Dihydropyridine Receptors Are Primarily Functional L-Type Calcium Channels in Rabbit Ventricular Myocytes

Wilbur Y.W. Lew, Larry V. Hryshko, and Donald M. Bers

We measured [3H]PN200-110 binding and patch-clamp currents in rabbit ventricular myocytes to determine if there is a disparity between the density of dihydropyridine-specific receptors and functional L-type calcium channels, as has been reported for skeletal muscle. The dihydropyridine receptor density was 74.7 ± 4.2 fmol/mg protein (mean ± SEM, Kd=1.73 ± 0.29 nM, n=6) in ventricular homogenates and 147 ± 6 fmol/mg protein (Kd=1.15 ± 0.16 nM, n=4) in myocytes. Ventricular homogenates contained 121 ± 9 mg protein/g wet wt (n=7). These values were used to calculate a dihydropyridine receptor density of 12.9 dihydropyridine sites/μm² for ventricular homogenates and 14.8 dihydropyridine sites/μm² for myocytes. The number of functional L-type calcium channels (N) was calculated from measurements of whole-cell current (I), single-channel current (i), and open probability (P0), where N=I/(i×P0). We measured sodium current through calcium channels (Ica) to avoid calcium-induced inactivation. Whole-cell (Iw) and single-channel (i0 and p0) measurements were obtained under similar ionic conditions at a test potential of -20 mV. In six cells, the peak Iw was ~105 pA/pF. The single-channel conductance was 40.8 ± 2.6 pS (n=12), and ic, at -20 mV was 1.96 pA. The mean p0 at -20 mV was 0.030 ± 0.002 in 16 patches in which only a single channel was evident. The calculated density of functional L-type calcium channels was ~18 channels/μm². Thus, in rabbit cardiac muscle, the number of L-type calcium channels (18 channels/μm²) is of similar magnitude to the density of specific dihydropyridine receptors (13–15 dihydropyridine sites/μm²). We conclude that the majority of dihydropyridine receptors in cardiac muscle are functional L-type calcium channels. For reasons discussed, this conclusion may also be valid for skeletal muscle. (Circulation Research 1991;69:1139–1145)

L-type calcium channels often are characterized by their sensitivity to 1,4-dihydropyridines (DHP). In skeletal muscle, the density of DHP receptors was reported to be 35–50 times that of functional L-type calcium channels.1 The number of DHP receptors agrees well with estimates of intramembrane charge movement in skeletal muscle,2 where the DHP receptor protein likely serves as the voltage sensor for excitation–contraction coupling.3 Sarcoplasmic reticulum calcium release in skeletal muscle does not require calcium influx but appears to be controlled by charge movements associated with the DHP receptor.

In cardiac muscle, sarcoplasmic reticulum calcium release requires calcium influx rather than simply the charge movements associated with calcium channel gating.4 It is unclear if a disparity exists between the DHP receptor density and the number of functional L-type calcium channels in cardiac muscle. The density of functional L-type calcium channels has been estimated from voltage-clamp studies as 2–5 channels/μm² in frogs5 and guinea pig6 ventricles. The DHP receptor density can be calculated from nitrendipine binding data in embryonic chick7 and rat8 myocytes (see DHP density equations in “Results”) to estimate ~14–33 sites/μm², but in canine sarcoplemmal vesicles, the density was estimated to be 1 site/μm².9 The density of DHP receptors also can be estimated from intramembrane charge movements associated with cardiac calcium channel gating and thus with DHP receptors. Assuming that six elementary charges move across the membrane for each DHP receptor and the capacitance is 1 μF/cm²

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surface area, then the charge movement (2.7–5.5 nC/µF) corresponds to a density of 37–57 channels/µm² in neonatal and adult rat, rabbit, and guinea pig ventricles.10–12 Although this suggests that cardiac muscle has an order of magnitude more specific DHP receptors than functional L-type calcium channels, data from different species and preparations are difficult to compare.

We directly compared the number of specific DHP receptors with the density of functional L-type cardiac calcium channels in rabbit ventricular myocytes. The DHP receptor density was measured using nifedipine-sensitive [³H]PN200-110 binding. The number of functional calcium channels (N) was determined using voltage-clamp techniques to compare whole-cell currents (I) with single-channel currents (i) and open probability (p_o) using the formula N=I/(i×p_o). We found the density of functional L-type cardiac channels to be comparable to the number of specific DHP receptors in rabbit cardiac muscle.

