Comparison of the Number of Dihydropyridine Receptors With the Number of Functional L-Type Calcium Channels in Embryonic Heart

Sumi Aiba and Tony L. Creazzo

We compared the density of dihydropyridine (DHP) receptor sites with the density of functional L-type calcium channels in ventricular myocytes from chick heart at embryonic day 11. DHP receptors were quantified by using the DHP antagonist (+)-[1H]PN200-110 and by competition binding with the agonist Bay K 8644. The number of agonist and antagonist binding sites per ventricle was similar (250±15 and 244±9 fmol, respectively; mean±SEM). The mean number of myocytes per ventricle was 8.57±0.65×10^6, as determined by histological methods. From these data, the number of DHP receptors was calculated to be approximately 17,000 per myocyte or 25 to 26 DHP receptors per square micron of cell membrane, based on a mean myocyte membrane capacitance of 6.7 pF and a specific membrane capacitance of 1 μF/cm². We next determined the number of functional L-type calcium channels by nonstationary fluctuation analysis with whole-cell patch clamp. The mean number of functional L-type channels per cell was 291±49 and 131±10 with Ca²⁺ and Ba²⁺ as the charge carriers, which yielded a channel density of 0.50±0.08 and 0.28±0.02 per square micron of cell membrane, respectively. From these data, the density of DHP receptor binding sites was determined to be from 50 to 100 times the density of functional L-type calcium channels. The function of the “excess” DHP receptors and the determination of whether the proportion of functional channels increases with development require further investigation. (Circulation Research 1993;72:396–402)

Key Words • L-type calcium channels • dihydropyridine receptors • heart • chick embryo

In skeletal muscle it appears that >90% of dihydropyridine (DHP) receptors function as voltage sensors for direct voltage control of Ca²⁺ release from the sarcoplasmic reticulum, whereas only a few serve as functional calcium channels. A direct mechanism for Ca²⁺ release from the sarcoplasmic reticulum is not generally accepted for heart muscle, although there is some evidence that a direct mechanism does supplement the more important Ca²⁺-induced Ca²⁺ release mechanism of the heart. Two reports of ventricular myocytes from rabbit and rat hearts indicate that the gating charge is much larger than expected for the number of L-type channels activated by depolarization, suggesting that the number of DHP receptors exceeds the number of functional L-type calcium channels. Further, in the embryonic chick ventricle, there are DHP receptors that do not correspond to functional calcium channels. In contrast, Bers and colleagues (Lew et al) conducted a systematic comparison in rabbit ventricle indicating that the densities of DHP receptors and functional L-type calcium channels are comparable. In their study, DHP receptors were assessed by ligand binding, and the number of functional channels was quantified by calculations based on experimental determinations of the whole-cell current and the open-channel probability (Pₒ) using Na⁺ as the charge carrier. Thus, the number of L-type calcium channels determined from charge movement in previous studies may have been too large because of overestimation of the Pₒ, which was not directly measured in the gating-charge studies.

Because there may be “silent” or nonfunctional calcium channels in the embryonic heart, we compared DHP receptor density to the number of functional L-type channels determined by nonstationary fluctuation (noise) analysis. An advantage of the noise-analysis technique is that the number of functional channels is extracted from the macroscopic current data without having to make a separate experimental determination of the Pₒ as in the study by Bers. Our results indicate that the number of DHP receptors is 50–100 times greater than the number of functional L-type calcium channels in chick ventricular myocytes at embryonic day 11.

Materials and Methods

Animals

Fertilized Arbor Acre chicken eggs (Seaboard Hatchery, Athens, Ga.) were incubated in forced-draft incubators maintained at 99% humidity and 38°C.

