Inhibition of Gap Junctional Conductance by Long-Chain Acylcarnitines and Their Preferential Accumulation in Junctional Sarcolemma During Hypoxia

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Electrophysiological and biochemical sequelae of myocardial ischemia occur within minutes of the onset of myocardial ischemia in vivo. Both conduction delay and conduction block occur rapidly within the same time interval as the accumulation of long-chain acylcarnitines. In the present study, double whole-cell voltage-clamp procedures were used to assess the influence of long-chain acylcarnitines on gap junctional conductance in isolated pairs of canine ventricular myocytes. Long-chain acylcarnitine (5 μM) decreased gap junctional conductance from 153 to 48 nS in a time-dependent and reversible manner. Although the amplitude of junctional current was reduced by 68%, the current continued to demonstrate a linear current–voltage relation. The extent of endogenous accumulation of long-chain acylcarnitines in junctional regions of the sarcolemma was assessed in isolated myocytes in which endogenous free, short-chain, and long-chain acylcarnitine pools had been equilibrated with [3H]carnitine. Under normoxic conditions, long-chain acylcarnitines were not detectable in junctional sarcolemma of myocytes as assessed using electron microscopic autoradiography. Exposure of myocytes to hypoxia (PO2, <15 mm Hg) for 10 minutes resulted in the preferential accumulation of endogenous long-chain acylcarnitines in junctional sarcolemma (173±5×10⁴ molecules/μm²), a concentration that was sevenfold greater than that found in nonjunctional sarcolemma. Therefore, endogenous long-chain acylcarnitines accumulate preferentially in junctional regions of the sarcolemma during short intervals of hypoxia. Exogenously supplied long-chain acylcarnitines can markedly decrease cellular coupling in a reversible manner, suggesting that this amphiphile may contribute to the marked slowing in conduction velocity in the ischemic heart in vivo, not only by suppressing the rapid Na⁺ inward current directly, as has been shown previously, but also by decreasing cellular coupling. (Circulation Research 1993;72:879–889)

KEY WORDS • arrhythmias • electrical uncoupling • gap junctions • myocardial ischemia

Sudden cardiac death associated with acute myocardial ischemia is often caused by spontaneous malignant ventricular arrhythmias.¹⁻⁵ Detailed three-dimensional intracardiac mapping has revealed that conduction delay and block occur within minutes of the onset of ischemia in vivo, even during normal sinus rhythm, thereby establishing the substrate for development of intramural reentry.³⁻⁴ In addition, studies performed in septal artery–perfused rabbit papillary muscle preparations⁶⁻⁸ and in isolated perfused septal preparations⁹ have demonstrated that a marked reduction in conduction velocity occurs during the early reversible phase of ischemia, which may ultimately lead to cellular electrical uncoupling.

A considerable body of evidence indicates that the rapid and reversible electrophysiological derangements during brief ischemia are caused by transient biochemical alterations at or near the sarcolemma.¹⁰ One of the early biochemical alterations during hypoxia in vitro and ischemia in vivo is the accumulation of long-chain acylcarnitines. Long-chain acylcarnitines have been shown to accumulate 3.5-fold in ischemic myocardium within 2 minutes of the onset of ischemia.¹¹ Inhibition of the accumulation of long-chain acylcarnitines markedly reduces the incidence of ventricular tachycardia or ventricular fibrillation in response to early ischemia in vivo.¹²

We recently demonstrated that isolated adult myocytes subjected to 10 minutes of hypoxia exhibited rapid and selective sarcolemmal accumulation of long-chain acylcarnitines.¹³ Accumulation of this membrane-active metabolite induces a variety of electrophysiological alterations, including suppression of sarcolemmal ion channels.¹⁴⁻¹⁹ However, the direct effect of long-chain acylcarnitines on gap junctional conductance (gj) has not been evaluated. Therefore, the purpose of the
present study was to test the hypothesis that long-chain acylcarnitines decrease g and may thereby contribute to slowing of the conduction velocity in ischemic myocardium. The experiments were designed to determine 1) whether exogenous long-chain acylcarnitines alter g in adult ventricular myocyte pairs and 2) whether endogenous long-chain acylcarnitines accumulate in the junctional regions of sarcolemma in isolated myocytes in response to hypoxia.

**Materials and Methods**

**Isolation of Adult Canine Myocytes**

Viable canine ventricular myocytes were obtained using a perfusion technique as described previously. Briefly, adult mongrel dogs of either sex were heparinized (3,000 units i.v.) and anesthetized with thiopental (30–35 mg/kg i.v.), and then each heart was excised rapidly and placed in cold oxygenated bicarbonate buffer solution consisting of (mM) NaCl 118, KCl 4.8, MgCl2 1.2, glucose 11, K2HPO4 1.2, NaHCO3 25, and CaCl2 1.2, saturated with 95% O2–5% CO2 at pH 7.4. A distal native coronary artery was cannulated and perfused with a nominally calcium-free oxygenated bicarbonate buffer solution (same composition as above without added calcium). The perfused tissue was excised along the blanded borders of the perfused region, placed inside a prewarmed (35–37°C) Nacpo incubator, and perfused from a height of 60 cm (gravity) for 15 minutes with a washout solution gassed with 95% O2–5% CO2 consisting of (mM) NaCl 118, KCl 4.8, MgCl2 1.2, glucose 11, K2HPO4 1.2, NaHCO3 25, and EGTA 0.03 and containing 0.01 mg/ml of 100 microgram medium Eagle amino acids (Flow Laboratories, Inc., McLean, Va.). At the end of the washout period, enzymatic digestion was initiated by perfusion for 30 minutes with a washout solution as indicated above but also containing CaCl2 (35 mM) and 0.1% collagenase (Boehringer Mannheim Corp., Indianapolis, Ind.).

