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ORIGINAL ARTICLE

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# Long-chain n-alkanols and arachidonic acid interfere with the $V_m$-sensitive gating mechanism of gap junction channels

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**Abstract** Experiments were carried out on preformed cell pairs and induced cell pairs of an insect cell line (mosquito *Aedes albopictus*, clone C6/36). The coupling conductance, $g_j$, was determined with the dual voltage-clamp method. Exposure of preformed cell pairs to lipophilic agents, such as long-chain n-alkanols (n-hexanol, n-heptanol, n-octanol, n-nonanol, n-decanol) or arachidonic acid, provoked a decrease in $g_j$. Hyperpolarization of both cells led to a recovery of $g_j$. Systematic studies revealed that this phenomenon is caused by a shift of the sigmoidal relationship $g_{j(ss)} = f(V_m)$ towards more negative values of $V_m$ (where $g_{j(ss)}$ = conductance at steady-state; $V_m$ = membrane potential). The shift was dose dependent, it developed with time and was reversible. The longer the hydrocarbon chain of n-alkanols, the lower was the concentration required to produce a given shift. Besides shifting the function $g_{j(ss)} = f(V_m)$, arachidonic acid decreased the maximal conductance, $g_{j(max)}$. Single-channel records gained from induced cell pairs revealed that the lipophilic agents interfere with the $V_m$-sensitive slow channel gating mechanism. Application provoked slow current transitions (transition time: 5–40 ms) between an open state of the channel (i.e. main state or residual state) and the closed state; subsequently, fast channel transitions (transition time: < 2 ms) involving the main state and the residual state ceased completely. Hyperpolarization of $V_m$ or washout of the lipophilic agents gave rise to the inverse sequence of events. The single-channel conductances $\gamma_{j(main\ state)}$ and $\gamma_{j(residual\ state)}$ were not affected by n-heptanol. We conclude that long-chain n-alkanols and arachidonic acid interact with the $V_m$-sensitive gating mechanism.

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## Introduction

Lipophilic agents, such as long-chain n-alkanols or fatty acids, impair the conductance of gap junctions in various tissues [9–11, 13, 15, 16, 19, 20]. However, despite extensive studies the molecular mechanism resulting in cell-to-cell uncoupling remains obscure. Niggli et al. [16] suggested that n-heptanol, n-octanol and halothane act by incorporation into the lipid bilayer of cell membranes. Bastiaanse et al. [3] reported that n-heptanol decreases the fluidity of cell membranes, preferentially in domains rich in cholesterol, and thereby provokes uncoupling. Burt et al. [10] proposed that lipophilic agents produce local disorder at the lipid–protein interface of cell membranes which leads to conformational changes of connexins and hence connexon dysfunction. Bastide et al. [4] recently reported that $[Ca^{2+}]_i$ is not involved in n-heptanol-dependent uncoupling.

Over the last decade, lipophilic agents have been used routinely to assess the properties of single gap junction channels (see [9, 11, 18]). The rationale was to reduce the number of operational channels in preformed cell pairs and thus to resolve single-channel events. It has been proposed that lipophilic agents do not affect the conductance of gap junction channels, but interfere with the open channel probability (see [9, 21]). However, so far quantitative data supporting this view have not been obtained.

The purpose of the present study has been to explore the effects of long-chain n-alkanols and arachidonic acid on voltage-sensitive gating of gap junction channels. The experiments were carried out on *preformed* cell pairs and *induced* cell pairs of an established insect cell line (mosquito *Aedes albopictus*, clone C6/36 [12]). In these preparations, the intercellular current flow is controlled by the voltage gradient across the gap junction, $V_j$, and the

voltage gradient across the cell membrane, $V_m$ [6, 7]. Our results demonstrate that the agents investigated act by interfering with the mechanism of $V_m$-sensitive slow gating of gap junction channels. Preliminary results have already been published [27].

## Materials and methods

### Cells and culture conditions

Cells of an insect cell line (clone C6/36, derived from larvae of the mosquito *Aedes albopictus*; ATCC code CRL 1660 [12]) were grown at 28°C in culture medium (RPMI 1640, Gibco, Paisley, UK) supplemented with 20% fetal calf serum, 100 µg/ml streptomycin and 100 U/ml penicillin (code 2212; Biochrom, Berlin, Germany). Once a week the cells were passaged and diluted 1:10. To carry out experiments, monolayers of cells ($\approx 4 \cdot 10^5$ cells/cm$^2$) were harvested and resuspended in RPMI 1640 containing 20% fetal calf serum at a cell density of $0.2 \cdot 10^6$–$1 \cdot 10^6$ cells/ml. Subsequently, the cells were seeded at a density of $\approx 10^4$ cells/cm$^2$ onto glass coverslips placed in multiwell culture dishes. Electrophysiological measurements were carried out 1–3 days after plating.

### Solutions and pipettes

All experiments were carried out using modified Krebs-Ringer solution (in mM): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 5 (pH adjusted to 7.4), glucose 5, pyruvate 2. Patch pipettes were pulled from glass capillaries (GC150TF-10; Clark Electromedical Instruments, Pangbourne, UK) using a horizontal puller (BB-CH; Mécanex, Geneva, Switzerland). Pipettes were filled with solution of the following composition (in mM): $K^+$ aspartate$^-$ 120, NaCl 10, $CaCl_2$ 1, $MgCl_2$ 1, MgATP 3, HEPES 5 (pH 7.2), EGTA 10 (pCa ≈8), filtered through 0.22-µm pores. Filled pipettes had direct current resistances of 3–5 MΩ (tip size: ≈1 µm). Long-chain n-alkanols (n-hexanol, n-heptanol, n-octanol, n-nonanol, n-decanol; Fluka, Buchs, Switzerland) were dissolved directly in Krebs-Ringer solution. Arachidonic acid (Sigma, St. Louis, Mo., USA) was mixed with hexane. The stock solution (10 mM) was divided into aliquots for storage at –20°C. Before use the solvent was removed under a stream of $N_2$ and the dry substance dispersed in Krebs-Ringer solution. Formation of fatty acid micelles was achieved by sonication at 35 kHz for 90 s.

### Electrical measurements

The experimental chamber consisted of a Perspex frame with a glass bottom. It was mounted on the stage of an inverted microscope equipped with phase-contrast optics (Diaphot-TMD, Nikon; Nippon Kogaku, Tokyo, Japan). The chamber was perfused with Krebs-Ringer solution at room temperature (22–26°C) by means of gravity (bath volume: 1 ml; flow rate: 1–2 ml/min). A video system (CCD camera, Panasonic WV-CD52; TV monitor, Panasonic WV-5410; Matsushita Electric Industrial, Osaka, Japan) facilitated the optical supervision of the cells and pipettes during an experiment.

