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Interaction between DPI 201-106 Enantiomers at the Cardiac Sodium Channel

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SUMMARY

The modification of cardiac sodium channels by DPI 201-106, its *S*-enantiomeric form (*S*)-DPI, and its *R*-enantiomeric form (*R*)-DPI was investigated with whole-cell voltage-clamp recording in single cultured ventricular myocytes obtained from late-fetal rats. From a holding potential of −100 mV, depolarizing pulses to −30 mV of 50-msec duration were applied at 0.2 Hz. Extracellular [Na] was reduced to 70 mM; temperature was 20°. Drugs were administered directly on the cell by a double-barrelled microsuperfusion system. Sodium current inactivation was progressively slowed when the concentration of DPI 201-106 was increased from 0.3 to 3 μM. At 10 μM DPI 201-106, this effect was followed by a blocking effect on peak inward sodium current (I_{Na}), and at 30 μM inward sodium current was fully blocked within 2 min. The slowing of inactivation was produced by (*S*)-DPI (maximally effective at 3 μM), whereas (*R*)-DPI had little effect on inactivation at 3 μM. Conversely, (*R*)-DPI reduced I_{Na} at 10 μM, whereas (*S*)-DPI did not reduce I_{Na} at 3 μM. The effects of both (*S*)-DPI and (*R*)-DPI were partially reversed by washout. (*R*)-DPI retained its blocking activity on I_{Na} when the interval between depolarizing pulses was prolonged to 90 sec. In order to test whether the different sodium channel modifications produced by (*S*)-DPI and (*R*)-DPI were mutually exclusive, the I_{Na}-reducing activity of (*R*)-DPI was measured in the absence of (*S*)-DPI and after equilibration with a maximally effective (*S*)-DPI concentration. In the absence of (*S*)-DPI, 3 μM (*R*)-DPI reduced I_{Na} by 35% and in the presence of 3 μM (*S*)-DPI, by 51%. Thus, modification by (*S*)-DPI of sodium channels did not prevent their block by (*R*)-DPI. The I_{Na}-reducing activity of (*R*)-DPI was even significantly augmented by (*S*)-DPI after a 1-sec depolarization to −30 mV. During such prolonged pulses, (*R*)-DPI accelerated the monoexponential decay of the (*S*)-DPI-induced slow phase of sodium current inactivation. The results are consistent with an irreversible binding reaction between (*R*)-DPI and (*S*)-DPI-modified open sodium channels (association rate constant, 4.7×10^5 $M^{-1}sec^{-1}$). We conclude that (*R*)-DPI reduces I_{Na} by interacting both with resting sodium channels and with (*S*)-DPI-modified open sodium channels. The corresponding receptor site is stereoselective and distinct from and allosterically coupled to the (*S*)-DPI receptor that mediates slowing of inactivation. Racemic DPI 201-106 is thus endowed with an auto-inhibitory mechanism that limits the number of conducting (*S*)-DPI-modified sodium channels. This may explain, in part, the lack of arrhythmogenicity of the drug in various animal models.

DPI 201-106 is a novel drug that combines positive inotropic with moderate negative chronotropic and peripheral and coronary vasodilating activities (1, 2). The positive inotropic effect is caused by a delay of sodium channel inactivation during the action potential (3, 4). The other actions can be explained by an additional blocking property on calcium channels in vascular smooth muscle and cardiac cells (3, 5–7). Romey *et al.* (8) have shown that the two enantiomers of DPI 201-106 have opposite actions on the sodium channel. The delay in sodium channel inactivation is due to the *S*-enantiomer, whereas the *R*-enantiomer is a blocker of the sodium channel. Racemic DPI 201-106 has also been shown indirectly to block sodium channels because it reduces the maximum rate of depolarization of the cardiac action potential (1, 9). Despite their different effects on the sodium channel, (*R*)- and (*S*)-DPI (Fig. 1) were indistinguishable in their interaction with another sodium channel ligand, batrachotoxinin A benzoate; both enantiomers inhibited the specific binding of this alkaloid to brain membranes allosterically, with a dissociation constant of 100 nM (8). These authors concluded "since (*R*)- and (*S*)-DPI and local anesthetics have similar effects on [^{3}H]batrachotoxinin A benzoate binding, DPI might act at the local anesthetic's receptor site." A common binding site for (*R*)-DPI and (*S*)-DPI at the sodium channel would imply an enantioselective substructure that

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ABBREVIATIONS: DPI 201-106, 4-[3-(4-diphenylmethyl-1-piperazinyl)-2-hydroxypropoxy]-1*H*-indole-2-carbonitrile; (*S*)-DPI, *S*-enantiomeric form of DPI 201-106; (*R*)-DPI, *R*-enantiomeric form of DPI 201-106; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

translates receptor occupancy into either block [(*R*)-DPI] or slowed inactivation [(*S*)-DPI]. We have tested this "one-receptor" hypothesis by investigating the effect of DPI 201-106 and its enantiomers on sodium current in single voltage-clamped myocardial cells.

Materials and Methods

Preparation. Cultured single ventricular myocytes were obtained from Wistar albino rats after 15–20 days of gestation, as described previously (10).

Electrophysiological experiments. Sodium current was recorded from single spherical cells adhering to the bottom of a 35-mm Petri dish, with an L/M-EPC 7 amplifier (List-Electronic, Darmstadt, FRG), employing the whole-cell configuration of the patch-clamp technique (11). Fire-polished, Sylgard-coated borosilicate glass micropipettes with 0.8–3-MΩ resistance were used. Membrane potentials are reported as the intracellular voltage with respect to the extracellular side. A sodium current under good voltage control fit the following two criteria: graded activation during pulses to between −60 and −40 mV and absence of notches in the current records. The experiments were carried out in the Petri dish used for culturing, which was filled with 2–3 ml of solution kept at 19.5–20.5° by a Peltier element device (npi Advanced Electronic Systems, Eching a. A., FRG). The pipette contained the following intracellular solution (in mM): CsCl, 108; NaCl, 14; $MgCl_2$, 2; $CaCl_2$, 1; EGTA, 11; glucose, 10; HEPES, 10; CsOH, ~25; NaOH, 0.3 (pH 7.3). Extracellular Na was reduced to improve the quality of the voltage clamp. The extracellular bath solution contained (in mM): NaCl, 69; CsCl, 69; KCl, 5.4; $MgCl_2$, 2; $CaCl_2$, 0.1; glucose, 10; HEPES, 10; NaOH, ~1.6; CsOH, ~1.6 (pH 7.3). The cell under investigation was continuously superfused by a microsuperfusion device (10) consisting of a double-barrelled glass tube mounted on a hydraulic micromanipulator and connected to two syringes in an infusion pump. The flow rate of 3 μl min^{-1} resulted in a flow velocity of 1 cm sec^{-1} through each ~80-μm orifice. One orifice was positioned as close as possible to the cell (diameter, 12–20 μm). The solution was changed by displacing the superfusion barrels rapidly under visual control with a video camera.

