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Heptanol-induced Decrease in Cardiac Gap Junctional Conductance Is Mediated by a Decrease in the Fluidity of Membranous Cholesterol-rich Domains

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Abstract. To assess whether alterations in membrane fluidity of neonatal rat heart cells modulate gap junctional conductance (g_j), we compared the effects of 2 mM 1-heptanol and 20 μM 2-(methoxyethoxy)ethyl 8-(cis-2-*n*-octylcyclopropyl)-octanoate (A_2C) in a combined fluorescence anisotropy and electrophysiological study. Both substances decreased fluorescence steady-state anisotropy (r_{ss}), as assessed with the fluorescent probe 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) by 9.6 ± 1.1% (mean ± SEM, n = 5) and 9.8 ± 0.6% (n = 5), respectively, i.e., both substances increased bulk membrane fluidity. Double whole-cell voltage-clamp experiments showed that 2 mM heptanol uncoupled cell pairs completely (n = 6), whereas 20 μM A_2C, which increased bulk membrane fluidity to the same extent, did not affect coupling at all (n = 5).

Since gap junction channels are embedded in relatively cholesterol-rich domains of the membrane, we specifically assessed the fluidity of the cholesterol-rich domains with dehydroergosterol (DHE). Using DHE, heptanol increased r_{ss} by 14.9 ± 3.0% (n = 5), i.e., decreased cholesterol domain fluidity, whereas A_2C had no effect on r_{ss} (−0.4 ± 6.7%, n = 5).

Following an increase of cellular "cholesterol" content (by loading the cells with DHE), 2 mM heptanol did not uncouple cell pairs completely: g_j decreased by 80 ± 20% (range 41–95%, n = 5). The decrease in g_j was most probably due to a decrease in the open probability of the gap junction channels, because the unitary conductances of the channels were not changed nor was the number of channels comprising the gap junction. The sensitivity of nonjunctional membrane channels to heptanol was unaltered in cholesterol-enriched myocytes.

These results indicate that the fluidity of cholesterol-rich domains is of importance to gap junctional coupling, and that heptanol decreases g_j by decreasing the fluidity of cholesterol-rich domains, rather than by increasing the bulk membrane fluidity.

Key words: Fluorescence anisotropy — Voltage clamp — Membrane fluidity — Gap junctional conductance — 1-heptanol — A_2C

Introduction

Gap junctions are plasma membrane specializations which allow the passage of ions and small molecules between adjacent cells. In heart, these structures are important for the conduction of the action potential from the sino-atrial node towards the working myocardium of atria and ventricles and, thus, for the coordinated contraction of the heart. They consist of hemichannels, called connexons, which cross the plasma membrane of one cell and connect tightly with connexons of the adjacent cell, leaving a narrow gap of about 2–4 nm. Each connexon is composed of six protein subunits, named connexins. In heart ventricle, the most abundant connexin is connexin43 (Beyer, Paul & Goodenough, 1987), although other connexins are reported to be expressed as well (Kanter, Saffittz & Beyer, 1992), recently even within the same gap junction (De Mazière et al., 1993).

The conductance of heart gap junctions is sensi-

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tive to a wide variety of agents, including $[Ca]_i$, pH_i, substances that regulate protein phosphorylation, several lipophilic substances and transjunctional voltage (for a review, *see* Spray & Burt, 1990; Page, 1992).

Since Johnston, Simon and Ramón (1980) first described the uncoupling action of higher linear alcohols on gap junctions between nerve cells from crayfish, it has been shown in different preparations that these and other lipophilic substances uncouple cell pairs (Veenstra & De Haan, 1986; Burt & Spray, 1988; Chanson et al., 1989; Niggli et al., 1989; Giaume et al., 1991; Pérez-Armendariz et al., 1991; Spray et al., 1991). These agents, of which halothane and heptanol are frequently used, appear to reduce gap junctional conductance (g_j) by reducing the open probability of the gap junction channels without affecting their unitary conductances or the number of channels in a gap junction (Burt & Spray, 1989; Takens-Kwak et al., 1992). However, the mechanism by which lipophilic substances alter the channel's activity is not known. Possible mechanisms are (i) alterations of the lipid environment around the channel; (ii) a direct effect on the gap junction channel through binding to the protein, and (iii) an indirect effect on the channel through a change in, for example, $[Ca]_i$ or pH_i. Meda et al. (1986) demonstrated in pancreatic acini that heptanol-induced uncoupling was not associated with changes in $[Ca]_i$ or pH_i. Furthermore, in several cell types heptanol-induced uncoupling still occurs in the presence of strong intracellular buffering of these ions (Burt & Spray, 1989; Chanson et al., 1989). The second possibility, a specific direct effect of heptanol on gap junction channels is considered when changes in conductance of nonjunctional channels are absent in the same cells (Burt & Spray, 1989; Chanson et al., 1989). The observation that in heart cells heptanol exerts effects in the same concentration range on gap junctional, as well as nonjunctional membrane channels, raised concerns about the specificity of this substance for cardiac gap junction channels (Niggli et al., 1989; Rüdisüli & Weingart, 1989; Takens-Kwak et al., 1992). These authors consider a nonspecific alteration of the lipid environment of the gap junction channels as the mechanism of action of heptanol.

To explore whether the lipid environment of gap junction channels is altered by lipophilic agents, we measured the effects of these agents, such as 1-heptanol and 2-(methoxyethoxy)ethyl 8-(cis-2-*n*-octylcyclopropyl)-octanoate (A_2C), on gap junctional current and nonjunctional membrane currents, as well as effects on steady-state anisotropy in different membrane domains. Fluorescence steady-state anisotropy is reciprocally related to membrane fluidity, which is known to affect the function of several membrane proteins (Yeagle, 1985). We found that an increase in the fluidity of the bulk membrane solely (by A_2C) does not affect gap junctional conductance. The fluidity of the cholesterol-rich domain, on the other hand, appears to influence gap junctional coupling. We conclude that in neonatal rat cardiomyocytes the uncoupling action of heptanol is related to a decreased fluidity of cholesterol-rich domains in which the gap junction channels reside.