The tissue was then minced into 20 ml fresh enzyme solution and 0.015 mg/ml trypsin for further enzymatic plus mechanical dissociation in a Dubnoff metabolic shaker at a rate of 100 per minute at 37°C for 15 minutes. At the end of the shaking harvest, 0.03 mg/ml trypsin inhibitor was added, and the contents were filtered through 350-μm nylon mesh (Small Parts, Inc., Miami, Fla.). The cell suspension was washed in a HEPES-buffed solution containing (mM) NaCl 133.5, KCl 4.8, MgCl2 1.2, K2HPO4 1.2, HEPES 10, glucose 10, and CaCl2 0.05, pH 7.2 with 1N NaOH. Extracellular [Ca2+] was increased to 500 μM over 1.5 hours. Percoll gradients were used to increase the percentage of elongated cells as described previously.

**Double Whole-Cell Voltage-Clamp Procedure**

Electrophysiological studies were performed by transferring cells to a small 0.5-ml bath mounted on the stage of an inverted microscope (Nikon Diaphot) and superfusing at 35°C with Tyrode’s solution containing (mM) NaCl 137, KCl 4, MgCl2 1, CaCl2 1.8, HEPES 10, and glucose 11, titrated with NaOH to pH 7.4. This extracellular solution also contained (mM) CsCl 4, BaCl2 1, 4-aminopyridine 1, and NiCl2 1 to block nonjunctional currents. In experiments designed to record propagation of action potentials from one cell to another, as shown in Figure 1, channel blockers were excluded from the extracellular solution. Glass pipettes were pulled with a programmable puller (P-87PC, Sutter Instrument Co., Novato, Calif.) and fire-polished with a microforge (Narishige). The intracellular pipette solution contained (mM) CsCl 80, potassium aspartic acid 40, MgCl2 1, HEPES 5, Na2-ATP 5, and EGTA 10, titrated with CsOH to pH 7.2. Under conditions in which the transmembrane action potential was recorded, CsCl was replaced with KCl, and the solution was titrated with KOH to pH 7.2. The whole-cell voltage-clamp procedure was performed using two voltage-clamp amplifiers (an Axoclamp-2A and an Axopatch-1D, Axon Instruments, Inc. Foster City, Calif.). Computer software (pClamp, Axon Instruments) was used to generate the voltage-clamp protocols and to acquire and analyze voltage and current signals. Current and voltage were sampled at 2–4 kHz by a 12-bit resolution analog-to-digital converter (Tecmar Labmaster) using a Zenith 386 computer. Data were also stored on videotape through a PCM-4 VCR adapter (Medical Systems Corp., Greenvale, N.Y.). During the experiments, cells were monitored by a video camera (Javelin) mounted to the microscope.

A junction potential of 10 mV was obtained by measuring the potential difference between the Tyrode’s solution and pipette solution when an Agar-KCl bridge was used as a reference ground and was corrected for in each experiment. The resistance of each pipette was compensated before establishing the seal. Access resistance was considered negligible because the internal pressure of the pipette was negative.

The method used for measuring g is essentially similar to that described by others. Elongated and quiescent cell pairs with significant side-to-side membrane contact were used in the study. An example of the double whole-cell recording procedure is shown in Figure 1. Each cell was connected to its respective amplifier via a suction pipette. The propagation of action potentials between the pair of cells indicated that the cells were well coupled and exhibited normal cellular action potentials. The cell pairs were distinguished from other cells by the presence of contact regions between opposed cells as observed under the inverted microscope. At the end of each experiment, the cell pair from which the recordings were obtained was injured by breaking the seals, resulting in two separate rounded cells and confirming that the two cells were joined in a pair. These criteria have been reported previously by others. The double whole-cell voltage-clamp procedures used in this study are shown in Figure 2. In voltage-clamp mode, when the membrane potential of cell 1 (pulsed cell) was changed and cell 2 (nonpulsed cell) was kept at the common holding potential, the value of potential change in cell 1 divided by the current recorded from cell 2 represented the junctional resistance (rj). The g was then determined by the value of 1/rj. When cell 2 was pulsed, analogous equations were used to obtain g in the opposite direction.

The exogenous long-chain acylcarnitine species used for these electrophysiological studies was palmitoyl-carnitine, the most abundant in ventricular cells. Palmitoyl-carnitine was added to 1 mM extracellular Tyrode’s solution for a final concentration of 5 μM. A Hamilton valve was used to switch perfusion solutions rapidly.
between the control solution and the solution containing long-chain acylcarnitine. To limit "rundown" of \( g_{L} \), initial voltage-clamp data were obtained within 7 minutes of rupturing the cell membranes. During this initial time interval, the current declined modestly (<10% decrease), which was not corrected for in the final result. Canine ventricular myocytes isolated in our laboratory did not exhibit a marked decrease in viability over 24-48 hours when placed in media and incubated as described below. For electrophysiological studies, cells were used within 12 hours after isolation. In our preliminary experiments, \( g_{L} \) values were not significantly different between cell pairs measured within 12 hours of isolation (153.4±25.3 nS) and those measured within 24 hours of isolation (154.0±28.1 nS), suggesting that the \( g_{L} \) in these cell pairs was well maintained in culture media. The resting membrane potential, input resistance, and the maximal current density of the L-type calcium current have also been evaluated in our laboratory and were not significantly different in elongated cells isolated either 12 or 24 hours previously. Thus, the cells used for electrophysiological studies were comparable to those prelabeled with \(^{3}H\)carnitine for 12-18 hours for the electron microscopic autoradiography studies as described below.

