extents. The calcium channel availability was estimated by measuring the peak I_{Ba} during a test pulse to +20 mV after a 6-s conditioning prepulse with an interval of 3 ms between conditioning and

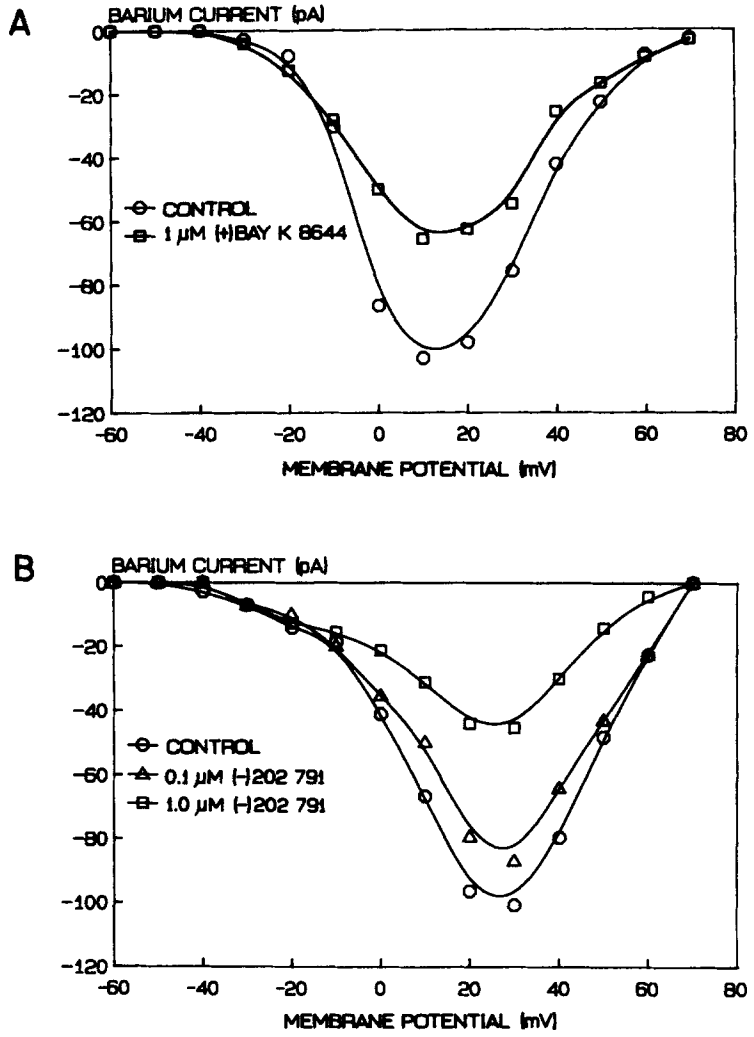

FIGURE 4. (*A*) The current–voltage relationships of peak inward currents in control 110 mM $BaCl_2$ solution (○) and after application of 1 μM (+) BAY K 8644 (□) currents were recorded from a holding potential of −60 mV. (*B*) The current–voltage relationship of peak inward currents in control 110 mM $BaCl_2$ solution (○) and after cumulative application of 0.1 μM (Δ) and 1 μM (−)202 791 (□) (same experiment as Fig. 3).

test pulses. The data were fitted to the function:

$$I/I_{max} = (1 - I_{ss})/\{1 + EXP[(V - V_h)/k]\} + I_{ss}$$

where I/I_{max} is the normalized peak inward current evoked during the test pulse after a 6-s conditioning pulse, V is the conditioning potential, V_h is the voltage where 50% of the channels are available, k is the slope parameter, and I_{ss} is a sustained component of I_{Ba} that did not inactivate during the 6-s depolarizing prepulse.