Materials and Methods

Cell Culture

Isolated heart cells were obtained from one- or two-day-old Wistar rats as described previously (De Bruijne & Jongsma, 1980). Briefly, the hearts of ether-anesthetized rats were excised aseptically, minced, and incubated in 0.75 ml dissociation medium per heart. Dissociation was achieved by gently stirring at 37°C. After 15 min, the dissociation medium was discarded and replaced by a fresh aliquot. A further 30 min of stirring resulted in complete dissociation of the remaining fragments. The cell suspension was then cooled to 0°C for 5 min. After centrifugation, the cell pellet was washed and suspended in growth medium. Aliquots of the cell suspension were pipetted into plastic petri dishes (Falcon 3001) and in plastic petri dishes (Falcon 3004) containing glass coverslips (10 × 32 mm). The cultures were incubated in a pH-controlled and humidity-controlled incubator at 37°C. Growth medium was refreshed after 4 hr and after 24 hr.

Solutions

The dissociation medium was a HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffered physiological saline containing 450 U/ml collagenase (Worthington, CLS type 1), 0.001% DNAse (Worthington), 0.01 mM Ca^{2+} and 0.6 mM Mg^{2+}. The growth medium consisted of Ham's F10 (Flow Laboratories) supplemented with 5% fetal calf serum and 5% horse serum (Flow Laboratories). During the electrophysiological experiments, the cells were bathed in a modified Tyrode solution composed of (in mM): 130 NaCl; 4 KCl; 2.2 $CaCl_2$; 0.56 $MgCl_2$; 10 glucose; 1.2 NaH_2PO_4; 14.3 HEPES (pH adjusted to 7.4). The electrodes were filled with a solution of the following composition (in mM): 130 K-gluconate; 10 KCl; 2 $MgCl_2$; 0.6 $CaCl_2$; 5 Na_2ATP; 10 EGTA; 10 HEPES (pH adjusted to 7.2). For perforated-patch experiments 140 μg nystatin (Sigma) and 140 μg pluronic F-127 (Molecular Probes) were added per ml of electrode solution. All fluorescence polarization measurements were performed in HEPES-buffered balanced salt solution (HBSS). HBSS consisted of (in mM): 125 NaCl; 5 KCl; 1 $MgSO_4$; 1 KH_2PO_4; 2.5 $CaCl_2$; 10 $NaHCO_3$; 5 sodium pyruvate; 20 HEPES (pH adjusted to 7.4).

Electrophysiology

Single cells and cell pairs were used for electrophysiological experiments after 18–40 hr of culturing. Before the experiments, the growth medium in the culture dish was replaced by modified

Tyrode. The dishes were placed on the stage of an inverted microscope (Nikon Diaphot TMD) and observed with phase-contrast optics at a total magnification of 400. The cells were superfused by means of a gravity-controlled flow system at ≥6 ml/min. The temperature was 20–22°C.

All recordings were made by using a set of two custom-built clamp amplifiers allowing both current-clamp and voltage-clamp recordings. Electrodes were pulled from 1 mm borosilicate capillaries containing a glass fiber and their tips were heat-polished. Electrode resistances ranged between 5 and 15 MΩ. The electrodes were lowered onto the cell surfaces by means of hydraulic micromanipulators (Narishige). Giga-ohm seals were formed on the cells, and the membrane patch under the electrode tip was broken to achieve the whole-cell configuration (Hamill et al., 1981). The access resistances generally ranged between 10 and 40 MΩ. In a few experiments, the perforated-patch configuration was used (Takens-Kwak et al., 1992). Giga-ohm seals were obtained with nystatin in the electrode tip, and the access resistance dropped within 8 min from 5–10 GΩ to stable values of 10–80 MΩ.

Membrane ionic currents were measured under voltage-clamp conditions by applying depolarizing and hyperpolarizing voltage steps from a holding potential of −60 or −35 mV. To determine gap junctional current (I_j), both cells of a cell pair were voltage-clamped at identical holding potentials near their resting potential (generally around −65 mV), resulting in a net zero junctional current and a net zero membrane current. The potential of one of the two cells was alternately depolarized 10 or 20 mV for 1 sec, resulting in a constant voltage drop across the intercellular junction during the command step. The current recorded in the cell kept at a constant potential is equal to $-I_j$.

Current and voltage signals of both cells were recorded on VCR-tape using a pulse-code modulation system (Sony HF 150, which had been modified to make it suitable for DC recording) in combination with a VCR (Sony). The sample rate was 22 kHz per channel. After low-pass filtering (2-pole Butterworth filter) at 0.5 or 1 kHz, the current and voltage recordings were played back into an IBM-compatible PC/AT (Acer 910) equipped with an A/D board (PCL-718, Advantech) and customized software. Digitized current recordings and *I-V* plots were plotted on an HP-plotter (ColorPro). Current signals, in which single channel events could be seen, were played back from tape and recorded on paper (HP 7402A) with a resolution of 5 pA/cm; the amplitudes of the current transitions in I_j were measured using a digitizing tablet (Morphomat 10, Zeiss) and customized software. Values were divided by the applied voltage step to obtain conductances associated with the current steps. The conductances were grouped in 4 pS bins and plotted as a step amplitude frequency histogram. The histogram was fitted with a double Gaussian using a nonlinear least-squares method to obtain the mean and SD of the measured single channel conductances.

