Morphological, Biochemical, and Electrophysiological Characterization of a Clonal Cell (H9c2) Line From Rat Heart

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Morphological, electrophysiological, and biochemical properties of H9c2 cells, a permanent cell line derived from rat cardiac tissue, were studied. Although the lectin binding pattern revealed similar sugar residues in the surface coat of H9c2 cells and isolated rat cardiocytes, heart-specific morphological structures could not be detected in H9c2 cells. Under physiological ionic conditions, H9c2 cells exhibited an outwardly rectifying, transient K⁺ current. When this current component was blocked by Ba²⁺ and Cs⁺, we observed an inward current through Ca²⁺ channels (15.8±2.2 pA/pF, n=18, measured as Ba²⁺ current) that showed all characteristics of cardiac L-type currents. The activation kinetics were fast, and the current was stimulated by isoproterenol. The effect of isoproterenol was mimicked by forskolin or intracellularly applied cAMP. In radioligand binding experiments, we identified a specific, saturable, stereoselective and reversible high-affinity [³²P]-[+]PN 200-110 binding with a dissociation constant K_d=0.53±0.28 nM and a maximal specific binding of B_max=129.3±16.1 fmol/mg protein. There was an additional low-affinity/high-capacity binding site, which is unlikely to be related to a Ca²⁺ channel protein. Signal-transducing G proteins in membranes were characterized by [³²P]ADP-ribosylation catalyzed by bacterial toxins and by the use of various antibodies. Cholera toxin substrates of 42 and 45 kd were identified that apparently correlated to Gα subunits. Pertussis toxin substrates of 40–41 kd were tentatively identified as Gα subunits. The G protein Ga was absent or at least extremely low in concentration. (Circulation Research 1991;69:1476–1486)

During the last decade, enzymatically dispersed cardiocytes have gained great importance for electrophysiological and biochemical studies (for review see Reference 1). Permanent cell lines are commonly used as model systems for many cell types, but until now studies have not been done on permanent cardiac cell lines. The reason may be the difficulty in establishing such a cell line, because freshly isolated cells are unable to multiply in culture and cardiac tumors are extremely rare. Nevertheless, Kimes and Brandt3 succeeded in breeding cells of cardiac origin by selective serial passaging. The established H9c2 cell line is a subclone of the original clonal cell line derived from embryonic BDIX rat heart tissue. In their original paper, Kimes and Brandt3 claimed that H9c2 cells had adopted features of skeletal muscle because the cells expressed nicotinic receptors and synthesized a muscle-specific creatine phosphokinase isoenzyme when the mononucleated myoblasts fused.

In the present study, we reinvestigated this permanent cardiac cell line, H9c2. We extended the previous investigations of Kimes and Brandt3 by characterizing lectin binding pattern, membrane currents under voltage-clamp conditions, 1,4-dihydropyridine binding sites, and the signal-transducing G proteins. The voltage-dependent Ca²⁺ channel current of these cells showed cardiac-specific characteristics. Therefore, the H9c2 cell line may provide a new tool to study the action of class 4 antiarrhythmic drugs and cardiotoxic hormones and neurotransmitters on the Ca²⁺ current.

Materials and Methods

Culturing Conditions

H9c2 cells (ATCC CRL1446, cardiac myoblasts from rat, passage numbers 16–21) purchased from the American Type Culture Collection, Rockville,
Md., were grown at a density of about 10^6 cells/cm² and cultured as monolayers in Dulbecco’s modified Eagle’s medium supplemented with fetal calf serum (10% [vol/vol]), glutamine (2 mM), nonessential amino acids (1%), penicillin (100 IU), and streptomycin (100 µg/ml) under an atmosphere of 5% CO₂ in air saturated with water vapor at 37°C. The medium was replaced by fresh medium every 2 days.

Light and Electron Microscopy

For counting the number of nuclei as well as for determination of lectin binding sites, we used an inverted microscope (IM 35, Zeiss, Oberkochen, FRG) equipped with epifluorescence illumination. Staining of the nuclei was performed on permeabilized and fixed H9c2 cells for 30 minutes in 0.5 mmol/ml 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). To characterize the sugar residues in the surface coat, H9c2 cells were incubated with various lectins (50 µg/ml for 30 minutes at 37°C) that were coupled to rhodamine isothiocyanate (Medac, Hamburg, FRG).