Data acquisition and analysis. Whole-cell transmembrane current, filtered to 3 kHz, was obtained from the L/M-EPC 7 amplifier, stored on videotape after processing with a PCM digital audio processor, and subsequently evaluated by computer. An MC 68020 processor-based system (Hewlett-Packard 9000/330), in combination with a fast analog-digital converter (AD200, Infotek Systems, Anaheim, CA), was used. The program for data acquisition and analysis was written in H-P Basic 5.1 by M.D. Exponential functions describing the slowly inactivating modified sodium current were fitted analytically using a weighted linear regression method (12). The cells were kept at a holding potential of −100 mV and pulsed to −30 mV for 50 msec every 5 sec. The computer program evaluated the level of holding current before each pulse, the maximum inward current during the pulse, and the current level 2 msec before the end of the pulse. The latter value was taken as a measure of leakage current at −30 mV under control conditions and was subtracted from the peak inward current to obtain the sodium current amplitude, I_{Na}. Leakage current was typically <3% of peak inward current. DPI 201-106 slows sodium channel inactivation such that inward current persists during 50-msec depolarizations. The level of inward current 2 msec before the end of the pulse, corrected for leakage current, was called "noninactivating" sodium current. Stability of leakage current throughout the experiment was verified by measuring the level of holding current at −100 mV.

Drugs. DPI 201-106 and its two enantiomers, (*R*)-DPI and (*S*)-DPI, were kindly supplied by Sandoz Ltd, Pharmaceutical Division, Preclinical Research (Basle, Switzerland). The purity of the enantiomers was >98.5% for (*R*)-DPI and >98.0% for (*S*)-DPI. Stock solutions of DPI 201-106 and its enantiomers were prepared in dimethyl sulfoxide at 0.3–30 mM. These were then added to the extracellular solution, resulting in a final dimethyl sulfoxide concentration of 0.1% (v/v), which was also added to the drug-free extracellular solution.

Statistics. Results are presented as mean value ± standard deviation.

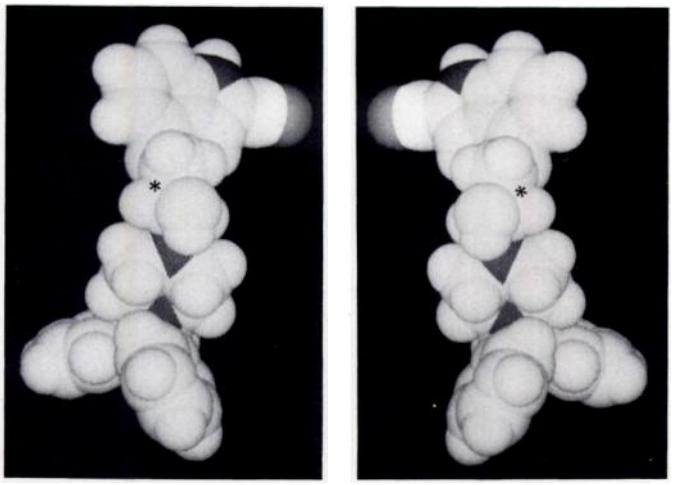


Fig. 1. Computer-generated molecular models of *S*- and *R*-enantiomeric forms of DPI 201-106. *Asterisks*, asymmetric carbon atoms. *White*, carbons, hydrogens, and oxygens; *gray*, nitrogens.

Results

Concentration-effect relationships. Original current records from a cultured rat ventricular myocyte exposed to 3 μM DPI 201-106 and from another cell treated with a 10-fold higher concentration are shown in Fig. 2A. Following a 15-sec exposure to 3 μM racemate, the inactivation of the sodium current showed two phases. After normal fast inactivation to about 2/3 of peak I_{Na}, a slow phase of inactivation followed, which was still present at the end of the 50-msec pulse. The component of slow inactivation increased only slightly during the following 45 sec. During application of 30 μM racemate, slowing of inactivation was a transient effect that was followed by complete suppression of I_{Na} within 1 min (Fig. 2A). A quantitative analysis of sodium current inactivation is shown for another cell in Fig. 2B. Before application of DPI 201-106, the decay of sodium current during a depolarizing pulse to −30 mV is well described by the sum of two exponential functions with time constants of 1.1 and 5.4 msec. Shortly after superfusion with 3 μM DPI 201-106 was started, sodium current failed to inactivate completely during the 50-msec pulse. The sodium current is then well described by the sum of a constant, i.e., noninactivating, current and a phasic current that decays with time constants that are nearly identical to those of the control (see legend to Fig. 2). Apparently, there are two populations of sodium channels in this situation, one with unchanged inactivation kinetics and another that fails to inactivate. As shown later, (*S*)-DPI-modified channels do in fact inactivate at a very slow rate (Fig. 9), but this is not resolved during 50-msec pulses. The time course of DPI 201-106 action is illustrated more fully in Fig. 3. During 3-min exposure times, concentrations up to 3 μM caused only a delay in sodium channel inactivation, whereas 10 or 30 μM DPI 201-106 acted biphasically, first delaying inactivation and later reducing I_{Na}. Concentration-effect rela-

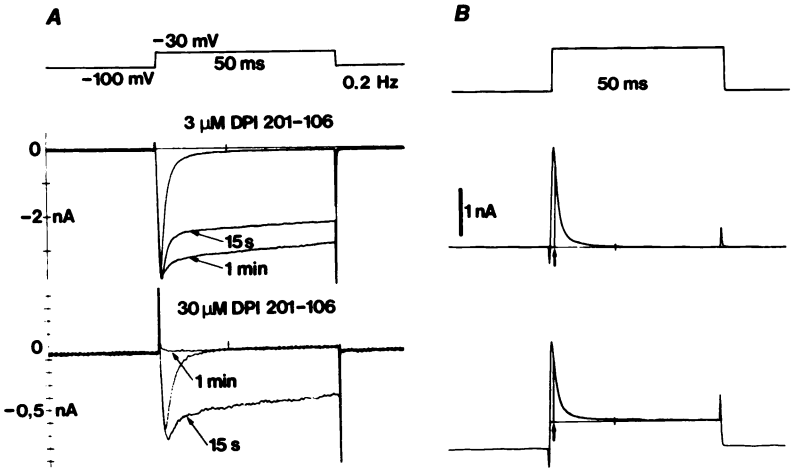


Fig. 2. A, Original records of transmembrane current from a single cultured ventricular myocyte before (*unlabeled trace*) and 15 sec and 1 min after starting superfusion with 3 μM DPI 201-106 and from a different cell before and 15 sec and 1 min after starting superfusion with 30 μM DPI 201–106. Temperature was 20°. DPI 201-106 at 3 μM slowed sodium current inactivation, whereas 30 μM blocked the sodium current after transiently only slowing its inactivation. B, Inverted transmembrane current from a different cell before (*upper*) and after 10 sec of superfusion with 3 μM DPI 201-106 (*lower*) under the same experimental conditions as A. Holding current was −0.12 nA for both records. The decaying phase of I_{Na} was fitted (starting at *vertical line* marked by *arrow* and ending at the end of the pulse) by the function $a \cdot \exp(-t/\tau_1) + b \cdot \exp(-t/\tau_2)$, with a = 1.4 nA, τ_1 = 1.1 msec, b = 0.3 nA, and τ_2 = 5.4 msec before DPI 201-106 and a = 1.1 nA, τ_1 = 1.2 msec, b = 0.26 nA, and τ_2 = 5.3 msec during DPI 201-106.