Fluorescence Polarization Measurements

Fluorescence polarization studies were performed according to Sumbilla and Lakowicz (1983). Briefly, the monolayers grown on coverslips were incubated for 10–15 min with 10 μM cPA (cis-parinaric acid, Molecular Probes), 10 μM tPA (trans-parinaric acid, Molecular Probes), or 10 μM TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluene-sulfonate, Molecular Probes) in HBSS at 37°C. Measurements of the fluorescence steady-state anisotropy of both cPA and tPA may reveal the existence of lipid domains (Van der Meer, 1988). In membranes with coexisting solid and fluid domains, tPA partitions preferentially into the solid domains, whereas cPA exhibits an almost equal distribution (Sklar, 1980; Van der Meer, 1988). TMA-DPH is a plasma membrane specific fluorophore, which is frequently used to sense the fluidity of the plasma membrane of intact cells (Kuhry et al., 1983; Sheridan & Block, 1988). After labeling the cells with the fluorophore, the coverslips with the monolayers were rinsed twice in HBSS, placed in a cuvette filled with 2.5 ml HBSS and used for the measurement of fluorescence steady-state anisotropy (r_{ss}), according to Lakowicz (1983):

$$r_{ss} = (I_{vv} - I_{vh} \cdot G)/(I_{vv} + 2I_{vh} \cdot G)$$

where I_{vv} and I_{vh} equal the fluorescence intensities parallel and perpendicular to the excitation plane (when placed vertically), respectively. G equals I_{hv}/I_{hh}, which is a correction factor for the monochromator's transmission efficiency for vertically and horizontally polarized light. The fluorescence measurements were carried out in a spectrofluorometer (Perkin-Elmer LS-3) equipped with a polarization accessory (Perkin-Elmer 5212-3269) at approximately 21°C.

Cholesterol Modulation of the Cultures

In this study the "cholesterol" content of the myocyte cultures was changed using two protocols:

(A) Cultures were incubated for 16–18 hr in Ham's F-10 medium supplemented with 8% lipoprotein deficient serum (LPDS) and 30 μM dehydroergosterol (DHE, Sigma) at 37°C. DHE is a fluorescent analogue of cholesterol. The biophysical properties and behavior in biological membranes of DHE are very similar to those of cholesterol (Schroeder et al., 1991). DHE has the advantage that it can be used for fluorescent anisotropy measurements and microscopy studies. LPDS was prepared (Havel, Eder & Bragdon, 1955) from 50% donor horse serum + 50% fetal calf serum. The density of the serum mix was brought to 1.23 g/ml by adding KBr, and centrifuged for 40 hr at 110,000 × *g* (4°C) in a fixed angle rotor (TFT 70.38, Kontron). LPDS was dialyzed three times against a 100-fold volume of HBSS at 4°C. Before use the lipoproteins were filtered through a 0.22 μm filter (Millipore).

(B) Incubation of the cultured myocytes with cholesterol-rich liposomes (cholesterol to phospholipid (C/PL) molar ratio = 2). All incubations were performed with a liposome content of 0.5 mg phospholipid per ml HBSS for a period of 5 hr (37°C, 95% air + 5% CO_2). Liposomes were prepared according to Sorisky, Kucera and Rittenhouse (1990), using egg yolk phosphatidylcholine (type XI-E, Sigma) as the phospholipid constituent. Free cholesterol and phospholipid contents in the liposomes were determined with commercial kits (Boehringer Mannheim).

Lipid Composition

Cultures which had been incubated with and without 30 μM cholesterol instead of DHE (because of the difficulty of measuring cellular DHE content) (protocol A) and cholesterol-rich liposomes (protocol B) were analyzed for free cholesterol content. The medium was poured off and the monolayers were rinsed two times with 2 ml HBSS containing 0.5% bovine serum albumin (BSA, Sigma), followed by rinsing three times with HBSS. The lipids were extracted from monolayers with 2-propanol, and were then evaporated under a stream of nitrogen and suspended in 150

μl ethanol. Free cholesterol was determined with a fluorescent cholesterol oxidase method (Gamble et al.,1978).

Results and Discussion

Effects of Heptanol and A_2C on Sarcolemmal Bulk Fluidity

In a first set of experiments, we used two different lipophilic agents: 1-heptanol, a well-known uncoupler of cell pairs and A_2C, which increases membranous fluidity nonspecifically (Kosower, Kosower & Faltin, 1974). To quantify membrane fluidity in neonatal rat cardiomyocytes cultured on glass coverslips, we measured the fluorescence steady-state anisotropy (r_{ss}), which is reciprocally related to membrane fluidity (Van der Meer, 1988). The sarcolemmal r_{ss} was assessed using the plasmalemma-specific membrane probe TMA-DPH (Kuhry et al., 1983; Sheridan & Block, 1988). A_2C produces a dose-dependent decrease in sarcolemmal r_{ss} (Fig. 1*A*). r_{ss} was measured before and 20 min after addition of A_2C. Δr_{ss} was calculated as percentual difference of its initial value. As demonstrated in Fig. 1*B*, heptanol also decreased r_{ss} in a dose-dependent manner.

Effects of Heptanol and A_2C on g_j

Pairs of cardiomyocytes were used to explore the effects of increased bulk membrane fluidity on gap junctional coupling. Under control conditions g_j ranged between 1 and 23 nS. As is shown in Fig. 2*A*, 2 mM 1-heptanol completely and reversibly abolished I_j. If the heptanol-induced decrease in g_j is mediated by an increase in sarcolemmal fluidity, one would expect that A_2C depresses g_j also. Thus, we exposed the myocytes to 20 μM A_2C, a concentration which increased sarcolemmal fluidity to the same extent as 2 mM heptanol did. Figure 2*B* shows that 20 μM A_2C had no effect on I_j. This is confirmed by five additional experiments with heptanol and four additional experiments with A_2C. A further increase in A_2C concentration to 40 μM did not change this result (*not shown*).