**Prelabeling of Cells With \(^{3}H\)Carnitine**

Isolated canine ventricular myocytes were incubated overnight at 35°C in a NaCp 6300 CO\(_2\) incubator in Joklik's modified minimum essential medium containing (mM) CaCl\(_2\) 0.5, NaHCO\(_3\) 23.8, and HEPES 15, along

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**Figure 1.** Isolated canine ventricular cell pair illustrating double whole-cell recording procedure (photomicrograph in center). Each cell of the pair was recorded using a suction pipette. The dimensions of the cells in this pair were 150x22 \( \mu \)m (left cell) and 129x24 \( \mu \)m (right cell), and transmembrane action potentials were obtained from each cell (left and right tracings, respectively) when channel blockers were excluded from extracellular and intracellular solutions. Panel A: Both cells were stimulated simultaneously (arrow). Panels B and C: Only one cell was stimulated (arrow), and the propagated impulse was recorded in the other cell. The dashed line in each action potential tracing indicates the level of 0 mV. The voltage and time scales in panels A, B, and C are the same in each panel, as shown at the right side of panel C.

**Figure 2.** Electrophysiological properties of junctional conductance in a control cell pair. Panel A: Voltage-clamp protocol used to determine the junctional conductance is shown at the top. Cell 1 (pulsed cell) was clamped at potentials from −80 to +60 mV in steps of 20 mV for 240 msec. At the same time, the voltage of cell 2 (nonpulsed cell) was kept at a common holding potential of 0 mV. Superimposed currents obtained from each cell are shown at the bottom. Currents recorded from nonpulsed cell represent the junctional current, which was used to calculate the junctional resistance and conductance (see "Materials and Methods"). Panel B: Graph showing corresponding current (I)–voltage (V) relation from the same cell pair shown in panel A.
with 1% penicillin G/streptomycin and 2% heat-inactivated horse serum, pH 7.4 with 10N NaOH. One million myocytes were incubated in each well in 20 ml media plus 150 μCi [3H]DL-carnitine (72 Ci/mmol). The following day, the myocytes were spun at 45g for 2 minutes to remove media and excess label, washed once with a HEPES-buffered solution (see discussion of cell isolation preparation) containing 500 μM Ca++, and then placed on a discontinuous Percoll gradient to increase the percentage of elongated cells, as described previously.30

**Induction of Hypoxia**

A glucose-free 500 μM Ca-HEPES buffer, pH 7.2, was degassed under vacuum for 1 hour and then bubbled with 100% prepurified N₂ for at least 2 hours to attain a PO₂ of <15 mm Hg. Prelabeled myocytes suspended in 500 μM Ca-HEPES (2 million cells per sample) in a glass vial were washed with glucose-free 500 μM Ca-HEPES and recentrifuged to obtain a loose pellet, which was then subjected to 10 minutes of hypoxia to induce accumulation of endogenous [3H]long-chain acylcarnitines. Room air was exchanged with 100% N₂ delivered into the glass vial for 1 minute. Hypoxic glucose-free 500 μM Ca-HEPES buffer (2 ml) was then transferred to the myocyte pellet via a spring-loaded glass syringe (Figure 3). The 100% N₂ atmosphere was maintained above the hypoxic solution and cells for the entire hypoxic interval. At the end of the hypoxic interval, aliquots of cell suspension were removed for mass measurement of carnitine fractions within the myocytes and for protein determination. The remainder of the myocytes were subsequently processed for electron microscopic autoradiography.

**Mass Measurement of Carnitine Fractions**

Total carnitine, long-chain acylcarnitine, total acylsoluble carnitine, and free carnitine fractions in the myocytes were separated according to the insolubility of long-chain acylcarnitine esters (i.e., those esters with an acyl group of more than 12 carbon atoms) in 7% perchloric acid (PCA) and the use of base-catalyzed hydrolysis of esterified carnitine. Immediately after each experiment, 2-ml aliquots of the cell suspension were added to 200 μl of 70% PCA and mixed well. A 300-μl aliquot of the suspension was removed for assay of the total carnitine fraction. The remaining suspension was centrifuged at 10,000g for 30 minutes, the supernatant was removed (free and short-chain carnitine), and the pellet was washed twice with 2 ml of 70% PCA and resuspended in 500 μl distilled water for assay of the long-chain acylcarnitine fraction. The supernatant and 7% PCA washes were combined, and one half of the total volume was used for assay of free carnitine. A 200-μl volume of 5 M KOH was added to the total carnitine, total acid-soluble carnitine, and long-chain acylcarnitine fractions, which were then hydrolyzed for 90 minutes at 70°C in a water bath to remove the acyl group. Fractions were neutralized by the addition of 1 ml of 1 M HEPES buffer (pH 8.0), and additional 70% PCA or 10N KOH was added as needed to adjust pH 7–8. The KClO₃ precipitate remaining after neutralization was removed by centrifugation of the samples at 10,000g for 30 minutes, after which the precipitates were washed twice with an additional 1-ml volume of distilled water.

