Postnatal Maturation of Excitation-Contraction Coupling in Rat Ventricle in Relation to the Subcellular Localization and Surface Density of 1,4-Dihydropyridine and Ryanodine Receptors

Maurice Wibo, Guadalupe Bravo, and Théophile Godfraind

To better understand excitation-contraction coupling in cardiac muscle, we investigated the main Ca\(^{2+}\) channels involved in that process in adult and neonatal rat ventricle. Voltage-dependent (L-type) Ca\(^{2+}\) channels and sarcoplasmic reticulum Ca\(^{2+}\) release channels were labeled by means of \(\text{[}^3\text{H}\text{]}\)(+)-PN200-110 and \(\text{[}^3\text{H}\text{]}\)ryanodine, respectively. The number of \(\text{[}^3\text{H}\text{]}\)ryanodine binding sites (per gram tissue) increased more than that of \(\text{[}^3\text{H}\text{]}\)(+)-PN200-110 binding sites over the postnatal period (2.1-fold versus 1.35-fold, respectively). After equilibration of microsomal fractions in density gradient, ryanodine receptors were characterized by a heavy distribution pattern that did not change appreciably between days 1 and 30 after birth. In neonatal tissue, 1,4-dihydropyridine receptors were found mainly in low-density subfractions, together with other sarcolemmal constituents, whereas in adult tissue, they were recovered predominantly in high-density subfractions, together with ryanodine receptors. Thus, after birth, and in parallel with the development of T tubules, there was a progressive concentration of L-type Ca\(^{2+}\) channels in junctional structures of high equilibrium density, where they were situated close to the Ca\(^{2+}\) release channels of the sarcoplasmic reticulum. In adult ventricle, L-type channels were, on an average, threefold more abundant in T tubules than in external sarcolemma. In parallel mechanical studies, we found that the inhibitory action of ryanodine on systolic contraction was much more pronounced in adult than in neonatal right ventricle, and that, conversely, neonatal tissue was more sensitive than adult tissue to inhibitors of L-type channels. We conclude that, in view of the presumed mechanism of Ca\(^{2+}\) release from the sarcoplasmic reticulum, that is, Ca\(^{2+}\)-induced Ca\(^{2+}\) release, the predominant localization in adult rat ventricle of the major Ca\(^{2+}\) entry pathway in the vicinity of the Ca\(^{2+}\) release pathway is of great functional significance. Furthermore, owing to the relative stoichiometry of Ca\(^{2+}\) entry and Ca\(^{2+}\) release channels in junctional structures (about 1:9), a physical link between these channels is not likely to be involved in the modulation of Ca\(^{2+}\) release from the sarcoplasmic reticulum in cardiac muscle. (Circulation Research 1991;68:662–673)

During the postnatal period, dramatic changes occur in the physiology of cardiac muscle, especially as regards excitation-contraction coupling. In contrast to neonatal heart, adult rat heart does not respond by an enhanced force development to an augmentation of stimulus frequency or to elevation of extracellular Ca\(^{2+}\) beyond physiological values. The shape of action potential is very different in adult and neonatal rat ventricle. In mechanically skinned rat cardiac cells, the threshold of free Ca\(^{2+}\) for Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) is lowered about 10 times between days 2 and 30 postpartum. These functional changes are paralleled by ultrastructural changes: from days 1 to 11 after birth, the volume percentages of myofibrils, mitochondria, and SR increase by 1.2-, 1.8-, and 2.1-fold, respectively, in rat ventricular myocytes.
In mammalian ventricle, most of the Ca\(^{2+}\) that activates the myofilaments is released from the SR Ca\(^{2+}\) stores.\(^5\) T tubules are expected to play a particularly important role in excitation–contraction coupling in rat ventricle, where, as shown by Page and Surdyk-Droske,\(^6\) 50% of the membrane surface area of T tubules is engaged in dyadic junctions with the terminal cisternae from which SR Ca\(^{2+}\) release occurs (see Fill and Coronado\(^7\)). The T-tubular system becomes clearly detectable only 2 or 3 weeks after birth,\(^8\)\(^,\)\(^9\) and it is likely that the development of the T-tubular junctional domain somehow is connected with the functional changes occurring in the postnatal period. To shed light on this question, we studied two key constituents involved in excitation–contraction coupling—sarcolemmal voltage-dependent Ca\(^{2+}\) channels and SR Ca\(^{2+}\) release channels—over the postnatal period. By means of appropriate radioligands, [\(^3\)H](+)-PN200-110 for sarcolemmal 1,4-dihydropyridine-sensitive channels\(^10\) and [\(^3\)H]ryanodine for SR channels,\(^7\) we compared the number of channels in neonatal and adult tissue, and we analyzed by density gradient centrifugation the postnatal evolution in the distribution of sarcolemmal and SR Ca\(^{2+}\) channels. We observed that the number of ryanodine receptors increased distinctly more than that of 1,4-dihydropyridine receptors after birth and that, concomitantly, a shift occurred in the subcellular localization of sarcolemmal Ca\(^{2+}\) channels, reflecting their progressive concentration in T-tubular areas physically linked to junctional SR. These changes in channel surface density and localization are likely to be involved in the profound modifications in excitation–contraction coupling observed over the same period, as reflected in the inotropic response of ventricular muscle to ryanodine and inhibitors of voltage-dependent Ca\(^{2+}\) channels.