These findings indicate that the fluidity of the sarcolemma is not a determinant for gap junctional conductance, and that the heptanol-induced increase in sarcolemmal fluidity is not responsible for the heptanol-induced uncoupling.

Existence of Membranous Lipid Domains in Neonatal Cardiomyocytes

It is generally accepted that the membrane bilayer is heterogeneous, in that it contains domains, such as cholesterol-rich domains (Yeagle, 1985; Van der Meer, 1988; Schroeder et al., 1991). To examine whether sarcolemma contains different lipid domains, we assessed the r_{ss} of tPA and cPA in cultured myocytes. It is known that cPA and tPA partition differently among lipid domains (Sklar, 1980; Van der Meer, 1988): tPA partitions preferentially into the solid domains, whereas cPA exhibits an almost equal distribution among the solid and fluid domains, as derived from combined information from fluorescence energy transfer, polarization, and quantum yield measurements (Sklar et al., 1979). So, a difference in r_{ss} values determined by these two fluorescent probes is an indication of the existence of membrane lipid domains (Van der Meer, 1988). The r_{ss} of cPA and tPA were 0.244 ± 0.009 and 0.266 ± 0.007, respectively (mean ± SEM; $n = 5$; paired *t*-test: $P < 0.05$). The difference between these values allows the conclusion that the sarcolemma of cultured cardiomyocytes contain various lipid domains.

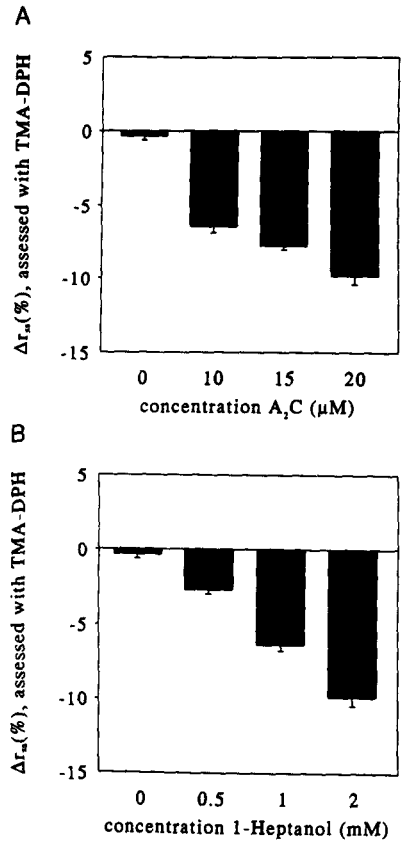

Fig. 1. The dose-dependent effect of A_2C (*A*) and 1-heptanol (*B*) on fluorescence steady-state anisotropy (r_{ss}) measured with TMA-DPH. r_{ss} was measured before and 20 min after addition of indicated concentrations of A_2C or 1-heptanol. The difference, Δr_{ss}, was expressed as a percentage of its initial value. Indicated are mean ± SEM of five measurements.

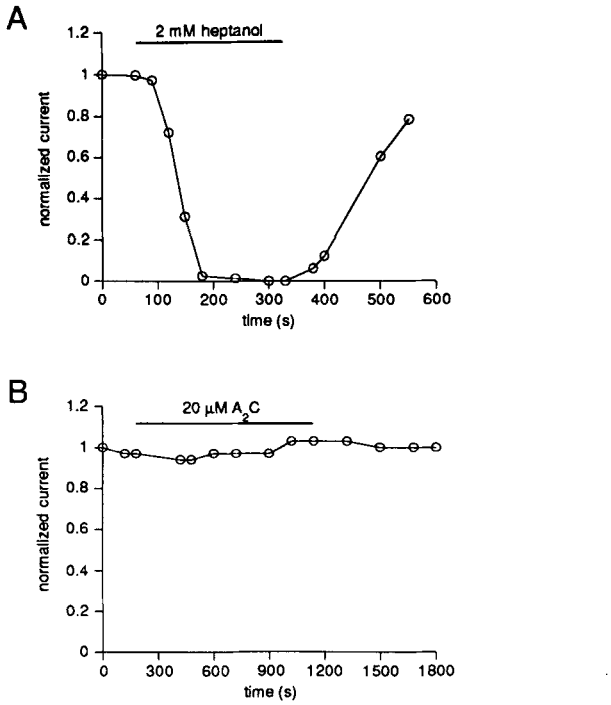

Fig. 2. Exposure to 2 mM heptanol reversibly uncoupled a pair of cardiomyocytes, as indicated by the decrease in normalized gap junctional current (*A*). Exposure to 20 μM A_2C had no effect on normalized gap junctional current (*B*). Both experiments were performed in the whole-cell configuration. Clamp pulses (10 mV, 1 sec) were delivered every 3 sec alternately to both cells of a cell pair. Junctional current was measured at the end of the step, normalized to the junctional current measured under control conditions at the beginning of the experiment, and plotted vs. time. Horizontal bars indicate period of exposure to the substances. *A*: $V_h = -59$ mV; *B*: $V_h = -60$ mV.

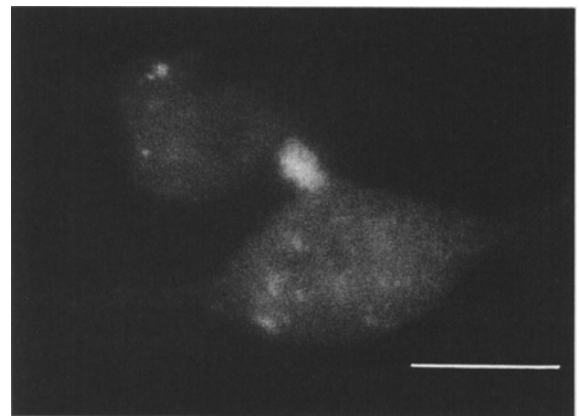

Fig. 3. Fluorescent microphotographs of cardiomyocytes, which were incubated for 16 hr with 30 μM DHE. Cells were fixed with 2% paraformaldehyde. Specific DHE labeling was observed in the contact area of this cell pair and a faint diffuse labeling is present in the cell membrane. Scale bar represents 10 μm.