To study the electrical properties of gap junction channels, the dual voltage-clamp method was used [24]. In brief, coverslips with adherent cells were transferred from the culture dishes to the experimental chamber. Experiments were carried out on *induced cell pairs* [6] and *preformed cell pairs* [7]. In the case of induced pairs, two single cells in close proximity were selected visually under the microscope. Each cell was attached to a patch pipette connected to a separate voltage-clamp amplifier (EPC 7; List Electronic, Darmstadt, Germany). After implementing the whole-cell, tight-seal recording conditions, the cells were manoeuvred against each other by moving the patch pipettes via micromanipulators (WR-88; Narishige Scientific Instrument, Tokyo, Japan). This led to the establishment of a physical cell-to-cell contact and subsequent channel formation. This approach enabled us to control the membrane potential of each cell ($V_1$, $V_2$) and to separately measure the associated currents through both pipettes ($I_1$, $I_2$). In the case of preformed pairs, cell pairs spontaneously formed in culture were selected visually. In the absence of intercellular communication, $I_1$ and $I_2$ represent currents through the membrane of cell 1 ($I_{m,1}$) and cell 2 ($I_{m,2}$), respectively. In the presence of intercellular communication, $I_1$ and $I_2$ correspond to $I_{m,1} + I_j$ and $I_{m,2}–I_j$, respectively ($I_j$: current through gap junction). Deflections in $I_1$ and $I_2$, coincident in time and opposite in polarity, reflect changes in $I_j$. The conductance of a gap junction, $g_j$, or a gap junction channel, $\gamma_j$, is given by the ratio $I_j/(V_2–V_1)$. The difference $V_2–V_1$ corresponds to the voltage gradient across the gap junction, $V_j$.

### Signal recording and analysis

Voltage and current signals were recorded on FM tape (SE 3000; SE Lab, Feltham, UK) at 90 mm (direct current bandwidth: 2.5 kHz). For off-line analysis with a personal computer, the current signals were filtered at 1 kHz (–3 dB) with an 8-pole Bessel filter (902LPF; Frequency Devices, Haverhill, Mass., USA) and digitized at 3.33 kHz with a 12-bit A/D converter (IDA 12120; INDEC Systems, Capitola, Calif., USA). Data acquisition and analysis were done with modular software (C-Lab; INDEC Systems). Measurements of membrane potentials were corrected for the liquid junction potential between pipette solution and bath solution (–12 mV).

## Results

### Dependence of coupling conductance on membrane potential

Previous studies of preformed pairs of C6/36 cells have shown that $g_j$ is sensitive to $V_m$ [7]. Figure 1A documents the experimental approach used to elucidate this property. The membrane potentials of cells 1 and 2 were clamped to a common holding potential, i.e. $V_{m,1} = V_{m,2} = –35$ mV (traces $V_1$ and $V_2$). Small test pulses were then administered repetitively to cell 2 (–15 mV; 100 ms; 1 Hz) to establish a junctional voltage gradient, $V_j = –15$ mV (downward spikes in $V_2$), and to elicite a junctional current, $I_j$ (upward spikes in $I_1$). The $I_j$ signals gained in this way were used to determine $g_j$, i.e. $I_j/V_j$. Simultaneous application of a hyperpolarizing conditioning pulse of 50 mV to both cells (left-hand panel) had virtually no effect on the amplitude of $I_j$. However, concomitant application of a depolarizing conditioning pulse of 80 mV (right-hand panel) gave rise to a pronounced time-dependent decrease in $I_j$. Within 25 s, $I_j$ declined virtually to zero. Return to the holding potential of –35 mV led $I_j$ to recover with time in an exponential manner. After 10 s, $g_j$ was back to the value prior to application of the conditioning pulse, i.e. 7.4 nS.

Figure 1B illustrates the behaviour of the same preparation after exposure to 1 mM n-heptanol (C-7). This corresponds to a submaximal dose for cell-to-cell uncoupling [18]. The n-alkanol was administered at a low flow rate to slow down the impairment of $I_j$. $V_{m,1}$ and $V_{m,2}$ were again clamped to the same holding potential (–35 mV) while a small test pulse was administered re-

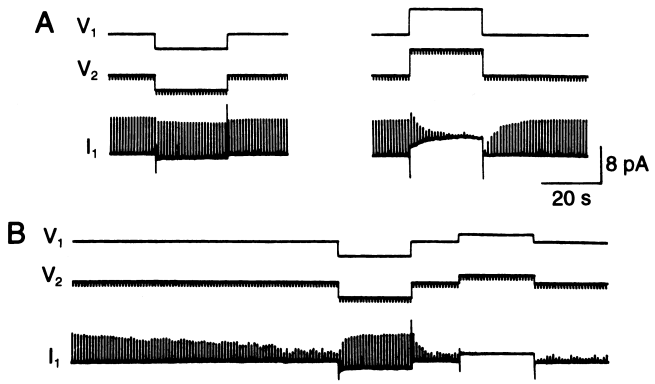


**Fig. 1A, B** Dependence of junctional current ($I_j$) on membrane potential ($V_m$) examined in a preformed cell pair. ($V_1$ and $V_2$ Membrane potential of cells 1 and 2, respectively, $I_1$ current of cell 1). Both cells were clamped to the same membrane potential (–35 mV), while a test pulse was applied repetitively to cell 2 (–15 mV; 100 ms; 1 Hz; downward deflections in $V_2$) to assay $I_j$ (upward deflections in $I_1$). **A** A conditioning pulse of –50 mV (*left-hand panel*) and 80 mV (*right-hand panel*) was administered concomitantly to cell 1 ($V_1$) and cell 2 ($V_2$). The former had no effect on $I_j$, the latter produced a reversible decrease in $I_j$ which developed with time. **B** Exposure to 1 mM n-heptanol led to a gradual decline in $I_j$. Conditioning pulses to –85 mV gave rise to a recovery of $I_j$, conditioning pulses to –10 mV led to complete uncoupling