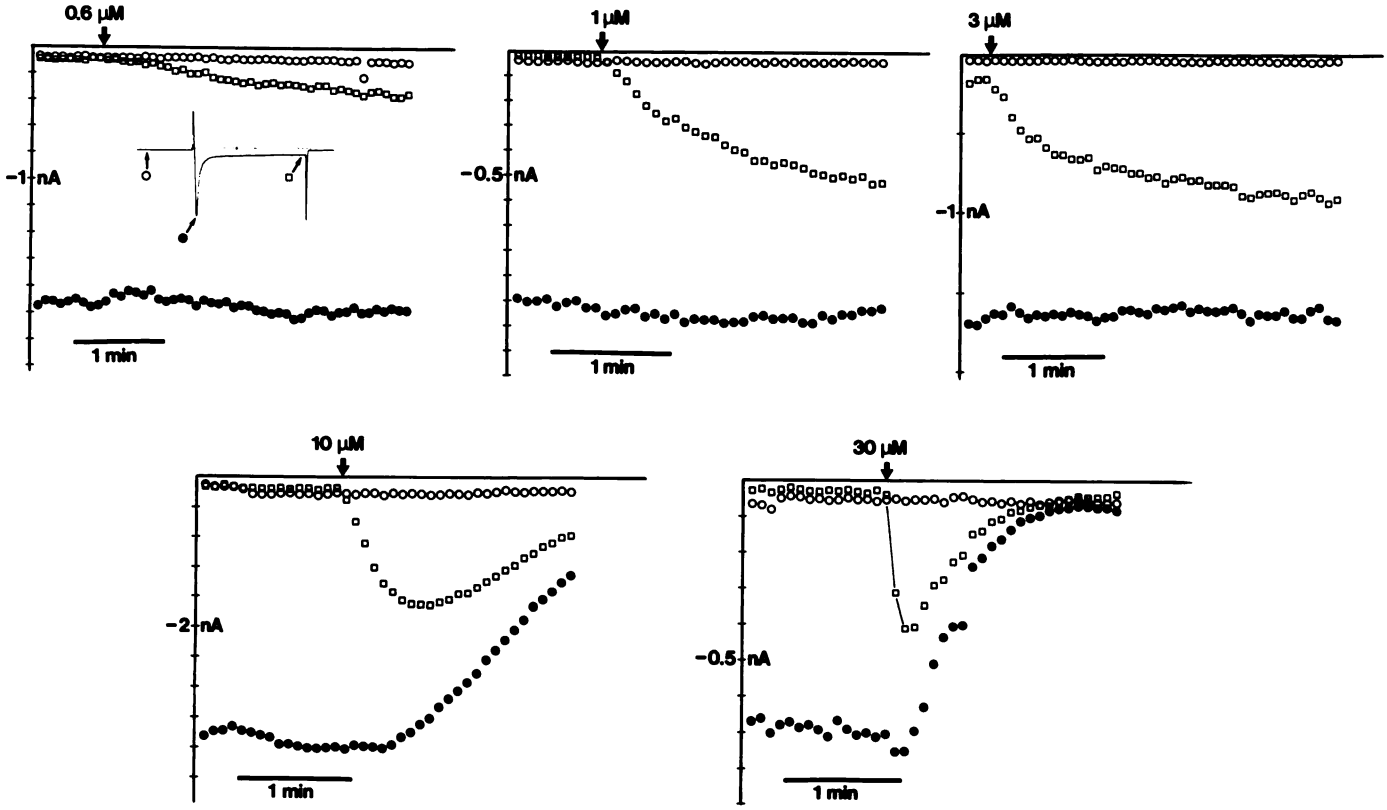


Fig. 3. Time course of peak inward current (●), residual inward current 2 msec before the end of the 50-msec depolarizing pulse to −30 mV (□), and holding current at −100 mV (○) in five different cells exposed to the indicated concentrations of DPI 201-106. Note the biphasic effect on noninactivating sodium current and inhibition of peak I_{Na} at 10 and 30 μM DPI 201-106. Pulse protocol is as in Fig. 2.

tionships, based on 2-min exposure times, are shown in Fig. 4 for DPI 201-106 and its enantiomers (*S*)-DPI and (*R*)-DPI. Racemic drug had little effect on peak I_{Na} up to 1 μM and reduced this parameter progressively at higher concentrations. The noninactivating component of the sodium current showed a bell-shaped concentration-effect relationship, with a maximum between 1 and 3 μM and complete suppression at 30 μM DPI 201-106. (*S*)-DPI increased peak I_{Na} slightly at 0.3–3 μM, whereas (*R*)-DPI progressively reduced this parameter at 0.3 to 10 μM, confirming the results of Romey *et al.* (8). (*S*)-DPI appeared to be 30 times more potent than (*R*)-DPI in delaying inactivation, whereas (*R*)-DPI was 8 times more potent than (*S*)-DPI as a blocker (Fig. 4). These eudismic ratios (13) are smaller than the ratios between nominal enantiomer and contaminant opposite enantiomer [(*R*)-DPI, >65; (*S*)-DPI, >49; see Materials and Methods], suggesting a small contribution of (*S*)-DPI to sodium channel block and, to a lesser extent, of (*R*)-DPI to slowing of inactivation.

Reversibility of enantiomer effects. As shown by Fig. 5A, the effect of a brief exposure (1.5 min) to a low concentration (0.3 μM) of (*S*)-DPI was partially reversible upon superfusion of the cell with drug-free solution. Two thirds of the noninactivating I_{Na} induced by (*S*)-DPI was reversed within 1 min; the remaining effect persisted. This result was confirmed in a second experiment with 0.3 μM (*S*)-DPI. The effect of 1 μM (*S*)-DPI was less readily reversed by wash-out (n = 3). The reduction of peak I_{Na} by 1 μM (*R*)-DPI (Fig. 5B) was reversed by 50% during a 4-min drug-free superfusion.

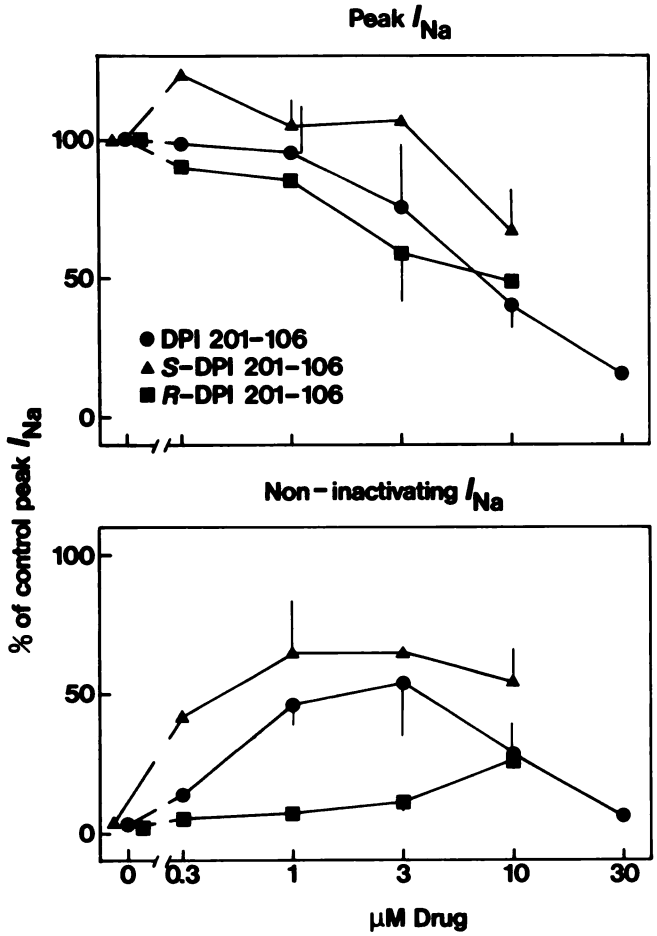


Fig. 4. Peak I_{Na}, measured during depolarization from −100 mV to −30 mV, and noninactivating I_{Na}, measured 2 msec before the end of the 50-msec depolarizing pulse, as affected by the concentration of DPI 201-106 (●), (*S*)-DPI (▲), and (*R*)-DPI (■), respectively. Each *point* is the mean of two to four cells; *vertical bar* gives standard deviation for $n = 3$ or 4. Cells were exposed to one or two (cumulatively increasing) drug concentrations for 2 min. Control peak I_{Na} was 2.3 ± 1.1 nA in 21 cells. Pulse protocol is as in Fig. 2.