Dihydropyridine Receptor Binding

Agonist and antagonist binding were as described by Maan and Hosey and as previously reported. DHP
antagonist (+)-[3H]PN200-110 (New England Nuclear, Boston) was used for equilibrium binding studies. The number of antagonist binding sites and the dissociation constant (Kd) was determined by transformation of the data by the method of Scatchard. Agonist binding was examined by (±)-Bay K 8644 competition with (+)-[3H]PN200-110. Crude homogenates were prepared from 10–20 ventricles, which were pooled and homogenized in assay buffer (see below) using a Polytron. The assays were in triplicate for 1 hour at 37°C. Each 2-ml tube contained 400 μg protein (determined by the Bradford method) with concentrations of (+)-[3H]PN200-110 ranging from 20 to 500 pM. With these concentrations, receptor saturation ranged from 15% to 91%. Nonspecific binding was determined for each point by competition with 1 μM nifedipine and ranged from 6% to 20% of the total bound (+)-[3H]PN200-110. The concentration of Bay K 8644 ranged from 1×10⁻¹⁰ to 1×10⁻⁵ M, and the concentration of competing [3H]PN200 was 600–700 pM (>95% saturation in the absence of agonist). The assay buffer consisted of (mM) MgSO4 2, EDTA 1, and HEPES (NaOH) 10 (pH 7.4). The assay was terminated by vacuum filtration, and GC/F filters (Whatman Inc., Clifton, N.J.) were equilibrated in wet fluor (toluene, 64%; Triton X-100, 36%; POPOP, 24 mM; and POPPOP, 0.25 mM) overnight before counting. The data were fitted with the Marquardt method of iterative least squares by the following equation: Bn = Bmax/(1 + [Bay K]/IC50), where Bn was the specific bound ligand and IC50 was the concentration of Bay K 8644 at which half of the DHP receptors were occupied. The number of binding sites per ventricle in fentomoles was determined by calculating fentomoles of bound ligand per unit volume of homogenate and dividing by the number of ventricles per unit volume.

**Cell Counting**

The number of myocytes per ventricle was determined by counting nuclei in tissue sections as described by Anversa and colleagues.10 Embryonic cardiac myocytes are typically mononucleated, whereas adult myocytes are generally binucleated. To verify that this was true for chick ventricle at embryonic day 11, freshly dissociated myocytes from several ventricles were spread on slides, fixed, and stained. Examination of these preparations showed that all of the myocytes were mononucleated (data not shown).

Embryos from embryonic day 11 were decapitated. The hearts were rapidly removed, trimmed of the atria and great vessels, weighed, fixed in 0.1 M Sorensen’s phosphate buffer (pH 7.2) containing 2% gluteraldehyde and 2% formaldehyde, and imbedded in araldite. Tissue blocks were sectioned at 0.75 μm and stained with toluidine blue. Morphometric sampling at ×1,000 consisted of counting the number of myocyte nuclear profiles (N) in an area (A) encompassed by an ocular grid covering an area of 5,900 μm² at ×1,000. A total of 75 fields were measured from the ventricles of each heart to determine the number of nuclear profiles per unit area of myocardium (N/A). Initially fields were taken from the apical, middle, and basal regions of the ventricles. Since no difference was found between these regions, subsequent measurements were made from approximately the middle region. The values for N/A were averaged to yield the numerical density of myocyte nuclear profiles per unit area of myocytes (N/A). The average nuclear length in microns (D) was determined from 85–100 measurements of each tissue sample in areas that were cut longitudinally. The number of myocyte nuclei per unit volume (μm³) of ventricle (Nv) was computed using the following equation: Nv = N/A/D. Assuming a tissue density of 1.05, the volumes of the ventricles were calculated from their measured weights.3 The average wet weight of the nine ventricles used for the morphometrics was 30.4±0.5 mg, which was equivalent to an average ventricular volume of 2.895±0.048×10⁶ μm³. From Nv, the total number of myocyte nuclei per ventricle, equivalent to the number of myocytes per ventricle, was calculated.