As shown in Fig. 5, *A* and *B*, the agonist enantiomers of both drugs induced small shifts in the midpoint voltage of the steady-state inactivation curve to more hyperpolarized voltages. In an additional series of experiments we studied the dose dependency of the shift in the midpoint voltage of the availability by agonists and antagonists using a pulse protocol with 10-s prepulses. Under these conditions 100 nM, 300 nM, and 1 μM of the agonist (+)202 791 induced mean shifts of 1.6, 6.8, and 10.5 mV (n = 3), respectively, in the hyperpolarizing direction. The correspond-

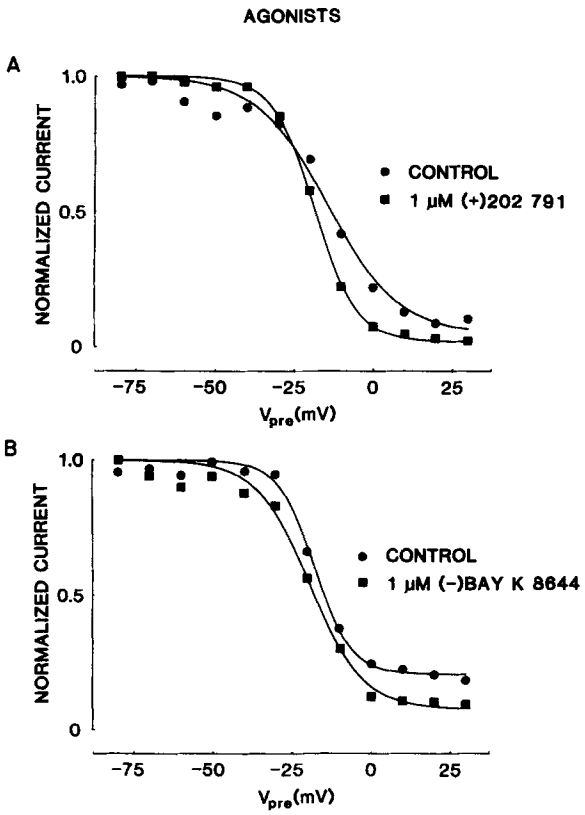

FIGURE 5. Effect of agonist DHP enantiomers on the voltage dependence of I_{Ba} availability. The peak currents during a 200-ms test pulse to +20 mV were plotted versus the voltage of a 6-s conditioning prepulse. Interpulse interval was 3 ms. Solid lines are drawn according to the equation $I/I_{max} = (1 - I_{ss})[1 + \exp[(V - V_h)/k]] + I_{ss}$. (*A*) Effect of (+)202 791 on the voltage dependence of I_{Ba} availability. Under control conditions (●) I_{max} = 87 pA, V_h = −14.5 mV, k = 11.2 mV, and I_{ss} = 5 pA, and after application of 1 μM (+)202 791 (■) I_{max} = 336 pA, V_h = −18.7 mV, k = 6.7 mV, and I_{ss} = 12 pA, respectively. (*B*) Effect of (−)BAY K 8644 on the voltage dependence of I_{Ba} availability. Under control conditions (●) I_{max} = 80 pA, V_h = −17.8 mV, k = 5.7 mV, and I_{ss} = 16 pA, and after application of 1 μM (−) BAY K 8644 (■) I_{max} = 190 pA, V_h = −19.3 mV, k = 8.36 mV, and I_{ss} = 15 pA, respectively.

ing shifts induced by 10 nM, 100 nM, and 1 μM of (−)202 791 were 5.3, 10.3, and 17.7 mV (n = 3), respectively. The antagonist enantiomers shifted the inactivation curve by a substantial amount in the hyperpolarizing direction even on application of low drug concentrations that had little effect on the peak current value (Figs. 3 and 6). The slowly inactivating component of the inward current was more affected by the antagonist enantiomers (Fig. 3) than by the agonist enantiomers (Fig. 1).

Action of the Enantiomers of Sandoz 202 791 and BAY K 8644 under Nonequilibrium Conditions

On and off rates for drug action can be approximated by monitoring of calcium channel currents and fast application of drug during the pulse train. In a biomolecular binding reaction, the rate for drug binding changes with concentration in a manner predicted by the law of mass action. The plot of $1/\tau$ against drug concentration will give a straight line with a slope K_1 and the intercept of the line with ordinate axis is K_{-1}. We investigated the onset rate of action of the agonist and antagonist