### Materials and Methods

#### Subcellular Fractionation

Twenty-five to 50 neonatal Wistar rats, seven to 25 30–35-day-old young rats, or two to 20 male 2–4-month-old adult rats were used in each experiment. Ventricles collected in warm, oxygenated Ringer’s solution were homogenized in 3–5 vol ice-cold solution (mM: sucrose 250, histidine 2, dithioerythritol 2, phenylmethylsulfonyl fluoride 0.2, and EDTA 0.2, pH 7.3)\(^11\) by means of an Ultra-Turrax (Janke and Kunkel KG, Staufen i. Br., FRG) with three 5-second bursts at 9,000 rpm followed by a 1-second burst at 20,000 rpm. After centrifugation at 1,000g for 10 minutes, the supernatant was collected, and the pellet was resuspended with a Dounce homogenizer. This suspension was recentrifuged at 1,000g, and the pellet was submitted to two additional cycles of resuspension and centrifugation under the same conditions. The washed pellet was resuspended in 0.25 M sucrose buffered at pH 7.4 with 5 mM Tris-HCl (buffered sucrose) and was designated N fraction. The combined supennatants were centrifuged at 10,000g for 20 minutes in a TFT 50.38 rotor (Kontron AG, Zurich, Switzerland), and the pellet was washed once by resuspension and recentrifugation. The washed pellet, resuspended in buffered sucrose, was designated M fraction. The combined supernatants were centrifuged at 110,000g for 35 minutes, and the microsomal pellet was resuspended in buffered sucrose (P fraction).

In some experiments, this microsomal fraction was subfractionated on a linear sucrose gradient, essentially as described previously.\(^12\) After overnight centrifugation at 100,000g, 10 fractions were collected from the gradient, and their density (at 0°C) was determined by refractometry. Density frequency histograms were constructed as described by Beaufays and Amar-Costesc.\(^13\)

#### Binding and Biochemical Assays

The specific binding of [\(^3\)H](+)-PN200-110 was measured by incubating fractions with the radioligand for 60 minutes at 37°C in a Tris-buffered physiological solution, as described previously.\(^14\) Nonspecific binding was estimated in the presence of 1 \(\mu\)M nifedipine. Membranes collected on GF/F filters (Whatman Biosystems Ltd., Maidstone, Kent, UK) were washed twice with 10 ml ice-cold 0.15 M NaCl. [\(^3\)H]Ryanodine binding was determined by incubating tissue samples with the radioligand (usually diluted with cold ryanodine to a specific radioactivity of 7,500 dpm \cdot \text{pmol}^{-1}) for 120 minutes at 37°C in a solution containing (mM) KCl 150, AMP 3, CaCl\(_2\) 0.3, and HEPES 10, pH 7.4. Nonspecific binding was estimated in the presence of 10 \(\mu\)M ryanodine. Membranes were collected on GF/F filters and washed twice with 10 ml of a chilled solution containing 150 mM KCl and 10 mM Tris-HCl, pH 7.4. [\(^3\)H]Ouabain binding was measured in an “Mg-P,” medium (37°C, 20 minutes) as previously described,\(^15\) except that in some experiments, tissue samples were preincubated for 20 minutes at room temperature with saponin (0.5 mg/ml; 0.33 mg/mg protein) to suppress the membrane permeability barrier. Nonspecific binding was estimated in the presence of 1 mM ouabain. [\(^3\)H]Quinuclidinyl benzilate ([\(^3\)H]QNB) binding was measured by incubating tissue samples with 0.2 nM radioligand for 60 minutes at 37°C in 10 mM Tris-HCl, pH 7.4. Nonspecific binding was determined in the presence of 1 \(\mu\)M atropine sulfate. Membranes were collected on GF/F filters and washed twice with 5 ml chilled 10 mM Tris-HCl (pH 7.4). In all binding assays, filters were immersed in Picofluor 15/toluene (1:4, vol/vol), and radioactivity was counted with an efficiency of 40–45%. Cytochrome c oxidase, S\(^\text{5}\)-nucleotidase, and protein were assayed as reported previously.\(^16\)

#### Contractility Studies

The contractile activity of right ventricular strips (10 mm length) from young and adult male Wistar rats was recorded as described by Finet et al.\(^17\) Tissues were incubated at 30°C in oxygenated (95%
O₂:5% CO₂ Tyrode’s solution (mM: NaCl 137, KCl 6, CaCl₂ 1.8, MgCl₂ 1, NaH₂PO₄ 0.42, NaHCO₃ 11.5, and glucose 5.5) under an initial tension of 5 mN and were stimulated with rectangular 10-msec pulses at a frequency of 1 Hz. Experimental conditions were identical for neonatal heart, except that the whole free wall from the right ventricle was used. Its approximate length (along the cardiac axis) was 5 mm, and its weight was 10–20 mg. Drugs were added after an initial equilibration period of 60 (neonatal tissue) or 90 (adult tissue) minutes. As stock solutions (10 mM) of nifedipine and verapamil were prepared in ethanol, appropriate controls were run in the presence of solvent.

