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Potential-Dependent Allosteric Modulation of 1,4-Dihydropyridine Binding by *d*-(*cis*)-Diltiazem and (±)-Verapamil in Living Cardiac Cells

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SUMMARY

We have studied allosteric effects of the Ca channel blockers *d*-(*cis*)-diltiazem, (±)-verapamil, and (*S*)- and (*R*)-devapamil on the specific binding of the 1,4-dihydropyridine derivative (+)-[^{3}H]PN 200-110 to intact tissue cultured rat heart cells. In polarized cells (membrane potential, −38 ± 4 mV) *d*-(*cis*)-diltiazem (5 μM) increased the affinity of the radiolabel 2–4-fold causing 100–187% enhancement of binding at (+)-PN 200-110 concentrations below 0.5 nM. (±)-Verapamil (0.1–3 μM) had a similar, although smaller, effect on (+)-PN 200-110 binding. The two enantiomers of devapamil were without effect. In depolarized cells (membrane potential, 0 mV) *d*-(*cis*)-diltiazem had a small and the phenylalkylamines a strong inhibitory effect on (+)-PN 200-110 binding, mainly due to a reduction of binding affinity. At 50% receptor occupation by the radioligand, (*R*)-devapamil, (±)-verapamil, and (*S*)-devapamil displaced 40, 55, and 75%, respectively, of specifically bound radiolabel. Half-maximal effects were reached with 50, 20, and 4.5 nM, respectively, of the three compounds. Compared with nominally Ca-free medium (containing 3–5 μM Ca), addition of 1.25 mM $CaCl_2$ caused an increase in the maximal binding capacity for (+)-PN 200-110 in both polarized and depolarized cells. However, Ca had only marginal effects on the allosteric interactions between (+)-PN 200-110, *d*-(*cis*)-diltiazem, and verapamil. We conclude from our results that positive cooperative interactions between Ca channel blockers prevail under conditions in which the voltage-dependent Ca channel can fluctuate between closed, open, and inactivated states. Negative cooperativity is usually observed under conditions in which all channels are inactivated (depolarized cells, fragmented membranes). Therefore, it is impossible to predict the type and the extent of allosteric interactions *in vivo* from studies in cell homogenates.

Several studies have shown that binding for 1,4-dihydropyridines, phenylalkylamines, and diltiazem on voltage-dependent L-type Ca channels are distinct but linked by heterotropic cooperative interactions (1–4). Verapamil and diltiazem were found to inhibit or to stimulate, respectively, equilibrium binding of dihydropyridine Ca channel blockers in membrane preparations from smooth muscle, skeletal muscle transverse tubules, and heart. All of this work has been performed in fragmented membrane preparations in which the membrane potential has collapsed and all Ca channels are presumably inactivated. In a recent study in intact cardiac cells, we have shown that at least within the dihydropyridine class of compounds the cooperative interactions between channel blockers and channel activators are highly voltage dependent (5). Positive cooperativity was observed in polarized cells (−38 mV resting potential) but was completely absent in depolarized cells. This result suggests that the potential-regulated state changes of the channel (resting, open, and inactivated states or groups of states) are associated with conformational changes of the dihydropyridine receptors and hence, their allosteric coupling. The available evidence indicates that both dihydropyridine and non-dihydropyridine Ca channel blockers have in common a high affinity for inactivated channels and rather low affinities for resting or open states of the channel (5–9). Therefore, it is a logical extension of our previous work to ask whether the well known allosteric interactions between the three main classes of Ca channel ligands, dihydropyridines, phenylalkylamines, and benzothiazepines, are also voltage dependent. Our results in the present study show that the membrane potential indeed affects the cooperative interactions of all three groups of channel-blocking compounds. The type and strength of these interactions in polarized cells and hence, their functional consequences *in vivo*, cannot be predicted from experiments in cell homogenates.

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Materials and Methods

Rat heart cell cultures. Primary cultures of cardiac myocytes were prepared after enzymatic dissociation of ventricles excised from 3–4-

ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)1-piperazineethane sulfonic acid; PN 200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridine carboxylate; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

day-old rats according to the procedure described by Porzig *et al.* (10) and Kokubun *et al.* (5). The cells were grown on glass coverslips in the Dulbecco modification of Eagles minimal essential medium supplemented with 10% fetal calf serum. Experiments were performed on day 3 of culture when a regularly beating monolayer of myocytes had formed. The cells stopped beating when the culture medium was replaced by a Ca-free experimental solution (see below).

Receptor binding assay. For binding assays we used coverslip-attached cell cultures at two different membrane potentials as described in Ref. 5. Cardiac cells maintained a mean steady state membrane potential of −38 ± 4 mV when studied in nominally Ca free Hanks' solution (contents in mM: NaCl, 137; KCl, 5.4; $MgSO_4$, 0.8; Na_2HPO, 0.34; KH_2PO_4, 0.44; $NaHCO_3$, 4.2; glucose, 5.5; HEPES, 20; pH 7.2; measured Ca contamination, 3–5 μM). The cells were depolarized (membrane potential close to 0 mV) by replacing most of the NaCl in the above solution by KCl (137 KCl, 5.4 NaCl, other components unchanged). In all binding experiments (+)-[^{3}H]-PN 200-110 was used as a radiolabel for dihydropyridine binding sites. Equilibrium binding was determined by incubating individual coverslip cultures with the radiolabel for 90 min at 37° in the presence or absence of non-dihydropyridine channel blockers. At the end of the incubation period the cultures were washed individually for 15 sec in a large volume (1200 ml) of hypotonic medium (10 mM K_2HPO_4, pH 7.5) at 37°. The cultures were then transferred into counting vials and digested with 0.8 ml of a 1:1 (v/v) mixture of absolute ethanol and Solutron (Kontron AG, Zürich, Switzerland). They were counted in a liquid scintillation counter (Kontron MR 300 or Intertechnique SL 4000) using a Triton/xylol-based commercial scintillation fluid (Kontrogel; Kontron AG, Zürich). Specific binding was defined as the fraction of total binding that could be displaced by 1 μM nonlabeled (+)-PN 200-110. Under control conditions with 0.1 nM (+)-[^{3}H]PN 200-110, nonspecific binding reached about 17% of total binding in depolarized and about 50% in polarized cells. The values for 1 nM (+)-[^{3}H]PN 200-110 were, respectively, 55 and 65% (see Fig. 1, *inset* for nonspecific binding in polarized cells as a function of (+)-[^{3}H]PN 200-110 concentration). Nonspecific binding of (+)-[^{3}H]PN 200-110 in membrane preparations was less than 10%. Because of the light sensitivity of dihydropyridines, all experiments were performed under sodium light. Nonspecific binding was not altered by membrane potential changes or by diltiazem or phenylalkylamine addition to the incubation medium. In individual experiments three to five coverslip cultures were analyzed for each ligand concentration used. Attempts to measure voltage-dependent binding in cardiac cell suspensions were not successful. Removal of the cells from the growth surface by mechanical means seemed to induce depolarization of the membrane potential.