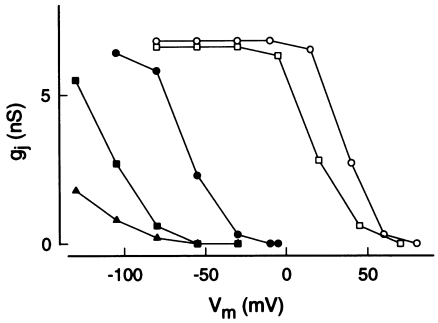


**Fig. 2** Effects of n-heptanol on the relationship of gap junction conductance ($g_j$) versus $V_m$. The junctional currents at the end of each conditioning pulse, $I_{j(ss)}$, were determined, the steady-state conductances, $g_{j(ss)}$, calculated and plotted versus $V_m$. During control (*open circles*) the relationship $g_{j(ss)} = f(V_m)$ was sigmoidal with $g_{j(max)} = 6.8$ nS, $V_{m,0} = 35$ mV, and the maximal slope of $g_{j(ss)} = f(V_m) = 0.14$ nS/mV. Exposure to 2 mM n-heptanol for 2–3 min (*filled circles*), 3–4 min (*filled squares*), and 4–5 min (*filled triangles*) provoked a leftward shift of the relationship ($V_{m,0} = –65$ mV, about –115 mV, less than –130 mV, respectively), and a slight decrease in $g_{j(max)}$ and the maximal slope of $g_{j(ss)} = f(V_m)$. Washout of n-heptanol for 8 min led to a recovery [*open squares*; $V_{m,0} = 18$ mV, $g_{j(max)} = 6.6$ nS, maximal slope of $g_{j(ss)} = f(V_m) = 0.11$ nS/mV]

petitively to cell 2 (downward spikes in $V_2$). The pharmacological intervention provoked a gradual decline of $I_j$ (upward spikes in $I_1$). About 9 min after the beginning of the intervention, a hyperpolarizing conditioning pulse of 50 mV was applied to both cells ($V_{m,1} = V_{m,2} = –85$ mV). This gave rise to a paradoxical increase in $I_j$ which developed with time. The increase in $I_j$ resembled the $V_m$-dependent recovery of $I_j$ shown in Fig. 1A, right-hand panel. After about 10 s, the restoration of $I_j$ was complete, i.e. its amplitude was close to control (compare trace $I_1$ in Fig. 1A: amplitude of $I_j$ at $V_{m,1} = V_{m,2} = –35$ mV). Return to the holding potential was associated with a time-dependent decrease in $I_j$. The amplitude of $I_j$ eventually reached a value that was lower than that prior to hyperpolarization. This suggests that the uncoupling caused by n-heptanol proceeded during the conditioning pulses. Subsequent application of a depolarizing conditioning pulse of 25 mV amplitude ($V_{m,1} = V_{m,2} = –10$ mV) provoked a small $I_j$ signal which disappeared completely with time. Return to the holding potential led to a partial recovery of $I_j$. Again, this indicates that the impairment of $I_j$ by n-heptanol had developed further in the meantime.

## Effects of n-heptanol on the relationship $g_j$ versus $V_m$

To establish the complete relationship between $g_j$ and $V_m$, the amplitude of the conditioning pulse was increased stepwise using increments of 25 mV or less. The conditioning pulses were administered simultaneously to both cells. Each step lasted 5–25 s, depending on the time course of $I_j$ decay. In each case $I_j$ was allowed to reach a steady-state, $I_{j(ss)}$. $V_m$ was increased until complete uncoupling. The test pulse applied repetitively to one of the cells was kept small and short (–15 mV; 100 ms; 1 Hz) to allow reliable estimates of $g_j$ [7]. The analysis of the current records obtained in this way revealed a decrease in $I_{j(ss)}$ with increasing depolarization of $V_m$. The time course of $I_j$ decay was faster during large depolarizing conditioning pulses. Similarly, the time course of $I_j$ recovery showed a slight dependence on the amplitude of the preceding depolarization. Furthermore, it increased slightly with an increasing duration of the conditioning pulse (data not shown).

Figure 2 summarizes the results from a complete experiment. The current records were assayed for $I_j$ prevailing at the end of each conditioning pulse, $I_{j(ss)}$. The values of $g_{j(ss)}$ were calculated and plotted versus $V_m$. In the presence of control solution (open circles), the relationship $g_{j(ss)} = f(V_m)$ was sigmoidal. For values of $V_m$ ranging from –80 mV to –10 mV, $g_{j(ss)}$ was maximal, i.e. $g_{j(max)} = 6.8$ nS. It started to decrease when $V_m$ was depolarized beyond –10 mV. At $V_m = 80$ mV, $g_{j(ss)}$ reached zero. The membrane potential at which $g_{j(ss)}$ had decreased by 50%, $V_{m,0}$, was 35 mV. At time $t = 0$ min, the bath was perfused at a slow rate with a solution containing 2 mM n-heptanol. This provoked a shift of the relationship $g_{j(ss)} = f(V_m)$ towards more negative values of $V_m$ which developed gradually with time. The data collected between 2 and 3 min (filled circles) and 3 and 4 min (filled squares) yielded $V_{m,0}$ values of –65 and approx. –115 mV, respectively. At time $t = 4$–5 min (filled triangles), the relationship $g_{j(ss)} = f(V_m)$ was further shifted to the left. However, due to the risk of electrical breakdown of the cell membranes, $V_m$ was not stepped beyond

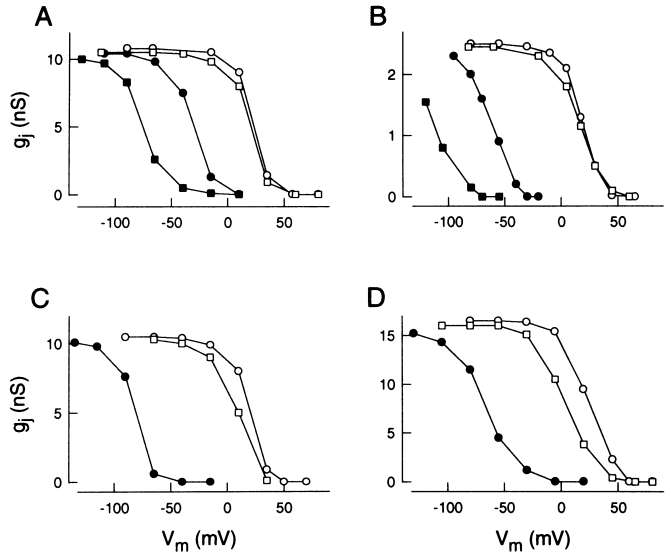


**Fig. 3A–D** Alterations of the relationship $g_{j(ss)} = f(V_m)$ caused by exposure to different n-alkanols. The *open circles* and *open squares* depict the situation before application and after washout of n-alkanol, respectively. Time $t = 0$ min corresponds to the beginning of an intervention. **A** Influence of 15 mM n-hexanol at time $t = 5–6$ min (*filled circles*) and 11–12 min (*filled squares*). **B** Influence of 0.5 mM n-octanol at time $t = 2–3$ min (*filled circles*) and 4–5 min (*filled squares*). **C** Influence of 200 μM n-nonanol at time $t = 4–5$ min (*filled circles*). **D** Influence of 20 μM n-decanol at time $t = 3–4$ min (*filled circles*)

–130 mV. Hence, estimates of $V_{m,0}$ were no longer possible. Associated with the shift of $V_{m,0}$ along the $V_m$ axis, the maximal slope of the function $g_{j(ss)} = f(V_m)$ became progressively more shallow. This phenomenon was prominent at time $t = 3–4$ min and 4–5 min. It suggests the involvement of more than one mechanism of action. Washout of n-heptanol shifted the relationship $g_{j(ss)} = f(V_m)$ back to the right-hand side. The relationship was close to control after 8 min (open squares). The small residues in $V_{m,0}$ shift and $g_{j(max)}$ decline may reflect rundown of the preparation and/or incomplete washout of the n-alkanol. The influence on $g_{j(ss)} = f(V_m)$ by n-heptanol was examined in 19 preparations by varying the concentration from 1 to 3 mM. The effects on the $V_m$- and time-dependent alterations of $g_{j(ss)}$ were comparable. Larger doses provoked more profound effects which developed faster.