For electron microscopy, H9c2 cells were fixed with 2.5% (wt/vol) glutaraldehyde in 100 mM Na⁺ cacodylate buffer or 0.14 M PIPES for 1 hour at room temperature (pH 7.2), postfixed in 1% (wt/vol) OsO₄ in 100 mM Na⁺ cacodylate, dehydrated in a graded series of ethanol, and embedded in Spurr’s low-viscosity epoxy resin (ERL-4206, Serva, Heidelberg, FRG). Ultrathin sections (70–100 nm) were cut on an LKB Ultrotome (LKB, Uppsala, Sweden) and stained with 1% (wt/vol) uranyl acetate in 70% (vol/vol) ethanol for 30 minutes at room temperature. The sections were examined using a Siemens 101A electron microscope at 60 or 80 kV.

Electrophysiology

For electrophysiological examinations, H9c2 cells were cultured for 2–6 weeks on small glass slides (4×5 mm), until they reached a density of about 10³ cells/mm². The whole glass slide was transferred into a small chamber (volume about 200 µl) mounted on an inverted microscope. The chamber was continuously perfused with Tyrode’s solution (E1) containing (mM) NaCl 140, CaCl₂ 1.8, MgCl₂ 1, KCl 5.4, glucose 10, HEPES 10 (pH 7.4) at 36°C. For determination of inward currents, K⁺ currents were blocked by Ba⁺⁺ and Cs⁺ in the external solution (E2) containing (mM) NaCl 125, BaCl₂ 10.8, MgCl₂ 1, CsCl 5.4, glucose 10, HEPES 10 (pH 7.4) at 36°C. Under these conditions Ba⁺⁺ was the divalent charge carrier permeating Ca²⁺ channels. Patch electrodes were prepared from Pyrex glass capillaries according to Hamill and coworkers; the capillaries had an inside tip diameter of 1–3 µm and a resistance of 3–6 MΩ when filled with pipette solution 11 containing (mM) K⁺ aspartate 80, KCl 50, MgCl₂ 1, EGTA 0.1, MgATP 3, HEPES 10 (pH 7.4) or solution 12 containing (mM) CsCl 120, MgCl₂ 3, MgATP 5, EGTA 10, HEPES 5 (pH 7.4).

Gigaohm seals were formed by suction with a negative pressure of about −30 cm H₂O, and whole-cell clamp configuration was achieved by disruption of the membrane patch. Under voltage-clamp conditions, cells were step-depolarized from a holding potential (usually −50 mV) to various test potentials (duration, 300 msec; stimulation rate, 0.5 Hz). Ba²⁺ inward currents through Ca²⁺ channels were determined as peak inward currents, outward currents as current at the end of the 300-msec test pulses. The membrane capacity, measured as current response to a ramp pulse, amounted to 21.1±2.7 pF (mean±SD, n=34). Assuming a cell surface of 1,880 µm² (calculated from the microscopically determined cell diameter of spherical cells, 24.5±2.8 µm [mean±SD, n=31], an underestimation by 10–20% has to be taken into account because of membrane enlargements below light microscopical resolution), the specific membrane capacity was 1.2 µF/cm², which is in quite good agreement with values obtained from other cells. The input conductance determined as ohmic conductance in the current–voltage relations amounted to 0.5±0.17 nS (mean±SD, n=17, measured under solution E2 supplemented with 5 mM Ni²⁺ and 1 µM tetrodotoxin).

The following pharmacological tools were used: D600 (1 µM; Knoll, Ludwigshafen, FRG), PN 200-110 (1 µM; Sandoz, Basel, Switzerland), Bay K 8644 (1 µM; Bayer, Leverkusen, FRG), ω-conotoxin (0.1 µM; Bissendorf, Hannover, FRG); isoproterenol (0.1 µM; Sigma, Deisenhofen, FRG), and forskolin (1 µM; Calbiochem, Frankfurt, FRG).

Preparation of Membranes and Binding Studies

H9c2 cells were harvested with a cell scraper, collected by centrifugation at 600g (4°C, 20 minutes), and disrupted by nitrogen cavitation in a buffer consisting of (mM) NaCl 100, EDTA 0.5, KH₂PO₄ 50 (pH 7.0). Immediately after cell disruption, the EDTA concentration was increased to 3 mM, and 2-mercaptoethanol was added to a final concentration of 15 mM. Nuclei were removed by short centrifugation at 1,000g (4°C), and sedimentation of membranes was achieved by centrifugation at 30,000g (4°C, 15 minutes). Total protein was determined according to Peterson. Membranes were stored at −70°C in 10 mM triethanolamine-HCl buffer (pH 7.4).