Free carnitine was measured in each fraction using a modification of the radioenzymatic method of McGarry and Foster.30 Assays were performed in disposable glass tubes (13×100 mm). The reaction mixture contained (in a volume of 1.0 ml) 120 μmol HEPES buffer (pH 7.4), 1.25 μmol EDTA, 2.0 μmol N-ethylmaleimide, 5.0 nmol (0.04 μCi) [14C]acetyl coenzyme A, and standards or samples to be analyzed containing 50–2,000 pmol free carnitine. The reaction was initiated by the addition of 50 μl of a solution prepared by diluting 200 μl carnitine acetyltransferase (10 mg protein [800 units]) in 2 ml of

**Figure 3. Schematic diagram of the airtight hypoxia apparatus consisting of the following:** 1) a glass vial (containing the cells) with a rubber stopper fitted with a port for continuous delivery of 100% prepurified N₂ and for delivering hypoxic buffer solution to the cells and a vent for releasing continuous positive N₂ pressure, 2) a spring-loaded glass syringe fitted with stainless-steel tubing connected to the hypoxic buffer solution reservoir, a glass vial containing the cells, and a waste receptacle used to purge all the lines of oxygen-contaminated solutions just before adding hypoxic solution to the cells, 3) a hypoxic buffer solution reservoir with gas dispersion tube delivering 100% prepurified N₂, and 4) a 100% prepurified N₂ tank connected to the solution reservoir and cell vial via a series of stopcocks. The spring-loaded syringe automatically fills by aspirating solution through the side port in the direction of the arrows.
2.9 M ammonium sulfate) and 150 μl of 0.83 M HEPES buffer (pH 7.4) to a total volume of 2.0 ml with distilled water. Samples were incubated at room temperature for 60 minutes. After incubation, 850 μl reaction mixture was transferred to a 13×100-mm serum separator tube with a 5-μm filter that contained 700–800 mg acid-washed Dowex AG1-X8 anion exchange resin for removal of unreacted [14C]acetyl coenzyme A from the reaction mixture. The serum separator tubes were placed inside a 13×100-mm glass culture tube to form an airtight seal, thereby allowing the reaction mixtures to filter through the anion exchange resin when the serum separator tube was partially withdrawn. The resin bed was washed with an additional 300-μl volume of distilled water, and the serum separator tube was withdrawn completely and discarded. A 900-μl volume of the filtrate containing the [14C]acetyl [3H]carnitine reaction product was added to 10 ml of 3a70 scintillation cocktail (Research Products International Corp., Mount Prospect, Ill.), and radioactivity in the samples was quantified by liquid-scintillation spectrometry. Sample carnitine concentration was determined from the least-squares linear regression curve from samples of carnitine standards containing 50–2,000 pmol carnitine. All fractions were assayed in triplicate.

Results were normalized according to the protein content of the cell suspension measured by a modification of the method described by Lowry using bovine serum albumin as the protein standard.

Preparation and Analysis of Autoradiographs

Both normoxic and hypoxic myocytes were processed for electron microscopy using a fixation technique that selectively retains and spatially fixes long-chain acylcarnitine while removing short-chain acylcarnitine and free carnitine. Radioactivity removed at each processing step was quantified by liquid-scintillation spectrometry. Autoradiographs were prepared as described previously with a modification of the flat substrate method of Salpeter and Bachmann. Pale gold sections (100 nm thick) were mounted on collodion-coated slides, covered with monolayers of Ilford L4 emulsion, and incubated at 4°C for 106 days. The emulsions were developed in 1.1% p-phenylenediamine and 12.6% sodium sulfite and fixed in 30% sodium thiosulfate as described previously. p-Phenylenediamine is a low efficiency developer that enhances resolution and hence results in very small grains that are not complex or coiled. This increased resolution is important, since the defined subcellular compartments are so small. The enhanced resolution is achieved at the expense of sensitivity of the emulsion. However, in the autoradiographic system used for this study, lowered sensitivity was not a concern. The half distance in this system is that distance from a radioactive point source that defines an area with a 50% probability of containing a developed grain (i.e., the radius of a circle around that source), and for this autoradiographic system, the half distance is 150 nm. A final print magnification of ×12,000 was selected to permit adequate resolution of cellular structures. Composite photographs of entire cells or large portions of cells were made to eliminate biases associated with photographing certain portions of selected cells. Only viable cells were analyzed; all hypercontracted cells were excluded. Hypoxia of 10-minute duration did not induce any significant ultrastructural changes in the myocytes, in agreement with previous findings from our laboratory indicating that this duration of hypoxia does not result in release of cellular enzymes, including lactate dehydrogenase and creatine kinase. A total of 66 composite photographs (33 photographs of 23 normoxic cells and 33 photographs of 16 hypoxic cells) containing 595 (254 normoxic and 341 hypoxic) grains were analyzed. Only viable myocytes were analyzed; all degenerated cells were excluded. The measured grain densities over the total myocyte area were 95 grains/100 μm² for normoxic myocytes and 268 grains/100 μm² for hypoxic myocytes.

Electron micrographs were analyzed by the maximum-likelihood method. This method requires the knowledge of the actual grain positions and the individual organelle geometries on each of the micrographs. Therefore, each enlarged autoradiograph was digitized with the EMA-MAP data acquisition program and segmented into 2.5×10⁶ pixels with the use of a graphics tablet with a resolution of 50 pixels per inch by tracing the boundary of each subcellular structure and locating the grains on each image. The precise location of each grain was defined as the center of the smallest circle surrounding the grain on the autoradiograph. Subcellular structures selected for analysis of radioactivity content included sarcolemma, mitochondria, nucleus, nuclear membrane, and a composite cytoplasmic compartment. This cytoplasmic compartment included all other intracellular structures such as the sarcoplasmic reticulum and other cytoplasmic membrane structures as well as unstructured cytoplasm. The extracellular space between myocytes was defined as a separate compartment, as required by the analytical algorithm. All of the subcellular structures selected for this study were easily identified at ×12,000 magnification.