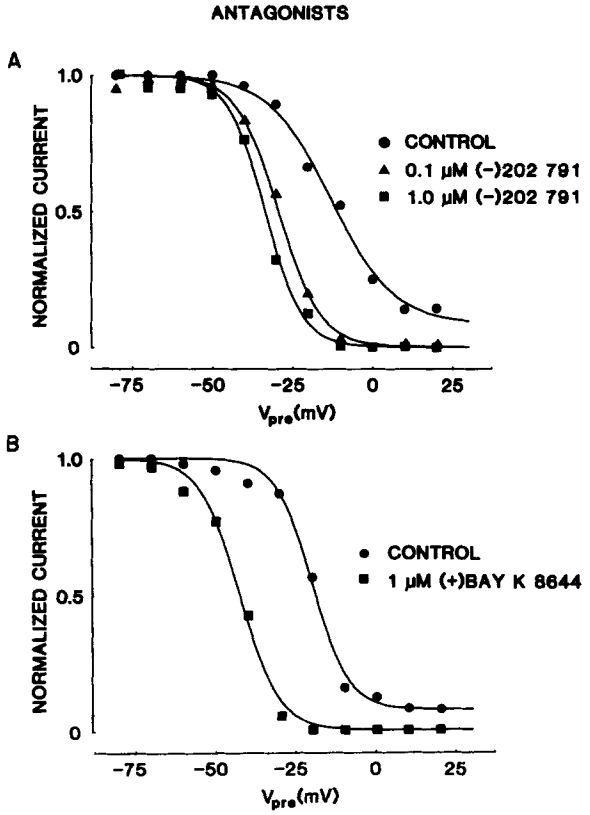

FIGURE 6. (*A*) Effect of the antagonist (−)202 791 on the voltage dependence of I_{Ba} availability. For the control (●) I_{max} = 72 pA, V_h = −12.9 mV, k = 9.7 mV, I_{ss} = 6 pA, after application (▲) of 0.1 μM (−)202 791 I_{max} = 42 pA, V_h = −29.4 mV, k = 6.6 mV, and I_{ss} = 0 pA, and (■) after application of 1 μM (−)202 791 I_{max} = 31 pA, V_h = −33.6 mV, k = 5.8, and I_{ss} = 0, respectively. (B) Effect of (+)BAY K 8644 on the voltage dependence of I_{Ba} availability. Under control conditions (●) I_{max} = 69pA, V_h = −20.0 mV, k = 5.5 mV and I_{ss} = 5pA and after application of 1 μM (+)BAY K 8644 (■) I_{max} = 34 pA, V_h = −42.8 mV, k = 6.0 mV and I_{ss} = 0 pA, respectively.

compounds at different membrane potentials. For this purpose the cell was depolarized from different holding potentials at a frequency of 1 Hz to a potential of +30 mV by short (20 ms) test pulses and the drug was applied in a concentration jump between two successive test pulses of the pulse train (Fig. 7). The experiments were designed to minimize the amount of inactivation during a test pulse and to observe at the same time the rate of onset of drug action at a negative holding potential. To exclude possible artifacts of a superimposed rundown of the currents, the peak current amplitude of the inward current was monitored during a 1-Hz train of 20-ms

pulses before each drug application. Only cells with stable current amplitudes over periods of 2 min were used in these studies. Fig. 7 *A* shows the increase in current amplitude after a concentration jump with 1 μM (+)202 791 during an experiment where the test pulses were applied from a holding potential of −60 mV to a test potential of +30 mV. The increase in peak current amplitude on application of a 100 times lower concentration of (+)202 791 (10 nM) is shown in Fig. 7 *B*. (−)BAY K