**Cell Preparation and Electrophysiology**

For each patch-clamp experiment, the ventricles from day-11 embryos (left and right) were cleaned of connective tissue and dissociated according to Nathan and DeHaan11 as previously described.9,10 Approximately 5×10⁶ cells were plated in DeHaan’s 2121 medium on plastic Petri dishes (Falcon 1008) that had previously been exposed to a drop of 25 M H2SO4. This resulted in cells that attached but remained spherical instead of flattening against the dish.12 The cells were cultured at 37°C in air containing 5% CO2 and were used within 12–24 hours after dissociation. Before use, the culture medium was decanted; this procedure was followed by gently rinsing the dish five times and replacing the medium with extracellular solution. With Ca²⁺ as the charge carrier, the extracellular solution consisted of (mM) CaCl2 10, CsCl 20, NaCl 22.5, tetraethylammonium chloride 110, MgCl2 1.8, glucose 28, tetrodotoxin 0.003 (approximately 500 times K, for the chick cardiac sodium channel),13 and HEPES (NaOH) 10 (pH 7.4). When Ba²⁺ was used as the charge carrier, the CaCl2 was replaced with 20 mM BaCl2, and tetraethylammonium chloride was reduced to 95 mM. The pipette solution contained (mM) CsCl 100, tetraethylammonium chloride 20, MgCl2 4, EGTA 5, HEPES (CsOH) 10 (pH 7.3), Na2-ATP 3, Na2-GTP 0.4, and phosphocreatine 5. All experiments were conducted at room temperature (22–24°C). Patch-clamp electrodes, pulled from Corning 7052 glass capillaries on a Flaming-Brown puller, coated with Sylgard, and fire-polished, had resistances ranging from 2 to 5 MΩ. Seal resistances were >40 GΩ. Single myocytes were voltage-clamped with a List EPC-7 amplifier (Medical Systems Corp., Greenville, N.Y.) using the whole-cell variation of the patch clamp14 as previously described.8,9 Offset potentials were compensated by zeroing the patch-clamp amplifier before seal formation with the electrode in the bath. Voltage-clamp protocols were elicited, and digitized data were acquired by a TL-125 Labmaster data acquisition system controlled by pClamp software (Axon Instruments, Burlingame, Calif.). Cell capacitance was determined by integration of capacitive transients as previously described.8,9 Electrode capacitance was canceled while in the cell-attached patch configuration immediately after seal formation, and cell capacitance was determined after achieving the whole-cell voltage clamp. The whole-cell configuration was obtained by rupturing the membrane patch with a combination of suction and small voltage pulses. The series resistance, estimated from the peak
current of the capacitive transient, was usually less than 10 MΩ. Series resistance and cell capacity compensation were not considered necessary and were not attempted. For fluctuation analysis, calcium currents were elicited from 400-msec voltage-clamp pulses to the test potential of +10 mV at 5-second intervals. The test pulse was preceded by a 500-msec prepulse to −40 mV from the holding potential of −80 mV to inactivate T-type calcium current.8 For current–voltage relations, the test pulse was varied from −30 to +50 mV in 10-mV intervals. The data were filtered at 1 kHz with an eight-pole Bessel filter and digitally sampled at 5 kHz.

Nonstationary Fluctuation Analysis

The nonstationary fluctuation analysis technique, originally developed by Sigworth,15 (see discussion by DeFelice16) and used by Bean17 in canine atrial myocytes, was used to count functional channels. Variance was calculated from an ensemble of whole-cell currents generated by a series of 100–120 identical voltage pulses. The digitized data were stored in files, each containing the data from five test pulses (20–24 files per cell). This approach minimizes the error due to a small amount of rundown of L-type calcium current17 that occurs in chick ventricular myocytes.8 Successive files were subtracted and averaged, and the standard deviation was calculated using the CLAMPFIT program from Axon Instruments. The standard deviations were subsequently squared to obtain the variance. The mean variances from each of the groups of five data sets were corrected for background variance by subtracting the mean variance of a segment immediately preceding the voltage pulse. These means were summed to obtain the mean variance for the entire data set and plotted against current and fitted to the following relation:

$$\sigma^2 = i(1 - (I^2/N))$$

where $\sigma^2$ is the variance, $i$ is the mean current, $I$ is the single-channel conductance, and $N$ is the total number of channels.15 In two cells, the analysis was repeated using the pairwise method of calculating variance from two consecutive tracings, as described by Bean,17 with identical results. $N$ and $i$ estimated from curve fitting, were used to calculate the maximum $P_e$ at peak current ($P_{e,\text{peak}}$) from the relation $P_{e,\text{peak}} = I_{\text{peak}}/N_i$, where $I_{\text{peak}}$ is peak whole-cell current. For calculating the number of functional channels, the fluctuation analysis technique was advantageous because it did not rely on a separate experimental determination of $P_e$ as do other methods.