Preparation of cell homogenates. Membranes from tissue-cultured cardiac myocytes were prepared according to the method of Lee *et al.* (11). Briefly, cells were mechanically removed from the growth surface and homogenized in 19 volumes of Hanks' solution with a Potter-type homogenizer equipped with a tightly fitting Teflon pestle. The homogenates were washed twice at 38,000 × *g* for 10 min and then resuspended in the Ca-free Hanks' solution used for binding assays (composition as above).

Protein determination. For each group of 12 coverslip cultures maintained together in a 10-cm diameter Petri dish, 2–4 were used to measure the mean protein content per culture. The cells were washed, removed from the coverslip by freeze-thawing, and homogenized in 30 mM NaCl medium. The protein content was estimated according to the method of Lowry *et al.* (12) with bovine serum albumin as a standard. The mean protein per coverslip culture varied between 0.125 and 0.212 mg. The mean variation between individual cultures within a group of 12 was 11%. Usually cultures in the center of the Petri dish contained somewhat more protein than those in the periphery. For binding experiments, cultures were systematically mixed in order to avoid any bias that could have been introduced due to this variability.

Data analysis. Wherever possible, binding data were analyzed using a computerized nonlinear least squares curve fitting procedure. The method is based on a BASIC version of the program MODFIT published by McIntosh and McIntosh (13) running on a HP 9816 computer. It uses the weighted means of replicate measurements to estimate maximal binding capacities (B_{max} values) and apparent equilibrium dissociation constants (K_d values) and their standard deviations. The error bars of individual data points in Figs. 1–7 give the standard errors of the mean with n being the number of coverslip cultures rather than the number of independent primary culture preparations. This seems justified because 1) for each such preparation 80–100 hearts were pooled and 2) the main source of experimental error was the differing protein content of individual coverslip cultures rather than differences between the various culture preparations. The significance of differences between binding parameters under various conditions was tested as follows. K_d and B_{max} values were estimated for each individual experiment by the described nonlinear regression analysis. The mean values of the individual parameter estimates for each set of experimental conditions were then compared, applying Student's t test with n being the number of independent experiments. A value of $p < 0.05$ was considered significant. K_d and B_{max} values with their standard deviations obtained from a nonlinear fit to the mean of all measurements in a group of experiments are given in the figure legends. They did not differ significantly from the values obtained by averaging the parameter estimates of individual experiments.

Material. Newborn rats (age 1–4 days) were purchased from the animal breeding facility of the Department of Pathophysiology, University of Bern. Tissue culture reagents were obtained from Boehringer Mannheim Corp. (Rotkreuz, Switzerland) GIBCO (Basel, Switzerland), and Flow Laboratories (Baar, Switzerland). (+)-[^{3}H]PN 200-110 (3.03–3.15 TBq/mmol) was purchased from Amersham International (Buckinghamshire, UK). Nonlabeled (+)- and (±)-PN 200-110 were gifts of Sandoz AG (Basel, Switzerland; Dr. Hof). Verapamil as well as (*R*)- and (*S*)-devapamil were gifts of Knoll AG (Ludwigshafen, FRG; Prof. Kreiskott). *d*- and *l*-(*cis*)-Diltiazem were donated by Goedecke AG (Freiburg, FRG; Dr. Bahrmann).

Results

Effect of *d*-(*cis*)-diltiazem on (+)-PN 200-110 binding in Ca-free media. In a first set of experiments we tested the effect of a maximally effective concentration of *d*-(*cis*)-diltiazem (5 μM) on (+)-PN 200-110 binding to polarized cells (Fig. 1). Binding equilibrium was established in nominally Ca-free Hanks' solution (Ca concentration, 3–5 μM) because earlier experiments had shown that with this medium cardiac myocytes are capable of maintaining a steady membrane potential of about −40 mV (5). *d*-(*cis*)-Diltiazem significantly decreased the apparent K_d value for (+)-PN 200-110 from a control value of 0.97 ± 0.17 nM to 0.41 ± 0.005 nM ($p < 0.05$) but left the maximal binding capacity nearly unchanged (see the legend to Fig. 1). The increase in binding affinity resulted in a doubling of specific (+)-PN 200-110 binding at low radioligand concentrations. A highly significant enhancement of (+)-PN 200-110 binding was observed at all (+)-PN 200-110 concentrations below 1 nM.

Fig. 2 shows the analogous set of experiments in K-depolarized cells. Under these conditions the same concentration of *d*-(*cis*)-diltiazem had no significant effect on the K_d value of the radioligand.