Drugs

[³H](+)-PN200-110 (isopropyl-4-[2,1,3-benzoaxadiazol-4-yl]-1,4-dihydro-5-methoxybenzyl-2,6-dimethyl-3-pyridinacarboxylate; 80 Ci/mmol) was from The Radiochemical Centre (Amersham, UK). [³H]Ryanodine (60 Ci/mmol), [³H]ouabain (15 Ci/mmol), and [³H]QNB (30 Ci/mmol) were from New England Nuclear Research Products (Boston). Ryanodine was purchased from AgriSystems International (Wind Gap, Pa.), and ouabain and atropine sulfate from Merck (Darmstadt, FRG). Nifedipine was kindly provided by Bayer AG (Leverkusen, FRG). Experiments with 1,4-dihydropyridines were carried out under yellow light.

Results

Characterization of [³H]Ryanodine Binding

[³H]Ryanodine binding had never been investigated previously in rat ventricle. In preliminary experiments (not shown), we found that under our assay conditions, specific binding was maximal in the presence of 0.2–0.5 mM CaCl₂. In saturation experiments (Figure 1), specific binding measured after 2 hours of incubation at 37°C reached a maximal value at a free concentration of approximately 100 nM. Scatchard and Hill plots of data obtained at free concentrations of 5–100 nM were linear (Hill coefficient close to 1), suggesting a single class of binding site with a K_d of approximately 20 nM (Figure 2). However, when incubation time was varied between 1 and 8 hours, apparent K_d values were inversely related to time (see Figure 2). Therefore, at low [³H]ryanodine concentrations, equilibrium was not reached after 2 hours. Moreover, at high [³H]ryanodine concentrations (50–100 nM), binding decreased when incubation time was longer than 2 hours, which indicated some decay of the binding site on prolonged incubation at 37°C. [³H]Ryanodine not only bound but also dissociated very slowly: when microsomal fractions that had been incubated for 2 hours with [³H]ryanodine (15 nM) were diluted 100-fold with incubation medium and further incubated for 2 hours, specific binding decreased by only 18% (±1.3%, three preparations).

Because binding was only slowly reversible under our assay conditions, and because prolonged incubation was deleterious for the ryanodine receptor, saturation experiments were carried out under non-equilibrium conditions (2 hours of incubation) and analyzed on the basis of a pseudo-first order model

\[ B = B_{\text{max}} (1 - e^{-ktL}) \]

where t is time (2 hours), L is ligand concentration (nM), and k is the association rate constant (hr⁻¹ · nM⁻¹). As illustrated in Figure 1, curves drawn according to this model (KINETIC program) fitted very well the experimental saturation data. It can be shown (for example, see Reference 18) that in first approximation, this model is formally equivalent to the equilibrium binding model, as confirmed by the linearity of Scatchard plots (Figure 2). However, the pseudo-first order model provides a more reliable estimate of the
number of binding sites, which is overestimated when the equilibrium binding model is applied to data obtained under nonequilibrium conditions.

Postnatal Changes in [3H]Ryanodine and [3H](+)-PN200-110 Binding in Rat Ventricle

In Figure 3, [3H]ryanodine binding data obtained with microsomal fractions from neonatal and adult rat ventricle have been plotted in a semilogarithmic manner (ln[B\textsubscript{max} - B] versus L). This graphic representation yielded straight lines, as expected from the proposed first-order model, and their slope (−k) was almost identical in adult (>30 days) and neonatal (1–8 days) rats. As for [3H](+)-PN200-110 binding, Scatchard analysis indicated similar K\textsubscript{d} values in adult and neonatal rats (Figure 4). When related to the amount of protein, the B\textsubscript{max} of [3H](+)-PN200-110 binding to microsomal fractions did not change with age (Table 1). In contrast, the B\textsubscript{max} of [3H]ryanodine binding was about twice lower in neonatal than in adult heart. In Table 1, results from rats older than 1 month were pooled, as no appreciable modification was noticed between 1 and 4 months.

Table 1. Specific Binding Parameters for [3H](+)PN200-110 and [3H]Ryanodine in Microsomal Fractions From Rat Heart

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1–8 days</th>
<th>&gt;30 days</th>
</tr>
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<tbody>
<tr>
<td><a href="+">3H</a>-PN200-110</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K\textsubscript{d} (pM)</td>
<td>73.0±7.3 (3)</td>
<td>85.6±5.2 (8)</td>
</tr>
<tr>
<td>B\textsubscript{max} (pmol · mg\textsuperscript{-1} protein)</td>
<td>0.51±0.07 (3)</td>
<td>0.54±0.03 (8)</td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>0.94±0.02 (3)</td>
<td>0.99±0.02 (8)</td>
</tr>
<tr>
<td>[3H]Ryanodine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>k (hr\textsuperscript{-1} · nM\textsuperscript{-1})</td>
<td>0.0265±0.0020 (4)</td>
<td>0.0288±0.0023 (5)</td>
</tr>
<tr>
<td>B\textsubscript{max} (pmol · mg\textsuperscript{-1} protein)</td>
<td>2.13±0.30 (4)*</td>
<td>4.04±0.56 (5)</td>
</tr>
</tbody>
</table>

Values are mean±SEM from n (in parentheses) microsomal preparations. [3H](+)-PN200-110 binding parameters were obtained by Scatchard and Hill analysis. [3H]Ryanodine binding parameters were estimated by a nonlinear curve-fitting technique (Kinetic program).