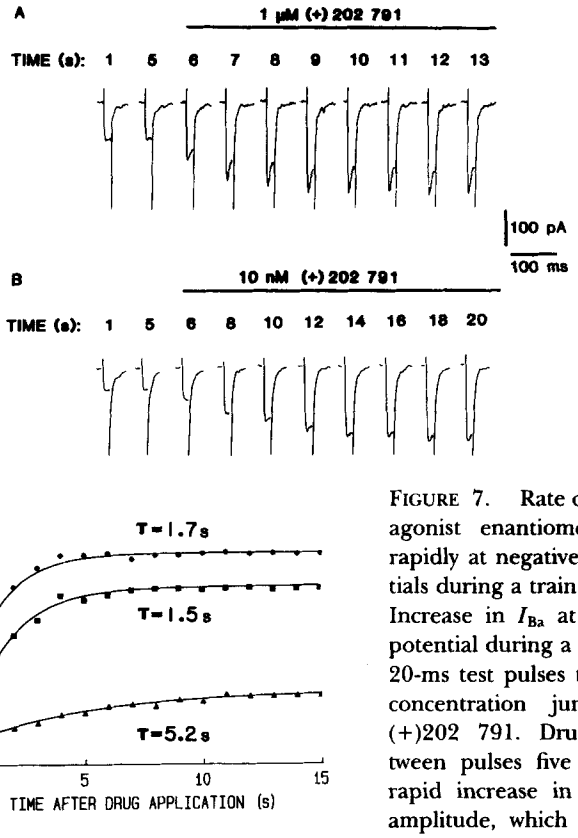

FIGURE 7. Rate of onset of action of agonist enantiomers when applied rapidly at negative membrane potentials during a train of brief pulses. (*A*) Increase in I_{Ba} at −60 mV holding potential during a 1-Hz pulse train of 20-ms test pulses to +30 mV after a concentration jump with 1 μM (+)202 791. Drug application between pulses five and six led to a rapid increase in the peak current amplitude, which reached a steady-state value after 12 s. Note the acceleration in the rate of current decay but no detectable change in the tail current kinetics after application of the agonist. (*B*) Increase in I_{Ba} on application of 10 nM (+)202 791. Same protocol as in *A*. (*C*) Exponential fits to the time-dependent increase in peak I_{Ba} on application of 1 μM(+)202 791 (■), 1 μM (−)BAY K 8644 (●), and 10 nM (+)202 791 (▲). The estimated time constants are indicated in Fig. 8 *B*.

8644 (1 μM) was also tested at −60 mV; its onset time constant was 2.3 ± 0.2 s (n = 4). The time course of the increase in peak I_{Ba} on application of agonist enantiomers during a pulse train could be fitted by a single exponential function (see Fig. 7 *C*). The mean time constants of the exponential increase in I_{Ba} when I_{Ba} was evoked from different holding potentials are given in Fig. 8 *B*. As shown, the $1/\tau_{increase}$ plot at −60 and −80 mV can hardly be approximated by a linear regression line. For (−)202 791 (Fig. 8 *A*) those plots are indistinguishable for large hyperpolar-

izations (−80 and −60 mV). Faster onset of drug action was observed at −40 mV holding potential. The estimated values for K_D compared with the values obtained from steady-state concentration–response relationships at various holding potentials are shown in Table II. There were no significant differences in the unbinding rates for the agonist drug within a voltage range from −80 to −40 mV. As shown in Table II, the off rates (K_{-1}) deduced from the drug onset kinetics (Fig. 8) are in good agreement with the drug off rates (K_{off}) estimated by rapid washout of drug in concentration jump experiments. To determine whether the action of the antagonist