Effect of *d*-(*cis*)-diltiazem in Ca-containing media. Earlier studies had shown that binding of *d*-(*cis*)-diltiazem to skeletal muscle transverse tubular membranes and cardiac vesicles is inhibited by Ca ion concentrations higher than 10 μM (3, 14). Therefore, it was of interest to assess whether Ca concentrations in the incubation medium within the physiological range would modify the allosteric enhancement of (+)-PN

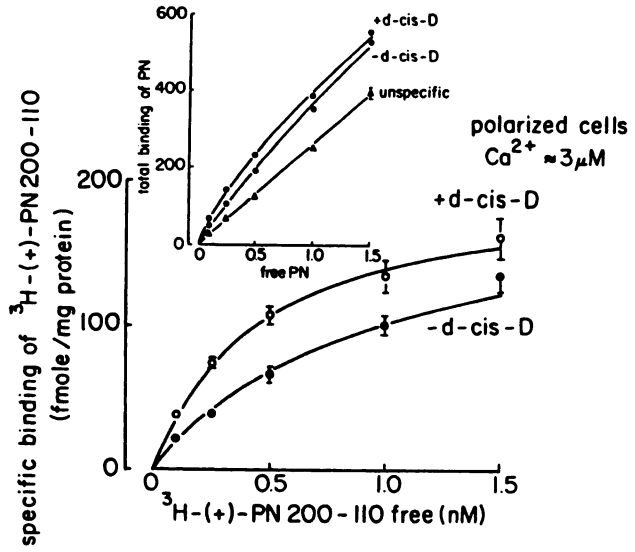

Fig. 1. Effect of *d-(cis)*-diltiazem (*d-cis-D*; 5 μM) on specific binding of (+)-[^{3}H]PN 200-110 to polarized cardiac cells in the absence of Ca. The experimental medium had a residual Ca contamination of 3–5 μM. The *curves* represent computerized nonlinear least squares regressions to the data points assuming binding to a single class of sites and a Hill coefficient of 1. K_d and B_{max} estimates for these curves were 0.91 ± 0.17 nM and 195 ± 24.3 fmol/mg of protein [− *d-(cis)*-diltiazem], 0.42 ± 0.06 nM and 198 ± 16.7 fmol/mg of protein [+ *d-(cis)*-diltiazem]. *Data points* represent the mean ± standard error of 46 cultures from 11 independent experiments [− *d-(cis)*-diltiazem] and 19 cultures from three independent experiments [+ *d-(cis)*-diltiazem]. *Inset*, total and unspecific binding for the same set of data.

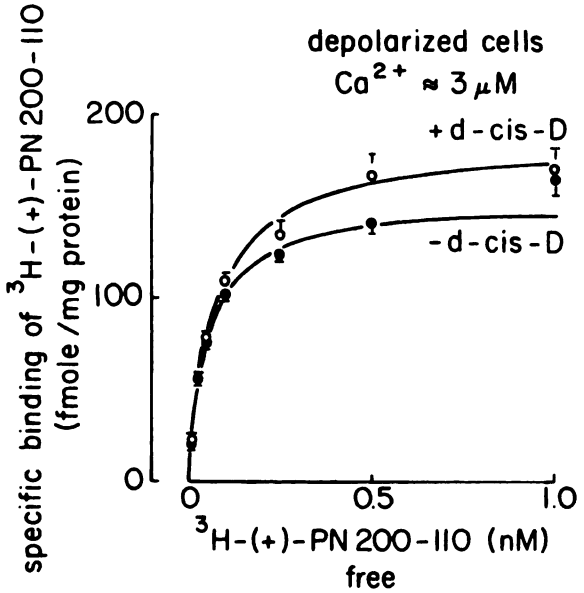

Fig. 2. Effect of *d-(cis)*-diltiazem (*d-cis-D*; 5 μM) on specific binding of (+)-[^{3}H]PN 200-110 to K-depolarized cardiac cells in the absence of Ca. Same conditions as in Fig. 1, except that the incubation medium contained 137 mM KCl instead of NaCl. K_d and B_{max} estimates for these curves were 0.05 ± 0.004 nM and 154.0 ± 4.1 fmol/mg protein [− *d-(cis)*-diltiazem] 0.071 ± 0.004 nM and 185.9 ± 7.2 fmol/mg of protein [+ *d-(cis)*-diltiazem]. The *data points* give the mean ± standard error of 5–31 cultures from seven independent experiments [− *d-(cis)*-diltiazem] and five cultures from one experiment [+ *d-(cis)*-diltiazem].