Dihydropyridine binding assays were performed according to Glossmann and Ferry with modifications. For the saturation experiments, membranes were incubated (37°C, triplicates) in 0.2 ml of Tris-HCl (50 mM, pH 7.4) buffer containing CaCl₂ (1 mM), MgCl₂ (1 mM), and increasing concentrations of [³H]-(+)-PN 200-110. For competition experiments, increasing concentrations of nonradioactive 1,4-dihydropyridines were added in the presence of 0.25 nM [³H]-(+)-PN 200-110. When the binding equilibrium was reached after 30 minutes, 2 ml ice-cold buffer (20 mM Tris-HCl, 10% [wt/vol] polyethylene glycol 6000, 10 mM MgCl₂) was added to each tube, and samples...
were rapidly filtered under vacuum through Whatman GF/B glass fiber filters. Filters were washed twice and dried under vacuum; the radioactivity was measured by liquid scintillation counting. Nonspecific binding was determined in the presence of 2 μM (+)PN 200-110. The specific binding was at least 80% of the total binding. Curves represent best fits as calculated by the iterative, nonlinear least-squares method of De Lean and coworkers.10

[123P]ADP-Ribosylation

Pertussis toxin– and cholera toxin–catalyzed [32P]ADP-ribosylation of G proteins, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and autoradiography of gels were performed as described11–13 with the following modifications. Pertussis toxin was preactivated in the presence of 1 mM ATP, and the incubation mixture contained 0.1% (wt/vol) Lubrol PX. To resolve subtypes of G protein α-subunits, polyacrylamide slab gels were composed of 9.6% (wt/vol) acrylamide, 0.25% (wt/vol) bisacrylamide, and 4 M urea.14

Immunoblotting Experiments

Sodium dodecyl sulfate–polyacrylamide slab gels used for immunoblots were composed as those used to separate [32P]ADP-ribosylated proteins (see above). Immunoblotting, detection of filter-bound antibodies with 125I-protein A, and autoradiography of nitrocellulose filters were performed as described.11,15 The sequences of peptides used for immunization, their coupling to keyhole limpet hemocyanine, immunization of rabbits, and the characterization of antisera generated against these peptides (with G proteins purified from various tissues) have been described elsewhere.12,13

Results

Light Microscopy Studies

H9c2 cells grown in culture dishes or on glass coverslips developed different forms: polygonal (Figures 1C–1E) and spindle-shaped flat cells, which were attached to the bottom at different spots, as well as spherical (Figures 1A, 1C, and 1E) and angular cells (Figure 1B), which were located above the level of the flat cells and in contact to the bottom by a few long cytoplasmic filopodia. The spindle-shaped cells were frequently arranged in parallel. All cells were mechanically quiescent. Evaluation of DAPI-stained cells revealed 95.3% mononucleated, 4.3% binucleated, 0.35% trinucleated, and 0.05% tetranucleated cells (n=4,033). Most nuclei appeared spherical and contained one to several nucleoli (Figures 1D and 1E; see also Figure 2A).

For characterization of the sugar residues of the cell surface, lectins were tested for their ability to bind to the cells (Table 1). Since both Limax flavus agglutinin (Figure 1B) and wheat germ agglutinin (Figure 1C) labeled the cells, sialic acid appears to be a prominent sugar residue. Fluorescence comparable to that achieved with Limax flavus agglutinin was observed with concanavalin A (Figure 1A) and Ricinus communis agglutinin-I (not shown), indicating the presence of α-mannose and galactose. The lectins Dolichos biflorus agglutinin, soybean agglutinin, peanut agglutinin, and Ulex europaeus agglutinin-I failed to bind to the cells (Figure 1E). In addition to the homogeneous binding to the cell surface, a spotted fluorescence was obtained in the case of succinylated wheat germ agglutinin and Ricinus communis agglutinin-I (Figure 1B). In general, the spherical cells exhibited a brighter fluorescence than the polygonal ones, possibly because of differences in geometry.

Electron Microscopy Studies

At low magnification, isolated H9c2 cells of polygonal type appeared in the cross section widely spread with numerous protrusions (Figure 2A). Cells grown in a confluent layer overlapped in their peripheral parts (Figure 2D) and formed two types of junctions: 1) the membranes of neighboring cells were indented and ran in parallel at a distance of less than 40 nm (not shown); and 2) the membranes were attached to each other (around 14 nm distance), forming spot desmosome–like contacts. Gap junctions were not observed (280 studied sections). The most prominent cell organelles were spherical or lobed nuclei (Figure 2A). In all cell types except the spherical ones, the cytoplasm was interlaced by a network of stress fibers16 (Figure 2B) attached to the cell membrane. Another element of the cytoskeleton was microtubules, which were sometimes arranged as a dense network (Figure 2F). The cells were rich in mitochondria and rough endoplasmic reticulum. Both were often parallel to each other (Figure 2G). Golgi cisternae were regularly found near the cell nucleus. The surface membrane was often enlarged by microvilli (Figure 2C). On the extracellular side, the cell membrane was partially covered by a surface coat (Figure 2H). Caveolae were absent.