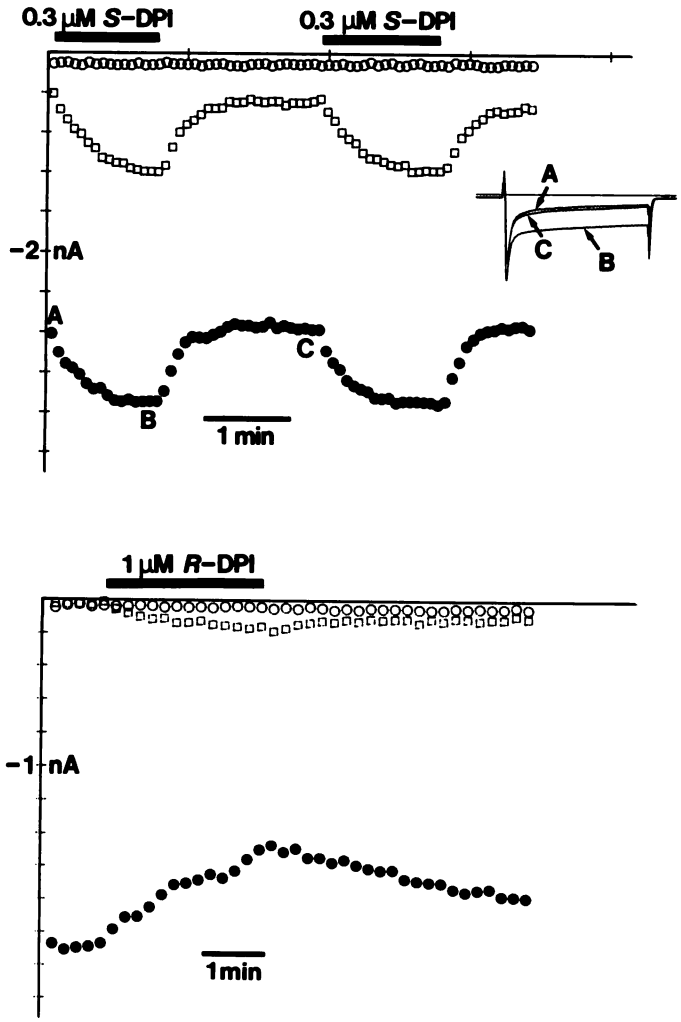


Fig. 5. Partial reversibility of the effects of a low concentration of (*S*)-DPI (*upper*) and of (*R*)-DPI (*lower*) on peak and noninactivating I_{Na}. *A*, *B*, and *C* refer to the times at which the original current traces shown in the *inset* were taken. *Symbols* are as in Fig. 3. The drugs were applied by a microsuperfusion system described in Materials and Methods. Pulse protocol is as in Fig. 2.

Persistence of (*R*)-DPI effect in nondepolarized cells. In the experiment of Fig. 6, we examined both reversibility and possible dependence on repetitive pulsing of the (*R*)-DPI effect. Because even the first depolarization elicited after a 90-sec superfusion with 3 μM (*R*)-DPI showed a reduced I_{Na} (by 26%; Fig. 6, *trace B* versus *trace A*), the blocking effect does not depend on the repetitive depolarizations (0.2 Hz) used in the preceding experiments. The effect progressed to 61% during a subsequent 90-sec period at −100 mV holding potential (Fig. 6, *trace C*). During a subsequent 4-min washout period, I_{Na} recovered to 74% of its control value (Fig. 6, *trace D*).

Are sodium channel modifications by (*S*)-DPI and (*R*)-DPI mutually exclusive? If the enantiomers of DPI 201-106 were competing for the same receptor site, a high (saturating) concentration of one enantiomer should drastically reduce the probability that the other enantiomer, applied at a lower non-saturating concentration, binds to that site. We have tested this prediction by measuring the effect of (*R*)-DPI (3 μM) in the absence and in the presence of a maximally effective (*S*)-DPI concentration (3 μM). In the absence of (*S*)-DPI, 3 μM (*R*)-DPI produced a slowly developing block of peak I_{Na} (Fig. 7A) that reached 35 ± 11% (mean ± SD) after 2 min in five

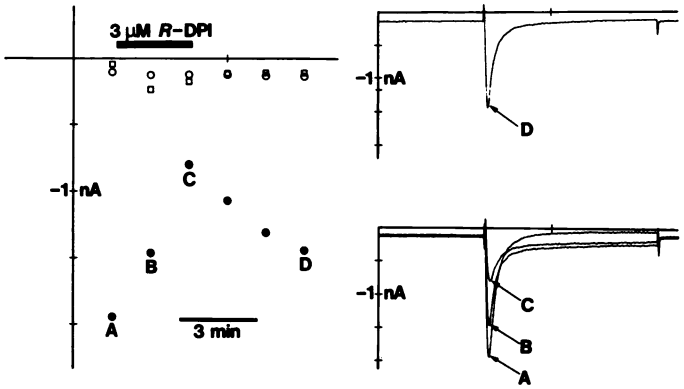


Fig. 6. Persistence and reversibility of the I_{Na}-blocking action of (*R*)-DPI at very low pulsing rate. This cell was pulsed (50 msec to −30 mV) every 90 sec and 3 μM (*R*)-DPI was applied from shortly after the first to shortly after the third pulse, as indicated. Currents elicited by the first (A), second (B), third (C), and sixth pulse (D) are shown on the *right*. Holding potential is −100 mV. *Symbols* are as in Fig. 3.