Materials and Methods

Myocyte Isolation

Myocytes were isolated from New Zealand White rabbits using previously described techniques.13 The hearts were perfused for 6–8 minutes with prewarmed (37°C) calcium-free Tyrode’s solution that contained (mM) NaCl 140, KCl 6, MgCl₂ 1, glucose 10, and HEPES 5 (pH 7.4) and then were perfused for 25–30 minutes with 50 μM calcium Tyrode’s with 0.01% albumin, 0.75 mg/ml collagenase (Type II, Worthington Biochemical Corp., Freehold, N.J.), and 0.16 mg/ml protease (Sigma Chemical Co., St. Louis, Mo.).

Dihydropyridine Binding

DHP binding studies with ventricular homogenates and homogenized isolated myocytes were done using methods similar to those described by Mann and Hosey.14 Isolated myocytes were cooled to ~4°C and homogenized in the incubation buffer for DHP binding in a 2-ml glass/Teflon homogenizer (~10 passes). For ventricular homogenates, hearts were perfused with 30 ml of either 50 mM Tris buffer (pH 7.4) or calcium-free Tyrode’s solution before homogenization. After incubation with [³H]PN200-110, the samples were filtered with GF/B or GF/C filters (Whatman Inc., Clifton, N.J.) and washed twice with 4 ml cold 10 mM NaHEPES buffer (pH 7.4); radioactivity was then measured.

Current Measurements

Whole-cell and single-channel currents were recorded with an Axopatch 1C patch clamp amplifier and analyzed with PCLAMP software (Axon Instruments, Burlingame, Calif.). Data were filtered at 1–5 kHz and digitized at 10 kHz. We used patch pipettes with a 1–6-µm-diameter tip and resistance of 0.2–2.0 MΩ to form 3–20-GΩ seals. For single-channel recordings, pipettes were coated with Sylgard (Dow Corning, Midland, Mich.) and fire polished.

To avoid calcium-induced inactivation, we measured the nonselective or nonspecific current of monovalent cations (sodium) through calcium channels, which has been termed I_{na}.15,16 Whole-cell capacitance was measured by applying small hyperpolarizing voltage steps (5–15 mV) from a holding potential of −90 mV. Current–voltage relations were measured using 5–7-mV increments. I_{na} was measured before and after addition of 10 μM isoproteanol from a holding potential of −50 mV to prevent activation of sodium channels. For comparison, we also measured calcium and barium currents (I_{Ca} and I_{Ba}) from holding potentials of −90 mV or −50 mV with sodium replaced by tetraethylammonium to prevent sodium channel currents. To compare our results with the single-channel data (i_{Ba}) of Yue and Marban,17 we measured I_{Ba} in the presence of Bay K 8644. Leak corrections were made by subtracting currents measured with 5 μM nifedipine (I_{n}) and with 90% of the barium replaced with cobalt (I_{Ba}).

For single-channel studies, a depolarizing superfuse containing 10 μM isoproteanol was used with 200-msec test potentials to −20 mV to measure p_o and test potentials ranging from −80 to +20 mV (in 10-mV increments) to determine conductance. The p_o was defined as the total open time divided by the total duration at −20 mV.

Results

Dihydropyridine Binding

Figure 1 shows data for nifedipine-sensitive [³H]PN200-110 binding of isolated myocytes and ventricular homogenates. The mean B_{max} was 75 fmol/mg protein from ventricular homogenates and 147 fmol/mg protein from isolated myocytes. This is similar to previous reports of nitrendipine binding with a B_{max} of 190 fmol/mg protein in embryonic chick ventricular homogenates7 and 147–159 fmol/mg protein in adult and neonatal rat myocytes.8,18 The protein content of ventricular homogenates was 121±9 mg/g wet wt (n=7). The DHP receptor density was calculated from the ventricular homogenate data by assuming a density of 1.06 g/cm³, 25% extracellular space,19 and a surface area to volume correction of 0.6 μm²/μm³:

\[
\text{DHP receptor density} = \frac{(75 \text{ fmol/mg homogenate protein}) \times (121 \text{ mg homogenate protein/g wet wt}) \times (1.06 \text{ g wet wt/cm³ heart}) \times (0.75 \text{ cm³ cell}) \times (\frac{\mu^3 \text{ cell volume}}{0.6 \mu^2 \text{ surface area}}) \times (10^{-12} \text{ cm}^3/\mu^3) \times (6.02 \times 10^{23} \text{ DHP sites/mol})}{12.9 \text{ DHP sites/μm}^2}.
\]