Effects of Heptanol and A_2C on Cholesterol-rich Domain Fluidity

In several cell types the molar ratio of cholesterol to phospholipid in the gap junctional membrane is generally much higher than that of the native plasma membranes (*see for refs:* Malewicz et al., 1990). Besides, cholesterol supplementation to cultured cells increases gap junction communication (Meyer et al., 1990; Zwijsen, Oudenhoven & de Haan, 1992).

In a following set of experiments we have used dehydroergosterol (DHE), a fluorescent analogue of cholesterol. The biophysical properties and behavior in biological membranes of DHE are very similar to those of cholesterol (Schroeder et al., 1991). DHE has the advantage that it can be used for fluorescent anisotropy measurements and microscopy studies. By incubating the cultured myocytes in lipoprotein-deficient medium supplemented with 30 μM DHE for 16–18 hr, the cholesterol analogue was introduced into the membrane. If this incubation procedure was performed with cholesterol instead of DHE (because of the difficulty of measuring DHE content), cellular free-cholesterol content had increased by 32.1 ± 4.1% (mean ± SEM; $n = 3$). Since DHE has properties very similar to those of cholesterol, including its incorporation into several membranes (*reviewed by* Schroeder et al., 1991), we assume that incubation of myocyte cultures with DHE also had resulted in an increase of "cholesterol" content by about 32%.

Fluorescence microscopy studies on DHE-loaded cell pairs showed a faint diffuse distribution of DHE in the cell membrane, and a marked concentration in contact areas of cell pairs (Fig. 3). This observation is consistent with the findings that the cholesterol/phospholipid molar ratio in gap junctional preparations is higher than that of the native plasma membranes (Malewicz et al., 1990). Visualization of cholesterol in gap junctional membranes has not been possible using freeze-fracture electron microscopy and filipin as a cholesterol probe. This was considered to be indicative for a low level of cholesterol in gap junctions (Severs, 1981; Robenek, Jung & Gebhardt, 1982). However, as recently reviewed by Malewicz et al. (1990), only a positive filipin test can be interpreted as evidence for the presence of cholesterol; a negative filipin test is inconclusive for the absence of cholesterol. In contrast, our experiments have visualized the cholesterol analogue DHE in the membrane without the ambiguity caused by the affinity of the probe for cholesterol, as is the case with filipin. Moreover, with our method the presence of the cholesterol analogue is demonstrated in intact cells, instead of in isolated gap junction membranes.

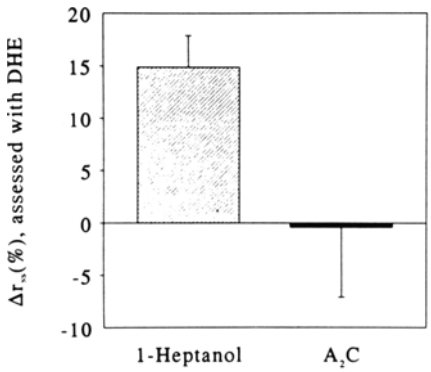

Fig. 4. The effect of 2 mM 1-heptanol and 20 μM A_2C on the fluidity of cholesterol-rich domains, as assessed with DHE anisotropy. The cells were labeled by incubating the cultures for 16 hr with 30 μM DHE in LPDS. r_{ss} was measured before, and 20 min after addition of indicated concentrations of A_2C or 1-heptanol. The difference, Δr_{ss}, was expressed as a percentage of its initial value. Indicated are mean ± SEM of five measurements.

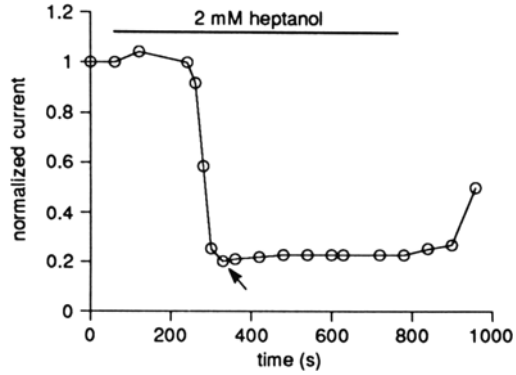

Fig. 5. Gap junctional current of a pair of cardiomyocytes loaded with DHE measured with the perforated-patch method before, during, and after exposure to 2 mM heptanol. Clamp pulses (10 mV, 1 sec) were delivered every 3 sec alternately to both cells of a cell pair. Junctional current was measured at the end of the step, normalized to the junctional current measured under control conditions at the beginning of the experiment, and plotted *vs.* time. Horizontal bar indicates period of exposure to heptanol. Arrow indicates time of maximal uncoupling, after which the coupling slightly improved. $V_h = -70$ mV.

Figure 4 shows the effect of 2 mM heptanol and 20 μM A_2C on the fluidity of the cholesterol-rich domains assessed with DHE. A_2C did not alter the fluidity of the cholesterol-rich domains. Heptanol, however, increased r_{ss}, which indicates that heptanol decreased the fluidity of the cholesterol-rich domains.