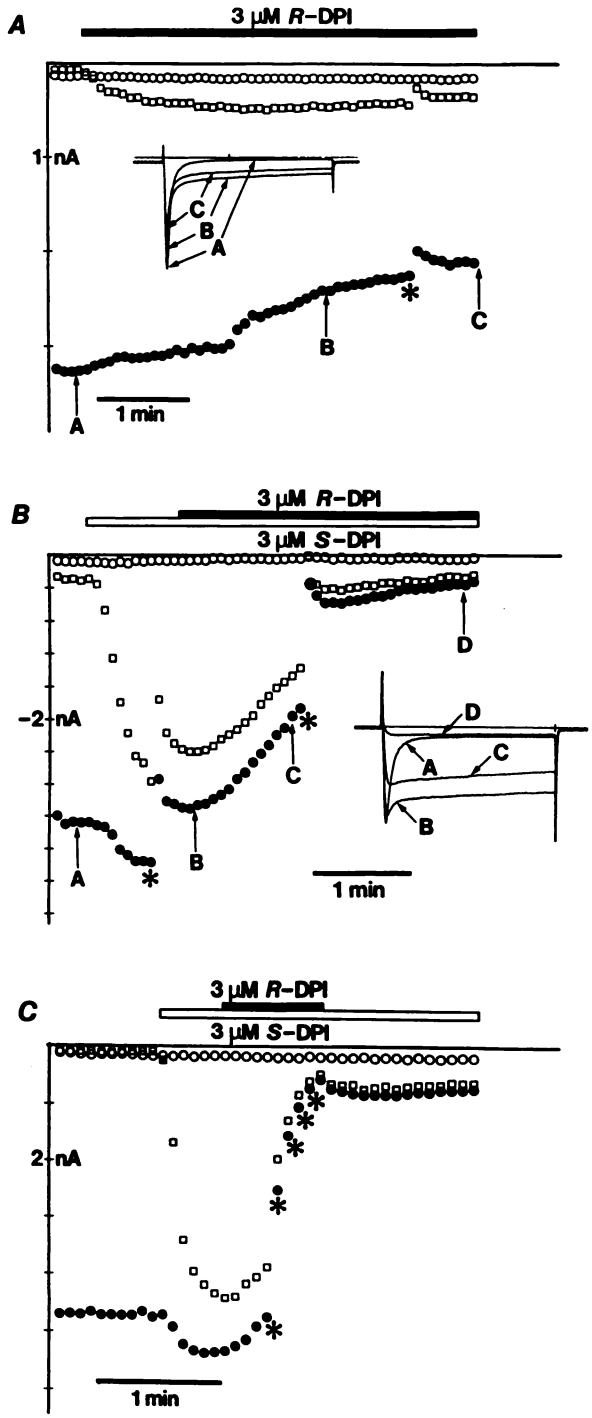


Fig. 7. Interaction between (*S*)-DPI and (*R*)-DPI. A, Time course of the effect of 3 μM (*R*)-DPI in the absence of (*S*)-DPI on sodium current. *Lettering* refers to the times of the original traces shown in the *inset*. B, Different cell. Time course of the effect of 3 μM (*R*)-DPI after preequilibration with and in the presence of 3 μM (*S*)-DPI. Note that (*R*)-DPI retained its I_{Na}-blocking activity. *Lettering* refers to the times of the original traces shown in the *inset*. C, Different cell. Lack of reversibility of the effect of 3 μM (*R*)-DPI if applied in the presence of 3 μM (*S*)-DPI during depolarizing pulses of 1-sec duration. The *asterisk* marks pulses (one in A, two in B, and five in C) that were prolonged from 50 msec to 1 sec; otherwise the pulse protocol was as in Fig. 2. *Symbols* are as in Fig. 3.

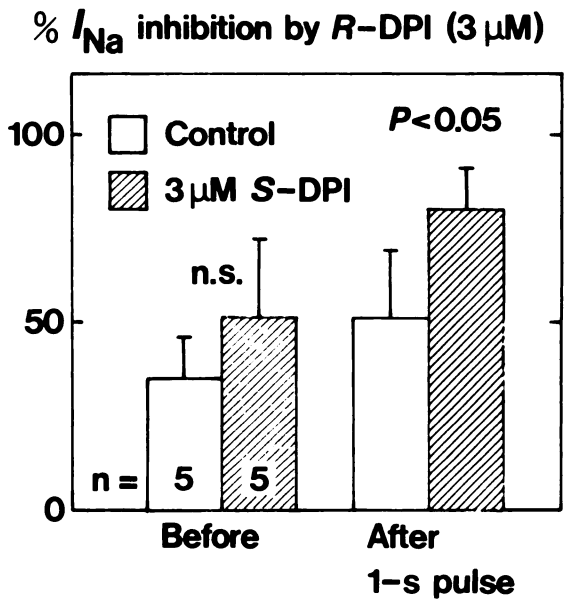


Fig. 8. Reduction of peak I_{Na} by a 2-min exposure to 3 μM (*R*)-DPI in the absence or presence (different cells) of 3 μM (*S*)-DPI. The block increased if, after 2 min, a single depolarizing pulse was prolonged to 1 sec and the block evaluated five pulses later (*right pair* of *columns*). Pulse protocol otherwise is as in Fig. 2. Means and standard deviation, as *vertical bar*, are shown. Significance of difference determined by *t* test for unpaired observations. *n.s.*, not significant. Control peak I_{Na} was 1.67 ± 0.9 nA in control and 2.06 ± 0.8 nA in (*S*)-DPI-treated cells.

cells, as summarized in Fig. 8. If the cell was preequilibrated with 3 μM (*S*)-DPI, (*R*)-DPI not only retained its blocking activity but also reduced I_{Na} to a greater degree (Figs. 7B, and 8). Although the larger effect (51 ± 22%; $n = 5$) did not reach statistical significance, it is clearly at variance with the prediction of *reduced* blocking activity of (*R*)-DPI during maximal (*S*)-DPI modification of the sodium channels.

Prolongation of a single (50-msec) pulse to 1 sec during our standard pulsing rate of 0.2 Hz caused a transient small depression of peak I_{Na} during the subsequent four regular pulses (not shown). If applied in the presence of 3 μM (*R*)-DPI, the effect was similar (Fig. 7A). In the combined presence of 3 μM (*S*)-DPI and 3 μM (*R*)-DPI, however, peak I_{Na} was strongly and irreversibly suppressed after a single 1-sec pulse (Fig. 7B). Under this condition, the block by (*R*)-DPI in the presence of (*S*)-DPI became significantly larger than that produced by (*R*)-DPI alone (Fig. 8). In the presence of 3 μM (*S*)-DPI alone, prolonging a single pulse to 1 sec induced only 12.5 ± 4.8% irreversible reduction of peak I_{Na} in four experiments (see also Fig. 7B). Thus, the block-enhancing effect occurred only in the presence of both (*S*)-DPI ınd (*R*)-DPI. In summary, (*S*)-DPI did not diminish but enhanced the blocking action of (*R*)-DPI, especially in combination with a depolarization pulse prolonged to 1 sec. This synergism argues against a common receptor site for (*R*)-DPI and (*S*)-DPI at the cardiac sodium channel.

Block by (*R*)-DPI of the (*S*)-DPI-induced slowly inactivating sodium current. The (*S*)-DPI-modified sodium current elicited by 1-sec pulses from −100 to −30 mV is shown in Fig. 9 for three different cells. The (*S*)-DPI-modified sodium current decayed monoexponentially, with time constants between 0.47 and 0.70 sec in these cells. In the additional presence of (*R*)-DPI, the sodium current was not simply scaled down but changed in kinetics. While the monoexponential nature of