Some 46% of the total sedimentable [3H](+)-PN200-110 and [3H]ryanodine binding sites were recovered in the microsomal (P) fraction from neonatal heart, as compared with 23–24% for adult heart (Table 2). As the percentage yield of protein was also twofold to threefold higher in neonatal heart, [3H](+)-PN200-110 and [3H]ryanodine binding sites of adult as well as neonatal heart were purified fourfold to fivefold in microsomal fractions with respect to sedimentable protein. The yield and purification factor were similar for [3H]QNB binding and 5'-nucleotidase, but somewhat lower for [3H]ouabain binding. Some 3–4% of cytochrome c oxidase was found in the microsomal fraction. As expected, the major part of this enzyme activity was recovered in the mitochondrial (M) fraction (data not shown).

Table 2. Percentage Amounts of Receptors, Cytochrome c Oxidase, and Protein in Microsomal Fractions From Rat Heart

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1–3 days</th>
<th>&gt;30 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><a href="+">3H</a>-PN200-110</td>
<td>46.2±7.1*</td>
<td>22.6±1.3</td>
</tr>
<tr>
<td>[3H]Ryanodine</td>
<td>46.3±10.0</td>
<td>23.9±2.8</td>
</tr>
<tr>
<td>[3H]Ouabain</td>
<td>30.8±2.2*</td>
<td>16.4±1.5</td>
</tr>
<tr>
<td>[3H]QNB</td>
<td>ND</td>
<td>21.6±1.1</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>ND</td>
<td>26.3±0.9</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>4.2±0.3</td>
<td>3.4±0.2</td>
</tr>
<tr>
<td>Protein</td>
<td>11.3±3.0</td>
<td>4.7±0.3</td>
</tr>
</tbody>
</table>

Results are amounts found in microsomal (P) fractions, expressed as percentage of total amounts found in the three particulate fractions (N+M+P). Values are mean±SEM from three (1–3 days) or five (>30 days) fractionation experiments. [3H]Ouabain binding was determined on fractions preincubated with saponin (see “Materials and Methods”). QNB, quinuclidinyl benzilate; ND, not determined.

*Significantly different from adult values (p<0.05; t test).
(×1.35) < protein (×1.5) < cytochrome c oxidase (×1.75) < [3H]ryanodine binding (×2.1). On the contrary, [3H]ouabain binding (measured at a concentration of 25 nM) was markedly lower in adult than in neonatal heart. We also have observed a decrease in the number of [3H]QNB binding sites over the postnatal period (data not shown), as reported recently by Kojima et al.20

Postnatal Changes in the Density Distributions of Receptor Sites in Rat Ventricle

To investigate the subcellular localization of 1,4-dihydropyrididine and ryanodine receptors over the postnatal period, we subfractionated microsomal fractions by density gradient centrifugation. Results obtained with adult tissue are shown in Figure 5. The shaded histogram reproduced on each panel is the distribution of [3H](+)-PN200-110 binding. It was characterized by a rather sharp peak centered around a density of 1.18 and a distinct shoulder at densities 1.10–1.16. The shoulder was somewhat less pronounced in older rats (right panels). This distribution pattern dissociated markedly from those of [3H]ouabain and [3H]QNB binding, which peaked at 1.13–1.14. With respect to the distribution of [3H](+)-PN200-110 binding, that of [3H]ryanodine binding was slightly shifted toward high densities and lacked a shoulder in low-density subfractions. Cytochrome c oxidase gave a sharp and symmetrical peak around 1.18. The distribution of protein was similar to that of [3H](+)-PN200-110 binding but was somewhat more flattened.

As illustrated in Figure 6, in which distribution patterns from neonatal (2–3 days) and young (30–35

![Figure 5. Subfractionation of microsomal fractions from adult rat ventricle by density gradient centrifugation. Density frequency histograms shown in left and right panels were obtained from young (30–35 days) and older (3–4 months) rats, respectively. Each subfraction is represented on the abscissa by its density boundaries, which were estimated from the measured average density and volume (see Beaufay and Amar-Costesecl23). The density frequency (ordinate) is the fractional amount of constituent (binding site, enzyme, protein) recovered in a given subfraction divided by the density increment across this subfraction. The shaded histogram reproduced on each panel is the density distribution of [3H](+)-PN200-110 binding. Total recoveries in gradient subfractions ranged 91–108% of amounts found in microsomal preparations layered on the gradient. QNB, quinuclidinyl benzilate; Cyt., cytochrome.]

<table>
<thead>
<tr>
<th>TABLE 3. Postnatal Evolution of Receptor Numbers and Cytochrome c Oxidase Activity in Rat Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–3 days</td>
</tr>
<tr>
<td>[3H] (+)-PN200-110 (pmol)</td>
</tr>
<tr>
<td>[3H]Ryanodine (pmol)</td>
</tr>
<tr>
<td>[3H]Ouabain (pmol)</td>
</tr>
<tr>
<td>Cytochrome c oxidase (units)</td>
</tr>
<tr>
<td>Protein (mg)</td>
</tr>
</tbody>
</table>