The DHP receptor density was calculated from the myocyte data using the assumption of ~100 mg cell
A. Isolated Myocyte DHP Binding

![Graph showing DHP binding in isolated myocytes.]

B. Ventricular Homogenate DHP Binding

![Graph showing DHP binding in ventricular homogenates.]

**Figure 1.** Homogenates from isolated myocytes (panel A) and whole ventricles (panel B) from rabbits were incubated for 90 minutes at 23°C in a medium containing 10 mM NaHEPES, 1 mM NaEDTA, 1.1 mM total MgCl₂, 3-9 mg/ml homogenate protein, and 0.1-10 nM [³H]PN200-110 in the absence and presence of 83 μM nifedipine. Nonspecific binding in the presence of 83 μM nifedipine was subtracted from the total [³H]PN200-110 bound. In isolated myocyte homogenates (n=4 different hearts), B_max=147±6 (mean±SEM) fmol/mg protein (pm) and K_d=1.15±0.16 nM. For ventricular homogenates (n=6 hearts), B_max=74.7±4.2 fmol/mg protein and K_d=1.73±0.29 nM. DHP, dihydropyridine.

**Protein/cm³ cell and the same surface area to volume correction:**

\[
\text{DHP receptor density} = \frac{(147 \text{ fmol/mg cell protein}) \times (100 \text{ mg cell protein/cm}^3 \text{ cell}) \times (\mu m^3 \text{ cell volume/0.6 } \mu m^2 \text{ surface area}) \times (10^{-12} \text{cm}^3/\mu m^3) \times (6.02 \times 10^{23} \text{ DHP sites/mol})}{14.8 \text{ DHP sites/um}^2}
\]

**Whole-Cell Voltage-Clamp Studies**

The whole-cell capacitance was 121±7 pF (mean±SEM, n=23). The peak Iにおける at -20 mV was 7.55±1.56 nA (n=6), or 35.7±6.3 pA/pF (normalized to membrane capacitance). After addition of 10 μM isoproterenol, peak Iにおける was 10.83±1.30 nA, or 52.3±5.4 pA/pF (a 1.7±0.2-fold increase in peak Iにおける). The small amount of extracellular magnesium (100 μM plus 4 mM EGTA) in these solutions decreased peak current amplitudes. However, these solutions improved the stability of electrode seals and reduced the large current amplitude, allowing better voltage control. We estimate the free [Mg] in to be ~65 μM, which is the same as the K_c for magnesium block of Iにおける reported by Matsuda and is consistent with our observation in the absence of extracellular magnesium (not shown). Thus, the peak Iにおける at -20 mV in the absence of magnesium block would be ~105 pA/pF.

The inactivation of Iにおける was slow (Figure 2). The average Iにおける during the 200-msec test pulse was 72.0±1.9% and 86.6±1.1% of the peak Iにおける value before and after 10 μM isoproterenol, respectively. This slow inactivation allowed Iにおける to be directly compared with single-channel currents throughout a 200-msec depolarizing pulse (see below). Iにおける was measured in the same six myocytes for comparison (Figure 2). From a holding potential of -90 mV, peak Iにおける was 1.06±0.15 nA, or 5.0±0.6 pA/pF, and occurred at 0.5±1.4 mV. Data were similar from a holding potential of -50 mV.

In 11 myocytes, the peak Iにおける at -20 mV with 1 mM barium was 1.46±0.10 pA, or 11.49±0.93 pA/pF. In eight myocytes, an increase to 5 mM barium increased Iにおける at -20 mV to 5.45±0.36 pA, or 43.69±3.35 pA/pF.