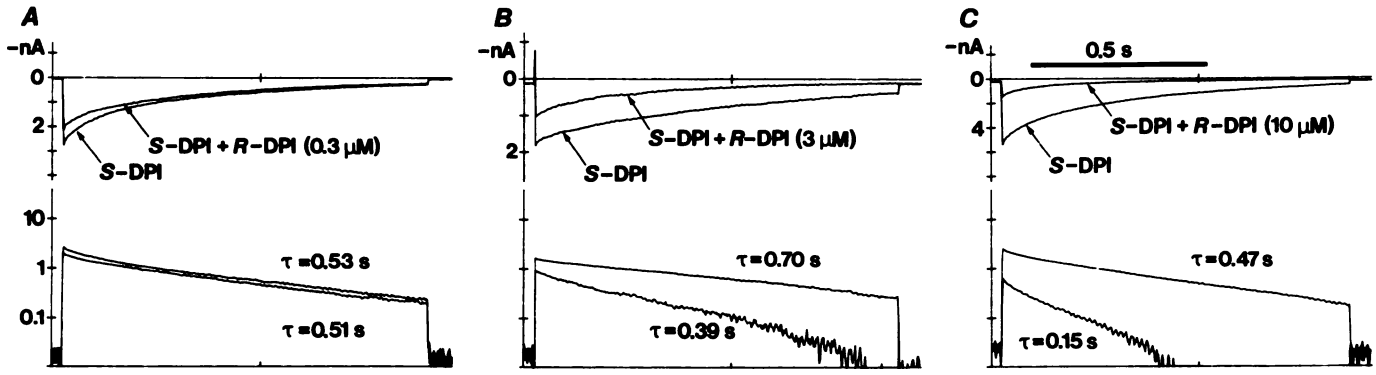


Fig. 9. Acceleration of decay of (*S*)-DPI-induced slowly inactivating I_{Na} by (*R*)-DPI. Three different cells (A–C) were preequilibrated with 3 μM (*S*)-DPI and subjected to a 1-sec depolarization to −30 mV from a holding potential of −100 mV (*traces* labeled *S-DPI*). Subsequently, the cells were additionally exposed to either 0.3 μM (A), 3 μM (B), or 10 μM (*R*)-DPI (C) for 2 min (A and B) or 1.5 min (C). At this time, a second 1-sec depolarization was applied, yielding the *currents* labeled *S-DPI + R-DPI*. *Lower traces* are semilogarithmic plots of *upper traces*. The time constants were obtained from monoexponential fits to I_{Na} (excluding the first 50 msec of the 1-sec pulse). Cells A and B were continuously pulsed at 0.2 Hz with 50-msec depolarizations; cell C was not pulsed during superfusion with (*R*)-DPI.

the decay was preserved, its time constant was reduced to 96, 56, and 32% by 0.3, 3, and 10 μM (*R*)-DPI, respectively (Fig. 9, A–C). The more rapid decay suggests that (*R*)-DPI binds progressively to (*S*)-DPI-modified open sodium channels during the 1-sec depolarizing pulse or, more correctly, to one or more of the conformations specifically associated with the (*S*)-DPI-modified bursting sodium channel (4). In contrast to the reversible nature of the sodium channel block induced by (*R*)-DPI in the absence of (*S*)-DPI (Figs. 5B and 6), the block induced by 1-sec pulses in the presence of (*S*)-DPI was irreversible (Fig. 7C). If, according to the scheme

$$(R)\text{-DPI} + O_{(S)\text{-DPI}} \xrightarrow{k_1} B\cdot(R)\text{-DPI} \qquad (1)$$

one (*R*)-DPI molecule binds, with an association rate constant k_1, irreversibly to one open and (*S*)-DPI-modified sodium channel, $O_{(S)\text{-DPI}}$, resulting in block of this channel, $B\cdot(R)$-DPI, the fractional occupancy, y, at time t is given by

$$y = 1 - \exp(-k_1((R)\text{-DPI})\cdot t). \qquad (2)$$

This time course of blockade is modified, however, by the spontaneous inactivation of (*S*)-DPI-modified sodium channels, which results in an exponential decay of the sodium current:

$$I_{Na_{(S)}} = \exp(-k_s\cdot t) \qquad (3)$$

The development of block observed during the slowly inactivating (*S*)-DPI-modified sodium current, y_0, is therefore only the fraction, defined by Eq. 3, of y:

$$y_0 = I_{Na_{(S)}}\cdot y \qquad (4)$$

Subtracting this fractional block from $I_{Na_{(S)}}$, we obtain the time course of sodium current in the presence of both (*S*)-DPI and (*R*)-DPI:

$$I_{Na_{(R+S)}} = \exp(-(k_s + k_1\cdot((R)\text{-DPI}))\cdot t) \qquad (5)$$

Hence, our model predicts that (*R*)-DPI should increase the rate constant of decay of the (*S*)-DPI-modified sodium current in proportion to (*R*)-DPI concentration.

Fig. 10 plots k_{on}, obtained as the difference between the sodium current decay rate constants in the absence and presence of (*R*)-DPI, as a function of (*R*)-DPI concentration for seven different cells. A linear relationship, which yields an

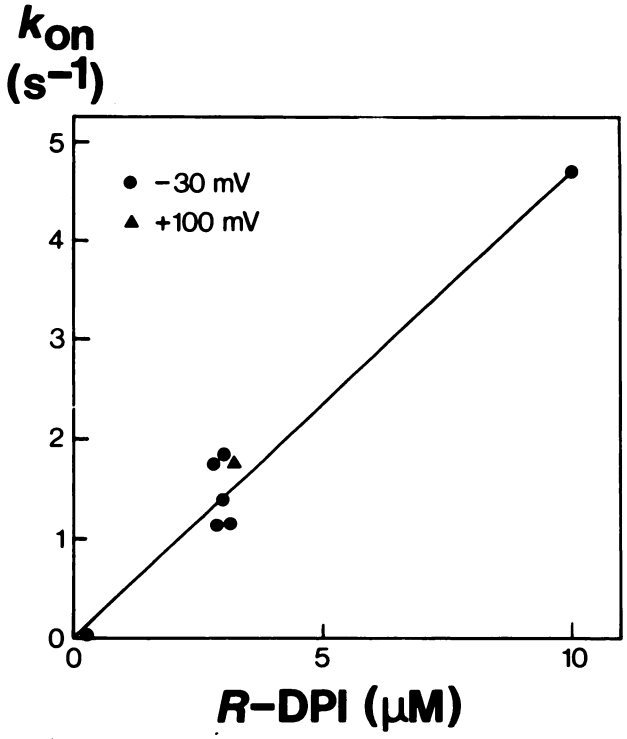


Fig. 10. Rate constant of block of (*S*)-DPI-induced slowly inactivating I_{Na} increases linearly with (*R*)-DPI. The blocking rate constant, k_{on}, was obtained from Fig. 9 and four analogous experiments by subtracting the rate constant of I_{Na} decay in (*S*)-DPI from that in the presence of (*S*)-DPI plus (*R*)-DPI (see text for details). The zero-crossing regression line relates k_{on} and molar (*R*)-DPI concentration: $k_{on} = 4.69 \times 10^5 \times (R)\text{-DPI}$. One additional experiment (▲) involved 1-sec pulses to +100 mV, yielding outwardly directed I_{Na} that was also blocked by (*R*)-DPI.

association rate constant $k_1 = 4.69 \times 10^5\ M^{-1}sec^{-1}$, is observed. These results are, therefore, in agreement with the reaction scheme (1). A single experiment with depolarizing pulses to +100 mV yielding *outward* sodium current showed a similar k_{on} (Fig. 10), suggesting that (*R*)-DPI block of modified open sodium channels is not affected by transmembrane potential or by the direction of the sodium current.