200-110 binding by *d-(cis)*-diltiazem. Increasing the extracellular Ca concentration under our conditions had the disadvantage of affecting the membrane potential. Compared with Ca-free solutions, the resting potential tended to assume more negative values (−50 to −60 mV). Moreover, in the absence of Ca channel blockers, action potentials and contractions occurred in irregular time intervals.[1] Judging from our previous results (5) we predicted that in polarized cells this would result in an increase in K_d for (+)-[^{3}H]PN 200-110 in the absence of *d-(cis)*-diltiazem. This was indeed observed. The mean K_d value from seven independent experiments was 2.8 ± 0.60 nM. There was also an increase in the mean B_{max} values to 398.5 ± 80.7 fmol/mg of protein. However, these values must be considered with caution. As a consequence of the reduced affinity, only a low degree of receptor saturation was reached with free PN concentrations up to 2 nM. Binding parameters could not be estimated reliably under these conditions because binding data were obtained only for free ligand concentrations at or below K_d. Unfortunately, a more precise estimate of B_{max} and K_d values could not be reached because the high level of unspecific binding made it impossible to extend the binding curve to (+)-PN 200-110 concentrations above 1.5 nM. Fig. 3 shows the result of three of these seven experiments in which we have measured simultaneously the effect of *d-(cis)*-diltiazem in the presence of 1.25 mM Ca. In the presence of a maximally effective concentration of *d-(cis)*-diltiazem (5 μM; see Fig. 3, *inset*), binding of (+)-PN 200-110 was markedly enhanced. With 0.25 nM (+)-PN 200-110 in the medium, specific binding increased by 187%, from 36.5 to 104.9 fmol/mg of protein. The K_d value for (+)-PN 200-110 decreased from 1.86 to 0.47 nM. The B_{max} value in the presence of *d-(cis)*-diltiazem reached 310.2 ± 19.8 fmol/mg of protein. Two other experiments with *d-(cis)*-diltiazem gave similar results. The mean B_{max} value from all five experiments with *d-(cis)*-diltiazem (286.1 ± 23.4 fmol/mg of protein) was significantly ($p < 0.025$) higher than the corresponding value in a Ca-free medium (196.7 ± 14.4 fmol/mg of protein) but not significantly different from the mean B_{max} value in Ca medium without *d-(cis)*-diltiazem. Ca had no effect on the K_d values for (+)-PN 200-110 in the presence of *d-(cis)*-diltiazem [0.46 ± 0.036 nM (with Ca) versus 0.41 ± 0.005 nM (without Ca)]. We conclude from these results that *d-(cis)*-diltiazem stimulates (+)-PN 200-110 binding in polarized cells mainly by increasing the binding affinity of this dihydropyridine compound.

Similar experiments were also performed under depolarizing conditions (Fig. 4). The analysis of the data showed that *d-(cis)*-diltiazem slightly decreased affinity and B_{max} values for (+)-PN 200-110, but both effects were not significant. Nevertheless, at low (+)-PN 200-110 concentrations (0.01 nM) the added small effects on K_d and B_{max} resulted in a significant displacement of (+)-PN 200-110 binding in the presence of increasing concentrations of *d-(cis)*-diltiazem (see Fig. 4, *inset*). At the level of 0.01 nM (+)-PN 200-110, specific binding was 24.5 ± 0.8 fmol/mg of protein in the absence of *d-(cis)*-diltiazem and decreased significantly to 17.3 ± 0.8 fmol in the presence of 3 μM *d-(cis)*-diltiazem. The half-maximal effect was reached with 0.6 μM *d-(cis)*-diltiazem. Under the same conditions *l-(cis)*-diltiazem was without effect. In the absence of *d-(cis)*-diltiazem Ca had a significant effect on the B_{max} value for (+)-

[1] H. Porzig and B. Prod'hom, unpublished observations.

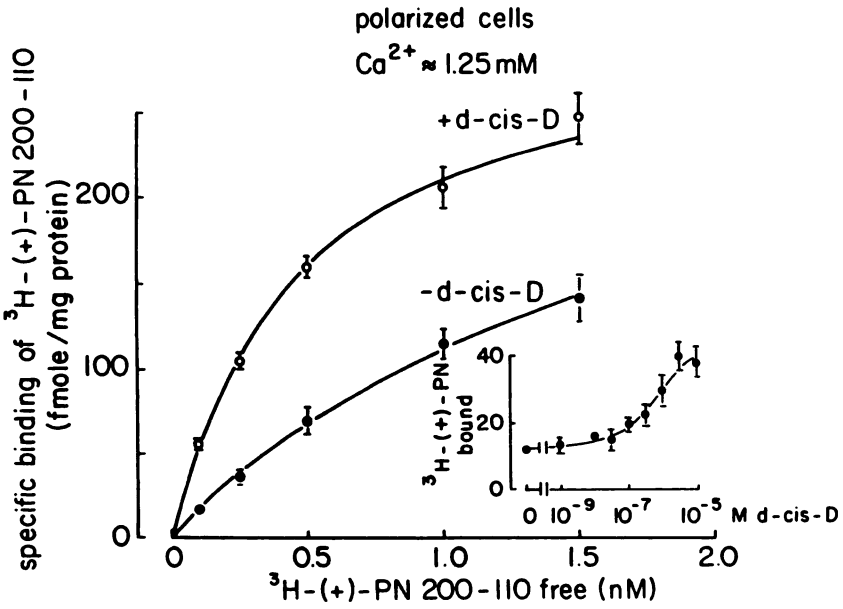

Fig. 3. Effect of *d*-(*cis*)-diltiazem (*d-cis-D*; 5 μM) on specific binding of (+)-[^{3}H]PN 200-110 to polarized cardiac cells in the presence of Ca (1.25 mM). *Main panel*, same data-fitting procedure as in Fig. 1. K_d and B_{max} estimates for these curves were 1.86 ± 0.55 nM and 323.3 ± 72.6 fmol/mg of protein [− *d*-(*cis*)-diltiazem], 0.47 ± 0.05 nM and 310.2 ± 19.8 fmol/mg of protein [+ *d*-(*cis*)-diltiazem]. The *experimental points* represent the mean ± standard error of 20 cultures from three independent experiments. Two other experiments with *d*-(*cis*)-diltiazem gave similar results. *Inset*, enhancement of specific (+)-[^{3}H]PN 200-110 binding as a function of *d*-(*cis*)-diltiazem concentration. *Data points* give the mean ± standard error of five cultures from one experiment. *Curve* was drawn freehand.