Statistics

Data were expressed as mean±SEM. Significance was determined by Student’s $t$ test for unpaired values; $p<0.05$ was considered significant.

Results

Density of DHP Receptors

Antagonist binding was measured by transformation of the data by the method of Scatchard using the radiolabeled ligand (+)-[3H]PN200-110, and agonist binding was determined in competition with Bay K 8644 (Figure 1). The data were well-fitted by single-site models, indicating the presence of a single high-affinity DHP binding site for both agonist and antagonist. The results, summarized in Table 1, indicated that the densities of agonist and antagonist DHP receptor sites were similar and in agreement with previous reports.5,9 From the number of pooled ventricles and the volumes used in the DHP receptor assays, the average number of receptors per ventricle was calculated (Table 1). To calculate the average number of DHP receptors per myocyte, the number of myocytes per ventricle was determined by a morphometric technique developed by Anversa and colleagues.10 The mean myocyte number per ventricle in the day-11 embryo was $8.57±0.65×10^7$ ($n=9$). This value was consistent with our tissue culture observations, which indicate that 3–5 million “viable

![Figure 1. Dihydropyridine receptor binding in the day-11 embryonic chick ventricle. Panel A: Saturation binding curve from a single experiment showing binding of the dihydropyridine antagonist [3H]PN200-110. Inset shows Scatchard plot from the same experiment. $B_{\text{max}}$, equivalent to the total number of receptors, and the radioligand affinity constant $K_D$ were determined by linear regression, where slope is $-1/K_D$ and $B_{\text{max}}$ is the intercept at the x axis. Panel B: Agonist binding plot from competition with [3H]PN200-110. The curve is a fit to $B_e = B_{\text{max}}/(1 + A/IC_{50})$, where $B_e$ is the specific binding at a specified agonist concentration (A) and $IC_{50}$ is the 50% inhibitory concentration. $K_D$, the binding constant for agonist binding in competition with the radioligand, was calculated as $K_D = IC_{50}/(1 + L/|K_D|)$, as described by Cheng and Prusoff.18 The total number of receptors (R) was calculated as $R = B_{\text{max}}/(1 + K_D/L)$, where L is the concentration of labeled ligand at 705 pM and R is 112 fmol/mg. The DHP receptor binding data are summarized in Table 1.](http://circres.ahajournals.org/lookup/fig/1)
TABLE 1. Dihydropyridine Binding

<table>
<thead>
<tr>
<th></th>
<th>Antagonist (n=5)</th>
<th>Agonist (n=3)</th>
</tr>
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<tbody>
<tr>
<td>DHP receptors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (fmol/mg)</td>
<td>116.0±2.6</td>
<td>122.7±8.6</td>
</tr>
<tr>
<td>Affinity (nM)</td>
<td>0.066±0.010 (Kd)</td>
<td>27.0±5.4 (K)</td>
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<td>Receptors per ventricle (fmol)</td>
<td>244±8</td>
<td>250±15</td>
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<td>Receptors per cell</td>
<td>17,327±758</td>
<td>17,558±1,032</td>
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<tr>
<td>Density (per μm²)</td>
<td>25.5±1.0</td>
<td>26.2±1.5</td>
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DHP, dihydropyridine; n, number of experiments (see “Materials and Methods”). Values are mean±SEM.