Results are total amounts found in the three particulate fractions (N+M+P), related to 1 g tissue wet wt. They are Bmax values for [3H](+)-PN200-110 and [3H]ryanodine, but not for [3H]ouabain. Specific binding of [3H]ouabain to saponin-treated membranes (see "Materials and Methods") was measured at a free concentration of 25 nM. Values are mean±SEM from n (in parentheses) experiments. Data at 1–3 days and >30 days are significantly different (p<0.02; t test).
days) rats are compared, a major influence of age was found only in the case of \(^{3}H\)(+)-PN200-110 binding. In neonatal heart, \(^{3}H\)(+)-PN200-110 receptor sites mainly were recovered at densities lower than 1.17, and the peak at 1.18 was absent. The peak of \(^{3}H\)ryanodine binding was broader but hardly less heavy than in adult heart. The distribution patterns of \(^{3}H\)QNB and \(^{3}H\)ouabain binding were almost superimposable in neonatal and adult heart. The peak of cytochrome c oxidase was slightly displaced toward low densities in neonatal heart. Data from five density gradient experiments are summarized in Table 4. As indicated again by median densities, \(^{3}H\)(+)-PN200-110 receptor sites were unique in their marked migration toward high densities in the early postnatal period. At 7–10 days, their median density value was about midway between those at 2–3 days and 3–4 months.

**Postnatal Changes in the Pharmacological Effects of Ryanodine and Calcium Antagonists**

As shown in Figure 7, the pharmacological effects of ryanodine and calcium antagonists (inhibitors of voltage-dependent Ca\(^{2+}\) channels\(^{10}\)) were dramatically different in neonatal (3–4 days) and adult ventricular muscle. As systolic tension decreased noticeably with time even in the absence of drug, each ventricle was exposed to only one drug concentration, and the tension decay of drug-treated preparations was compared with that of control preparations run under identical conditions. After incubation for 35–40 minutes with 100 nM nifedi-

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**TABLE 4. Median Densities of Receptors and Cytochrome c Oxidase in Microsomal Fractions From Rat Heart: Postnatal Evolution**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>1–2 days</th>
<th>2–3 days</th>
<th>7–10 days</th>
<th>30–35 days</th>
<th>3–4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{3}H)(+)-PN200-110</td>
<td>1.141</td>
<td>1.146</td>
<td>1.166</td>
<td>1.175</td>
<td>1.179</td>
</tr>
<tr>
<td>(^{3}H)ryanodine</td>
<td>&gt;1.17</td>
<td>1.188</td>
<td>1.191</td>
<td>1.191</td>
<td>ND</td>
</tr>
<tr>
<td>(^{3}H)ouabain</td>
<td>1.134</td>
<td>1.138</td>
<td>1.139</td>
<td>1.134</td>
<td>1.145</td>
</tr>
<tr>
<td>(^{3}H)QNB</td>
<td>ND</td>
<td>1.128</td>
<td>1.132</td>
<td>1.124</td>
<td>1.135</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>ND</td>
<td>1.172</td>
<td>1.175</td>
<td>1.178</td>
<td>1.179</td>
</tr>
</tbody>
</table>

Median densities were estimated from density distribution histograms such as those shown in Figure 5. QNB, quinuclidinyl benzilate; ND, not determined.
pine, a 1,4-dihydropyridine calcium antagonist, systolic tension was inhibited by more than 50% in neonates but was hardly affected in adults. The reverse was true for ryanodine, which, at a concentration of 100 nM, depressed systolic tension by more than 60% in adults, as compared with approximately 15% in neonates. When drug concentration was increased to 1 \( \mu M \), systolic contraction was almost abolished by nifedipine in neonates and by ryanodine in adults, whereas the inhibitory effect (at 40 minutes) of ryanodine in neonates and nifedipine in adults was approximately 40%. Thus, the nifedipine concentration that inhibited contraction by 50% (IC\(_{50}\)) was approximately 0.1 \( \mu M \) in neonatal tissue, as compared with 1 \( \mu M \) in adult tissue.\(^{17}\) The inhibitory patterns observed with nifedipine and verapamil were similar, but the latter drug was somewhat less potent.

In adult heart, the inhibitory action of 100 nM ryanodine was still increasing after 40 minutes. When incubation was prolonged for up to 3 hours, systolic tension eventually was almost totally inhibited by 100 nM ryanodine (Figure 8). At that time, inhibition by 10 nM ryanodine seemed to reach a maximal value of approximately 70%, whereas the inhibitory effect of 1 nM ryanodine did not attain a plateau value. Thus, IC\(_{50}\) was probably between 1 and 10 nM, that is, of the same order of magnitude as the apparent \( K_d \) values observed in binding experiments (6–20 nM; Figure 2). The very slow kinetics of inhibition is probably attributable in part to slow binding but also to slow depletion of SR Ca\(^{2+}\) stores, because at nanomolar concentrations, ryanodine is thought to maintain the Ca\(^{2+}\) SR release channel in an open state of low conductance.\(^{21}\)

