Relationship of Binding of a Calcium Channel Blocker to Inhibition of Contraction in Intact Cultured Embryonic Chick Ventricular Cells

James D. Marsh, Evan Loh, Daniel Lachance, William H. Barry, and Thomas W. Smith

From the Cardiovascular Division, Department of Medicine, Brigham and Women’s Hospital, and Harvard Medical School, Boston, Massachusetts

SUMMARY. To study the mechanism of action of calcium channel-blocking drugs in intact, functioning myocardial tissue under physiological conditions, we related the inhibition of contraction of spontaneously beating monolayers of cultured chick embryo ventricular cells to studies of binding of four calcium channel-blocking drugs to the same preparation under the same physiological conditions. Nitrendipine produced a concentration-dependent decrease in amplitude of contraction (IC50 = 23 nm) as determined by a computer-assisted optical-video system. Studies using [3H]nitrendipine and equilibrium-binding techniques revealed a high affinity binding site in ventricular homogenates (KD = 0.15 nm) and on intact cultured heart cells (KD = 0.26 nm, B_max = 51 fmol/mg of protein). These dissociation constants are similar to those reported by other workers for ventricular homogenates, but 100-fold lower than the IC50 for the negative inotropic effect in intact cells. Computer analysis of displacement of [3H]nitrendipine by unlabeled nitrendipine yielded a slope factor of 0.76 and identified an additional low affinity binding site for nitrendipine with KD = 19 nm, in good agreement with the IC50 for inhibition of contraction. Verapamil partially interacted with nitrendipine binding, whereas diltiazem showed no interaction. We conclude that, in intact myocardial cells, there are two classes of binding sites for nitrendipine; interaction of dihydropyridines with the low affinity site appears to mediate their effects on contractile function. (Circ Res 53: 539-543, 1983)

CALCIUM influx across the cell membrane via the slow Ca²⁺ channel is essential for activation and modulation of contraction of the heart and of smooth muscles (Henry, 1980; Reuter et al., 1982). A series of drugs has been developed which alter calcium entry into the cell; this family of compounds is variously termed calcium channel blockers or calcium antagonists. Although there are many similarities in clinical applications of these drugs (Braunwald, 1982), they are structurally diverse. Three drugs of this group are available for clinical use in the United States: nifedipine, which is a dihydropyridine, verapamil, which is related structurally to papaverine, and diltiazem, which is a benzothiazepine. It is becoming increasingly clear that accompanying the diversity of structure is a diversity of mechanism and site of action of these drugs. Thus, whereas some calcium channel blockers modulate the transarcolemmal flux of calcium, others may also alter the uptake and release of calcium by intracellular organelles (Triggle, 1981; Pang and Sperelakis, 1982; Murphy and Snyder, 1982; Ehler et al., 1982; Colvin et al., 1982). Radioligand-binding techniques have been developed and applied extensively to studies of the interaction of hormones and neurotransmitters with cellular receptors. Recently, these ligand-binding techniques have been extended to the examination of interactions of radiolabeled calcium channel blockers with binding sites in various membrane preparations, although the molecular pharmacology of the calcium channel in the heart may differ substantially from that of a hormone receptor (Williams and Tremble, 1982; Reuter et al., 1982; Sarmiento et al., 1983). There have been recent descriptions of binding sites for calcium channel blockers in several preparations, with a wide range of affinities for the drugs studied (Murphy and Snyder, 1982; Ehler et al., 1982; Williams and Tremble, 1982; Yamamura et al., 1982; Colvin et al., 1982; Sarmiento et al., 1983). Because binding studies were performed in homogenates or membrane preparations, it has been difficult to determine what relation binding affinities bear to pharmacological effect; in some cases, dissociation constants appear to differ by 1–3 orders of magnitude from drug concentrations that are presumed to elicit a half-maximal pharmacological effect (Murphy and Snyder, 1982; Colvin et al., 1982; Sarmiento et al., 1983). To gain insights into the regulation of the Ca²⁺ channel under physiological conditions, we examined the pharmacological action and binding properties of nitrendipine in an intact, functioning cultured heart cell preparation that obviates limitations inherent in homogenate binding studies and permits direct comparison of binding properties and contractile function under identical conditions.
conditions. We also examined displacement of [H]-nitrendipine by four different Ca channel-blocking drugs with the aim of determining the extent to which binding of calcium channel blockers to a specific site modulates the physiological contractile state of the intact myocardial cell.