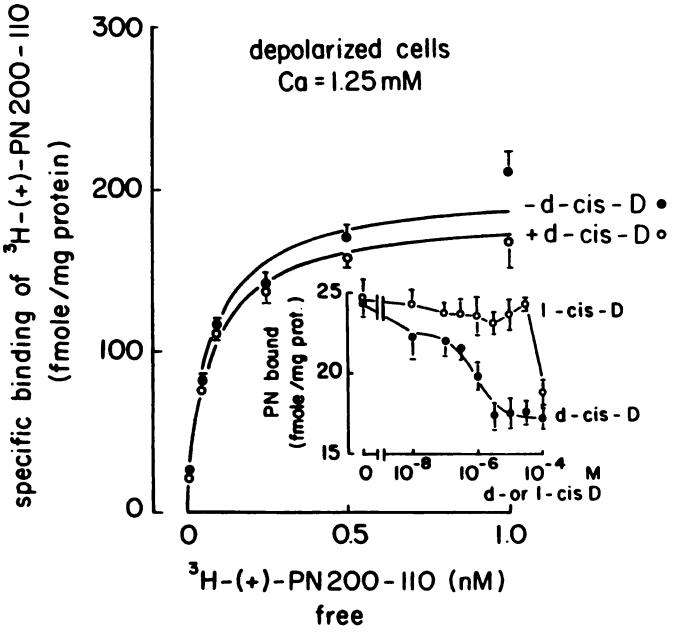

Fig. 4. Effect of *d*-(*cis*)-diltiazem (*d-cis-D*; 5 μM) on specific binding of (+)-[^{3}H]PN 200-110 to depolarized cells in the presence of Ca (1.25 mM). *Main panel*, same data-fitting procedure as in Fig. 1. Calculated K_d and B_{max} estimates for these curves were 0.071 ± 0.005 nM and 200.0 ± 6.5 fmol/mg of protein [− *d*-(*cis*)-diltiazem], 0.074 ± 0.005 nM and 185.7 ± 6.6 fmol/mg of protein [+ *d*-(*cis*)-diltiazem]. The *data points* represent the mean of 10–15 cultures from three independent experiments. *Inset*, displacement of specifically bound (+)-PN 200-110 (0.01 nM) by increasing concentrations of *d*- or *l*-(*cis*)-diltiazem. The *points* represent the mean ± standard error of eight cultures from two independent experiments. An additional experiment with *d*-(*cis*)-diltiazem gave similar results. Curves were drawn freehand.

PN 200-110. The binding capacity increased from 146.7 ± 9.2 (nine experiments) to 197.0 ± 9.0 (five experiments) fmol/mg of protein ($p < 0.005$). This difference was lost in the presence of *d*-(*cis*)-diltiazem.

Effect of *d*-(*cis*)-diltiazem on (+)-PN 200-110 binding in fragmented membranes. Previous studies on interactions of *d*-(*cis*)-diltiazem with dihydropyridine binding in cardiac cells have all been performed in cell homogenates (2, 11, 15). Therefore, in additional experiments we have measured the effects of *d*-(*cis*)-diltiazem also in cell homogenates prepared from cardiac cell cultures according to the method of Lee *et al.* (11). This allowed us to compare our intact cell data with binding parameters from fragmented membranes under identical conditions. The result of these experiments is shown in Fig. 5. The membranes were equilibrated with 0.04 nM (+)-PN 200-110 in the presence of increasing concentrations of *d*-(*cis*)-diltiazem in a medium containing 1.25 mM Ca. Confirming the reports cited above, *d*-(*cis*)-diltiazem stimulated (+)-PN 200-110 binding maximally by about 25% at 37°. Binding experiments (not shown) with (+)-PN 200-110 concentrations ranging between 0.01 and 1 nM indicated that this effect was entirely

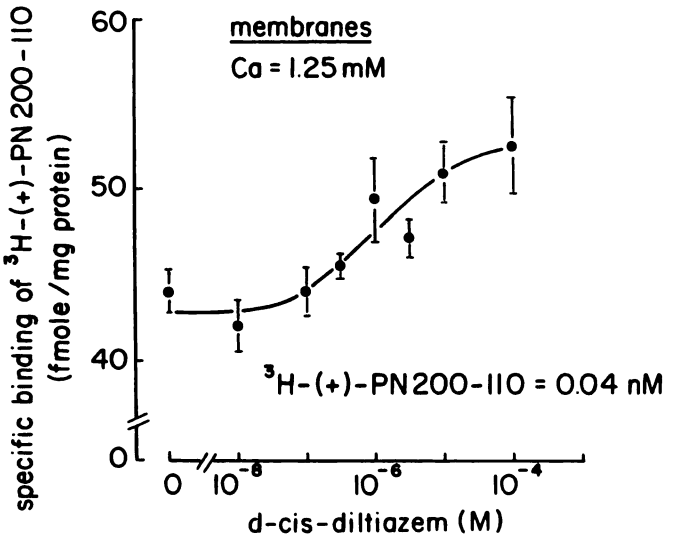

Fig. 5. Stimulating effect of *d*-(*cis*)-diltiazem on the specific binding of (+)-[^{3}H]PN 200-110 to cardiac cell homogenates. The cell homogenate was prepared from tissue-cultured rat heart cells and was equilibrated with 0.04 nM (+)-[^{3}H]PN 200-110. The binding medium (Hanks' solution) contained 1.25 mM $CaCl_2$. The *experimental points* give the mean ± standard error of eight cultures from two independent experiments. A third experiment gave similar results. Curve was drawn freehand.

due to an increase in affinity for (+)-PN 200-110. The mean K_d value was reduced from 0.33 ± 0.03 to 0.26 ± 0.02 nM or by 27% in the presence of *d*-(*cis*)-diltiazem whereas the mean B_{max} value reached 245.8 ± 8.6 and 225.2 ± 8.6 fmol/mg of protein in the absence and presence of *d*-(*cis*)-diltiazem, respectively. Note that under both conditions the K_d value for (+)-PN 200-110 binding in membranes was significantly higher ($p < 0.001$) than the corresponding value in depolarized cells. It is clear from these experiments that a stimulatory effect of *d*-(*cis*)-diltiazem on (+)-PN 200-110 binding can be observed in fragmented membranes whereas no such effect was obtained in depolarized intact cells.