Effects of Heptanol on g_j of DHE-loaded or Cholesterol-enriched Cardiomyocytes

In an attempt to measure g_j with the whole-cell method in cell pairs cultured with DHE, each time we tried to rupture the membrane under the patch to get access to the cells, we destroyed the cells. Although we finally measured g_j of DHE-loaded cells using the perforated-patch method with a double concentration of nystatin (compared to control cells), we were still confronted with rather high access resistances (40–80 MΩ). Further increase of the nystatin concentration in the electrodes, however, made it more difficult to obtain giga-ohm seals, while the access resistance to the cells did not decrease markedly. In DHE-loaded cardiomyocytes, g_j ranged between 3 and 11.5 nS ($n = 5$), which was not different from g_j in control cell pairs. It was reported previously that cholesterol enrichment increases gap junctional coupling (Meyer et al., 1990; Zwijsen, et al., 1992). However, in the neonatal cardiomyocyte pairs we used, large variations in g_j exist under both conditions, which can easily mask possible differences in g_j given the relatively small number of experiments we performed. Unfortunately, because the incubation procedure took several hours, it was not possible to measure g_j during the incubation on the same cell pair. Another experimental limitation was the high access resistances we observed in the DHE-loaded cells. This produces a large drop in the applied transjunctional voltage, causing considerable underestimation of g_j (Wilders & Jongsma, 1992).

In DHE-loaded cells, the uncoupling action of heptanol was diminished considerably: 2 mM heptanol decreased g_j by 80 ± 20% only (mean ± SEM; range 41–95%, $n = 5$). Figure 5 illustrates the time course of I_j before, during and after addition of heptanol. After a short delay, which was mainly due to a delay in the perfusion system, 2 mM heptanol caused a considerable decrease in I_j. After I_j had reached minimal values rapidly (*see arrow* in Fig. 5), a small recovery was observed under continuing heptanol superfusion. The decrease in I_j was completely reversible following heptanol washout (*not shown* in Fig. 5).

The decrease in I_j can be explained by (i) a decrease in the number of gap junction channels between the cells; (ii) a decrease in the conductance of the individual channels or (iii) a decrease in the open probability of the channels. Because both uncoupling and recoupling rates were fast, removal of gap junction channels from the junctional membrane is not likely the mechanism by which I_j was decreased in these DHE-loaded cardiomyocytes. Moreover, we observed complete reversibility of the heptanol-induced uncoupling in two of the five cell pairs, a phenomenon which is also less likely if chan-

nels were removed from the junctional membrane. To resolve the mechanism of action of heptanol in these cells, cell pairs should be uncoupled to the single channel level. Exposure of the DHE-loaded cell pairs to heptanol concentrations > 3 mM destroyed the cells, while with lower heptanol concentrations single channel events could not be resolved. Therefore, we increased the cellular cholesterol content alternatively by incubating the cardiomyocyte cultures with cholesterol-rich liposomes. This way, the cellular cholesterol content was increased by 27.6 ± 2.8% (mean ± SEM; n = 3). Using cell pairs from these cultures, we were able to obtain lower access resistances to the cells (10–50 MΩ) and to decrease I_j to values where single channel events could be observed (Fig. 6*A*), although complete uncoupling was never reached. These liposome-treated cells also showed a small recovery after minimal I_j had been reached. To determine the unitary gap junction channel conductance (γ_j), we constructed frequency histograms of the conductance changes underlying the current steps in I_j. Under control conditions unitary conductance values exhibited Gaussian distributions centered around amplitudes of about 20 and 40–45 pS (Fig. 6B, dotted line; *see also* Fig. 6*B* in Takens-Kwak et al., 1992). In experiments on cell pairs with increased cholesterol content, we observed the same two γ_j peaks (Fig. 6*B*, unbroken line). Besides, the distribution of the two peaks in the histogram relative to each other did not change compared to the distribution obtained under control conditions. Thus, we conclude that in pairs of cholesterol-enriched cardiomyocytes, the heptanol-induced decrease in g_j is not caused by a decrease in the number of channels comprising the gap junction or a decrease in the unitary conductance of the channels. The only remaining explanation for the heptanol effects, therefore, is a decrease in the open probability of the channels, as was suggested before for heptanol-induced uncoupling of control cardiomyocytes (Takens-Kwak et al., 1992). A decrease in open probability can be due to a decrease in mean open time or an increase in mean closed time of the channels or a combination thereof. Unfortunately, we were not able to obtain recordings from the cholesterol-enriched cardiomyocytes which showed openings and closings of one single channel for a sufficiently long period of time to calculate the mean open time. In control cardiomyocytes uncoupled by heptanol, we measured mean open times of approximately 20 msec (*unpublished results*), being roughly 45 times shorter than mean open times observed in naturally weakly coupled cardiomyocyte pairs (Burt & Spray, 1988; Rook, Jongsma & van Ginneken, 1988). Measurement of the mean closed time is impossible in our preparation, because opening of a channel after a previous closure cannot be ascribed with any certainty to the same channel because the gap junction contains numerous channels.

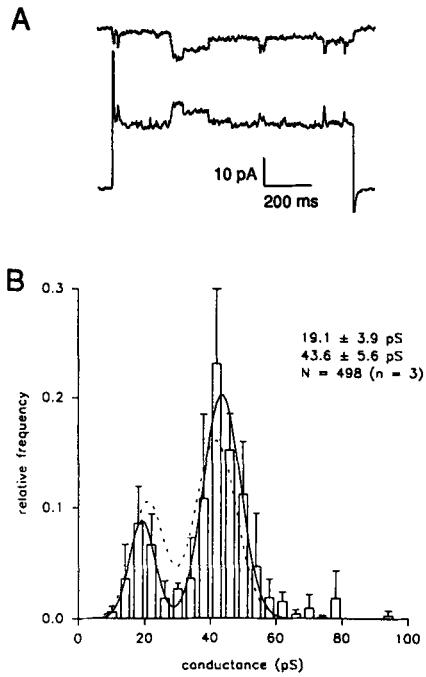

Fig. 6. (*A*) Current recordings under voltage-clamp conditions from both cells of a pair of cholesterol-enriched cardiomyocytes. g_j was decreased by applying 2 mM heptanol. Top trace: current recorded in cell held at constant potential (= $-I_j$); bottom trace: current recorded in cell whose membrane potential was changed from −55 to + 45 mV (= $I_m + I_j$). Gating of individual channels can be observed as steplike current transitions, which occur simultaneously in both traces and are of equal amplitude but of opposite sign, (*B*) Frequency histogram of conductance changes determined from steps in I_j recorded with the perforated-patch method in pairs of cholesterol-enriched cardiomyocytes. g_j was decreased to the single channel level with 2 mM heptanol. ΔV_j = 100 mV. Binwidth = 4 pS. Bars indicate SD per bin. Mean and SD of the unitary conductances were obtained by fitting the data to a double Gaussian distribution using a least-squares method, and are indicated in the figure. N = number of events; n = number of experiments. The dotted line indicates the distribution of conductance changes obtained from control cardiomyocytes exposed to 2 mM heptanol, as reported earlier (Takens-Kwak et al., 1992).