Organelles that are linear, such as the sarcolemma or nuclear membrane, are “traced” from the photograph mounted on the digitizing pad using a “mouse” and are expressed as a line. Organelles that occupy a region, such as the mitochondria, are traced around their boundary, connecting the first and last points and thereby creating a closed contour. Verification of the position of organelles on the photograph can be made simultaneously through visual comparison of the video display. The information inherent in the relative juxtaposition of grains and organelle structures was used to compute grain–organelle probabilities by the maximum-likelihood method and estimate the radiolabel concentration in each organelle studied. For each grain and for each organelle type on an image, a grain–organelle probability was computed. This probability depends on the half distance (which depends on the particular radioactive label and the distance between the tissue sample’s midplane and the emulsion’s midplane) and on the geometry of the image. For the autoradiographic system used in these experiments, the half distance is 150 nm, which, when multiplied by the print magnification, corresponds to a half distance of 1.8 mm on the autoradiographs. The grain–organelle probabilities are computed for each image in the experiment. The probability density function is based on the universal curve experimental value and the half-distance. The universal curve experimental values were fit to a Poisson distribution. The probability density function permits
determination of the probability that any pixel on any image adjacent to any particular grain contains the source that gave rise to that grain. This function is applied to each actual grain location in the context of the unique juxtapositions of the organelle structures and the grains. The maximum likelihood estimator then predicts the probability that any pixel within a 20×half-distance radius of that grain contains the point source. This is computed for each actual grain.

To measure the concentration of endogenous long-chain acylcarnitine in junctional and nonjunctional sarcotendinous compartments in normoxic and hypoxic cells, the electron microscopic autoradiographs were digitized, and junctional sarcotendina was defined as any region of the cell surface membrane that formed an intercellular junction with another myocyte. This included all gap junctions and adhesive components of the intercalated disc. These junctional regions were readily identified by their characteristic ultrastructural features, such as desmosomes (macula adherens) and intermediate junctions (fascia adherens), which were apparent on the autoradiographs. Previous studies have indicated that 24% of the total exterior cell surface is intercalated disk area. In this study, junctional sarcolemma constituted 19% of the total sarcolemma in normoxic myocytes and 14% of the total sarcolemma in hypoxic myocytes. Gap junction length within the intercalated disk has been shown to vary between species and method of analysis but is between 5% and 15% of the intercalated disk length (for review, see Reference 38). All remaining sarcolemma was designated nonjunctional and was characterized ultrastructurally by a well-developed glycocalyx and the absence of any junctional structures.

The computer-generated grain densities were used to calculate values expressed as molecules of long-chain acylcarnitine per cubic micrometer. By using the number of pixels occupied on the digitizing pad and the magnification of the autoradiographs, the number of grains per cubic micrometer was calculated using the following formula:

$$G = gpt$$

where G is grains per cubic micrometer, g is grains per pixel, p is the number of pixels per square micrometer on the image, and t is the thickness of the section (100 nm).

The number of endogenous molecules of long-chain acylcarnitine per unit volume of each compartment was then calculated from the grains per cubic micrometer using the following formula:

$$M = GdA/ES$$

where M is the total number of molecules per cubic micrometer, d is the inverse of emulsion efficiency (15 decays per grain), A is Avogadro’s number, E is exposure time in minutes, and S is specific activity of the radiolabeled long-chain acylcarnitine pool in the myocytes (35.65 Ci/mol). Statistical analysis of maximum-likelihood estimates of the intensity of radioactivity in a subcellular structure (grain density values) was performed using a real data-based simulator system, a novel method developed to define the statistical significance of maximum-likelihood intensity estimates.

One hundred realizations of the actual maximum-likelihood grain density estimates were performed, and standard deviations were computed for each density value. The concentration of long-chain acylcarnitine, expressed as a percentage of total membrane phospholipids, was calculated by dividing the number of endogenous long-chain acylcarnitine molecules per cubic micrometer by $4 \times 10^7$ phospholipid molecules/μm$^2$. The latter value was obtained by assuming that a pair of phospholipids in a bilayer forms a cylinder 0.8 nm in diameter and 10 nm in length, and, hence, occupies a volume of approximately $0.5 \times 10^7$ nm$^3$, which corresponds to $4 \times 10^7$ molecules/μm$^2$. No comparison was made between the phospholipid content of junctional compared with nonjunctional sarcolemma. Since the protein/phospholipid ratio is higher in junctional regions, any estimate of concentration of long-chain acylcarnitine expressed as a percentage of total sarcolemmal phospholipids will be underestimated.

All chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., unless indicated otherwise. Data are expressed as mean±SEM. Statistical analyses were performed on electrophysiological and autoradiographic data using Student’s t test. The criterion for statistical significance was $p<0.05$.

**Results**

**Influence of Exogenous Long-Chain Acylcarnitine on $g_j$**

Initial experiments were performed to characterize the electrophysiological properties of gap junctions in isolated pairs of ventricular myocytes. Both cells in each cell pair studied were voltage-clamped to evaluate $g_j$. Results of a typical experiment are presented in Figure 2. The pulsed cell was clamped at potentials from −80 to +60 mV while the nonpulsed cell was held at 0 mV (Figure 2A, top). The currents from the nonpulsed cell represented the junctional currents evoked from the pulsed cell through the gap junctions. The cell pair represented in Figure 2 was well coupled and exhibited an $r_j$ of 6.25 MΩ corresponding to a $g_j$ of 160 nS. The junctional current elicited during the 240-msec step pulse was stable and did not exhibit any time-dependent decay (Figure 2A). The current–voltage relation was linear between −80 and +60 mV (Figure 2B). Idiometric responses were observed when voltages were fixed or pulsed in either of the cells, indicating no directional dependence of $g_j$. Similar results were observed in four additional experiments. The mean value of $r_j$ was 7.35±1.2 MΩ, and the corresponding $g_j$ was 154±28.1 nS ($n=5$), indicating that the cell pairs were healthy and well coupled.