Methods

Tissue Culture

Primary monolayer cultures of beating chick embryo ventricular cells were prepared as previously described (Marsh et al., 1982). Briefly, fragments of embryonic chick ventricles 10 days in ovo were dissociated by repeated cycles of trypsinization, and the resulting cell suspensions were plated in 100-mm culture dishes and grown in culture medium consisting of a bicarbonate-buffered physiological salt solution containing 54% M199 and 6% fetal calf serum (FCS). The final calcium concentration of the culture medium was 0.9 mM. Spontaneously and synchronously contracting confluent monolayers were present by 3 days in culture. For contractility experiments, cells were permitted to attach to 25-mm glass cover slips. When cells were to be utilized for calcium channel blocker binding experiments, they were harvested by gentle scraping of the culture plates to suspend sheets of cells. Eighty-five percent of the suspended cells excluded trypan blue.

Ventricular Homogenate

For binding studies in homogenates of embryonic ventricle, ventricles were removed from 10-day-old chick embryos and homogenized in medium at pH 7.7 containing 50 mM Tris and 1% bovine serum albumin (BSA) by 20 strokes of a tight-fitting Dounce homogenizer. After discarding a 1,000 g × 10-minute pellet from this homogenate, a 48,000 g × 20-minute pellet was prepared and resuspended in Tris buffer.

Contractility Measurements

To determine the contractile response of cultured heart cells to exposure to the dihydropyridine calcium channel blocker, nitrendipine, measurements of the amplitude of contraction of individual cells in a spontaneously contracting monolayer at 3 days in culture were conducted (Marsh et al., 1982), using a phase contrast microscope-video motion detector system. The medium bathing cells during contractility measurements was culture medium containing 0.6 mM calcium. Previous studies from this laboratory have demonstrated that this method of assessing the contractility measurements of cells in a monolayer correlates closely with other physiological parameters known to relate to the contractile state, such as alterations in monovalent and divalent cation fluxes (Barry et al., 1981). The contractility measurements were made on one cell per coverslip. Several coverslips were used from one plating; all experiments of nitrendipine or nifedipine were assiduously protected from light.

Binding Studies

Nine hundred microliters of ventricular homogenate or intact cell suspension were added to 50 μl of [H]-nitrendipine and 50 μl of buffered medium containing desired amounts of nifedipine or other calcium channel-blocking agents. Nifedipine (10⁻⁶ M) was used to define nonspecific binding (Ehlert et al., 1982; Williams and Tremble, 1982). The medium in which the experiments were conducted was identical to the physiological salt solution in which contractility experiments were conducted, except that medium for binding studies was buffered by 4 mM HEPES and contained 1% BSA rather than FCS. Incubation was for 15 minutes at 37°C in the dark. Binding was terminated by adding 10 ml of wash buffer (50 mM potassium phosphate, pH 7.5) at 37°C to the assay mixture, incubating 5 seconds, then rapidly filtering through a Whatman GF/C filter, followed by two rapid 5-ml washes with buffer at 37°C. Filters were dried and counted at 49% efficiency in a Beckman liquid scintillation counter. Equilibrium binding data and displacement curves were analyzed using the iterative, nonlinear least squares method of Munson and Rodbard (1980) on an IBM 370 computer. This program solves the system of multiple linear equations describing the interaction of hormone H 1 with receptor R 1 with equilibrium constant K D 1 . It incorporates an additional term for nonspecific binding N 1 . Because the program uses as input only the untransformed experimental data, total hormone concentration, and bound hormone concentration, analysis is not confounded by reduction in free hormone concentration by internalization or high receptor concentration, as may be the case for conventional Scatchard analysis. Further simplifying assumptions to relate IC 50 to K D 1 which may not always be valid (Strickland and Loeb, 1981) are also unnecessary, as K D 1 for each effector is calculated directly. To test the hypothesis that ligand displacement curves are better described by a one-site than a two-site model, the K D 1 for nitrendipine was fixed at the value determined by analysis of equilibrium-binding isotherms, and the system of equations solved for number of receptors and nonspecific binding. Then the equations were solved again with a second binding affinity and variable receptor number included in the model (Munson and Rodbard, 1980). An F-test was used to compare goodness of fit for the two models. If the two-site model described the data better at the P < 0.05 level, then the one-site hypothesis was rejected.

Materials

[H]-Nitrendipine (specific activity, 88 Ci/mmol) was obtained from New England Nuclear, nifedipine from Pfizer, nitrendipine from Miles, verapamil from Knoll, and diltiazem from Marion Laboratories. Other reagents were obtained from Fisher Scientific and were the highest grade commercially available. Tissue culture media were from sources previously described (Marsh et al., 1982).