## Effects of other n-alkanols on the relationship $g_j$ versus $V_m$

Using the same protocol, we explored the effects on $I_j$ of n-alkanols of different hydrocarbon chain length. The substances examined include n-pentanol (C-5), n-hexanol (C-6), n-octanol (C-8), n-nonanol (C-9), and n-decanol (C-10). The substance with the shortest hydrocarbon chain, n-pentanol, exerted no measurable effects on $I_j$ at concentrations up to 15 mM (data not shown). The other n-alkanols reversibly impaired $I_j$ as shown in Fig. 3.

The C-6 compound was administered at concentrations of 5–15 mM. Exposure to 5 mM for 10 min provoked a left-hand shift of the function $g_{j(ss)} = f(V_m)$ by 10–20 mV without affecting $g_{j(max)}$ and the maximal slope of $g_{j(ss)} = f(V_m)$ ($n = 3$; data not shown). The result of an experiment performed in the presence of 15 mM n-hexanol is illustrated in Fig. 3A. It shows the relationship $g_{j(ss)} = f(V_m)$ during control (open circles), at time $t = 5–6$ min (filled circles), and $t = 11–12$ min (filled squares) after application of the n-alkanol. The analysis yielded a shift of $V_{m,0}$ from approximately 22 mV to –31 mV and –77 mV, respectively. This shift was accompanied by a small decrease in $g_{j(max)}$ and the maximal slope of $g_{j(ss)} = f(V_m)$. After washout for 12 min, the three parameters were restored almost completely (open squares). Similar results were obtained in four additional experiments.

The C-8 compound was examined at concentrations of 0.15–0.5 mM. As shown in Fig. 3B, the largest dose shifted the relationship $g_{j(ss)} = f(V_m)$ rather quickly. $V_{m,0}$ was changed from approximately 18 mV (control; open circles), to –60 mV ($t = 2–3$ min; filled circles), and approx. –110 mV ($t = 4–5$ min; filled squares). At the same time, $g_{j(max)}$ and the maximal slope of $g_{j(ss)} = f(V_m)$ were slightly impaired. The three parameters recovered almost completely within 8 min of washout (open squares). Similar results were observed in five additional experiments.

The C-9 compound was tested at concentrations of 100–300 μM. Figure 3C illustrates that exposure to 200 μM n-nonanol provoked a substantial shift of the function $g_{j(ss)} = f(V_m)$ within 4 min, i.e. $V_{m,0}$ was changed from approximately 20 mV (control; open circles) to –82 mV ($t = 4$ min; filled circles). At that time the effects on $V_{m,0}$ and the maximal slope of $g_{j(ss)} = f(V_m)$ were marginal only. Washout for 8 min restored $V_{m,0}$ to 10 mV (open squares). Comparable results were obtained in four additional experiments.

The C-10 compound was examined at concentrations of 10–50 μM. In all experiments, we observed a time- and dose-dependent shift of $g_{j(ss)} = f(V_m)$ along the $V_m$ axis which recovered during washout. Figure 3D documents the result of a typical example. Exposure to 20 μM n-decanol shifted $V_{m,0}$ from about 25 mV (control; open circles) to –67 mV ($t = 3–4$ min; filled circles). This n-alkanol also produced a small decrease in $g_{j(max)}$ and the maximal slope of $g_{j(ss)} = f(V_m)$. Washout for 8 min restored the three parameters almost completely ($V_{m,0} = 10$ mV; open squares). Similar behaviour was found in four additional experiments.

## Actions of n-heptanol on single channels

The effects of n-heptanol on single gap junction channels were examined using the induced cell pair approach (see Materials and methods, Electrical measurements). For this purpose, coverslips with adherent cells were screened visually for two single cells in close proximity.

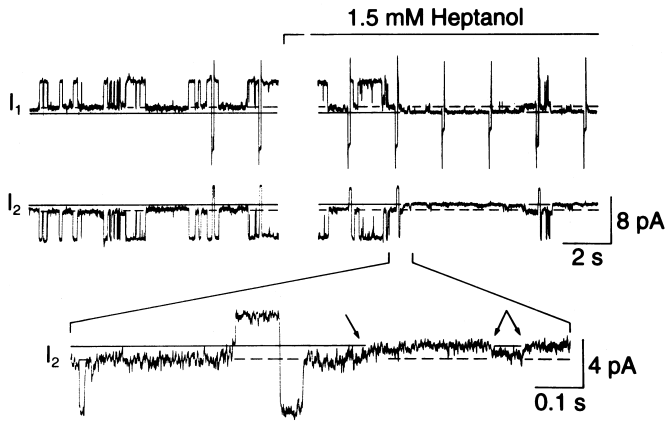


**Fig. 4** Influence of n-heptanol on a single gap junction channel studied in an induced cell pair. The current signals $I_1$ and $I_2$ were recorded simultaneously from cell 1 and cell 2. Synchronous transitions in $I_1$ and $I_2$, identical in amplitude and of opposite polarity, reflect gap junction events. Channel openings gave rise to upward deflections in $I_1$ and downward deflections in $I_2$. $V_1$ = –40 mV, $V_2$ = –57 mV. The first channel opening occurred at time $t$ = 0 min. At time $t$ = 21 s, 2 mM n-heptanol was applied. The *inset* shows a segment of $I_2$ displayed on an expanded time scale. It documents slow transitions between the residual state and the closed state (see *arrows*). The small upward deflections in $I_2$ resulted from hyperpolarizing test pulses applied to cell 1 (–25 mV, 100 ms, 0.5 Hz). The *dashed lines* indicate the residual current, the *solid lines* the zero-current level

Each cell was then brought into contact with a patch pipette to form a giga-ohm seal. After disruption of the membrane patches, the whole-cell recording conditions were established. The membrane potentials of cell 1 and cell 2 were clamped to different levels ($V_{m,1} \neq V_{m,2}$). Thereafter, the cells were pushed against each other by gently moving the pipettes via micromanipulators. Once the cell-to-cell contact was achieved, the sequential formation of gap junction channels began. In each preparation, there was a variable time window with only one operational channel present. During this period the junctional currents were suitable for studying single-channel events.