We have demonstrated previously that exogenously added long-chain acylcarnitine (1–10 μM) produces profound electrophysiological alterations in isolated ventricular myocytes, including a marked and reversible reduction in the voltage-dependent calcium current. In the present study, the influence of exogenous long-chain acylcarnitine (5 μM) on $g_j$ was evaluated. The results of a representative experiment are shown in Figure 4. The amplitude of the junctional currents from the nonpulsed cell were significantly diminished in the presence of long-chain acylcarnitine, indicating a reduction in $g_j$ (Figure 4A, left). These currents, although reduced, remained constant during the 240-msec pulse...
steps (Figure 4A), and the current–voltage relation remained linear (Figure 4B). The average results of five experiments demonstrated a 67.9±2.6% (p<0.01) reduction of g_i from 153.4±25.3 to 47.5±6.6 nS elicited by long-chain acylcarnitine.

To evaluate the time course of inhibition of g_i by long-chain acylcarnitine as well as the reversibility of the response, junctional currents from the nonpulsed cell were monitored every 15 seconds during exposure to long-chain acylcarnitine (5 μM) and subsequent washout. Currents were elicited in the nonpulsed cell by clamping the pulsed cell to −60 mV (Figure 5A). Within 45 seconds of exposure to long-chain acylcarnitine, g_i began to fall and continued to decrease throughout the 4-minute exposure period (Figure 5B). Within minutes after washout of long-chain acylcarnitine, g_i returned to 131 nS, which was within 14.9% of the control value. This modest reduction in g_i after washout was due to spontaneous rundown of g_i over the 12-minute time course. The time-dependent nature of inhibition of g_i by long-chain acylcarnitine was confirmed in three additional experiments. The mean time for the half-maximal inhibition of g_i by long-chain acylcarnitine (5 μM) was 1.7±0.1 minutes (n=4). The maximal percent recovery of g_i after 4 minutes of exposure to long-chain acylcarnitine (5 μM) and 6 minutes of washout in the absence of long-chain acylcarnitine was 14.5±0.8% (n=4) lower than the control values. The mean time required for this recovery was 5.1±0.4 minutes (n=4). Previous findings have indicated that action potentials occur virtually simultaneously between cell pairs when r_i ranges from 5 to 265 MΩ. Although long-chain acylcarnitine significantly decreased the g_i by 68% within 5 minutes, the value of r_i in the presence of long-chain acylcarnitine was still <30 MΩ. Thus, the delay of action potential propagation between cell pairs was too small to be detected. However, this certainly does not exclude the potential pathophysiological role of long-chain acylcarnitine to delay the conduction in the intact heart when the extent of delay at each cell is magnified over millions of sequential cells.

Electron Microscopic Autoradiographic Analysis of the Extent of Accumulation of Endogenous Long-Chain Acylcarnitines in Junctional Sarcolemma

To verify that incubation overnight with [3H]carnitine resulted in uniform and equilibrium labeling of each carnitine pool within the myocytes, long-chain acylcarnitine, short-chain acylcarnitine, and free carnitine fractions were measured by assay of the mass of carnitine in each fraction as well as quantification of radioactivity in each fraction. Normoxic myocytes were found to contain 42.3±3.3 pmol/mg protein (n=10) of long-chain acylcarnitines, which constituted 1.4±0.2% of the total carnitine content by mass measurement. Quantification of radioactivity within the long-chain acylcarnitine fraction in these myocytes demonstrated that 1.8±0.2% of the total label was present in the long-chain acylcarnitine fraction. The percentage of total carnitine radioactivity in the short-chain and free fractions was nearly identical to the relative mass measurements of each pool. Therefore, uniform labeling was achieved, analogous to that reported previously in neonatal rat myocytes.15

Initial studies were performed in prelabeled myocytes that were not subjected to Percoll gradients on the second day. In these cells, exposure of the myocytes to
hypoxia (PO₂ < 15 mm Hg) for 10 minutes resulted in a ninefold increase in the mass of long-chain acylcarnitine to 374±42 pmol/mg protein (n=8). Quantification of 3H label in the hypoxic myocytes showed a sixfold increase in label in the long-chain acylcarnitine fraction. The distribution of radiolabel in the different carnitine pools within the myocytes corresponded closely to that observed using mass measurements. Because it was essential to maximize the percentage of elongated myocytes for autoradiographic analysis, myocytes used for autoradiographic studies were passed through Percoll gradients to increase the percentage of elongated cells after overnight incubation with [3H]carnitine. This resulted in 84% elongated myocytes in the cell population. The long-chain acylcarnitine content in these normoxic myocytes, subjected to Percoll gradients on the second day, was 44.3 pmol/mg protein and increased fourfold to 185.5 pmol/mg protein after 10 minutes of hypoxia. These values are very close to those observed in canine myocytes subjected to hypoxia on the same day of isolation, with normoxic levels of 39.2±3.9 pmol/mg protein (n=10) increasing to 183.7±22 pmol/mg protein (n=10) after 10 minutes of hypoxia.

The amount of acid-soluble and acid-insoluble radioactivity was measured in the aqueous and organic processing reagents and in the remaining cell pellet at the completion of processing to verify that processing of the myocytes for autoradiography selectively removed short-chain acylcarnitine and free carnitine, leaving long-chain acylcarnitine intact within the myocytes. This evaluation was performed on a separate aliquot of cells from the hypoxic group, and during cell processing, all of the radioactivity could be accounted for. During cell processing for electron microscopic autoradiography, 99.97% of the short-chain acylcarnitine and free carnitine fractions were removed, whereas only 5% of the long-chain acylcarnitine fraction was removed from the myocytes. Therefore, of the retained radioactivity in the cell pellet, 97% of the radioactivity originated from the long-chain acylcarnitine fraction. A sample of autoradiographs of normoxic and hypoxic myocytes are shown in Figure 6.