Results

Inhibition of Contraction

The amplitude of contraction of spontaneously beating myocytes was inhibited by nitrendipine in a concentration-dependent fashion. Serial concentration-effect curves were determined by exposing monolayers to progressively increasing concentrations of nitrendipine. Observations at each concentration were repeated in 5–6 different preparations (Fig. 1). Inhibition of contraction by nitrendipine was rapid in onset. A plateau in effect for each nitrendipine concentration was reached within 5 minutes. When nitrendipine was washed out, inhi-
Concentration-effect curve for inhibition of contraction of cultured heart cells by nitrendipine. Spontaneously contracting cultured myocytes were superfused with increasing concentrations of nitrendipine. Percent inhibition of contraction amplitude is indicated on the vertical axis with 100% inhibition defined as the point at which the cell completely ceases to contract. Bars indicate SEM; n = 5–6 for each point.

Inhibition of contraction was fully reversible, with return to control levels of contractility within 5–10 minutes. Long-logit transformation of the sigmoid-shaped concentration-effect curve revealed an IC\textsubscript{50} of 23 nM for inhibition of contraction by nitrendipine. The slope factor for the concentration-effect curve was 1.26 ± 0.19 (SEM).

[3H]Nitrendipine Binding

To confirm the observation from other laboratories that a high affinity binding site for nitrendipine could be identified in heart homogenates (Ehlert et al., 1982; Bristow et al., 1982; Williams and Tremble, 1982; Bolger et al., 1982), binding isotherms for nitrendipine were assessed in homogenates of embryonic ventricle using 0–500 pM [3H]nitrendipine. Binding was rapid, reaching equilibrium within 5 minutes and was saturable. At concentrations of nitrendipine >500 nM, there was substantial nonspecific binding. Computer analysis of the binding isotherms disclosed a single binding site with KD = 0.15 nM and B\textsubscript{max} = 190 fmol/mg of protein.

[3H]Nitrendipine also bound rapidly to intact cultured cells, reaching equilibrium in 5 minutes at 37°C. Binding was saturable (Fig. 2) and reversible with a half-time of about 5 minutes (data not shown). Simultaneous computer analysis of three such binding isotherms indicated the presence of a single binding site with KD = 0.26 nM and B\textsubscript{max} = 51 fmol/mg of protein. Binding was approximately 70% specific at the KD; this degree of specific binding was achieved by washing the cells for 5 seconds at 37°C at the conclusion of binding prior to filtration. During this 5-second period, less than 5% of specifically bound nitrendipine dissociated from the cells.

The contractility studies indicated that a 100-fold greater drug concentration was necessary to half-maximally inhibit contraction than to half-maximally interact with this high affinity binding site, suggesting that a lower affinity site for drug interaction might be present. To address this problem, binding properties of nitrendipine were studied by examining the displacement of 0.2–0.5 nM [3H]nitrendipine from the binding site by increasing concentrations of unlabeled nitrendipine in eight separate experiments. In addition, we studied the effects of nifedipine, verapamil, and diltiazem on [3H]nitrendipine binding in intact cells. This method minimized the confounding effect of nonspecific binding that occurs at high concentrations of labeled ligand. Analysis of [3H]nitrendipine displacement curves indicated the presence of an additional binding site with lower affinity for nitrendipine (KD = 19 ± 2 nM, n = 8). Analysis of four detailed, 15-point nitrendipine displacement curves (Fig. 3) simultaneously demonstrated the presence of the high affinity binding site (KD = 0.26 nM) with the low affinity binding site. In each case, the two-site model described the data better than the one-site model (P < 0.05). The ratio of number of high affinity to low affinity binding sites was 8:92. Nifedipine displaced [3H]nitrendipine to the same degree as did unlabeled nitrendipine; the KD for nifedipine was 10 nM (Table 1). The slope factor for nitrendipine displacement was 0.76 ± 0.06, indicating either negative cooperativity or two (or more) binding sites. Verapamil at maximal concentration displaced only 50% as much [3H]nitrendipine as did nitrendipine or nifedipine; the KD for verapamil was 8 nM. Diltiazem, in concentrations up to 10 μM, did not displace [3H]nitren-
Nitrendipine at all, nor did it enhance \([^{3}H]\)nitrendipine binding.