Effects of (+)-verapamil on (+)-PN 200-110 binding. The results of binding experiments in membrane preparations classify (±)-verapamil as a negative allosteric modulator of dihydropyridine binding (1, 16). We have tested whether the membrane potential and, hence, the functional state of the channel could modify the interaction between (±)-verapamil and dihydropyridines.

In experiments documented in Fig. 6 we have measured (+)-PN 200-110 binding in polarized and in depolarized cells in the presence of increasing concentrations of (±)-verapamil. In depolarized cells, verapamil inhibited specific binding of (+)-PN 200-110 noncompetitively. When cells were equilibrated in the presence of 0.06 nM (+)-[^{3}H]PN 200-110 (corresponding to the mean K_d value for (+)-PN 200-110 under these conditions) maximally effective concentrations of (±)-verapamil (10 μM) displaced about 55% of the specifically bound (+)-PN 200-110. Half-maximal displacement was reached with 20 nM (±)-verapamil. The Hill coefficient of the displacement curve was significantly lower than 1 (0.58, see Fig. 7). Comparable results have been obtained in isolated cardiac membrane preparations (11; for review see Ref. 17). Thus, in contrast to the results with *d*-(*cis*)-diltiazem, depolarized cells and membranes behaved similarly with respect to the interaction between (±)-verapamil and (+)-PN 200-110. However, in polarized cells the effect of (±)-verapamil on (+)-PN 200-110 binding was stimulatory rather than inhibitory. For the experiments in Fig. 6 the cells were equilibrated with 0.7 nM (+)-[^{3}H]PN 200-110, the concentration that corresponded to its mean K_d value under these conditions. Concentrations of (±)-verapamil higher than 10 nM enhanced (+)-PN 200-110 binding with a half maximal effect at 0.6 μM (±)-verapamil. With the maximally effective concentration of 10 μM (±)-verapamil, (+)-PN 200-110 binding reached 170% of the control level in the absence of (±)-verapamil. Still higher concentration of (±)-verapamil reduced the stimulatory effect. Compared with depolarized cells, the concentration of (±)-verapamil required for a half maximal effect increased by a factor of 30. This finding suggests that the binding of (±)-verapamil is itself voltage dependent. (±)-Verapamil enhanced (+)-PN 200-110 binding exclusively by reducing the mean K_d value for (+)-PN 200-110 from 0.91 ± 0.17 to 0.28 ± 0.04 nM (three independent experiments). The B_{max} value for (+)-PN 200-110 binding was not significantly altered in the presence of (±)-verapamil (181.8 ± 12.7 versus 195.7 ± 24.3) but small effects may have been obscured by the large scatter of the data. The experiments shown in Fig. 6 were performed in nominally Ca-free media. However, control experiments showed that the stimulatory effect of (±)-verapamil in polarized cells was not significantly modified in the presence of 1.25 mM $CaCl_2$.

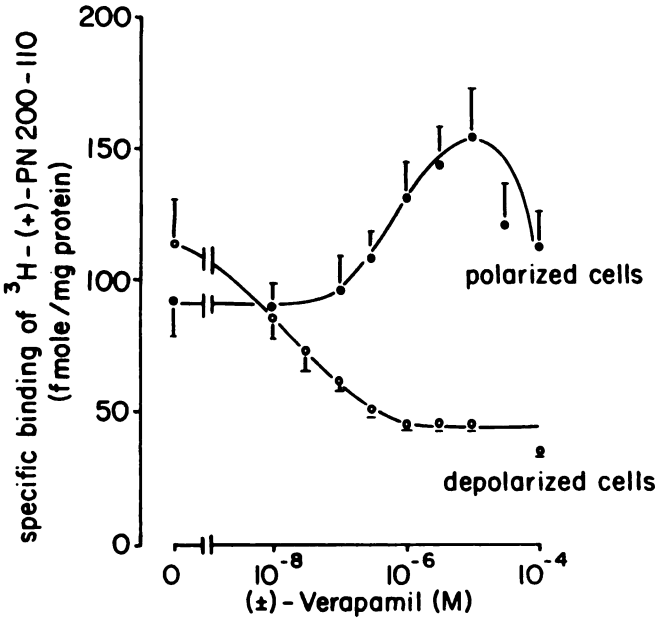

Fig. 6. The effect of (±)-verapamil on the specific binding of (+)-[^{3}H]PN 200-110 in polarized and depolarized cells. Binding equilibrium was established in Ca-free Hanks' solution containing 0.7 nM (polarized cells) and 0.06 nM (depolarized cells). These concentrations correspond to the mean K_d values for (+)-PN 200-110 under the two conditions. Half-maximal stimulation and inhibition required 0.63 μM and 20 nM (±)-verapamil, respectively. *Data points* for polarized cells give the mean ± standard error for 11–15 cultures from four independent experiments; for depolarized cells they give the mean of eight cultures from two independent experiments. Curves drawn freehand.