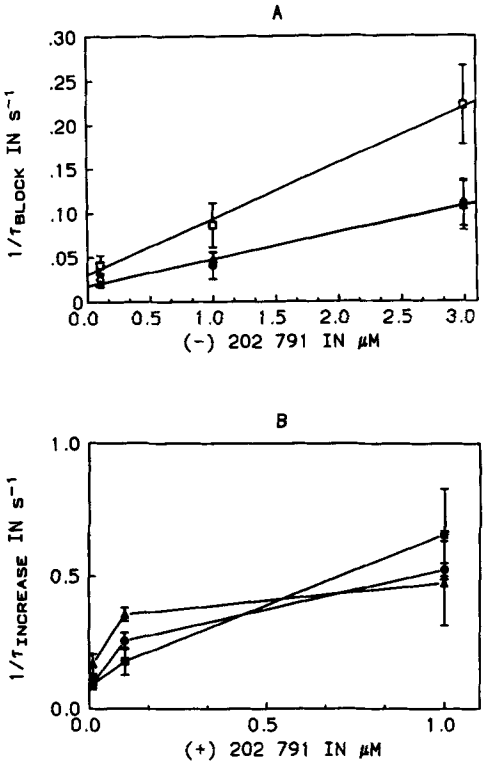

FIGURE 8. Rate of development (onset) of antagonist ((−)202 791) and agonist ((+)202 791) action at −40, −60, and −80 mV as a function of applied drug concentration. Drug was applied in concentration jumps during a train of brief test pulses. (*A*) $1/\tau_{block}$ of peak I_{Ba} inhibition at −40 (□), −60 (△), and −80 mV (○) holding potential during a 1-Hz pulse train of 20-ms test pulses to +30 mV after application of 0.1, 1, and 3 μM (−)202 791. Estimated drug on rates are $K_1[D] = 0.6 \times 10^5\ M^{-1}\ s^{-1}$ at −40 mV, $K_1[D] = 0.3 \times 10^5\ M^{-1}\ s^{-1}$ at −60 mV, and $K_1[D] = 0.3 \times 10^5\ M^{-1}\ s^{-1}$ at −80 mV. (*B*) $1/\tau_{increase}$ of peak I_{Ba} at −40 (■), −60 (▲), and −80 mV (●) holding potential during a 1-Hz pulse train of 20-ms test pulses to +30 mV; application of 0.01, 0.1, and 1 μM (+)202 791. Points are connected by lines; linear regression analysis at −40 mV (■) reveals $K_1[D] = 5 \times 10^5\ M^{-1}\ s^{-1}$. Data points in *A* and *B* represent mean values ± SD of three to seven experiments. The values for K_1, which were estimated from the y-intercept of the linear regression lines, are shown in Table II. In most of the studied cells we could observe 80–90% recovery from drug action on rapid application of drug-free high-barium solution.

was dependent on the opening of the calcium channels, we tested the rate at which peak current was reduced on application of 2 μM (−)202 791 during 20-ms pulses to +30 mV applied from a holding potential of −50 mV at different pulse frequencies. In Fig. 9 *A* drug was applied during a 1-Hz pulse train and in Fig. 9 *B* the drug was applied 2 s after the last control current during a 0.2-Hz pulse train. Fig. 9 *C* displays the time course of the peak current inhibition after the drug application in this experiment. The rates of onset of antagonism at the two pulse frequencies were very similar.

Currents evoked from a holding potential of −60 mV to a test potential of +20 mV displayed a biphasic inactivation time course with a fast inactivation time constant of 156 ± 49 ms and a slow inactivation time constant of 5,250 ± 2,709 ms (n = 4). The agonist enantiomer (+)202 791 (1 μM) was applied during the slowly inactivating component of the inward current during a 6-s voltage jump to +20 mV (Fig. 10 *A*).