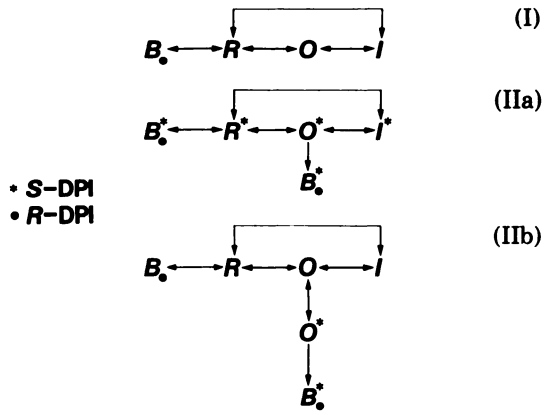
Discussion

Biphasic effect of DPI 201-106. Romey *et al.* (8) were the first to show that the two enantiomers of DPI 201-106 have

different effects on the voltage-dependent sodium channel in heart and neuronal cells; (*S*)-DPI delays inactivation, (*R*)-DPI blocks the channel. We have confirmed this result and show in addition that the effect of (*S*)-DPI predominates up to a concentration of 3 μM of the racemic mixture DPI 201-106, whereas that of (*R*)-DPI predominates at 10 and 30 μM, eventually causing complete suppression of the sodium current in single rat ventricular myocytes. The characteristic prolongation of the cardiac action potential under the influence of DPI 201-106 has previously been found to be associated with a moderate reduction of the maximum rate of depolarization, suggesting simultaneous sodium channel inhibition (1, 9). Reversal of the cardiac effects by high concentrations of DPI 201-106 has not been described in previous work (1, 3, 14), probably because the concentration range did not exceed 3 μM in these studies. The biphasic effect of racemic DPI 201-106 reported here is of interest in several respects. The drug-induced additional sodium influx and consequent intracellular calcium accumulation via sodium/calcium exchange, which explains the positive inotropic action (3), is expected to reach a maximum near 3 μM and to decline again at higher drug concentrations, because DPI 201-106 then inhibits its own effect on sodium influx. This "autoinhibition" may explain, in part, the lack of arrhythmogenicity of DPI 201-106 (1), which is exceptional among agents that interfere with sodium channel inactivation (15). On the other hand, although sodium channel inhibition is a major antiarrhythmic mechanism, high concentrations of DPI 201-106 may share the potential to induce reentrant tachyarrhythmias (16) with the class 1 antiarrhythmic drugs.

Reversibility. Previous studies on multicellular cardiac preparations failed to show reversibility of the DPI 201-106 effect on repolarization (1, 3), even after up to 1.5 hr of washout. The highly lipophilic nature of the drug (4) is expected to cause a time- and concentration-dependent accumulation in cellular membranes, including the microenvironment of each sodium channel macromolecule. During washout, DPI 201-106 may, therefore, continuously reassociate with the sodium channel from a membrane pool that does not readily equilibrate with the extracellular aqueous phase. By using a low concentration (0.3 μM) of (*S*)-DPI and a short exposure time of 1.5 min, we have shown that the effect on sodium channel inactivation was indeed largely, although not fully, reversed during a 1-min superfusion with drug-free solution. The slowing of sodium channel inactivation induced by (*S*)-DPI (8, 17) must, therefore, be attributed to a dynamic rather than irreversible interaction of the drug with its receptor site. The same considerations apply to (*R*)-DPI. The true dissociation rates of the DPI 201-106 enantiomers may be faster than those observed experimentally because of possible reassociation of drug from a membrane pool during drug-free superfusion.

Interaction between (*R*)-DPI and (*S*)-DPI. The major aim of this study was to analyze the interaction between (*S*)-DPI and (*R*)-DPI at the cardiac sodium channel. Specifically, we tested the hypothesis that (*S*)-DPI and (*R*)-DPI bind to a common receptor site (or two overlapping receptor sites) but produce different effects on sodium channel function. A similar concept has been proposed for the benzodiazepine receptor, which can accept functionally bidirectional drugs, i.e., agonists and inverse agonists, although these ligands differ in chemical composition (benzodiazepines versus β-carbolines) rather than in chirality (18). If (*R*)-DPI were an inverse agonist at the (*S*)-DPI receptor, a saturating concentration of (*S*)-DPI should minimize the blocking effect of a moderately effective (*R*)-DPI concentration. There were two different manifestations of sodium channel block produced by (*R*)-DPI. In the absence of (*S*)-DPI, (*R*)-DPI (1–3 μM) produced a block of I_{Na} that increased gradually during 3 min of application, both in the presence and absence of 50-msec depolarizing pulses. This block was slowly reversible upon washout. We interpret this block as a reversible reaction of (*R*)-DPI with resting closed sodium channels, denoted by *R* in Scheme I below:



Inactivated sodium channels did not reveal a higher affinity for (*R*)-DPI, as indicated by the ineffectiveness of a 1-sec depolarization to produce additional block (Fig. 7A). In the presence of 3 μM (*S*)-DPI, which modified sodium channel inactivation maximally, (*R*)-DPI retained its blocking activity in both repetitively depolarized (Fig. 8) and nondepolarized cells (Fig. 9C). We conclude that (*R*)-DPI binds to resting sodium channels despite saturation of the (*S*)-DPI receptor sites and, therefore, at its own distinct enantioselective receptor site, as depicted by Scheme IIa. A second type of sodium channel block by (*R*)-DPI became apparent *during* the slowly inactivating I_{Na} induced by (*S*)-DPI. During depolarizing pulses of 1-sec duration, (*R*)-DPI was shown to accelerate the decay of the modified sodium current. This block can be explained quantitatively by an irreversible binding reaction of (*R*)-DPI with (*S*)-DPI-modified open sodium channels, denoted by *O** in Scheme IIa. Because of this second type of sodium channel block, (*S*)-DPI augmented the blocking action of (*R*)-DPI if prolonged depolarizing pulses were used. Such a potentiating interaction can only be explained by allosterically coupled and therefore separate receptor sites for the two enantiomers. Scheme IIa assumes a continuously (but reversibly) (*S*)-DPI-bound form of the sodium channel with altered gating such that the transition from open to inactivated state is slowed. In Scheme IIb, the sodium channel binds (*S*)-DPI only during activation, and the slow transition to the inactivated state results from dissociation of the drug molecule. Again, the acceleration of inactivation rate by (*R*)-DPI can only be explained by allosteric interaction. However, the persistence of resting block by (*R*)-DPI in the presence of (*S*)-DPI (Fig. 9C) cannot be taken as evidence against competition, because (*S*)-DPI does not bind to sodium channels in the *R* state in this scheme. Is the (*S*)-DPI receptor site separate from the open channel-blocking (*R*)-DPI receptor site but identical to that for resting channel block? In this case, (*R*)-DPI block should be diminished by frequent brief pulses that activate sodium channels and induce (*S*)-DPI binding but little open channel block by (*R*)-DPI. This was not observed in our experiments

with 50-msec pulses at 0.2 Hz (Fig. 8), but shorter pulses at higher frequency will be necessary to settle this point. Taken together, these findings suggest different binding sites for the enantiomers of DPI 201-106 at the cardiac sodium channel, which are allosterically coupled such that opening of an (*S*)-DPI-modified sodium channel facilitates the block of this channel by (*R*)-DPI. The site at the sodium channel mediating the latter type of block and that of resting sodium channels by (*R*)-DPI may or may not be identical; if not, we cannot exclude the possibility that the (*S*)-DPI binding site and (*R*)-DPI binding site for resting block are identical. Synergistic, rather than competitive, interactions have also been observed with oppositely acting dihydropyridine enantiomers at cardiac calcium channels (19). Although (*R*)-DPI and (*S*)-DPI exert opposite actions on the sodium channel (8) and although our data suggest different enantioselective receptor sites for sodium channel block and slowing of inactivation, respectively, both enantiomers share one type of effect at neuronal and cardiac sodium channels, allosteric inhibition of the binding of a batrachotoxin derivative (8). The relation of the nonstereospecific receptor underlying this effect to those mediating sodium channel block or inhibition of inactivation is not clear at present.