Membrane Currents

Membrane currents of H9c2 cells were recorded in the whole-cell configuration during voltage-clamp pulses from −50 mV to various test potentials. With the external solution E1 and the internal solution I1, cells exhibited outwardly rectifying currents, which developed a transient inactivation during the 300-msec test pulse. The outward current–voltage relation could be approximated by two straight lines with slope conductances of 0.6 and 7.2 nS, negative and positive to −20 mV, respectively (results not shown; for further characterization see Reference 17). No inwardly rectifying K+ current could be detected.

Inward currents through voltage-dependent Ca2+ channels were determined as Ba2+ currents under blockade of K+ currents (solutions E2 and I2) and Na+ currents (0.1 μM tetrodotoxin). H9c2 cells that were cultured for several days on glass coverslips showed no voltage-dependent current under these conditions. The only detectable current was an ohmic component that may have been due to nonspecific cation channels (see
REFERENCES 17). However, if the cells were cultured for longer periods of time (e.g., several weeks), voltage-dependent calcium current became detectable, suggesting that some kind of autocrine factor is necessary for the expression of this type of channel. The shape of currents (Figure 3A) was similar to that observed in freshly prepared ventricular cardiocytes from the guinea pig. The activation occurred with a fast time course (1–2 msec) in contrast to the time course of activation in skeletal muscle (about 50 msec; see references 19 and 20). The inactivation time constant was about 150 msec at 10 mV. Currents recorded at potentials positive or negative to this maximum declined with a slower time course. The current–voltage relation was bell shaped (Figure 3B). The current had an apparent threshold at $-28.6 \pm 1.8$ mV, a maximum at $8.3 \pm 1.9$ mV, and an apparent reversal potential at $46.1 \pm 2.2$ mV ($n=18$; estimated according to Reference 21). The maximum mean peak current density (measured at 10 mV) was $15.8 \pm 2.2$ pA/pF ($n=18$). The steady-state
inactivation curve was determined by clamping the cells from various prepotentials to the test potential of 0 mV. The inward currents measured during the test pulse were normalized to the maximum current and plotted versus the holding potential (Figure 3D, triangles). Original recordings for current tracings are shown in Figure 3C. The average inactivation curve measured from 18 cells showed a half-maximal value at -21.3 mV and a steepness of 7.2. From the data shown in panel B, the average activation curve of the inward current was evaluated and normalized in the same way as the inactivation curve (Figure 3D, triangles). It reached 50% at -3.6 mV and had a steepness of 6.9.

**Effects of Ca\(^{2+}\) Channel Blockers and \(\beta\)-Adrenergic Agonists**

The inward current of H9c2 cells was further characterized using pharmacological tools (mean±SD of the Ba\(^{2+}\) current densities are summarized in Table 2). In the experimental results shown in Figure 4, cells were voltage-clamped from -50 to 0 mV. Superfusion with the inorganic Ca\(^{2+}\) channel blocker Cd\(^{2+}\) (0.1 mM) resulted in a complete blockage of inward currents (panel A). Similar effects were seen with 0.1 mM Ni\(^{2+}\) and 1 mM Co\(^{2+}\) (not shown). A Ca\(^{2+}\) channel blocker of the phenylalkylamine type, D600 (1 μM), and a Ca\(^{2+}\) channel blocker of the dihydropyridine type, PN 200-110 (1 μM), partially reduced the inward current (panels C and D). An increase was seen with 1 μM Bay K 8644, a Ca\(^{2+}\) channel agonist of the dihydropyridine type (panel B). o-Conotoxin, which was previously reported to block N-type Ca\(^{2+}\) channels of neuronal but not L-type Ca\(^{2+}\) channels of muscular origin,\(^{22,24}\) did not affect the Ba\(^{2+}\) current of H9c2 cells (0.1 μM; see Table 2).

The L-type Ca\(^{2+}\) current of cardiac cells is augmented by \(\beta\)-adrenergic agonists via cAMP-dependent phosphorylation (for review see Reference 25). To test whether the current through Ca\(^{2+}\) channels in H9c2 cells is also sensitive to \(\beta\)-adrenergic stimulation, cells were superfused with 0.1 μM isoproterenol (Figure 5, left panel). Isoproterenol caused about a twofold increment of the inward current within 2 seconds. The effect was fully reversible after washing out the agonist. In two of 27 cells, a reversible inhibition of the Ba\(^{2+}\) inward currents was observed. The reason for this inhibitory effect is unknown.