Ki calculated for each experiment as described in the Figure 1B legend.

cells” are obtained per completely dissociated day-11 ventricle (T.L. Creazzo, unpublished data). The average number of DHP receptors per cell was calculated by dividing the number of DHP sites per ventricle (Table 1, femtomoles times Avogadro’s number) by the number of myocytes per ventricle. The average density of DHP receptors, calculated by dividing the number of binding sites per cell by 670 μm² (calculated from an average cell capacitance of 6.7 pF per cell and a specific membrane capacitance of 1 μF/cm²),⁹ was approximately 25–26 per square micron of cell membrane.

Density of Functional L-Type Calcium Channels

For comparison with the DHP binding data, the number of functional L-type calcium channels was determined by nonstationary fluctuation analysis.¹⁵,¹⁶ By plotting the mean variance versus the whole-cell current, the single-channel current (i) and the number of functional channels (N) were calculated. From the parameters N and i and Ipeak, P_{o(peak)} was calculated as

$$I_{\text{peak}}/N_i.$$

Examples of data from fluctuation analysis for two day-11 ventricular myocytes are given in Figure 2, and the data are summarized in Table 2. From these data, it is apparent that there are 50–100 times fewer functional L-type calcium channels than there are DHP receptors. The dashed lines indicate fits to the data in which the number of functional channels was fixed at 15,000 and 1,000. These fits correspond to low P_{o(peak)} values ranging from 0.01–0.02 for 15,000 channels to 0.18–0.20 for 1,000 channels. Note that the theoretical fits assuming 15,000 functional channels are essentially straight lines. For comparison, the coefficients of determination (r²) for the nonlinear curve fits shown in Figure 2 were computed. The r² values were larger, and better fits to the data were obtained if the number of functional channels was low. This is especially evident when 10 mM Ca²⁺ was the charge carrier. Finally, fixing the number of channels at a high value reduces the

**Figure 2.** Examples of nonstationary fluctuation analysis from two cells, one in 10 mM [Ca²⁺], and the other in 20 mM [Ba²⁺]. Panels A and C are example tracings. The upper tracings are the mean variances (pA²), and the lower tracings show the mean L-type calcium current from the same cells. The test potential was to +10 mV. Panels B and D are plots of variance vs. current. P_{o(peak)} is open-channel probability at peak current. The solid lines are theoretical fits of the data to $$\sigma^2 = i F/N$$, where $$\sigma^2$$ is variance, i is single-channel conductance, I is the mean current, and N is the total number of channels. The coefficients of determination (r²) for these fits were 0.65 and 0.49 for Ca²⁺ and Ba²⁺, respectively. The dashed lines are curves fitted to the data, with N fixed at 15,000 (topmost lines) and 1,000 channels. The i value was –0.08 (0.42) and –0.09 pA (0.51) in Ca²⁺ and –0.26 (0.26) and –0.27 pA (0.37) in Ba²⁺, respectively. The data are summarized in Table 2.
value calculated for the single-channel current by as much as 50%. With Ba$^{2+}$ as the charge carrier, the value we calculated for the single-channel current, without fixing the number of channels, was very close to the reported value for the single-channel current obtained directly from single-channel measurements in 20 mM Ba$^{2+}$ and at +10 mV in the chick ventricle (≈−0.4 pA).9 In summary, our results indicate that the great majority of DHP receptors are not functional calcium channels in the embryonic ventricle. Although intriguing, it is not known why there appeared to be fewer functional L-type calcium channels when 20 mM Ba$^{2+}$ was used as the charge carrier. A possible explanation is that a test potential to +10 mV is not on the same position on the $P_{o(peak)}$-voltage relation in the Ba$^{2+}$ and Ca$^{2+}$ solutions. Hence, the data may be explained by a shift of the Ba$^{2+}$ current–voltage relation to the right of the Ca$^{2+}$ current–voltage relation, with the peak currents at the +10 mV position on the descending limb of both current–voltage relations. However, Figure 3 shows that the 20 mM Ba$^{2+}$ current–voltage relation was maximum at +10 mV and was actually to the left of the current–voltage relation in 10 mM Ca$^{2+}$. The data suggest that the difference in the number of functional channels between Ba$^{2+}$ and Ca$^{2+}$ may actually be slightly larger than indicated by the fluctuation analysis. Another possibility may be related to Ba$^{2+}$ toxicity. The embryonic chick myocytes were sensitive to Ba$^{2+}$, since we observed that many cells died shortly after perfusing the culture dish with the Ba$^{2+}$-containing solution. We have never observed this phenomenon when using Ca$^{2+}$-containing solutions regardless of concentration. Thus, it was possible that Ba$^{2+}$ selected for myocytes with fewer calcium channels or damaged the remaining myocytes in such a way as to reduce the number of functional channels.