The percentage of total micrograph area occupied by each subcellular compartment did not differ significantly between the micrographs derived from normoxic compared with hypoxic myocytes, indicating that each set represented a comparable sampling of myocytes (Table 1). By using the maximum likelihood method, no grains were assigned to the junctional sarcolemma of normoxic myocytes (Figure 7). The calculated concen-

![Figure 6](http://circres.ahajournals.org/)

**Figure 6.** Electron microscopic autoradiographs of the end regions of normoxic (left panel) and hypoxic (right panel) myocytes. The hypoxic cell is associated with more autoradiographic grains in the region of the intercalated disk. Distinctive structural features of the intercalated disk are readily apparent (small arrows show the fascia adherens). An annular gap junction is seen in the normoxic cell (large arrow), but gap junctions were not always clearly identified in these preparations. Bar, 2 μm.

**Table 1. Number of Grains Assigned to Each Subcellular Compartment Computed By the Maximum-Likelihood Method in Normoxic and Hypoxic Myocytes**

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Normoxia</th>
<th>Hypoxia*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Photographic area (%)</td>
<td>Assigned grains (n)</td>
</tr>
<tr>
<td>Junctional sarcolemma</td>
<td>0.08</td>
<td>0.00</td>
</tr>
<tr>
<td>Nonjunctional sarcolemma</td>
<td>0.50</td>
<td>2.12</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>12.34</td>
<td>51.92</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>41.60</td>
<td>181.45</td>
</tr>
<tr>
<td>Nuclear membrane</td>
<td>0.01</td>
<td>0.00</td>
</tr>
<tr>
<td>Nucleus</td>
<td>0.30</td>
<td>5.50</td>
</tr>
<tr>
<td>Extracellular space</td>
<td>45.17</td>
<td>5.01</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td>246.00</td>
</tr>
</tbody>
</table>

*Hypoxia was for a 10-minute duration.
tration of long-chain acylcarnitine in nonjunctional sarcoclemma of normoxic myocytes was very low (2.54±0.04×10⁶ molecules/µm²). Long-chain acylcarnitines increased more than 100-fold in the total sarcoclemmal (junctional and nonjunctional) compartment of myocytes subjected to 10 minutes of hypoxia. The concentration of long-chain acylcarnitine observed in nonjunctional sarcoclemma of hypoxic myocytes increased 10-fold over that observed in nonjunctional sarcoclemma of normoxic myocytes to 26.1±0.08×10⁶ molecules/µm² (Figure 7). Remarkably, after 10 minutes of hypoxia, selective accumulation of long-chain acylcarnitine was found to occur in junctional sarcoclemma. The concentration of long-chain acylcarnitine observed in junctional sarcocemma, 173±5×10⁶ molecules/µm² (Figure 7), was sevenfold greater than that observed in nonjunctional sarcoclemma of hypoxic myocytes. The concentration of long-chain acylcarnitine in the junctional sarcocemma of hypoxic myocytes corresponded to approximately 4 mol% of total phospholipids in the sarcocemma (see “Materials and Methods” for calculation). During exposure to hypoxia, the concentration of long-chain acylcarnitines in the mitochondria increased 1.5-fold, whereas the concentration in the other subcellular compartments was not significantly increased after 10 minutes of hypoxia.

**Discussion**

In the present study, gj was found to exhibit the following electrophysiological properties. First, an rj of 4−11 nS corresponded to a mean conductance of 154 nS, indicating that the cell pairs were very well coupled with little variability. Second, a linear current−voltage relation between −80 and +60 mV was obtained, and no voltage dependence of gj was observed. Third, a time independence of gating behavior was observed: current recorded during the potential steps showed no relaxation. Fourth, equal conductance in both directions indicated the absence of directional dependence of gj. These results are similar to those reported by other investigators evaluating ventricular cell pairs isolated from guinea pig and rat.25,43 The time independence of junctional current was different from that reported in neonatal cardiac myocytes.28 This might be due to the relatively short duration (240 msec) of the voltage-clamp pulse used in the present study, because the voltage dependence of the junctional current seen in neonatal cardiac cell pairs expressing connexin 43 develops slowly. In the present study, we chose a pulse duration of 240 msec to more accurately reflect the normal action potential duration in canine ventricular myocytes (Figure 1).

We have previously shown that long-chain acylcarnitines accumulate rapidly after the onset of ischemia in vivo11,12 and that prevention of the accumulation of long-chain acylcarnitines during ischemia markedly reduces the incidence of lethal ventricular arrhythmias.11,12 Since long-chain acylcarnitines influence ionic channel function,18,19 we reasoned that the amphiphilic properties of the compound might also influence cellular coupling. Therefore, we tested the hypothesis that long-chain acylcarnitines alter gj in isolated pairs of ventricular myocytes. Exogenous long-chain acylcarnitine produced a profound decrease of gj within minutes of exposure to the amphiphile. This inhibition was time dependent and reversible. The concentration of 5 µM used in the present study was comparable to those concentrations of palmitoyl carnitine required to suppress ion channels18,19 and induce delayed afterdepolarizations in isolated myocytes.16 Although the amplitude of junctional current was reduced by 68%, gj still demonstrated time independence and a linear current−voltage relation. These findings indicate that long-chain acylcarnitine might decrease the whole-cell junctional current by reducing the number of channel openings or open channel probability. Indeed, there are data indicating that the gap junction channels in adult cardiac myocytes do not reside in subconductive states but, rather, that changes in gj reflect variations in open time.44,45