Effects of Heptanol on Nonjunctional Membrane Currents of Cholesterol-enriched Cardiomyocytes

To establish whether an increase in cellular cholesterol content affected the sensitivity of nonjunctional membrane channels to heptanol, we studied current-voltage (*I-V*) relations of single cardiomyo-

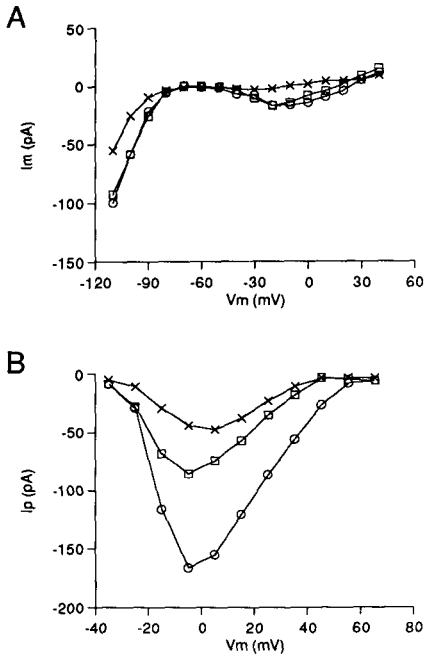

Fig. 7. (*A*) Quasi-steady-state current-voltage (I_m-V_m) relations recorded in a single cholesterol-enriched ventricular cardiomyocyte before (o), during (x), and after □) exposure to 2 mM heptanol. Current amplitudes plotted were determined at the end of a 1-sec clamp pulse. Holding potential (V_h) = −60 mV. (*B*) Peak current-voltage (I_p-V_m) relations recorded in the same myocyte before (o), during (x), and after □) exposure to 2 mM heptanol. Current amplitudes plotted were determined at a maximum negative peak of inward current. V_h = −35 mV.

cytes. To prevent "rundown" of the Ca^{2+} current due to washout of intracellular constituents, we used the perforated-patch configuration in these experiments. To obtain low access resistances to the cells (10–40 MΩ), we used cardiomyocyte pairs whose cellular cholesterol content was increased by means of cholesterol-rich liposomes.

Figure 7*A* shows quasi-steady-state *I-V* curves obtained before, during, and after exposure to 2 mM heptanol. Before and after heptanol treatment, the curve showed the N-shape typical for ventricular cardiomyocytes, whereas during heptanol the curve was flattened. Thus, the inward rectifying K^+ current (I_{K1}; observed in response to voltage-clamp steps to −110 to −80 mV from a holding potential of −60 mV), as well as the delayed rectifying K^+ current (I_K; observed in response to voltage-clamp steps to +10 to +40 mV from the same holding potential), were reduced by heptanol. The effects of 2 mM heptanol on other inward currents were assessed by comparison of peak *I-V* curves obtained before, during, and after exposure. If positive voltage-clamp steps were applied from a holding potential of −60 mV the peak current reflected a combination of Na^+ current (I_{Na}) and Ca^{2+} current (I_{Ca}). Due to large current flow, it was not possible to perform a reliable voltage clamp on these combined inward currents (Beeler & McGuigan, 1978). However, exposure to heptanol was associated with a large decrease in peak current, which was partly reversible after heptanol was washed out (*not shown*). If positive voltage-clamp steps were applied from a holding potential of −35 mV, peak current reflected I_{Ca} only (Fig. 7*B*). Exposure to heptanol decreased the peak current. This was partly reversible after heptanol was washed out. These effects of heptanol on nonjunctional membrane currents were comparable to, or even slightly larger than the effects of heptanol on myocytes cultured under control conditions (see Fig. 2 in Takens-Kwak et al., 1992) and were confirmed by two additional experiments. Unlike the case with gap junction channels, cholesterol enrichment does not affect the sensitivity of nonjunctional membrane channels against heptanol-induced closure. Therefore, we hypothesize nonjunctional membrane channels to be localized in membrane domains which differ from gap junction domains with respect to lipid composition.

To establish whether the effects of heptanol on nonjunctional membrane channels were due to a change in bulk membrane fluidity, we performed some experiments with A_2C on control cardiomyocyte pairs. Figure 8*A* shows quasi-steady-state *I-V* curves obtained before and during exposure to 20 μM A_2C. The curve showed the N-shape typical for ventricular cardiomyocytes, and during A_2C exposure, I_{K1} as well as I_K were slightly reduced. Peak *I-V* curves obtained before and during A_2C exposure showed that I_{Ca} was reduced during A_2C exposure (Fig. 8*B*) and that I_{Na} might be reduced also during the exposure (*not shown*), although we were not able to determine I_{Na} properly due to an inadequate voltage clamp. The complete reversibility of the A_2C effects could not be studied as washout of A_2C took a very long period of time which can be explained by its strong lipophilic character. These results were confirmed by two additional experiments. The effects of 20 μM A_2C on nonjunctional membrane currents were much smaller compared to the effects of 2 mM heptanol, which increased bulk membrane fluidity to the same extent. Therefore, we conclude that increased bulk membrane fluidity does affect nonjunctional membrane channels, but is certainly not solely responsible for the heptanol-induced decrease in these currents.