One possible explanation for the large discrepancy in the number of functional channels versus DHP receptors was that there was substantial rundown of L-type calcium current. However, in a previous report we found rundown of L-type calcium current in chick ventricular myocytes to be minimal. For the present report, we compared the means of the first five current tracings with the last five for each data set. With Ca$^{2+}$ as the charge carrier, the current decreased by 12±6% by the end of the experiment. In two cells the current actually increased. The range was from −21% to +27%. With Ba$^{2+}$ as the charge carrier, the current decreased by 8±1% with a range from −30% to +27%, with one cell showing an increase in current. We conclude that it is not likely that such a large disparity in the number of functional channels versus DHP receptors is due to an experimental error in the calculation of the number of channels because of rundown of the whole-cell current during the experiment.

**Discussion**

Although it has been reported that in adult ventricle the number of DHP receptors and functional calcium channels are comparable, it appears that not all DHP receptors represent functional calcium channels in the embryonic ventricle. In the early embryonic heart, there are few Na$^+$ channels; hence, there is a slow upstroke velocity of the cardiac action potential that is due largely to calcium conductance. Yet, Lazdunski and colleagues (Renaud et al) found that DHP antagonists showed little effect on action potentials or contractile activity in the embryonic chick ventricle on day 3 of development despite the presence of a relatively high density of DHP receptors; whereas, these parameters were substantially inhibited by DHP antagonists at day 11. It should not be surprising that the early embryonic cardiac action potential is not sensitive to DHPs, since it is known from several studies conducted at later ages (days 7–14) that chick ventricular myocytes have both L-type calcium current and a comparatively large DHP insensitive T-type calcium current. It may be that the day-3 heart has mostly T-type current, although this remains to be determined. These investigators concluded that there are nonfunctional or “silent” calcium channels in the embryonic ventricle. The present results are consistent with this conclusion, because we find that the vast majority of DHP receptors in the chick embryonic ventricle are not functional calcium channels while the density of functional channels is quite low.

In evaluating the results, a potential consideration is that not all of the functional channels are counted by the fluctuation analysis technique. This would occur if $P_o$ is very low, as in the study by Bers and coworkers. With a low $P_o$, there may be many sweeps or long periods of time when some channels may not open. It is possible that functional channels that open rarely may be missed by the fluctuation analysis method. In fact, fluctuation analysis will not give an accurate measure of channel number if the $P_{o(peak)}$ is less than 0.18.17 However, we calculate the average $P_{o(peak)}$ to be approximately 0.5, which well exceeds this “margin of safety.” This value is 16–17 times the $P_o$ of 0.03 determined by single-channel measurements in the Bers study. Although the $P_{o(peak)}$ that we calculate is relatively high compared with observations in the adult heart, it is in agreement with two recent reports of direct single-channel measurements in the embryonic chick heart. Tohse and Sperelakis show that DHP-sensitive calcium channels in the day-3 embryonic chick heart exhibit long openings with $P_o$ > 0.65 in 20% of sweeps with only 18% blank sweeps. Although not showing the

**Figure 3.** Graph showing peak L-type calcium current–voltage relations in 10 mM Ca$^{2+}$ and 20 mM Ba$^{2+}$. Measurements are from 17 and nine myocytes for Ca$^{2+}$ and Ba$^{2+}$, respectively. The currents were normalized to cell capacitance. Bars are ±SEM.
data, the investigators further state that a high P_o was also evident in day-17 ventricular myocytes. Mazzanti and coworkers, also using 20 mM Ba^{2+}, found P_o at +10 mV to be between 0.5 and 0.6, which is in full agreement with our fluctuation analysis data. Finally, our variance versus current plots are poorly fitted by theoretical curves if it is assumed that P_o is low and that the number of functional channels is high (Figure 2). Thus, we conclude that we have not significantly underestimated the number of functional L-type calcium channels in chick ventricular myocytes.