To elucidate whether cAMP is involved in the \(\beta\)-adrenergic stimulation of the Ba\(^{2+}\) current in H9c2 cells, the adenylyl cyclase stimulator forskolin\(^{26,27}\) (1 μM) was applied from the outside (Figure 5, right panel). The observed increment was comparable to that caused by isoproterenol. An increment of the inward currents was also observed when cAMP (0.1 mM) was intracellularly infused via the patch pipette (see Table 2). At the applied concentrations, the effects of isoproterenol, forskolin, and cAMP were not additive.

**1,4-Dihydropyridine Binding Sites**

Among radiolabeled 1,4-dihydropyridines, which are commonly used to determine specific binding to the \(\alpha_1\)-subunit of voltage-dependent Ca\(^{2+}\) channels, \(^{[3H]}(+)-\)PN 200-110 is known as a radioligand with high affinity for the dihydropyridine-sensitive Ca\(^{2+}\) channels, especially in heart (\(K_d\leq1\) nM) and brain and with lower affinity in skeletal muscle.\(^{9,28,29}\) In saturation experiments with \(^{[3H]}-(+)\)PN 200-110, we detected high- and low-affinity binding sites (Figure 6A) with \(B_{max}\) values of 129.3±16.1 and 986.3±113.0 fmol/mg protein and \(K_d\) values of 0.53±0.28 and 31.7±2.0 nM, respectively (mean±SEM, \(n=4\)). In competition experiments with 0.25 nM \(^{[3H]}-(+)\)PN 200-110 (Figure 6B), we measured IC\(_{50}\) values of 0.31±0.16, 87.0±8.6, and 6.7±1.4 nM for (+)PN 200-110, (–)PN 200-110, and nimodipine, respectively. We calculated an affinity ratio of 280 for (+)PN 200-110 versus (–)PN 200-110 and of 22 for nimodipine versus (+)PN 200-110. Similar high-affinity \(K_d\) and \(B_{max}\) values and affinity ratios for the (+) and (–) isomers of PN 200-110 have been reported for rat heart membranes and rat heart primary cell cultures.\(^{30,31}\)

**Pattern of G Proteins**

We also investigated the pattern of membranous signal-transducing heterotrimeric G proteins.\(^{32}\) Their
α-subunits are substrates of ADP-ribosylating bacterial toxins (i.e., cholera toxin and pertussis toxin). In H9c2 cell membranes, cholera toxin mainly \([^{32}\text{P}]\)ADP-ribosylated a 42-kd protein and much less a 45-kd protein. In cholate extract from brain membranes used for comparison, cholera toxin \([^{32}\text{P}]\)ADP-ribosylated equally well 45- and 42-kd proteins. The data suggest that H9c2 cells contain two forms of the

**FIGURE 2.** Transmission-electron microscopic images of H9c2 cells. Panel A: Section through the cell body of a flattened cell; prominent elements are the nucleus with nucleoli (n), mitochondria (dark spots), vacuoles, and cytoskeletal fibers. Bar, 5 μm. Panel B: Cytoskeletal fibers. Bar, 1 μm. Panel C: Microvilli on the cell surface. Bar, 1 μm. Panel D: Cross section through a densely grown cell layer. The upper and lower cells are connected by a mechanical cell contact (arrowhead). Bar, 1 μm. Panel E: Mechanical cell contact shown at higher magnification. Bar, 0.2 μm. Panel F: In some regions of the cells numerous microtubules appear as a network of thin lines. Bar, 2 μm. Single microtubules are illustrated in the inset (bar, 0.1 μm). Panel G: Rough endoplasmic reticulum is often in parallel with mitochondria. Bar, 1 μm. Panel H: The membrane is partly covered by a fuzzy coat. Bar, 0.6 μm.
TABLE 2. Modification of the Ca\textsuperscript{2+} Current of H9c2 Cells by Various Agents