The interpretation of the results with (±)-verapamil is complicated by the racemic nature of the compound. The bell-shaped concentration-response curve in polarized cells could have resulted from differential effects of the (+)- and (−)-enantiomers. To clarify this point we studied the effects of (*S*) and (*R*)-devapamil (desmethoxyverapamil), derivatives of verapamil that were available as optically pure enantiomers. (−)-(*S*)-[^{3}H]Devapamil has been used to label (±)-verapamil binding sites at voltage-dependent Ca channels in skeletal and heart muscle preparations (18–20). In polarized cells, concentrations of the two enantiomers ranging between 10^{-9} and 10^{-5} M did not exert any significant stimulatory or inhibitory effect on (+)-PN 200-110 binding. Yet, in depolarized cells (Fig. 7) both (*R*)- and (*S*)-devapamil inhibited specific (+)-PN 200-110 binding noncompetitively. In cells equilibrated with 0.06 nM (+)-[^{3}H]PN 200-110, half maximal displacement of the radiolabel was observed with 4.5 and 50 nM (*S*)- and (*R*)-devapamil, respectively. As in the case of (±)-verapamil the displacement by (*S*)- and (*R*)-devapamil was incomplete. The *S*-enantiomer displaced maximally 75%, the *R*-enantiomer 40% of specifically bound (+)-PN 200-110. The Hill coefficients of the displacement curves were both lower than 1 and similar to the one for (±)-verapamil. We obtained values of 0.6 for (*R*)-devapamil and of 0.54 for (*S*)-devapamil. Binding curves for (+)-PN 200-110 measured in the presence or absence of 100 nM (*S*)-devapamil indicated that the apparent inhibitory effect on (+)-PN 200-110 binding could be fully explained by the observed increase in the K_d value for (+)-PN 200-110.

Discussion

The most interesting result of our study is the observation that the voltage-dependent changes of Ca channel conforma-

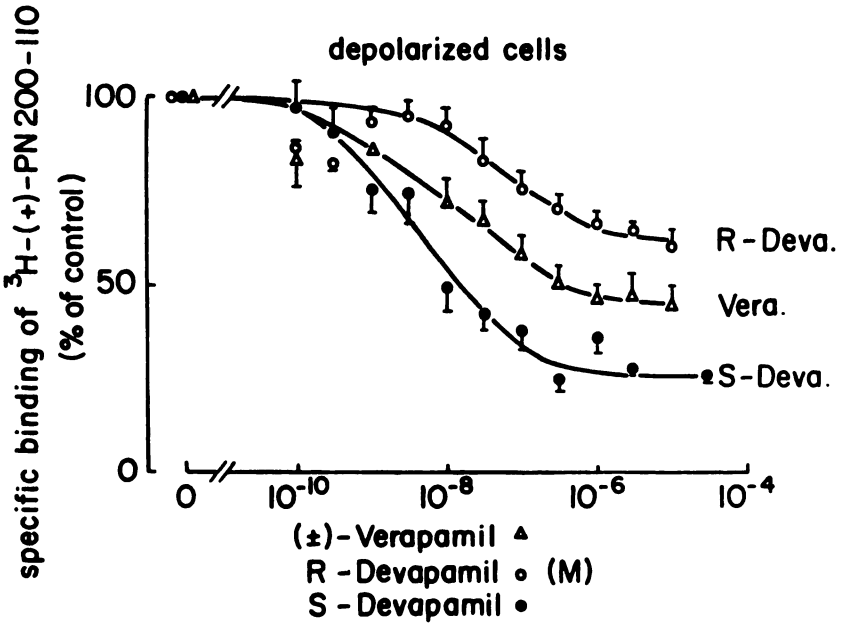

Fig. 7. Competitive displacement of specifically bound (+)-[^{3}H]PN 200-110 by (*R*)-devapamil, (±)-verapamil, and (*S*)-devapamil in depolarized cells. The cells were equilibrated with 0.06 nM radiolabel in K-rich (137 mM), Ca-free Hanks' solution. The *data points* give mean values ± standard error for 5–13 cultures from three independent experiments. The *curve* for verapamil was taken from Fig. 6. The Hill coefficients for the curves are 0.6, 0.58, and 0.54, respectively. Curves were fitted freehand.

tion significantly modulate the allosteric interactions between the three main groups of blocking ligands. All the identified allosterically coupled binding sites seem to sense changes of the electric field across the membrane, because their accessibility and affinity for specific ligands is different in resting, open, and inactivated channels. A voltage-dependent change in binding affinity associated with a transition from the resting to the inactivated states of the channel has been directly demonstrated for dihydropyridines in mammalian heart (5), smooth muscle (21), and PC 12 cells (22). For *d*-(*cis*)-diltiazem and (±)-verapamil, high affinity binding to inactivated channels can be inferred from the fact that the strength of their channel blocking action is frequency dependent (6, 23, 24). Moreover, the dissociation constants reported for the two compounds in cardiac membrane preparations (in which channels are inactivated) are lower than the concentrations required for pharmacological effects in the same tissue (24, 25). These findings together with our observation of a voltage-dependent allosteric coupling between the different sites suggest that the ligands are all bound close to the voltage-sensitive gating part of the channel protein. The increase in the binding affinity of channel blockers when channels are inactivated could provide an explanation for the positive cooperativity observed in polarized cells between dihydropyridines and either (±)-verapamil or *d*-(*cis*)-diltiazem. Positive cooperativity will be exerted by any ligand that is capable of shifting the steady state frequency distribution of channel states at a given membrane potential in favor of an increased fraction of inactivated states. Because drug-occupied inactivated channels represent a relatively stable state such a shift will cause a steady state increase in the affinity of all allosterically coupled sites and, hence, stimulate simultaneous binding of a second ligand. A similar interpretation has been given for the positive cooperativity observed between channel-blocking and channel-activating dihydropyridine derivatives (5). It could be argued that the observed cooperative effects between Ca channel ligands might have resulted simply from a depolarizing effect of verapamil or diltiazem. Such mechanism is unlikely for two reasons. 1) Prolonged exposure of myocardial preparations from newborn rats to dihydropyridine Ca channel blockers had no effect on the steady state membrane potential in Ca-free solution (5). Hence, Ca channel blockade is not associated with depolarization. 2) In Ca-containing solution Ca channel blockers tended to suppress spontaneous action potentials and contractile activity and, hence, *reduced* the probability of channels to cycle through open and inactivated states. Therefore, the differences in spontaneous voltage oscillations in the presence and absence of a channel blocker would favor a weaker rather than a stronger effect of diltiazem on (+)-PN 200-110 binding.