It is probable that some of the DHP binding sites may be L-type channels located on other cell types, such as smooth muscle cells and postganglionic cholinergic neurons. However, nonmuscle cells are sparse in the embryonic chick heart and are not observed at all until day 4 of development. A second possibility is that some DHP receptors are probably intracellular, as they may be in transit to or from the sarcolemma and would not be detected by the fluctuation analysis. Even if we assume the unlikely possibility that only half of the DHP receptors are located on the sarcolemma of cardiac myocytes, the number of DHP receptors is still at least 25 times in excess of functional L-type calcium channels. Most likely, intracellular DHP binding and binding in other cell types accounts for only a very small fraction of the total DHP receptor content in our ventricular homogenates.

Is it possible that other ion channels are receptors for DHPs? That this is probably true for heart has recently been reported by Giles and colleagues (Gotoh et al.). In their study, both the DHP calcium channel antagonist nicardipine and Bay K 8644 inhibited a transient outward current in frog atrial myocytes, although 50% inhibitory concentrations (IC_{50}) were relatively high at 630 nM and 1 μM, respectively. Other DHP antagonists inhibited this current but with less potency. Interestingly, nicardipine has been shown to inhibit a transient outward current in embryonic mammalian sensory neurons but not in the adult. Again, the effect was at relatively high concentrations (0.5–5 μM), indicating a low-affinity binding site. It is not likely that this low-affinity DHP binding site is related to the one observed in the present study, because the binding constants for both the antagonist and Bay K 8644 are well below this range (Table 1) and the data were well-fitted by single-site models. If a low-affinity binding site had been present, it would not have been detected in the range of ligand concentrations that were tested. Nevertheless, our binding experiments do not rule out the possibility there may be more than one high-affinity DHP receptor type.

Do all the DHP receptors in the embryonic chick heart represent membrane proteins with voltage sensors as has been described in skeletal muscle? Since there is an average of about 17,000 DHP receptors per myocyte, then there should be a measurable gating charge movement on depolarization. Assuming a cell capacitance of 6.7 pF, a density of 25 DHP receptor sites/μm^2 and movement of four electron charge units per DHP receptor, there should be a measurable gating charge of approximately 1.6 nC/μF. This calculated gating charge is close to the value for calcium channel gating charge measured by Josephson and Sperelakis' (4.45 nC/μF in a 6.5-pF day-17 chick heart cell), suggesting that all of the DHP receptors maintain their voltage-sensing capacity, whether or not they are functioning calcium channels.

Is it possible to recruit some of these “silent” channels by mechanisms that increase calcium current? Since I_{0(peak)}=0.5 in the present study, drugs that stimulate L-type current should not increase the peak magnitude of the current more than twofold. This contention is supported by recent findings in our laboratory showing that β-adrenergic stimulation of the peak calcium current is less than twofold in the day-11 chick ventricle, as might be expected if it is assumed that β-receptor stimulation acts by increasing channel open times and not the number of channels. In contrast, we have found that Bay K 8644 increases peak calcium current by fourfold in chick ventricular myocytes. Further, Sperelakis and colleagues (Tohse et al.) have also reported that Bay K 8644 increases the peak magnitude of ensemble-averaged calcium current in embryonic chick heart cells by fourfold but without prolongation of open times. Taken together, these results support the hypothesis that there are “silent” L-type calcium channels and that these channels may be capable of becoming functional. Whether the proportion of functional channels increases with development remains to be determined.

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