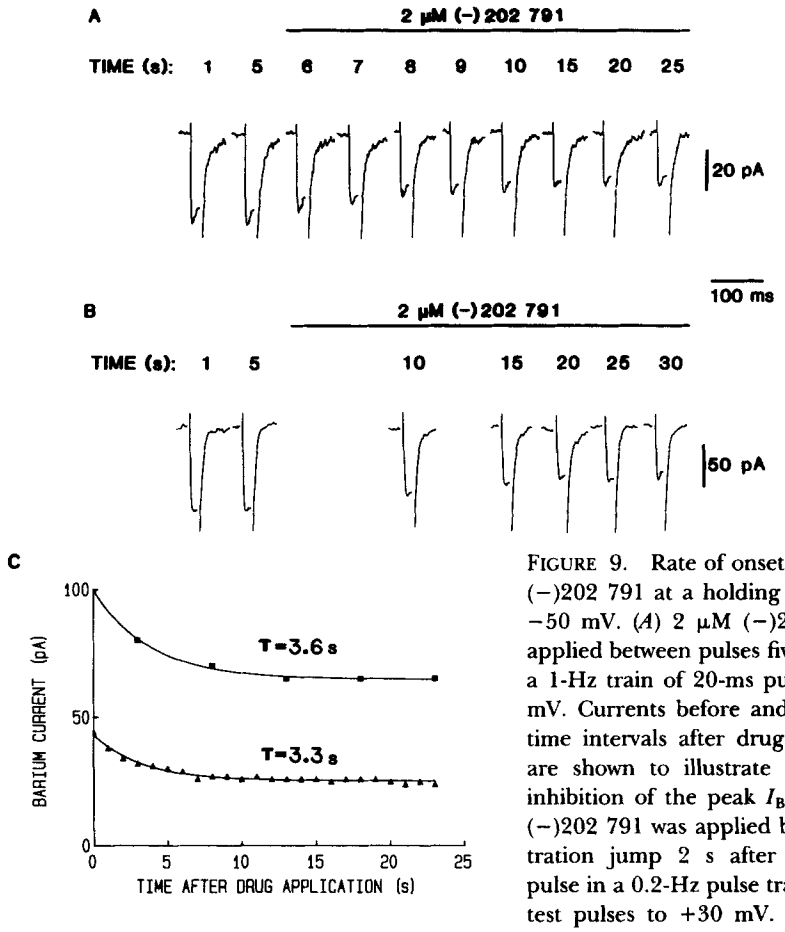

FIGURE 9. Rate of onset of action of (−)202 791 at a holding potential of −50 mV. (*A*) 2 μM (−)202 791 was applied between pulses five and six of a 1-Hz train of 20-ms pulses to +30 mV. Currents before and at selected time intervals after drug application are shown to illustrate the gradual inhibition of the peak I_{Ba}. (*B*) 2 μM (−)202 791 was applied by a concentration jump 2 s after the second pulse in a 0.2-Hz pulse train of 20-ms test pulses to +30 mV. Inward currents at selected time intervals are displayed. (*C*) Time course of the inhibition of the inward current during a pulse train at −50 mV in two cells of *A* and *B* stimulated at different rates (0.2 and 1 Hz). The rate of block is very similar, suggesting that brief activations of the inward current do not significantly affect the rate of block.

The increase in inward current produced by 1 μM (+)202 791 is shown with a monoexponential fit in Fig. 10 *B*. A mean time constant of 380 ± 80 ms (n = 3) was estimated for the net increase in inward current on application of 1 μM (+)202 791 2–3 s after the voltage step to 20 mV during the slowly inactivating component of I_{Ba} (Fig. 10; see also Fig. 8 in Hering et al., 1989*b*). However, shortly after application of

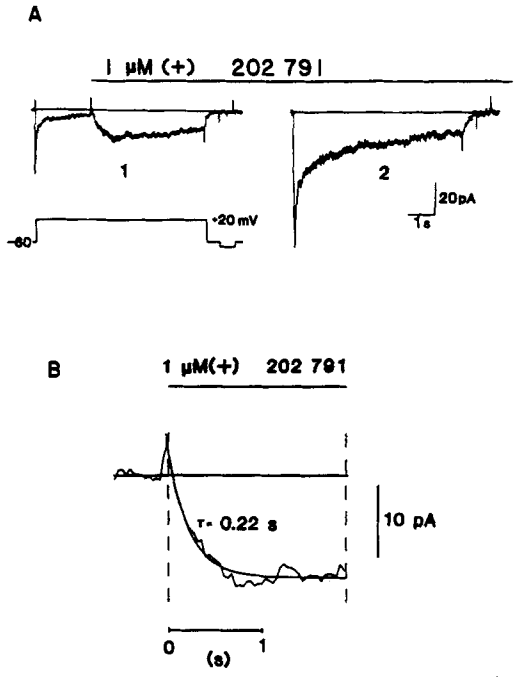

FIGURE 10. Application of (+)202 791 at a depolarized membrane potential. The barium current was activated by a 6-s voltage steps from −60 to +20 mV. (*A*) 1 μM (+)202 791 was applied by a concentration jump 2 s after the voltage jump (*1*). *2* shows the barium current at the second pulse in the presence of the drug after a rest period of 10 s at the holding potential of −60 mV. (*B*) The net increase in I_{Ba} is shown. This was obtained by digital subtraction of the control current decay during the previous pulse in the absence of (+)202 791 from the current after application of 1 μM (+)202 791. The activation of the agonist-induced current was fitted to a monoexponential function and yielded a time constant of 220 ms.

the drug, its effect on channel kinetics rapidly reached equilibrium, and thus the amount of current at the end of the 6-s-depolarizing step when the drug was applied was 12 pA; it was 13 pA at the end of the next depolarizing pulse applied after a 10-s rest at −60 mV in the presence of the drug (cf. Fig. 10 *A*, *1* and *2*). The decay of current during the last 3 s of the pulse was virtually the same. This latter observation implies that the rate of channel transitions out of or into the open state may be unchanged.