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>Before drug application</th>
<th>After drug application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bay K 8644</td>
<td>1.0 (\mu)M</td>
<td>17.5±1.2</td>
<td>29.1±2.1 (n=5)</td>
</tr>
<tr>
<td>(\text{Ca}^{2+})</td>
<td>0.1 mM</td>
<td>15.7±2.1</td>
<td>0.4±0.8 (n=5)</td>
</tr>
<tr>
<td>D600</td>
<td>1.0 (\mu)M</td>
<td>15.0±2.4</td>
<td>0.6±0.3 (n=4)</td>
</tr>
<tr>
<td>PN 200-110</td>
<td>1.0 (\mu)M</td>
<td>14.1±1.9</td>
<td>0.9±0.4 (n=6)</td>
</tr>
<tr>
<td>(\omega)-CT</td>
<td>0.1 (\mu)M</td>
<td>14.6±2.2</td>
<td>13.7±2.1 (n=6)</td>
</tr>
<tr>
<td>ISP</td>
<td>0.1 (\mu)M</td>
<td>12.9±1.6</td>
<td>25.0±1.5 (n=7)</td>
</tr>
<tr>
<td>Forsk</td>
<td>1.0 (\mu)M</td>
<td>16.5±1.6</td>
<td>30.6±1.5 (n=3)</td>
</tr>
<tr>
<td>cAMP (intra)</td>
<td>0.1 (\mu)M</td>
<td>13.9±2.3</td>
<td>25.9±2.1 (n=4)</td>
</tr>
</tbody>
</table>

Values (given in pA/pF) represent mean±SD. \(\omega\)-CT, \(\omega\)-conotoxin; ISP, isoproterenol; Forsk, forskolin; intra, intracellulary applied.

G, \(\alpha\)-subunit.\textsuperscript{33} Pertussis toxin catalyzed \([^{32}\text{P}]\text{ADP-ribosylated of a 40/41-kd doublet in H9c2 cell and brain membranes. Only the latter preparation contained a 39-kd pertussis toxin substrate.}

For identification of pertussis toxin substrates, the two preparations were probed with antisera generated against synthetic peptides that were derived from sequences of cDNAs encoding G protein \(\alpha\)-subunits (Figure 7). Since modification of G protein \(\alpha\)-subunits by bacterial toxins changes their mobility in urea-containing sodium dodecyl sulfate gels,\textsuperscript{14} the relative molecular masses of \([^{32}\text{P}]\text{ADP-ribosylated G protein \(\alpha\)-subunits (see Figure 8) differ from those of the unmodified \(\alpha\)-subunits detected by the various antibodies (see Figure 7). The \(\alpha\text{common}\) peptide antiserum, which reacts with \(\alpha\)-subunits of \(G_i\), \(G_o\), and \(G_s\), recognized 40-kd proteins in membranes of H9c2 cell and 39–41-kd proteins in cholate extract from membranes of porcine brain. The \(\alpha\text{common}\) antiserum did not detect proteins corresponding to the 42- and 45-kd cholera toxin substrates; this observation may be due to the low abundance of \(G\), \(\alpha\)-subunits compared with that of \(G_i/G_o\), \(\alpha\)-subunits. The \(\alpha\text{common}\) peptide antiserum, which recognizes the \(\alpha\)-subunits of \(G_{15}, G_3\), or \(G_{13}\), recognized 40-kd proteins in membranes of H9c2 cells and in cholate extract from membranes of porcine brain. The finding that the \(\alpha\text{common}\) peptide antiserum and the \(\alpha\text{common}\) Peptide antiserum failed to detect a doublet that may account for the 40/41-kd pertussis toxin substrates found in either preparation is most likely due to the poor resolution of these proteins. An

**FIGURE 3.** Electrophysiological characterization of the Ca\textsuperscript{2+} inward current. Panel A: Recordings of inward currents during 200-msec voltage-clamp pulses from -80 mV to the indicated test potentials. The dotted line corresponds to zero current. Panel B: Current–voltage relation of the \(\text{Ba}^{2+}\) inward current. Mean±SD are shown (n=18). Panel C: Original recordings of inward currents measured during voltage-clamp pulses from various holding potentials to 0 mV. Panel D: Steady-state activation (circles) and inactivation (triangles) curves of 18 cells are shown (mean±SD). Inward currents were measured during 200-msec voltage-clamp pulses from -80 mV to the test potentials varying from -60 to 50 mV.

**FIGURE 4.** Pharmacological characterization of Ca\textsuperscript{2+} inward current. Pulses from -50 to 0 mV were applied. Dotted lines correspond to zero current. Con, control currents. Cells were superfused with \(\text{Cd}^{2+}\) (0.1 mM, panel A), Bay K 8644 (1 \(\mu\)M, panel B), D600 (1 \(\mu\)M, panel C), or PN 200-110 (1 \(\mu\)M, panel D). Tetrodotoxin (0.1 \(\mu\)M) was present in all experiments.
α2 peptide antiserum, which specifically reacts with the 40-kd G2 α-subunit, detected a 40-kd protein in either preparation. The α2 peptide antiserum, which specifically reacts with the two forms of the G2 α-subunit, failed to detect proteins in the H9c2 membranes but clearly detected proteins of 39 and 40 kd in brain cholate extract.