Binding of Ca channel blockers has close analogies to the binding of local anesthetics to sodium channels conceptualized in the modulated receptor hypothesis by Hille (26) and Hondeghem and Katzung (27). Allosteric interactions between different Ca channel ligands have their analogy in the interaction between the local anesthetic site and the toxin binding site 2 (batrachotoxin site) in Na channels (28). The recent cloning of the dihydropyridine-binding protein with its close relationship to the Na channel protein (29) provides a rational biochemical basis for these analogies in pharmacological mechanisms.

If the above interpretation is correct, positive cooperativity should not be observed under conditions in which all channels are inactivated, i.e., in fully depolarized cells. This is indeed the case. The only observation that does not fit into this picture is the consistent stimulation of dihydropyridine binding by *d*-(*cis*)-diltiazem in fragmented membrane preparations (1, 2, 11, 15). In the absence of cytosolic regulatory control, Ca channels seem to assume a state that differs from the voltage-induced inactivated state by a lower affinity for (+)-PN 200-110. This may be a prerequisite for a *d*-(*cis*)-diltiazem-induced decrease in K_d whereas the affinity of the channel in depolarized intact cells cannot be further inhanced. Similarly, the observation of negative cooperativity between phenylalkylamines and dihydropyridines in depolarized cells and fragmented membranes (15, 30, 31) suggests that the Ca channel can assume drug-induced low affinity states in addition to voltage-induced resting or open states. The transition into such low affinity states appears to be favored by prior inactivation because devapamil enantiomers, which lowered the dihydropyridine affinity in

depolarized cells, were ineffective in polarized cells. The effects of phenylalkylamines on dihydropyridine binding in polarized cells probably result from two competing allosteric interactions, a positive cooperative action due to channel inactivation and a negative cooperative action due to a drug-induced transition of channels into a voltage-independent low affinity state. A small shift in the balance between the two antagonistic effects may explain the differential effects of verapamil and devapamil. Theoretically, a drug-promoted transition of inactivated channels into low affinity resting channels could also result in apparent negative cooperativity (32). However, such an effect is not compatible with the pronounced leftward shift of the steady state inactivation curve for Ca currents that is observed in intact cardiac cells with dihydropyridines and phenylalkylamines alike (5, 7, 9). An inactivated to resting transition is similarly unlikely to occur in membrane fragments. The available electrophysiological evidence suggests that Ca channels in isolated membrane patches inactivate rapidly and irreversibly in media used for binding experiments (33). Therefore, the low and high affinity states in isolated membranes must be functionally distinct from voltage-induced states.

Does Ca^{2+} affect the allosteric interactions between different Ca channel blockers in intact cells? Taken at face value our results seemed to indicate an effect of *d*-(*cis*)-diltiazem on the maximal binding capacity for (+)-PN 200-110 in addition to its effect on affinity (Figs. 1 and 3). However, closer analysis suggested that *d*-(*cis*)-diltiazem may have merely uncovered an effect of Ca on the B_{max} values for (+)-PN 200-110 that is obscured under conditions in which the affinity for (+)-PN 200-110 is low. At concentrations below 1.5 nM, (+)-PN 200-110 binding to polarized cells was not significantly affected by increasing the Ca concentration from 3 μM to 1.2 mM (5). This finding is confirmed in the present experiments. However, with 1.5 nM (+)-PN 200-110 only 50% (or less) of the binding sites in polarized cells are occupied. Consequently, estimation of maximal binding capacities by computerized curve fitting is subject to a large error. Individual B_{max} estimates for (+)-PN 200-110 were always higher (and K_d lower) in the presence of Ca than in its absence. However, due to the inherent weakness of the fitting procedure, these changes could not be exactly quantified. The *d*-(*cis*)-diltiazem-induced increase in the affinity for (+)-PN 200-110 simultaneously reduced the standard error of the B_{max} estimates without affecting their mean value. Therefore, we conclude that the binding capacity is sensitive to the Ca concentration but is not affected by diltiazem. This conclusion is supported by the observation of a significant Ca-induced but diltiazem-independent increase in the B_{max} value of depolarized cells.

A stimulatory effect of Ca on dihydropyridine binding has been repeatedly observed in various tissues including heart (34; for review see Ref. 17). Usually these effects became manifest only after prior Ca depletion with chelators. We have deliberately avoided the use of EGTA because polarized cells did not maintain a steady membrane potential at extracellular Ca concentrations below 1 μM. Moreover, we found it difficult to discriminate the effects of Ca removal from direct effects of the chelator. In electrophysiological studies, variations of the extracellular Ca concentration between 1 and 10 mM did not affect the blocking action of dihydropyridines (8). On the other hand, Ca has been found to inhibit the binding of (±)-verapamil and of *d*-(*cis*)-diltiazem. Most of these studies have been performed in skeletal muscle T tubules and brain tissue (14, 35, 36) but, for *d*-(*cis*)-diltiazem, data from cardiac membranes are also available (3, 37). Usually more than 80% inhibition of binding was reached with 1 mM Ca in the medium. In our experiments heterotropic allosteric interactions between (+)-PN 200-110 and (±)-verapamil or *d*-(*cis*)-diltiazem were neither lost nor significantly reduced in the presence of 1.25 mM Ca. Therefore, it is questionable whether Ca affects the binding of the two allosteric modifiers in intact polarized cells. Possibly the inhibitory effect of Ca requires its binding to a site on the internal surface of the membrane that is not accessible in intact cells. The available evidence suggests that allosteric interactions as defined in binding studies have functional consequences in intact cardiac tissue contracting at physiological Ca concentrations. Thus, nimodipine potentiated the negative inotropic effect of diltiazem on cardiac contractility (3, 38). Interestingly, a coupling between phenylalkylamine and benzothiazepine receptors could not be observed in these functional studies (3).

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