**Single-Channel Recordings**

The single-channel conductance from 12 patches was 40.8±2.6 pS (Figure 3, inset). The calculated Iにおける at -20 mV was 1.96 pA. The possibility of contamination by sodium channel currents (I_n) was excluded, because 50 μM tetrodotoxin was present in the pipette solution, sodium channels inactivate very rapidly, and typical I_n conductance under similar conditions is much smaller (15 pS). As expected, the Iにおける conductance (41 pS) at pH 7.4 (same pH used for whole-cell Iにおける measurements) was lower than that reported at pH 9.0 (85 pS) because of rapid flickering block by protons at the lower pH. Increasing pH to 9.0 in our pipettes increased single-channel conductance approximately twofold and in six cells increased whole-cell Iにおける by 3.3±0.2-fold, in agreement with previous studies.

The p_0 at -20 mV was recorded in 25 patches using ~400 consecutive sweeps in each patch (range, 120–520) (Figure 3). In 16 patches in which only a single channel was evident, the open time averaged 1.8±0.9 seconds during a total observation period of 81.1±6.5 seconds, producing a p_0 of 0.030±0.002 (i.e., the channel was open 3% of the time). The p_0 ranged from 10^-5 to 0.234, with a median p_0 of 0.005–0.004. Table 1 lists the percent open time (p_0×100) for each patch. Even within a single patch, p_0 varied markedly between individual sweeps (Figure 3). In nine patches in which at least two channels were evident, the mean p_0 was 0.107±0.034 (range, 0.004–0.314).

**Functional L-Type Calcium Channels**

The number of functional calcium channels (N) was determined using whole-cell and single-channel...
data with test pulses of \(-20\) mV. At \(-20\) mV, the mean whole-cell \(I_{\text{ns}}\) in the absence of magnesium would be \(-105\) pA/pF, the calculated \(I_{\text{ns}}\) was 1.96 pA, and the mean \(p_0\) was 0.03 (patches with a single channel evident). Thus, \(N=I/(i\times p_0)=17.8\) channels/\(\mu\text{m}^2\) (assuming 1 \(\mu\text{F/cm}^2\)).

**Discussion**

This study demonstrates that the number of functional L-type calcium channels (18 channels/\(\mu\text{m}^2\)) is of similar magnitude to the DHP receptor density (13–15 sites/\(\mu\text{m}^2\)) in rabbit cardiac muscle. The number of functional calcium channels was calculated using the formula \(N=I/(i\times p_0)\). Whole-cell and single-channel data were recorded under comparable ionic conditions in the absence of calcium and with very low intracellular sodium. We used sodium (\(I_{\text{ns}}\)) rather than calcium (\(I_{\text{Ca}}\)) as the charge carrier for measuring calcium channel currents to avoid calcium-induced inactivation, providing a better estimate of peak or maximal whole-cell currents. Rapid inactivation of \(I_{\text{Ca}}\) (Figure 2) precludes a comparison between whole-cell and single-channel data, unless \(p_0\) is measured only during the first few milliseconds (corresponding to peak \(I_{\text{Ca}}\)). In contrast, the inactivation of \(I_{\text{ns}}\) is slow.\(^{16}\) The average \(I_{\text{ns}}\) during a 200-msec pulse was only 13% lower than the peak \(I_{\text{ns}}\) (Figure 2), making it reasonable to compare whole-cell \(I_{\text{ns}}\) with single-channel \(I_{\text{ns}}\) and \(p_0\) values measured throughout the 200-msec test pulse.

We used \(\beta\)-adrenergic stimulation both to maximize currents and to slow inactivation. \(\beta\)-Adrenergic stimulation or cAMP does not alter the amplitude of the single-channel current \((i)\) or conductance\(^{5,25–27}\) but produced a twofold increase in peak \(I_{\text{ns}}\). Outward currents at large positive potentials were not altered by isoproterenol\(^{28}\) (Figure 2C), making it unlikely that the increase in \(I_{\text{ns}}\) was due to an increase in the number of functional channels. \(\beta\)-Adrenergic agonists shift calcium channel gating to increase \(p_0\)\(^{28,29}\) because of longer-duration openings,\(^{25,26}\) fewer blank sweeps,\(^{25,29,30}\) and/or a change in calcium channel gating favoring more active modes.\(^{27}\) Increasing \(p_0\) improved the accuracy of \(p_0\) estimates and made it easier to exclude multichannel patches. Although Bay K 8644 also increases calcium currents by promoting longer-lasting channel openings,\(^{29}\) Bay K 8644 was not used because it is a partial calcium channel antagonist at holding potentials of \(-50\) mV.\(^{31}\)