**Discussion**

**Postnatal Evolution in Numbers of Receptor Sites in Rat Ventricle**

The binding properties of the ryanodine receptor seem to remain unchanged after birth, as judged
from the apparent association rate constant \((k)\). In contrast, the number of \([3H]\)ryanodine receptors increased markedly in the postnatal period (Tables 1 and 3). The 2.1-fold increase in number per gram tissue between days 1–3 and 30, as determined from binding measurements in the three particulate fractions, is in line with the reported postnatal proliferation of SR membranes.\(^4\) Our \(B_{\text{max}}\) value in microsomes from adult rat ventricle was 4 pmol \(\cdot\) mg\(^{-1}\) protein. As shown in Figure 5, some 90% of the microsomal \([3H]\)ryanodine binding sites was recovered in high-density subfractions (density greater than 1.17) as compared with 60% of the total protein, indicating that \([3H]\)ryanodine binding sites were purified 1.5-fold in heavy subfractions. The average \(B_{\text{max}}\) in these subfractions, 6 pmol \(\cdot\) mg\(^{-1}\) protein, is similar to the values reported by others\(^{22-24}\) in canine cardiac heavy SR preparations (5–9 pmol \(\cdot\) mg\(^{-1}\) protein). Under our assay conditions, we have not been able to detect a second, low-affinity binding site, as reported in some studies.\(^{25,26}\)

In agreement with previous investigators\(^{30,27}\) who used \([3H]\)nitrendipine, we did not observe any modification in the affinity of the 1,4-dihydropyridine receptors over the postnatal period. The number of 1,4-dihydropyridine receptors in the sum of particulate fractions increased somewhat when referred to 1 g tissue (Table 3), but this augmentation was smaller than that of total protein. Our \(B_{\text{max}}\) values at different ages are almost identical to those reported by Kojima et al.\(^{20}\) In contrast, Kazazoglou et al.\(^{27}\) found a threefold increase in \(B_{\text{max}}\), expressed in fentomoles per milligram protein, between days 1 and 10. These results were obtained on fractions prepared by low-speed centrifugation (1,000g, 10 minutes), which might not be representative of the whole tissue.

\([3H]\)Ouabain binding measured at a concentration of 25 nM decreased markedly between days 13 and 30 (Table 3). At this concentration, binding in adult and neonatal heart should be predominantly to a high-affinity Na\(^+\),K\(^+\)-ATPase isoform (\(\varepsilon 2\) or \(\varepsilon 3\) isoform), even though the low-affinity \(\varepsilon 1\) isoform is the most abundant Na\(^+\),K\(^+\)-ATPase isoform in cardiac tissue.\(^{15,28,29}\) Our results agree with those of Charlemagne et al.\(^{28}\) showing a twofold higher number of high-affinity sites (expressed in picomoles per milligram protein) in sarcolemmal preparations from neonatal rat heart as compared with adult heart. In this study, we made no attempt to fully characterize high- and low-affinity \([3H]\)ouabain binding sites in our membrane fractions.

**Density Distributions of Receptor Sites in Adult and Neonatal Ventricle**

The postnatal changes in the subcellular localization of 1,4-dihydropyridine and ryanodine receptors were investigated by subtractioning microsomal fractions in sucrose density gradient. The choice of microsomes as starting material was guided by the conflicting requirements of adequate sampling and partial purification (which improved the signal-to-noise ratio in binding measurements). Microsomal fractions indeed contained a fair proportion (25–50%) of reasonably purified (fourfold to fivefold) receptors (Table 2). On the whole, the composition of the microsomal fractions obtained from adult and neonatal tissue appears similar, except that the percentage yield of constituents was higher in the microsomal fraction from neonatal tissue. It is likely that this higher yield is related to the less tough structure of the neonatal ventricle.

The density distributions of \([3H] (+)\)-PN200-110, \([3H]\)ouabain, and \([3H]\)QNB binding sites in adult rat ventricle (Figure 5) are in good agreement with data reported by Brandt\(^{11}\) on rabbit and cat ventricle. Noël et al.\(^{30}\) recently have shown that in microsomal fractions from adult heart, the density distribution of \([3H]\)ouabain (high-affinity) binding cannot be distinguished from that of the total Na\(^+\),K\(^+\)-ATPase activity. In contrast to "classical" sarcolemmal constituents such as 5'-nucleotidase (data not shown), Na\(^+\),K\(^+\)-ATPase \(([3H]\)ouabain binding), or muscarinic receptors \(([3H]\)QNB binding), which peaked at densities less than 1.15, \([3H](+)-PN200-110 binding peaked at 1.18. \([3H]\)Ryanodine binding also was characterized by a heavy distribution pattern, with a modal density at 1.19. These results agree with the view that the 1,4-dihydropyridine receptors found in high-density subfractions are associated with dyadic structures, that is, T-tubular and external sarcolemmal fragments that are linked to SR vesicles via the so-called feet.\(^{11,31,32}\) However, the distribution of the mitochondrial enzyme cytochrome \(c\) oxidase also showed a peak in the subfractions enriched in \([3H](+)-PN200-110 binding. Although mitochondria are devoid of high-affinity, 1,4-dihydropyridine receptors,\(^{33}\) the presence of low-affinity binding in purified mitochondria of guinea pig ventricle has been reported recently.\(^{34,35}\) Under our assay conditions, we did not detect low-affinity \([3H](+)-PN200-110 binding in our microsomal fractions, which contained only 3–4% of the total cytochrome \(c\) oxidase activity (Table 2). Moreover, in gradient experiments on neonatal tissue, the distribution of \([3H](+)-PN200-110 binding dissociated from that of cytochrome \(c\) oxidase (Figure 6).