Enantioselective receptor sites at the sodium channel. Although DPI 201-106 appears to be unique in acting on (at least) two different enantioselective receptor sites that mediate opposite effects on the sodium channel and interact allosterically, stereoselective binding sites as such are not unique to the interaction of DPI 201-106 with the sodium channel. Local anesthetics or class 1 antiarrhythmic agents display a moderate degree of stereoselectivity at sodium channels (20). The *R*-enantiomer of tocainide has a 2- to 3-fold higher potency in blocking cardiac sodium channels (21, 22) and in reducing their affinity to batrachotoxin benzoate (22). An eudismic ratio of up to 5.3 has been determined in a series of chiral class 1 antiarrhythmic drugs with regard to inhibition of batrachotoxin benzoate binding to cardiac sodium channels (23). A 7–8-fold difference in dissociation rate from sodium channels has been found in enantiomeric pairs of local anesthetics (24). A much higher degree of stereoselectivity (eudismic ratio > 1000) is observed among pyrethroid enantiomers when their stimulation of veratridine-dependent Na uptake by brain synaptosomes is taken as a measure of sodium channel modification (25). Thus, both blockers and activators have been shown to act on stereoselective receptor sites of the sodium channel. This is consistent with the existence of enantioselective and therefore distinct receptor sites for (*R*)-DPI, a blocker, and (*S*)-DPI, an activator, at the cardiac sodium channel.

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References

1. Scholtysik, G., R. Salzmann, R. Berthold, J. W. Herzig, U. Quast, and R. Markstein. DPI 201-106, a novel cardioactive agent: combination of cAMP-independent positive inotropic, negative chronotropic, action potential prolonging and coronary dilatory properties. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **329**:316–325 (1985).
2. Takahashi, K., M. Endoh, and N. Taira. Inotropic versus chronotropic, dromotropic, and coronary vasodilator actions of DPI 201-106, a novel positive inotropic agent, in the dog heart. *J. Cardiovasc. Pharmacol.* **9**:451–460 (1987).
3. Buggisch, D., G. Isenberg, U. Ravens, and G. Scholtysik. The role of sodium channels in the effects of the cardiotonic compound DPI 201-106 on contractility and membrane potentials in isolated mammalian heart preparations. *Eur. J. Pharmacol.* **118**:303–311 (1985).
4. Kohlhardt, M., U. Fröbe, and J. W. Herzig. Modification of single cardiac Na^+ channels by DPI 201-106. *J. Membr. Biol.* **89**:163–172 (1986).
5. Hof, R. P., and A. Hof. Mechanism of the vasodilator effects of the cardiotonic agent DPI 201-106. *J. Cardiovasc. Pharmacol.* **7**:1188–1192 (1985).
6. Holck, M., and W. Osterrieder. Interaction of the cardiotonic agent DPI 201-106 with cardiac Ca^{2+} channels. *J. Cardiovasc. Pharmacol.* **11**:478–482 (1988).
7. Siegl, P. K. S., M. L. Garcia, V. F. King, A. L. Scott, G. Morgan, and G. J. Kaczorowski. Interactions of DPI 201-106, a novel cardiotonic agent, with cardiac calcium channels. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **338**:684–691 (1988).
8. Romey, G., U. Quast, D. Pauron, C. Frelin, J. F. Renaud, and M. Lazdunski. Na^+ channels as sites of action of the cardioactive agent DPI 201-106 with agonist and antagonist enantiomers. *Proc. Natl. Acad. Sci. USA* **84**:896–900 (1987).
9. Kohlhardt, M., U. Fröbe, and J. W. Herzig. Removal of inactivation and blockade of cardiac Na^+ channels by DPI 201-106: different voltage-dependencies of the drug actions. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **335**:183–188 (1987).
10. Dugas, M., P. Honerjäger, and U. Masslich. Tetrodotoxin block of single germitrine-activated sodium channels in cultured rat cardiac cells. *J. Physiol. (Lond.)* **411**:611–626 (1989).
11. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch. Eur. J. Physiol.* **391**:85–100 (1981).
12. Leaver, R. H., and T. R. Thomas. *Analysis and Presentation of Experimental Results.* Macmillan Press, London (1974).
13. Ariens, E. J., E. W. Wuis, and E. J. Veringa. Stereoselectivity of bioactive xenobiotics. *Biochem. Pharmacol.* **37**:8–19 (1988).
14. Scholtysik, G., U. Quast, and A. Schaad. Evidence for different receptor sites for the novel cardiotonic S-DPI 201-106, ATX II and veratridine on the cardiac sodium channel. *Eur. J. Pharmacol.* **125**:111–118 (1986).
15. Honerjäger, P. Cardioactive substances that prolong the open state of sodium channels. *Rev. Physiol. Biochem. Pharmacol.* **92**:1–74 (1982).
16. Josephson, M. E., A. E. Buxton, F. E. Marchlinski, J. U. Doherty, D. M. Cassidy, M. G. Kienzle, J. A. Vassallo, J. M. Miller, J. Almendral, and W. Grogan. Sustained ventricular tachycardia in coronary artery disease: evidence for reentrant mechanism, in *Cardiac Electrophysiology and Arrhythmias* (D. P. Zipes and J. Jalife, eds.). Grune & Stratton, Orlando, FL, 409–418 (1985).
17. Nilius, B., J. Vereecke, and E. Carmeliet. Properties of the bursting Na channel in the presence of DPI 201-106 in guinea-pig ventricular myocytes. *Pflügers Arch. Eur. J. Physiol.* **413**:234–241 (1989).
18. Polc, P., E. P. Bonetti, R. Schaffner, and W. Haefely. A three-state model of the benzodiazepine receptor explains the interactions between the benzodiazepine antagonist Ro 15-1788, benzodiazepine tranquilizers, β-carbolines, and phenobarbitone. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **321**:260–264 (1982).
19. Kokubun, S., B. Prod'Hom, C. Becker, H. Porzig, and H. Reuter. Studies on Ca channels in intact cardiac cells: voltage-dependent effect and cooperative interactions of dihydropyridine enantiomers. *Mol. Pharmacol.* **30**:571–584 (1986).
20. Akerman, S. B. A., G. Camougis, and R. V. Sandberg. Stereoisomerism and differential activity in excitation block by local anesthetics. *Eur. J. Pharmacol.* **8**:337–347 (1969).
21. Block, A. J., D. Merrill, and E. R. Smith. Stereoselectivity of tocainide pharmacodynamics *in vivo* and *in vitro*. *J. Cardiovasc. Pharmacol.* **11**:216–221 (1988).
22. Sheldon, R. S., N. J. Cannon, A. S. Nies, and H. J. Duff. Stereospecific interaction of tocainide with the cardiac sodium channel. *Mol. Pharmacol.* **33**:327–331 (1988).
23. Hill, R. J., H. J. Duff, and R. S. Sheldon. Determinants for stereospecific binding of type I antiarrhythmic drugs to cardiac sodium channels. *Mol. Pharmacol.* **34**:659–663 (1988).
24. Yeh, J. Z. Blockage of sodium channels by stereoisomers of local anesthetics. *Prog. Anesthesiol.* **2**:35–44 (1980).
25. Ghiasuddin, S. M., and D. M. Soderlund. Pyrethroid insecticides: potent, stereospecific enhancers of mouse brain sodium channel activation. *Pestic. Biochem. Physiol.* **24**:200–206 (1985).

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