Figure 4 shows paired current traces ($I_1$ and $I_2$) recorded during application of 1.5 mM n-heptanol. Discrete steps in $I_1$ and $I_2$, concomitant in time and opposite in polarity, reflect the activity of a gap junction channel. On the left-hand side, the signals represent the situation shortly after the first channel inserted had begun to operate (time $t$ = 0 s: incidence of first channel opening). A maintained $V_j$ gradient of –17 mV was present throughout ($V_{m,1}$ = –40 mV, $V_{m,2}$ = –57 mV; not shown). The associated currents exhibited fast transitions (< 2 ms) between two discrete levels corresponding to the main state and the residual state (dashed lines) of the channel. The respective conductances $\gamma_{j(\text{main state})}$ and $\gamma_{j(\text{residual state})}$ approximated 375 and 80 pS. $I_1$ and $I_2$ did not reach the zero-current level (solid lines) indicating that the channel failed to close completely. At time $t$ = 18 s, superimposed on the maintained $V_j$ gradient, a hyperpolarizing test pulse was administered repetitively to cell 1 (–25 mV, 100 ms, 0.5 Hz) giving rise to a $V_j$ of 8 mV and a small $I_j$ (upward deflections in $I_2$).

At time $t$ = 21 s, 2 mM n-heptanol was added to the bath. The test solution was administered via a blunt glass pipette positioned close to the preparation to reduce the delay (dead time for local fluid exchange: few seconds). As a result, shortly after time $t$ = 38 s, $I_1$ and $I_2$ simultaneously underwent a slow transition between the residual current level (dashed lines) and the zero current level (solid lines) indicative of a complete channel closure. The channel then remained in the closed state for most of the time. As a consequence, $I_j$ signals elicited by the test pulse were absent at time $t$ = 40 s, 42 s, and 46 s. Most strikingly, starting from the closed state, there was no fast channel flickering. On a few occasions, $I_1$ and $I_2$ synchronously returned to the residual current level for short periods of time. Such episodes always began and ended with a slow transition. During the most prominent episode ($t \cong$ 44 s), the fast flickering between the residual state and the main state resumed temporarily. Furthermore, the test pulse again produced an $I_j$ signal (upward deflection in $I_2$).

To document the differences between fast and slow current transitions, the inset in Fig. 4 repeats a segment of the $I_2$ signal on an expanded time scale. The channel first exhibited fast transitions passing from the residual state (dashed line) to the main state and back to the residual state again. At time $t$ = 38 s, a test pulse was applied to cell 1. This led $V_j$ to change from –17 mV to 8 mV, thus inverting its polarity and decreasing its amplitude. As a result, $I_2$ altered from being "net inward", attributable to the residual state, to "net outward", corresponding to the main state. This is consistent with the idea that inversion of $V_j$ polarity, with virtually no delay, provokes a re-opening of a previously closed $V_j$-sensitive gate [8]. At the end of the test pulse, $V_j$ returned from 8 mV back to –17 mV. This provoked $I_2$ to change instantaneously from "net outward" to "net inward", both reflecting the main state. The channel then returned to the residual state by means of a fast transition. From there, $I_2$ decreased to the zero reference level (solid line) via slow transition, thereby closing the channel completely (see arrow). After some time, $I_2$ transiently returned back to the residual state involving slow transitions (see arrows). Fast current transitions were complete in 1–2 ms, i.e. within the frequency response of the experimental set-up. In contrast, the slow transitions lasted 5–40 ms ($n$ = 16). They were only observed after exposure to n-heptanol, not in the presence of control solution.

Up to the time $t$ = 52 s, the channel dwelled mainly in the closed state. At this time, both cells were hyperpolarized by 35 mV ($V_{m,1}$ = –75, $V_{m,2}$ = –92 mV). As a result the fast channel activity recovered for about 1 s (not shown). This observation is consistent with the data presented in Fig. 1. At time $t$ = 58 s, there was the first opening of the second channel newly inserted. The resulting current transition exhibited a slow time course (≈20 ms), comparable to that of the first opening of the

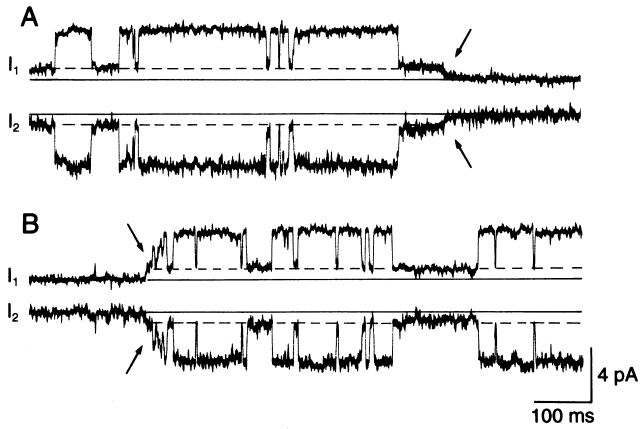


**Fig. 5A, B** Effects of 2 mM n-heptanol on a single gap junction channel examined in a preformed cell pair. $I_1$ and $I_2$ were recorded simultaneously in the presence of a sustained $V_j$ of –10 mV ($V_1$ = –50 mV, $V_2$ = –60 mV). **A** After 10 s, application of n-heptanol led to a slow transition between the residual state and the closed state (see *arrows*) which terminated the fast channel flickering. **B** After 6 s, washout of n-heptanol led to a slow transition between the closed state and the main open state (see *arrows*), thereby re-establishing fast channel activity. The slow transition showed multiple fragmentations. The *dashed lines* indicate the residual current, the *solid lines* the zero-current level

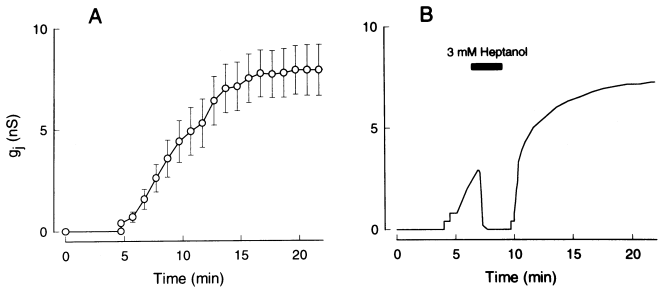


**Fig. 6A, B** De novo formation of gap junctions between two single cells. Time $t$ = 0 min marks the time of physical contact between the cells. **A** Plot of $g_j$ versus time, summarizing the data from 32 experiments carried out under control conditions. *Circles* and *bars* represent means ± 1 SEM, respectively. **B** Plot of $g_j$ versus time, gained from a single experiment. Exposure to 2 mM n-heptanol for 2.5 min (*black bar*) had no effect on the processes underlying formation of a gap junction (*interrupted curve*)

first channel in the absence of n-heptanol. This suggests that the reopening of a channel previously impaired by n-heptanol and the first opening of a newly formed channel resemble each other.