The proportion of 1,4-dihydropyridine receptors associated with dyadic structures in microsomal fractions from adult rat ventricle can be estimated to 75%, as illustrated in Figure 9. In this figure, we show the distribution of \([3H](+)-PN200-110 binding that one should obtain if 75% of the sites followed the distribution of \([3H]\)ryanodine binding and 25% followed that of \([3H]\)ouabain binding. The agreement between experimental and calculated data is reasonably good, especially at densities below 1.15. Above 1.15, the calculated distribution is slightly shifted toward high densities with respect to the actual distribution. This suggests that the ratio of junctional SR to junctional sarcolemma in the dyadic structures recovered after homogenation is not constant and is an important determinant of equilibrium density.
In contrast to that of [3H]ryanodine binding, the heavy distribution pattern of [3H](+)−PN200-110 binding was not found in neonatal ventricle, as evidenced by Figure 6. At days 1–3, the [3H](+)−PN200-110 distribution still peaked below 1.15 and was only slightly heavier than those of [3H]ouabain and [3H]QNB binding. Thus, the distribution of [3H](+)−PN200-110 binding was quite distinct from that of [3H]ryanodine binding in neonatal ventricle, but both distributions overlapped markedly in adult ventricle. These results indicate that early after birth most of the 1,4-dihydropyridine receptors are present at the cell surface together with the other sarclemmal constituents and that at least a large part of them are not physically associated with the ryanodine receptors. Thereafter, SR elements proliferate and, perhaps with some delay, T tubules develop progressively. The doubling of ryanodine receptors reflects the maturation of SR terminal cisternae, whereas the growth of T tubules is accompanied not only by a moderate increase in the number of 1,4-dihydropyridine receptors (per gram tissue) but principally by a concentration of these receptors in the T tubules, where most of the junctional complexes between sarcolemma and SR cisternae are localized. Interestingly, in cultured skeletal muscle cells, the differentiation of typical triads coincides with a migration of the slow Ca2+ channels to the T-tubular membrane, without detectable change in the number of 1,4-dihydropyridine receptors.

### Table 5. Estimated Surface Density of 1,4-Dihydropyridine and Ryanodine Receptors in Adult Rat Ventricle

<table>
<thead>
<tr>
<th>Membrane domain</th>
<th>Membrane surface area (μm²·μm⁻³ tissue)</th>
<th>No. of receptors (pmol·g⁻¹ tissue)</th>
<th>Surface density (μm⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sarcolemma</td>
<td>0.362</td>
<td>13.5</td>
<td>...</td>
</tr>
<tr>
<td>Junctional</td>
<td>0.073</td>
<td>10.1</td>
<td>84</td>
</tr>
<tr>
<td>Nonjunctional</td>
<td>0.289</td>
<td>3.4</td>
<td>7</td>
</tr>
<tr>
<td>External sarcolemma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Junctional</td>
<td>0.018</td>
<td>2.5</td>
<td>...</td>
</tr>
<tr>
<td>Nonjunctional</td>
<td>0.228</td>
<td>2.7</td>
<td>...</td>
</tr>
<tr>
<td>Total</td>
<td>0.246</td>
<td>5.2</td>
<td>...</td>
</tr>
<tr>
<td>T tubules</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Junctional</td>
<td>0.055</td>
<td>7.6</td>
<td>...</td>
</tr>
<tr>
<td>Nonjunctional</td>
<td>0.061</td>
<td>0.7</td>
<td>...</td>
</tr>
<tr>
<td>Total</td>
<td>0.116</td>
<td>8.3</td>
<td>...</td>
</tr>
<tr>
<td>Sarcoplasmic reticulum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Junctional</td>
<td>0.073</td>
<td>...</td>
<td>92.7 765</td>
</tr>
</tbody>
</table>

Membrane surface areas reported by Page and Surdyk-Droske were expressed as μm²·μm⁻³ tissue by assuming that myocytes occupy 80% of the myocardial volume. The surface area of junctional sarcoplasmic reticulum is identical to that of junctional sarcolemma. The partition of 1,4-dihydropyridine (DHP) receptors between the junctional and nonjunctional sarcolemmal domains was assumed to be 75% and 25%, respectively. All ryanodine (RYA) receptors were ascribed to the junctional sarcoplasmic reticulum.
in 1 g of adult ventricle might be associated with the junctional domain of the sarcolemma, which yields a surface density of about 84 sites/μm². Our estimate of the surface density in the nonjunctional domain, 13 sites/μm², must be considered as an upper limit, because it neglects 1) the possible presence of 1,4-dihydropyridine receptor sites in nonmyocardial cells, which should provide plasma membrane fragments of low equilibrium density, and 2) the possible disruption of some junctional complexes, which would lead to equilibration in low-density fractions of T-tubular fragments enriched in 1,4-dihydropyridine receptors. Although nerve endings or varicosities are too scarce to be taken into account in morphometric studies, capillaries are an important component of ventricular tissue, at least as regards the plasma membrane surface area.37 However, it is generally considered that endothelial cells,38,39 as well as sympathetic nerve endings,40 are devoid of, or at least poorly equipped with, L-type calcium channels. Taking into account the surface area of the junctional and nonjunctional domain in T tubules and external sarcolemma in myocardial cells, their average surface density of 1,4-dihydropyridine receptors would be 43 and 13 sites/μm², respectively. Our estimate of the surface density of 1,4-dihydropyridine receptors in cardiac T tubules, approximately 40/μm², is distinctly lower than previous estimates in skeletal muscle T tubules, which range from 200 to 300/μm².41,42