Discussion

In the present study we show that H9c2 cells lack morphological properties of freshly prepared cardiocytes. The cells did not express gap junctions, caveolae, T tubules, or myofibrils with organized sarcomeres, but were rich in rough endoplasmic reticulum, and the cell surface was often enlarged by microvilli. H9c2 cells did not form confluent, multinuclear cells (myotubes) as were observed regularly by Kimes and Brandt. Since the cell density was high enough to allow formation of myotubes, the difference between our cells and those of Kimes and Brandt may point to a dedifferentiation of this cell line caused by increased passage number. Whether the dedifferentiation of H9c2 cells is a progressive process or arrested at this stage needs to be demonstrated by further studies.

To classify H9c2 cells by morphological criteria, they have to be compared with other types of cultured heart cells. According to the literature, cardiocytes of the adult, neonatal, or embryonic heart undergo various changes when cultured for longer periods of time (see, e.g., References 34 and 35). Soon after the isolation procedure heart cells transform from rod-shaped to rounded cells with disorganized ultrastructural features, that is, sarcomeric arrangement is lost, former Z lines appear as focal densities, and mitochondria have compact cristae and are often found in the numerous blebs at the surface. In addition, microvilli are found on the surface of these cells. After the fifth day in culture, nontransformed cells start to reorganize ultrastructure; the cells spread out and build up new myofibrils with complete sarcomeres. This is accompanied by formation of new intercalated disks and T tubules. During this phase of redifferentiation, embryonic cultured cardiocytes build up stress fibers that are progressively replaced by myofibrils. In our study, H9c2 cells did not develop myofibrils but only stress fibers. Thus, the only cultured cells of mammalian heart comparable to H9c2 cells are immature embryonic cardiocytes with stress fibers. It may be concluded that H9c2 cells have lost nearly all morphological characteristics of cultured cardiocytes and have dedifferentiated to a high degree.

In contrast to these discrepancies between H9c2 and freshly prepared cardiocytes, the composition of the surface coat is greatly preserved. The lectins soybean agglutinin and Dolichos biflorus agglutinin, known to preferentially bind to the cell poles of adult

Figure 6. 1,4-Dihydropyridine binding sites. Panel A: Specific [3H]-(+)-PN 200-110 binding to membranes of H9c2 cells. The inset shows a Scatchard analysis. B, bound; F, free. Panel B: Inhibition of [3H]-(+)-PN 200-110 binding by (+)-PN 200-110 (filled circles), (-)PN 200-110 (open circles), and nimodipine (triangles). One of four experiments which yielded similar results is shown. Values represent mean ± SEM of triplicates.
guinea pig cardiocytes, did not bind to adult rat cardiocytes nor to H9c2 cells. Similarly, the reaction of peanut agglutinin was negative in both H9c2 cells and freshly isolated adult rat heart cells. The only difference occurred in the case of *Ulex europaeus* agglutinin-I, which binds to isolated rat cardiocytes to a small extent but failed to bind to H9c2 cells.

Another cell type-specific parameter is voltage-dependent Ca++ channels (for review see References 39 and 40). Cardiac cells exhibit an L-type Ca++ current, which is characterized by a slow time course of inactivation (using Ba+ as charge carrier) and by its sensitivity to organic Ca++ channel blockers of the dihydropyridine and phenylalkylamine type. Furthermore, the open probability is enhanced by β-adrenergic agonists acting via G, and cAMP-dependent phosphorylation (for review see Reference 25). All these properties of cardiac Ca++ channels were found in H9c2 cells, opposing the statement of Kimes and Brandt that H9c2 cells have adopted all features of

**Figure 7.** G protein α-subunits in membranes of H9c2 cells and cholate extract from porcine brain membranes. H9c2 cell membranes (150 μg protein) or cholate extract from porcine brain membranes (30 μg protein) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of urea and transferred onto nitrocellulose filters. Filters were incubated with an α_common peptide antiserum AS8 (α_common), an α_common peptide antiserum AS19 (α_common), an α2 peptide antiserum AS64 (α2), and an α peptide antiserum AS6 (α). Dilutions of antisera were 1:150 to 1:300. After the incubation with 125I-protein A, filterbound antibodies were visualized by autoradiography. One of three experiments that yielded comparable results is shown. As was tested in various systems (membranes and purified proteins), the interaction of antisera with proteins in the 40-kd region was blocked or considerably reduced if the antisera had been incubated with the respective peptide (5 μg/ml) used for immunization. The experiment was performed in duplicate. DF, dye front.