The precise mechanisms whereby long-chain acylcarnitines inhibit gj have not yet been elucidated. It is likely that this amphiphile interacts directly with the gap junction channel. This potential mechanism has been suggested previously by Burt and colleagues46,47 in studies in which free fatty acids modulate gj. The preferential accumulation of long-chain acylcarnitines in junctional sarcocemma is likely due to the high protein/lipid ratio in this region compared with nonjunctional sarcocemma, further supporting the concept that this amphiphile may affect channel gating at the gap junction because of direct interaction with the channel protein. Agents that have been shown to decrease gj in other cell systems include general anesthetics (halothane, heptanol, and octanol),2,27,44,45,48,49 arachidonic acid,50 and doxyl stearic acids.46 These agents could also alter channel gating within the gap junctions. Long-chain acylcarnitines are membrane-active amphiphiles that accumulate during hypoxia in vitro and ischemia in vivo and have been shown to incorporate selectively into cardiac myocyte sarcolemma.14,16 Therefore, it is also possible that long-chain acylcarnitines might indirectly influence the function of gap junction channels through
a nonspecific effect on membrane disordering by decreasing $g_j$ secondary to altering sarcolemmal membrane fluidity. The hypoxia-induced accumulation of long-chain acylcarnitine in the total sarcolemmal pool represents a concentration of approximately 1 mol%. In contrast, the concentration of long-chain acylcarnitine incorporated in the junctional sarcolemma corresponds to approximately 4 mol% of total phospholipid in the membrane. This level exceeds the 1–2 mol% long-chain acylcarnitine concentration, which was shown to elicit increases in membrane fluidity using electron spin-resonance spectroscopy. This also exceeds the 1–2 mol% $[{\text{H}}]$lso phosphatidylcholine concentration incorporated into the sarcolemma after exogenous delivery that was sufficient to elicit electrophysiological derangements. Thus, the concentration of long-chain acylcarnitines attained within the sarcolemma that was due to endogenous accumulation occurring during hypoxia may be high enough to cause membrane perturbations and electrophysiological alterations. The rapid onset and reversibility of the decrease in $g_j$ indicates that incorporation of long-chain acylcarnitines does not cause irreversible membrane damage via a potential detergent-like interaction.

It is not clear whether long-chain acylcarnitines may be exerting specific or nonspecific effects on gap junctional proteins, leading to a reduction in $g_j$. Long-chain acylcarnitines suppress sarcolemmal Na$^+$ and Ca$^{2+}$ channels in a reversible manner. In addition, although long-chain acylcarnitine has been shown to inhibit the voltage-gated L-type Ca$^{2+}$ channel, it has been shown indirectly to increase intracellular Ca$^{2+}$. Previous studies in guinea pig ventricular cell pairs and have indicated that $g_j$ is reduced when intracellular Ca$^{2+}$ increases. Thus, increased intracellular Ca$^{2+}$ may be one mechanism whereby long-chain acylcarnitines inhibit $g_j$. However, this mechanism cannot explain the present findings, since the intracellular pipette solution contained EGTA (10 mM). Free intracellular Ca$^{2+}$ should have been chelated by EGTA once the membrane was perforated and the intracellular pipette solution was equilibrated with the intracellular contents. Another potential mechanism may relate to the extra-cellular Ca$^{2+}$, which might cross the junctional membrane in the presence of long-chain acylcarnitine and thereby decrease $g_j$. However, several lines of indirect evidence suggest that this mechanism is not likely to be responsible for the inhibition of $g_j$ by long-chain acylcarnitine. In preliminary experiments, we have shown that long-chain acylcarnitines increase intracellular Ca$^{2+}$ in isolated myocytes, but this effect was mediated through enhanced Na$^+$–Ca$^{2+}$ exchange and was blocked by Ni$^{2+}$. The extracellular solution used in the present study contained NiCl$_2$ (1 mM), thereby precluding this effect of long-chain acylcarnitine.

Cardiac gap junctions provide a low-resistance pathway to facilitate impulse propagation between adjacent cells. Several lines of experimental evidence indicate that $g_j$ decreases during myocardial ischemia. By using detailed three-dimensional recordings from 232 simultaneous transmural sites in situ, profound degrees of conduction delay and block were seen within minutes of the onset of ischemia. Ischemia-induced conduction delay progressing to electrical uncoupling, as manifested by an increase of intracellular longitudinal resistance, has been shown to occur in blood-perfused rabbit papillary muscles after 15–20 minutes of ischemia. It is difficult to compare the time required for the development of conduction delay under different experimental conditions. However, these findings indicate that conduction delay develops during the early phase of ischemia. This degree of conduction delay and uncoupling can lead to malignant ventricular arrhythmias by a mechanism that we have termed intramural reentry. Although $g_j$ in ventricular cell pairs is decreased markedly by long-chain acylcarnitines that accumulate selectively in the junctional regions of sarcolemma during early hypoxia, further studies are required to measure $g_j$ during hypoxia and to determine the precise membrane concentration required to induce cellular uncoupling.

The findings of the present study indicate indirectly that inhibition of $g_j$ by long-chain acylcarnitines could lead to a slowing of conduction velocity and further to functional uncoupling if exposure to long-chain acylcarnitine were sustained. We have demonstrated washout and reversibility of the effect on $g_j$ after minutes of exposure to long-chain carnitine. Interestingly, long-chain acylcarnitines have been shown to decrease the sodium current and induce early and delayed afterdepolarizations in isolated myocytes. The mechanism for activation of the transient inward current has not yet been elucidated. However, together, these data suggest that sarcolemmal accumulation of long-chain acylcarnitines may contribute to both reentrant and nonreentrant mechanisms, leading to the malignant arrhythmias observed during myocardial ischemia in vivo.

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