Current traces gained late during uncoupling by n-heptanol or early during recovery from n-heptanol uncoupling were evaluated to determine the single-channel conductances. The analysis of three cell pairs yielded the following values: $\gamma_{j(\text{main state})}$ = 364 ± 7 pS ($n$ = 31); $\gamma_{j(\text{residual state})}$ = 88 ± 3 pS ($n$ = 30). The time intervals between application of n-heptanol and complete uncoupling or vice versa were too short to perform a complete analysis of the open channel probability, $P_o$. However, preliminary experiments ($I_j$ records lasting 10–30 s) revealed that $P_o$ is not affected by n-heptanol (F. F. Bukauskas and R. Weingart, unpublished).

Figure 5 shows current records from a preparation whose gap junction consisted of a single channel. To facilitate the identification of gap junction channel events, paired records ($I_1$ and $I_2$) were displayed on an expanded time scale. The records document the effects of application and washout of 2 mM n-heptanol, gained 10 s after application and 6 s after withdrawal, respectively. After exposure to the agent (see Fig. 5A), initially the channel exhibited fast transitions between the residual state (dashed lines) and the main state. Substates were present occasionally as well (not shown). Suddenly, the channel underwent a slow transition between the residual state and the closed state (solid lines) which developed over 14 ms (see arrows). As a result, the fast channel flickering ceased completely. Transient hyperpolarization of $V_1$ and $V_2$ resumed the fast channel activity via slow reopening (not shown). This finding is consistent with the observations made of cell pairs whose gap junction contained many channels (see Fig. 1A, right-hand panel, or Fig. 1B). Shortly after withdrawal of n-heptanol (see Fig. 5B), the channel underwent a slow transition between the closed state and the main state (see arrows). It showed a fragmented behaviour which developed over approximately 40 ms. As soon as the main state was reached, the channel restarted with fast transitions involving the main state and the residual state. In three out of nine experiments, the first transition after n-heptanol washout occurred between the closed state and main state, in the other cases between the closed state and residual state.

## Gap junction formation in the presence of n-heptanol

To examine the effects of n-heptanol on formation of gap junctions, we used the induced cell pair approach (see Materials and methods, Electrical measurements). Early during these experiments, i.e. when only few channels were present (≤ 2), a sustained $V_j$ gradient was applied to determine $I_j$. Later on, when more channels were involved, a $V_j$ pulse was administered repetitively (≈12 mV, 100 ms, 1 Hz). The values of $g_j$ obtained in this way were plotted versus time.

Figure 6A summarizes the results from 32 experiments carried out under control conditions. Time $t$ = 0 min indicates the moment of physical cell-to-cell contact. The very first channel opening ensued after a delay of 4.7 ± 0.6 min. After this event, $g_j$ was determined every minute. The values obtained were averaged and plotted versus time. The graph shows that channel insertion followed a sigmoidal time course. The maximal rate of channel insertion was about 2.5 channels/min. At time $t$ = 20 min, steady-state coupling was reached with $g_j$ = 8 ± 1 nS, corresponding to 21 channels with a $\gamma_{j(\text{main state})}$ of 375 pS (see [6]; see also Actions of n-heptanol on single channels).

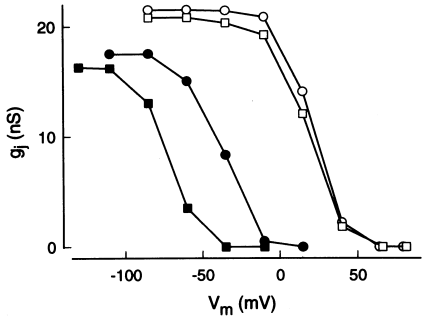


**Fig. 7** Effect of arachidonic acid (*AA*) on the relationship between $g_{j(ss)}$ and $V_m$. During control (*open circles*), the function $g_{j(ss)} = f(V_m)$ was sigmoidal [$g_{j(max)}$ = 21 nS; $V_{m,0}$ = 22 mV; maximal slope of $g_{j(ss)} = f(V_m)$ = 0.37 nS/mV]. Exposure to 10 μM AA for 6–7 min (*filled circles*) and 10–11 min (*filled squares*) gave rise to a leftward shift of the curve ($V_{m,0}$ = –40 mV and –70 mV), a decrease in $g_{j(max)}$ (18 nS and 17 nS), and a decrease in maximal slope of $g_{j(ss)} = f(V_m)$ (0.29 nS/mV and 0.27 nS/mV). Washout of AA for 16 min led to a recovery (*open squares*)

Figure 6B illustrates the result of a single experiment. Channel insertion began at time $t \approx 4$ min. Shortly thereafter, the preparation was exposed to 3 mM n-heptanol for a period of 2.5 min using rapid flow rates (see black bar). This provoked a reversible impairment of $g_j$ exhibiting a fast "on" and "off" response (approximately 1 min). During de novo formation of channels, the rate of change of $g_j$ was significantly lower than that during washout of n-heptanol. Furthermore, the global time course of the $g_j$ increase was comparable in Fig. 6A and 6B. These observations are consistent with the view that docking of hemichannels and formation of gap junction channels both continue in the presence of n-heptanol. Similar findings were obtained in five other experiments.

In three experiments, after initiation of the process of gap junction formation, 2 mM n-heptanol was added to the bath untill full uncoupling. At this stage, the cells were separated mechanically by gentle dislocation of the patch pipettes. Thereafter, n-heptanol was washed out and the cells were brought into physical contact again. This resulted in the de novo formation of gap junction channels comparable to that described in Fig. 6A.