DISCUSSION

In this study we observed a pronounced similarity between the actions of the DHPs Sandoz 202 791 and BAY K 8644 on the calcium channel current in rabbit ear artery cells. The agonist enantiomers of both drugs induced a larger increase in peak current during small than during large depolarizations, and also caused a shift in the peak of the current–voltage relationship and activation curve to more hyperpolarized voltages (see Figs. 1 and 2 and Table I). In contrast, the antagonist enantiomers of both drugs were more potent during depolarizations to membrane potentials where the channels have a high open probability and undergo inactivation. A smaller inhibitory effect on the peak current values of I_{Ba} was observed in the voltage range of −30 to −10 mV (see Figs. 3 and 4 and Table II). The agonist enantiomers, in contrast, had less effect on the sustained component of the barium inward current. In this respect our results confirm the findings of earlier electrophysiological studies where similar changes in the current–voltage relationship of the calcium channel current caused by DHPs have been reported for various tissues (Hess et al., 1984;

Sanguinetti, Krafte, and Kass, 1986; Caffrey, Josephson, and Brown, 1986; Bean, Sturek, Puga, and Hermsmeyer, 1986; Markwardt and Nilius, 1988). The observed dissimilar effects of agonist and antagonist DHPs under steady-state conditions (Figs 1, 3, 5 and 6) may indicate selective binding of agonists to closed available or open channel conformations, while antagonists bind with a higher affinity to inactivated (Bean, 1984; Sanguinetti and Kass, 1984) and/or open conformational states of calcium channels or promote inactivation by accelerating the state transition rates to inactivated channel states (Hering et al., 1989*b*).

Voltage-dependent Onset Rates for Agonist Action: Implications for State-dependent Agonist DHP Binding

Similar agonist concentrations applied at different membrane potentials induce different rates of current increase. The rate of increase of inward current on rapid application of 1 μM (+)202 791 during a single test pulse at +20 mV in the concentration jump experiment in Fig. 10 *A* is nearly monoexponential and faster than the current increase induced by a similar agonist concentration applied at more negative membrane potentials during repetitive application of short depolarizing pulses (Fig. 7). A slower onset of agonist action during weak compared with strong depolarizations has been previously shown in heart cells (Hering et al., 1989*b*). Lacerda and Brown (1989) speculated that a rate-limiting step is partitioning of DHPs into the membrane. The observed difference in the time constants for agonist action at different membrane potentials suggests, however, that the accessibility of the receptor is not rate limiting. This finding is supported by recent findings of Kass, Arena, and Chin (1991), with evidence for an extracellular localization of the DHP receptor. Different onset rates of agonist action at different membrane potentials may reflect state-dependent drug binding, i.e., a higher affinity of agonist DHPs to open states. Alternatively, the observed differences in onset kinetics of drug action may reflect voltage dependence of drug association and/or dissociation, or a redistribution of unbound and drug-bound channels between different channel states. Hence, under nonsaturating drug concentrations there will be a mixture of both nonmodified and drug-modified channels. The former may have normal rate constants and the latter rate constants modulated by drug. Thus, deviations of the association rate constants from a simple bimolecular kinetics (Fig. 8 *B*) may reflect the fact that movement of channels into the DHP-bound state depends on both concentration-dependent and concentration-independent rate constants. At high drug concentrations channel state transition rates might become rate limiting. Thus, in concentration jump expertiments the onset picture could be camouflaged by the redistribution of channels between states of drug-bound channels with modified gating. In other words, monitoring of drug onset by peak current measurement during a train and subsequent interpretation of the data assuming simple bimolecular kinetics may not always be adequate.