Interestingly, both nonjunctional and gap junctional currents are reduced by heptanol—nonjunctional current by an increase in bulk membrane fluidity and gap junctional current by a decrease in cholesterol-rich domain fluidity. In our opinion, this

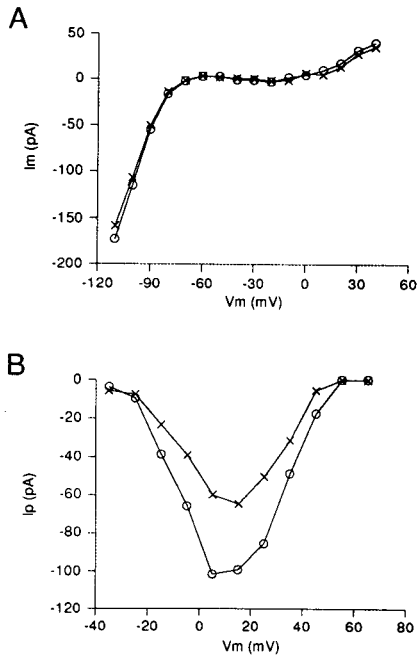

Fig. 8. (*A*) Quasi-steady-state current-voltage (I_m-V_m) relations recorded in a single ventricular cardiomyocyte before (o) and during (x) exposure to 20 μM A_2C. Current amplitudes plotted were determined at the end of a 1-sec clamp pulse. Holding potential (V_h) = −60 mV. (*B*) Peak current-voltage (I_p-V_m) relations recorded in the same myocyte before (o) and during (x) exposure to 20 μM A_2C. Current amplitudes plotted were determined at a maximum negative peak of inward current. V_h = −35 mV.

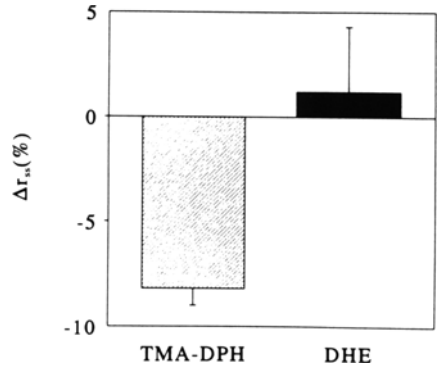

Fig. 9. The effect of 5 mM 1-butanol on sarcolemmal fluidity, as assessed with TMA-DPH anisotropy, and on the fluidity of cholesterol-rich domains, as assessed with DHE anisotropy. r_{ss} was measured before, and 20 min after addition of 5 mM 1-butanol. The difference, Δr_{ss}, was expressed as a percentage of its initial value. Indicated are mean ± SEM of five measurements.

difference is due to a difference in lipid environment, i.e., gap junctions are embedded in cholesterol-rich domains, whereas the nonjunctional channels probably are not.

Mechanism of Heptanol-induced Uncoupling

The molecular mechanism that leads to heptanol-induced cell uncoupling is not resolved yet. A direct effect on the gap junction channel through binding to the channel protein or alterations of the lipid environment around the channel are plausible explanations. Combining the results of r_{ss} measurements in the different lipid domains with the results obtained with measurements of g_j after exposure to heptanol and A_2C suggests that the fluidity of cholesterol-rich domains is of importance to cell coupling. Moreover, the results suggest that the heptanol-induced uncoupling of cell pairs is mediated by a decrease in the fluidity of cholesterol-rich domains, rather than by an increase in the bulk fluidity of the sarcolemmal membrane. The structure and concentration of the applied lipophilic agents, A_2C and 1-heptanol, differ markedly. The possibility that heptanol-induced uncoupling is mediated via direct interactions of the hydroxyl group of heptanol with amino acid residues of the gap junction protein (hydrogen bond), as suggested by Burt (1989), thus, cannot be excluded. Unlike heptanol, butanol does not affect g_j (Chanson et al., 1989), but its hydroxyl group is expected to behave similar to the hydroxyl group of heptanol. Therefore, we performed experiments with 1-butanol to study its effect on r_{ss} of TMA-DPH and DHE. Butanol (5 mM) increased the sarcolemmal bulk fluidity to the same extent as heptanol and A_2C, but did not alter the fluidity of cholesterol-rich domains (Fig. 9). This observation suggests that an interaction of the hydroxyl group of the alcohol with gap junction channel protein is not a likely mechanism to explain heptanol-induced uncoupling.

Heptanol induces rigidification of the gap junction lipid domain, leading to a decrease in g_j. Rigidification is known to increase lateral pressure on proteins, which interferes with protein conformation in the membrane (Shinitzky, 1984). Quantitative freeze-fracture experiments revealed that heptanol-induced uncoupling in heart tissue is accompanied by a reduction in gap junction particle diameter as well as center-to-center particle spacing (Délèze & Hervé, 1983), which is consistent with increased lateral pressure.

An increase in cellular cholesterol content may induce an increase in the size of the cholesterol-rich lipid domains. Consequently, more heptanol will be needed to achieve the same increase in r_{ss} of cholesterol-rich lipid domains compared to these domains in control cells. Thus, one will need more heptanol to uncouple cholesterol-enriched cell pairs completely.

Unfortunately, we were not able to apply heptanol concentrations higher than 3 mM because these concentrations destroyed the DHE-enriched neonatal rat cardiomyocytes (Fig. 5).

In summary, this study demonstrates that the fluidity of cholesterol-rich domains is of importance to cell coupling, and that heptanol decreases g_j by decreasing the fluidity of these cholesterol-rich domains. Cholesterol enrichment partly protects pairs of cardiomyocytes against heptanol-induced uncoupling.

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