### Influence of arachidonic acid on the relationship $g_j$ versus $V_m$

At this stage we were wondering whether or not fatty acids act on gap junctions in the same way as n-alkanols. Hence we tested the influence of arachidonic acid (AA) on the relationship $g_{j(ss)} = f(V_m)$ using preformed cell pairs. Figure 7 illustrates the result of a typical experiment. Exposure to 10 μM AA shifted the function $g_j = f(V_m)$ to the left-hand side. $V_{m,0}$ was approximately 22 mV during control (open circles). At time $t$ = 6–7 min (filled circles) and 10–11 min (filled squares), it was shifted to about –40 mV and –70 mV, respectively. The maximal slope of the function $g_{j(ss)} = f(V_m)$ was barely affected. In contrast to n-alkanols, AA considerably impaired $g_{j(max)}$, i.e. it decreased from 21 nS to 18 nS and 17 nS, respectively. Washout of AA for 16 min (open squares) almost completely restored $V_{m,0}$ (25 mV), $g_{j(max)}$ (20.5 nS), and the maximal slope of $g_{j(ss)} = f(V_m)$. The recovery from exposure to AA was considerably slower than that from exposure to n-alkanols presumably because of the high lipophilicity of the former. Similar results were obtained from four other cell pairs.

## Discussion

### Influence of n-alkanols on macroscopic currents

Insect gap junctions possess two types of voltage-sensitive gates, a $V_j$ gate controlled by the transjunctional voltage gradient, and a $V_m$ gate controlled by the membrane potential (see [7, 23, 29]). The $V_j$ gate gives rise to the bell-shaped relationship $g_{j(ss)} = f(V_j)$. At large $V_j$, $g_{j(ss)}$ does not decline to zero. The $V_m$ gate is responsible for the sigmoidal relationship $g_{j(ss)} = f(V_m)$. At positive values of $V_m$, $g_{j(ss)}$ decreases to zero. This model sets the framework for the discussion of the data presented in this paper.

We found that n-alkanols impair $g_j$ in a dose- and time-dependent manner. At supramaximal concentrations, each compound tested produced a *complete* inhibition of $g_j$. Most strikingly, the decrease in $g_j$ caused by n-alkanols readily recovered when $V_m$ was hyperpolarized (see Fig. 1B). This prompted us to hypothesize that n-alkanols affect the $V_m$-sensitive gating mechanism. To verify this possibility, we examined systematically the effects of n-alkanols on $V_m$ gating.

A convenient way to express the effects of n-alkanols on $g_{j(ss)}$ is by means of the function $g_{j(ss)} = f(V_m)$. If one uses the Boltzmann formalism, the relevant parameters are: $g_{j(max)}$, the maximal gap junction conductance at steady-state; $V_{m,0}$, the membrane potential at which $g_{j(ss)}$ decreases to 50%; and $z$, the equivalent number of unitary positive charges moving through the electric field applied. The key finding was that n-alkanols provoke a concentration-dependent shift of $V_{m,0}$ to more negative values (see Fig. 3). However, it was difficult to obtain reliable dose/response curves. At a given concentration, $V_{m,0}$ shifted continuously with time, presumably due to accumulation of n-alkanol in the lipid bilayer of the cell membrane. The effects on gap junctions were dependent on the structure of the n-alkanol examined. The longer the lipophilic moiety, the more pronounced were the changes of the Boltzmann parameters. For example, the concentration required to shift $V_{m,0}$ by 50 mV decreased with increasing length of the hydrocarbon chain (n-hexanol: tens of mM; n-decanol: tens of μM; compare Fig. 3A and 3D). The parallelism between the lipophilic property of the agents and their efficacy on gap junction impairment suggests that n-alkanols act by incorporation into the lipid bilayer of the cell membrane (see Site of action of lipophilic agents). The prompt reversibility of

the effects supports this view. The n-alkanols examined did not interfer with $V_j$ gating. Gentle application or washout of n-heptanol gave rise to a proportional decrease in $g_j$ at all values of $V_j$. Hence, the bell-shaped relationship between $g_{j(ss)}$ and $V_j$ was not affected (not shown).

Johnston et al. [13] provided the first hint of a correlation between the structure of n-alkanols and gap junction impairment. Later on, others contributed additional evidence in favour of this concept [1, 16]. Rüdisüli and Weingart [18] reported for n-heptanol a dose-dependent decrease of $g_j$ in neonatal rat heart cells (see also [21]).

## Effects of n-alkanols on single gap junction channels

Gap junction channels of insects exhibit multiple conductance states, a main state [$\gamma_{j(\text{main state})}$], a residual state [$\gamma_{j(\text{residual state})}$], several substates [$\gamma_{j(\text{substates})}$], and a closed state [6, 26]. Kinetic analyses revealed that these channels operate in two modes. One mode gives rise to fast $I_j$ transitions controlled by $V_j$ gating [transition times between open states, i.e. $\gamma_{j(\text{main state})}$, $\gamma_{j(\text{substates})}$, and $\gamma_{j(\text{residual state})}$: < 2 ms]. The other mode leads to slow $I_j$ transitions governed by $V_m$ gating (transition times between closed state and an open state: 15–60 ms). Hence, the residual state and closed state may be regarded as the ground state of $V_j$ gating and $V_m$ gating, respectively. This concept serves as a guideline for the discussion of modification of channels by lipophilic agents.

Examining single-channel current records, we noticed slow transitions between an open state and the closed state late during application of n-heptanol (see Figs. 4 and 5A). Conversely, we saw such transitions between the closed state and an open state early during washout (see Fig. 5B). Under these conditions, the slow events lasted 5–40 ms. In the absence of n-alkanol, there were no slow transitions in conjunction with hyperpolarizing conditioning pulses – they were only present with strong depolarizing conditioning pulses [6]. This suggests that the $V_m$ gating mechanism is involved in the uncoupling caused by n-alkanols. This conclusion is consistent with the following observations. In the presence of a submaximal dose of n-alkanol, hyperpolarizing conditioning pulses led to a restoration of fast channel flickering. This phenomenon was always triggered by a slow transition between the closed state and an open state.

An effect of n-alkanols on fast $V_j$ gating can be excluded. The single-channel conductances were not altered late during application and early during washout. In the case of n-heptanol, $\gamma_{j(\text{main state})}$ was 364 ± 7 pS ($n$ = 31) and $\gamma_{j(\text{residual state})}$ was 88 ± 3 pS ($n$ = 30). These values are not significantly different from those previously found in control solution {$\gamma_{j(\text{main state})}$ = 375 ± 6 pS ($n$ = 61); $\gamma_{j(\text{residual state})}$ = 77 ± 5 pS ($n$ = 13; data at $V_j$ = 15 mV); Student's $t$-test: $P$ < 0.01 and 0.01; see [6]}. Furthermore, preliminary experiments indicated that n-heptanol does not affect $P_o$ (F.F. Bukauskas and R. Weingart, unpublished).

Slow channel transitions induced by lipophilic uncoupling agents have also been observed to occur in neonatal rat heart cells [18, 25, 28] and HeLa cells transfected with various murine connexins (F.F. Bukauskas and R. Weingart, unpublished data). Similar events were found in RIN cells transfected with rat connexin 43 (C×43) in conjunction with large $V_j$ gradients, but in the absence of uncoupling agents [2]. These observations are intriguing since mammalian gap junctions are thought to have no $V_m$-sensitive gate.