In 1-day-old rats, the external surface of myocardial cells in left ventricle is higher than in adult rats and has been estimated to be 0.46 μm² · μm⁻³ tissue.4 We found that the number of 1,4-dihydropyridine receptors in neonatal ventricle was 10 pmol g⁻¹ tissue (Table 3). Therefore, the average number of receptor sites per unit membrane surface area would be about 13 μm⁻², a value identical to that estimated for the external sarcolemma in adult ventricle. This estimate, which should be taken as an upper limit (see above), is somewhat higher than the surface density (0.5–5 μm⁻²) of voltage-dependent Ca²⁺ channels in cardiac cells from neonatal rats.43

As shown in Table 5, the surface density of ryanodine receptors in the junctional SR of adult rat ventricle can be estimated to 765 μm⁻². (A similar calculation is not possible for neonatal rat ventricle because we do not know the surface area of junctional SR in that case.) The surface density of ryanodine receptors in the junctional SR of skeletal and cardiac muscle might be of the same order of magnitude, as inferred from the B₅₀ of [³H]ryanodine binding in heavy SR fractions.25 According to the model proposed by Bock et al44 (their Figure 11), the upper limit for the surface density of feet in skeletal muscle junctional SR would be 1,070 μm⁻². Thus, our data support the idea that a tetrameric foot contains only one high-affinity ryanodine receptor.45 In junctional complexes of skeletal muscle SR, the ultrastructural observations of Bock et al44 suggest that the surface density of 1,4-dihydropyridine receptors might be twice that of feet. According to our data, this stoichiometric ratio would be much lower (approximately 0.1) in the junctional T-tubular domain of rat ventricle.

**Functional Implications**

Postnatal changes in receptor number and localization are accompanied by profound modifications in excitation-contraction coupling, as evidenced by changes in the sensitivity of systolic contraction to external Ca²⁺ (see Reference 2) and to modulators of Ca²⁺ channels (Figure 7). Our contractility data agree with those reported recently by Tanaka and Shigenobu46 on rat papillary muscle. These authors observed that both ryanodine and the calcium antagonist nicardipine were able to completely inhibit systolic contraction of adult tissue, whereas in neonatal tissue, part of the contraction (approximately 40%) was resistant to ryanodine. Moreover, neonatal tissue displayed a lower sensitivity to ryanodine than adult tissue but a higher sensitivity to nicardipine (IC₅₀ of approximately 0.3 versus approximately 2 μM).

The difference in the response of neonatal and adult tissue to ryanodine has been interpreted as indicating a postnatal shift from extracellular Ca²⁺ to SR Ca²⁺ stores as a source of activator Ca²⁺.3,46,47 This view is in line with the postnatal proliferation of SR4 and the marked increase in the number of ryanodine receptors that we have observed. Although most of the activator Ca²⁺ in adult tissue comes from the SR, its release is triggered and largely controlled by Ca²⁺ entry via the process of Ca²⁺-induced Ca²⁺ release.3,48 The difference in sensitivity to 1,4-dihydropyridines between adult and neonatal ventricle is not attributable to a difference in the affinity of the receptor, as indicated by binding data. As the plateau phase of the action potential is especially short in adult rat ventricle but much longer in neonatal tissue,1 a potency difference is not unexpected in view of the voltage-dependence of the interaction of 1,4-dihydropyridine inhibitors with L-type Ca²⁺ channels and the relatively slow kinetics of this interaction.49,50 Furthermore, comparison of Iᵦ and the [Ca²⁺], transient in adult rat ventricular myocytes suggests that only a small Ca²⁺ current is needed to initiate Ca²⁺ release and that not all the Ca²⁺ current is needed to achieve maximal release.51 It is likely that the spatial concentration of the channels involved in Ca²⁺ entry and release at the level of the junctional areas, as shown in this study, favors the amplification mechanism of Ca²⁺-induced Ca²⁺ release in adult rat ventricle, and thereby renders contraction less sensitive to inhibition of Ca²⁺ entry. The relative importance of direct activation of myofilaments by inflowing Ca²⁺ in other species (e.g., rabbit52) might possibly be related to the existence in those species of a relatively higher density of Ca²⁺ channels at the cell surface (see Doyle et al52). Nevertheless, even in adult rat ventricle, because Ca²⁺ entry is required for Ca²⁺ release, it is not surprising that calcium antagonists
are able to block contraction when used at appropriately high concentrations.

Although 1,4-dihydropyridine receptors of adult rat ventricle are concentrated in junctional areas, the stoichiometric ratio between these receptors and ryanodine receptors in junctional areas appears rather low (approximately 0.1). This suggests that a physical link between these receptors is not likely to be involved in the regulation of SR Ca\(^{2+}\) release in cardiac muscle, in contrast with the situation prevailing in skeletal muscle, where such a physical link is essential to the functioning of 1,4-dihydropyridine receptors as voltage sensors.\(^{53}\)

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**KEY WORDS** • 1,4-dihydropyridine • ryanodine • T tubule • sarcoplasmic reticulum • rat ventricle
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