**Discussion**

The observations reported here indicate that in intact, spontaneously contracting cultured heart cells, there are two classes of binding sites for \([^{3}H]\)nitrendipine. The high affinity binding site we identified in heart homogenates and in intact cells has properties similar to the site previously reported in heart homogenates (Ehlert et al., 1982; Bolger et al., 1982) and PC12 cell homogenates, but discounted this site as having an unknown physiological role. They did not correlate binding with function in intact preparations. Nitrendipine binding to a low affinity site in intact cells has not previously been reported. The \(K_D\) for nitrendipine binding to the low affinity site in the intact, contracting cultured heart cell reported here (19 nm) correlates well with the concentration producing a contractility effect (IC\(_{50} = 23\) nm) in the identical preparation, in the same physiological salt solution at the same temperature. Furthermore, the time course for association and dissociation of nitrendipine binding to cell suspensions at 37°C is quite similar to the time course of negative inotropic effect (3–5 minutes), supporting the hypothesis that the site of nitrendipine binding mediating the pharmacological effect on contractile state is the low affinity site on the cell membrane. If, indeed, the low affinity site of nitrendipine binding is the site at which the pharmacological effect is mediated, then one can calculate that at the concentration of nitrendipine causing 50% of the low affinity sites to be occupied (the \(K_D\)), 39% of the maximally negative inotropic effect is produced (Furukawa et al., 1982). However, if the high affinity site that we identified on the intact cell or the site we and other investigators identified in heart homogenate (Ehlert et al., 1982; Bolger et al., 1982) mediated the negative inotropic effect, then 99% of the high affinity sites would need to be occupied by nitrendipine to produce a 50% maximal negative inotropic effect. These concentration–effect relations argue strongly that the low affinity binding site for nitrendipine on intact cells is the site that is related to the \(Ca^{++}\) channel-blocking effects of the drug. The slope factor for the physiological effect is near 1, suggesting that a single class of binding sites may control \(Ca^{++}\) entry that modulates contractile amplitude. This finding is consistent with the hypothesis that, whereas the high affinity binding site may be associated with the \(Ca^{++}\) channel, only the low affinity binding site modulates the gating function for \(Ca\).

The binding site on the intact cells for the dihydropyridine class of calcium channel antagonists, which includes nitrendipine and nifedipine, differs from the site of action of verapamil and diltiazem. In our preparation, verapamil interacted only partially with \([^{3}H]\)nitrendipine binding; this observation is consistent with the suggestion by Ehlert et al. (1982) that the interaction with the nitrendipine binding site may be allostERIC. It is likely that verapamil interacts at least partially with the high affinity binding site for nitrendipine in the intact heart cell because at a nitrendipine concentration of 0.5 nm, it is predominantly the low affinity site from

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**Table 1**  
**Binding and Contractility Effects of Ca Channel Blockers**

<table>
<thead>
<tr>
<th>Dissociation constants*</th>
<th>Homogenate</th>
<th>Intact cell</th>
<th>Slope factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrendipine (K_D)</td>
<td>0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrendipine (K_I)</td>
<td>0.26</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Nitrendipine (K_I)</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nifedipine (K_D)</td>
<td>10</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Verapamil (K_D)</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diltiazem (K_D)</td>
<td>&gt;1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contractility†</td>
<td>IC(_{50}) = 23</td>
<td>1,26</td>
<td></td>
</tr>
</tbody>
</table>

* Dissociation constants (\(K_D\)) expressed in nanomolar. \(K_I\) are constants for dissociation from high and low affinity sites, respectively. The \(K_D\) for nifedipine presumably reflects the affinity for the low affinity, high capacity dihydropyridine-binding site.

† IC\(_{50}\) is concentration of drug which reduces amplitude of contraction to 50% of control amplitude.
which nitrendipine is being displaced. Further experiments in which the displacement of [3H]vera-
pamil from binding sites on intact cells by a dihy-
dropyridine is studied will be neede
more fully the nature of the binding site interactions.
Diltiazem had no interaction with the dihydropyri-
dine binding site on intact cells, and in contrast to
its binding to synaptosomes (Yamamura et al.,
1982), it did not increase binding of nitrendipine.

Previous work from this laboratory (Barry and
Smith, 1982) has described a close relationship be-
tween the concentration of a Ca++ channel blocker (in this case verapamil) that inhibits contractility,
with the concentration that inhibits cellular uptake
of rapidly exchangeable Ca++ in intact, spontaneously
contracting heart cells. Of interest, the Kd for vera-
pamil binding reported here (8 nM) correlates very
well with the concentration that half-maximally in-
hibits Ca++ uptake (10 nM) and contraction (10 nM).
Thus, it appears that there is a close linkage between
Ca++ channel blocker binding, inhibition of Ca++
 influx, and inhibition of contraction.

Taken together, the observations reported here
demonstrate that there are two binding sites of different
affinities on intact myocardial cells for a member of
the dihydropyridine class of calcium channel block-
ers. Binding to the low affinity site correlates well
with the pharmacological, negative inotropic effect
of these drugs, and drug binding at this site likely
modulates Ca++ entry into the cell. As reviewed by
Reuter (1983), Ca++ channels, unlike Na+ channels,
are not functional in isolated membrane vesicles,
and binding studies should preferably be done in
intact cells. The identification of a low as well as a
high affinity binding site in intact cells resolves the
dilemma in part (Reuter et al., 1982; Sarmiento et
al., 1983) of the previously described disparity be-
tween Ca++ channel binding and functional effect.

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Address for reprints: Dr. James D. Marsh, Brigham and Women’s
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