## Effects of arachidonic acid

Arachidonic acid acts on gap junctions in a similar way to n-alkanols. At the macroscopic level, it shifted the function $g_{j(ss)} = f(V_m)$ to more negative voltages (see Fig. 7). This change was dose dependent and reversible. Due to a progressive shift of $V_{m,0}$ with time, it was difficult to obtain a reliable dose/response curve. Arachidonic acid also decreased $g_{j(max)}$ without affecting $z$. The change in $g_{j(max)}$ was dose dependent, time dependent, and reversible. At the microscopic level, application of arachidonic acid provoked slow channel transitions between an open state and the closed state followed by abolition of the fast channel activity. The inverse sequence of events was observed during washout of arachidonic acid (not shown). We conclude from these observations that arachidonic acid may act via different mechanisms, an alkanol-type of mechanism (see above) and another mechanism (see [20]). For comparison, a dose/response curve $g_j = f([AA]_o)$ (where $[AA]_o$ = external concentration of arachidonic acid) has been reported previously for cell pairs from neonatal rat hearts ($K_d$ = 4 μM; $z$ = 0.75 [19]).

## Site of action of lipophilic agents

The effects of lipophilic agents (n-alkanols, arachidonic acid) on arthropod gap junctions may involve several mechanisms: (1) a direct action on channel proteins; (2) an indirect action mediated by subcellular intermediates; (3) an indirect action mediated via the lipid bilayer. Mechanism 1 appears difficult to reconcile with the following observations. The efficacy of n-alkanols on $g_j$ was inversely related to the hydrocarbon chain length, suggesting that the lipophilic property of the uncoupling agents plays a pivotal role. In addition, hyperpolarization of $V_m$ provoked a quick recovery of n-alkanol- and arachidonic-acid-dependent uncoupling. With respect to mechanism 2, lipophilic substances may affect the level of intracellular ions (e.g. $H^+$, $Ca^{2+}$) or second messengers (e.g. cyclic nucleotides) and thus modify $g_j$. However, considering the $H^+$- and $Ca^{2+}$-buffering capacity of our pipette solutions (see Materials and methods, Solutions and pipettes) and the speed of $g_j$ recovery after $V_m$ hyperpolarization (see Fig. 1B), it seems unlikely that n-alkanols and arachidonic acid act via intracellular $H^+$ and/or

$Ca^{2+}$. This conclusion agrees with previous observations that n-heptanol and arachidonic acid do not elevate $[Ca^{2+}]_i$ in rat heart cells and lacrimal gland cells ([4, 11, 15]; but see also [14]. It is also consistent with the time course of uncoupling and recoupling during rapid application and removal of n-heptanol (0.5 s [4]).

With respect to mechanism 3, lipophilic agents incorporate readily into the lipid bilayer of cell membranes. Hence, they may affect $g_j$ by disturbing the bulk properties of the lipids and thereby alter the fluidity of the membrane. However, the low dose of fatty acid required to impair gap junctions seems to rule out this possibility [9]. It may be rescued by the observation that n-heptanol impairs $g_j$ by decreasing the fluidity of local membrane domains embedding the gap junction channels [3]. The finding that n-alkanols and arachidonic acid affect various ion channels also suggests a non-specific action (see e.g. [16, 19]). Alternatively, lipophilic substances may act by interfering with the lipid–protein interface. It has been proposed that lipophilic agents cause local disorders in the membrane-channel domains leading to conformational changes of gap junction proteins and channel dysfunction [10]. According to this model, the uncoupling potency of an agent depends on its ability to induce disorders in the C1–C9 region of membrane lipids (short-chain saturated fatty acids, n-alkanols), or the C9–C18 region (kinked chains of unsaturated fatty acids). This concept predicts that n-alkanols and arachidonic acid are effective uncoupling agents. The same conclusion was drawn from a study aimed at identifying the eicosanoids responsible for arachidonic acid-dependent uncoupling of neonatal rat heart cells [20].

## Mode of chemical uncoupling

Based on the current concept of an arthropod gap junction channel, the effects of n-alkanols and arachidonic acid can be explained as follows. We may assume that the slow gating mechanism involves a gating element and a voltage-dependent sensor. In this case, n-alkanols and arachidonic acid could interfere with the voltage sensor and thereby alter its $V_m$ sensitivity. Alternatively, we may assume that the slow gating mechanism consists of a gating element but different sensing devices. In this case, the lipophilic agents could interact with a sensor for lipophiles and $V_m$ with a voltage sensor. Interestingly, our experiments with C6/36 cells revealed that intracellular $H^+$ and n-heptanol provoke similar modifications of both the macroscopic and microscopic currents ([27, 28]; R. Weingart and F.F. Bukauskas, in preparation). This supports the view that the slow gating mechanism involves several sensors – a $V_m$ sensor, a sensor responsive to lipophilic agent such as n-alkanols or arachidonic acid, and a sensor responsive to ions such as $H^+$. Our intracellular pH results are consistent with earlier observations of *Chironomus* salivary glands [17]. Elevation of $[Ca^{2+}]_i$ or $[H^+]_i$ in these cells leads to uncoupling which is readily restored by hyperpolarization of $V_m$. This raises the possibility that $H^+$ and $Ca^{2+}$ may act in a similar way.

The modification of gap junctions by n-alkanols and arachidonic acid is not limited to arthropods [6, 13]. These agents also impair $g_j$ in mammalian cells via slow current transitions between an open state of the channels and the closed state [20, 22]. This observation is interesting because mammalian gap junctions generally are thought to have no $V_m$-sensitive gate (see e.g. [5]). It opens the possibility that these channels also have a $V_m$ gate, yet their voltage sensor is not operational under physiological conditions.

## De novo formation of gap junctions

De novo formation of gap junctions can be induced by forcing two cells into physical contact. Previously, we have used this approach for insect cell lines [6], HeLa cells transfected with murine connexins [8] and neonatal rat cardiomyocytes [22]. In C6/36 cells, $g_j$ increased in a sigmoidal manner during gap junction formation, suggesting that channel formation is a cooperative process (see Fig. 6A; see also [6]). Our experiments also indicate that n-heptanol has no effect on de novo formation of gap junctions (compare Fig. 6A with 6B). Channel formation continued during application of the agent, i.e. the process underlying channel insertion and channel recruitment were not affected by n-heptanol. This suggests that lipophiles do not interfere with the docking of hemichannels. Pretreatment of cells with n-heptanol did not affect the time course of gap junction formation. This may mean that the lipophilic agents do not interfere with the synthesis and trafficking of gap junction proteins, or the assembly of hemi-channels.

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