Voltage-dependent Onset Rates for Antagonist Action: Implications for State-dependent Antagonist DHP Binding

These studies demonstrate that the affinity for the antagonist (−)202 791 is increased at a depolarized membrane potential (−40 mV; see Table II and Fig. 8). These

findings are consistent with the hypothesis proposing that DHP antagonists possess high affinity for inactivated states of the calcium channel (Bean, 1984). Under conditions of microscopic reversibility, an increased affinity of drug for the inactivated state implies that the drug alters the transition rate of channels into or out of inactivated states, since under these conditions binding rate constants and channel transition rates are interdependent. However, the evidence that antagonists bind selectively to inactivated (as opposed to open) states is not unequivocal. Hence, the inhibition of the sustained current component and faster apparent inactivation (see Lee and Tsien, 1983; Gurney, Nerbonne, and Lester, 1985), but also increased potency at depolarized voltages can be explained by open channel block. On the other hand, an additional component of block for drugs with high affinity to inactivated channel states would be expected in such a case during high frequency depolarization. In this study the observed rates of antagonist-induced current decay at −50 mV were very similar during pulse trains of 0.2 and 1 Hz. Similar observations were made for other antagonist DHPs in smooth muscle (Terada et al. 1987; Hering et al., 1988) under different experimental conditions. This obvious discrepancy of 1,4-DHP action in smooth muscle with the suggested similarity in 1,4-DHP action to the effect of local anesthetic on sodium channels could be explained by a very rapid unbinding of drug at negative holding potentials between the individual test pulses of a train. Various experimental findings do not, however, agree with such an assumption: (*a*) Hess, Lansman, and Tsien (1985) have shown that nitrendipine and nimodipine do not affect the mean open time of calcium channels in ventricular cells but increase the percentage of null sweeps. Thus, if the drugs bind selectively to used channels (in the O or I state) the channels remain blocked for several sweeps. (*b*) Kinetic studies of drug action in whole-cell experiments also reveal slow unbinding rates of drug (rate constant of $\sim 0.02\ s^{-1}$; see Fig. 8 and Table II; see also results of Bean [1984] with $K_{-1} = 0.0012\ s^{-1}$). The correlation between the values for unbinding of drug estimated in concentration jump experiments (K_{off}) and the values calculated with the y-intercept method (K_{-1}; Fig. 8) indicates that the time constants observed in concentration jump experiments reflect binding and unbinding of drug to the DHP receptor.

The interpretation of antagonist DHPs induced changes in whole-cell current kinetics (i.e., accelerated apparent inactivation time course; Fig. 3) is also complicated by the possibility that the transient component of current decay is at least partially determined by inactivation of T-type channels (Benham et al., 1987). The pronounced increase in the transient component of current in smooth muscle in the presence of agonist DHPs (Fig. 10; see also Bean et al., 1986) suggests, however, that the rapidly decaying current is not entirely T-type current but represents a fast kinetic component of L-type channels. It has been shown by Hering et al. (1988) that nifedipine acts very slowly in smooth muscle cells when applied during a single voltage clamp step to depolarized voltages. In contrast in cardiac cells, (−)202 791 (1 μM) acts fairly rapidly with an onset time constant of $\tau = 200$ ms (Hering et al., 1989*b*). It is unclear whether this difference is a pecularity of nifedipine or a general difference between calcium channels in smooth muscle and heart cells. Further studies will be necessary to resolve this question. Nevertheless, it is worth noting that the association kinetics of different DHPs vary considerably in radioligand binding

studies with purified calcium channels even between two antagonist enantiomers of the same compound (Knaus, Striessnig, Hering, Marrer, Schwenner, Höltje, and Glossmann, 1992).

In conclusion, we believe that the kinetic analysis of agonist and antagonist DHP action in concentration jump experiments has great potential for revealing new aspects of their state-dependent action and drug-induced changes in channel gating. This approach may be helpful in answering a number of unresolved questions regarding the interaction of DHPs with voltage-dependent calcium channels, such as whether different compounds can be distinguished by specific on and off rates in electrophysiological experiments; whether DHPs act by binding to a single receptor site or multiple receptor sites (Kamp, Sanguinetti, and Miller, 1989; Hughes, Hering, and Bolton, 1990; Mironneau, Yamamoto, Sayet, Arnaudeau, Rakotoarisoa, and Mironneau, 1992); and whether differences exist between DHP action on voltage-dependent calcium channel in cardiac and smooth muscle.

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