**Figure 8.** Cholera and pertussis toxin substrates in H9c2 cell membranes and cholate extract from porcine brain membranes. Cholate extract from porcine brain membranes was prepared as described. H9c2 cell membranes (100 μg protein) or cholate extract from porcine brain membranes (30 μg protein) were incubated with [32P]NAD and, if indicated, with cholera toxin (CT) or pertussis toxin (PT). [32P]ADP-ribosylated proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of urea and visualized by autoradiography. One of three experiments that yielded comparable results is shown. DF, dye front.
a skeletal muscle cell line. The L-type Ca\(^{2+}\) current of skeletal muscle activates more than one order of magnitude more slowly and inactivates very slowly with a time constant of about 1 second.\(^{41}\) The difference between the Ca\(^{2+}\) current of cardiac and skeletal muscle has been related to the different mechanisms of depolarization-contraction coupling. Whereas in cardiac cells the L-type Ca\(^{2+}\) current represents the major pathway for entry of extracellular Ca\(^{2+}\) and subsequently triggers the release from the sarcoplasmic reticulum, depolarization of the skeletal muscle releases Ca\(^{2+}\) from the sarcoplasmic reticulum without the requirement for entry of extracellular Ca\(^{2+}\). The dihydropyridine-sensitive L-type Ca\(^{2+}\) current of other cell types, such as smooth muscle, neuronal, and endocrine cells, exhibits kinetics similar to that found in H9c2 cells but lacks a regulation by cAMP-dependent phosphorylation.\(^{39,40}\) A specific feature of the neuronal and endocrine Ca\(^{2+}\) current is its sensitivity toward \(\omega\)-conotoxin.\(^{22-24}\)

Another argument for the similarity of H9c2 cells to cardiocytes comes from binding studies. 1,4-Dihydropyridine binding sites showed all heart-specific characteristics, that is, much lower capacity and higher affinity than in skeletal muscle tissues.\(^{28}\) The use of the enantiomeric pair of (+) and (−)PN 200-110 in our binding assays reveals strong stereoselectivity for the pure radioligand [\(^{3}H\)](+)-PN 200-110. Low-affinity binding sites for the 1,4-dihydropyridines, some of which are discussed to be functionally relevant in heart, have been reported by several laboratories.\(^{42,43}\) However, in saturation experiments, we found that EGTA (1 mM) added to the incubation medium or heating of membranes (56°C, 10 minutes) strongly diminished the [\(^{3}H\)](+)-PN 200-110 high-affinity binding site but did not affect the \(K_a\) or \(B_{max}\) values of the low-affinity binding site (unpublished data). Therefore, we consider the high-affinity [\(^{3}H\)](+)-PN 200-110 binding site reported here as the 1,4-dihydropyridine-sensitive Ca\(^{2+}\) channel.\(^{44,45}\)

From their pattern of G proteins, H9c2 cells show all characteristics of a striated muscle cell.\(^{46}\) H9c2 cells possess two forms of the G\(_{\alpha}\)-subunit and two forms of the G\(_{\beta\gamma}\)-subunit, one of which apparently corresponds to the \(\alpha\)-subunit of G\(_{\alpha}\). G\(_{\alpha}\) plays an important role in the signal transduction of cardiotoxic hormones since G\(_{\alpha}\) proteins functionally couple \(\beta\)-adrenergic receptors to adenylate cyclase and possibly also directly to Ca\(^{2+}\) channels.\(^{25,32}\) G\(_{\alpha}\) proteins of the G\(_{\alpha}\) family are involved in the hormonal inhibition of adenylate cyclase and stimulation of \(\kappa\) channels in atrial cardiocytes. H9c2 cells lack G\(_{\alpha}\), which is apparently involved in the inhibition of Ca\(^{2+}\) current in neuronal and endocrine cells (for review see Reference 47). In line with this observation we found no hormone or neurotransmitter (e.g., acetylcholine, epinephrine, somatostatin) that significantly reduced the amplitude of the Ca\(^{2+}\) channel current in H9c2 cells. The data are consistent with the finding that adult cardiac myocytes from rat do not (or at very low levels) express G\(_{\alpha}\).\(^{46,48}\)

In conclusion, the present study demonstrates that H9c2 cells show morphological characteristics similar to those of immature embryonic cardiocytes but have preserved several elements of the electrical and hormonal signal pathway found in adult cardiac cells. Therefore, this cell line may be useful as a model for cardiocytes in aspects of transmembrane signal transduction.

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