Despite the wide variability in \(p_0\), it is unlikely that we missed a great disparity between the density of specific DHP receptors and functional L-type calcium channels. We would have had to underestimate \(p_0\) by an order of magnitude (e.g., if \(p_0\) is 0.3 rather than 0.03) to conclude that there was an order of magnitude more DHP receptors than functional calcium channels. Indeed, because of the modest amount of inactivation of \(I_{\text{ns}}\) during the 200-msec test pulse, we may have underestimated \(p_0\) by 13%. However, a greater underestimate of \(p_0\) is unlikely. It was rare to observe \(p_0\) values as high as 0.3 except in multichannel patches. In fact, we may have overesti-
FIGURE 3. Representative single-channel tracings and open probability (pₒ) data for a patch with only a single channel evident. Cells were depolarized in a solution containing (mM) potassium aspartate 120, MgCl₂ 20, EGTA 10, HEPES 10, and 10 μM isoproterenol, pH 7.4 at 22°C. The pipette solution contained (mM) NaCl 150, EDTA 5, HEPES 5, and 50 μM tetrodotoxin, pH 7.4 at 22°C with NaOH. Each sweep comprised a test pulse to −20 mV for 200 msec from a holding potential of −90 mV. Sweeps were repeated every 2 seconds for a total of 500 sweeps. Left panel: Total open time (Tₒ, msec) for each 200-msec sweep. There was a wide variability in Tₒ, ranging from 0 to 77 msec (pₒ range, 0–0.387). In 418 of 500 sweeps, there were no channel openings (Tₒ=0). Overall, the channel was open a total of 435 msec during 100 seconds of observation at the −20-mV test pulse, yielding a pₒ value of 0.0043. Right panel: Representative tracings of single-channel recordings with four consecutive sweeps (as marked) at four different time periods. Inset (left panel): All single-channel iₕₒ conductance measurements from 12 patches (mean±SD). Conductance was measured by plotting mean iₕₒ amplitude vs. test potential. Twenty sweeps (200-msec pulses) were produced from a holding potential of −90 mV to test potentials ranging from −80 to +20 mV to produce several channel openings. The mean conductance calculated from the 12 individual patches was 41 pS (or 35 pS if all raw iₕₒ values are pooled, as shown).

TABLE 1. Percent Open Time for 16 Patches With Only a Single Channel Evident

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Percent open time=open probability (pₒ)×100.
by Yue and Marban (Reference 17 and E. Marban, personal communication).

Our finding of a comparable number of DHP-specific receptors and functional L-type calcium channels in cardiac muscle contrasts with the disparity reported by Schwartz et al1 for skeletal muscle. They estimated 2 FA of calcium current per DHP site in skeletal muscle (site density, ~230 sites/μm²), which was 35–50 times lower than the anticipated single-channel current through L-type calcium channels (0.07–0.1 pA). However, concluding that there are 35–50 times more DHP-specific receptors than functional L-type calcium channels implicitly assumed that p0 is 1.0 at the peak of Iω. Estimates of p0 from intact skeletal muscle are unavailable. When incorporated into planar bilayers, skeletal muscle T tubule calcium channels have a conductance approximately half that of cardiac muscle,32,33 and p0 at potentials corresponding to peak Iω is approximately 0.04–0.10.34 These values are lower than assumed by Schwartz et al1 and would make the density of DHP receptors and functional calcium channels in skeletal muscle about the same. The presence of DHP receptors, which also function as L-type calcium channels, does not contradict the hypothesis that excitation–contraction coupling in skeletal muscle depends on the voltage sensor function of the DHP receptors.3 Calcium currents develop in skeletal muscle with such a slow time course that they are unlikely to participate in twitch contractions.35

In conclusion, we found a comparable number of DHP-specific receptors and functional L-type calcium channels in cardiac muscle.

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