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Biphasic effect of linoleic acid on connexin 46 hemichannels

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Abstract

Connexins form hemichannels at undocked plasma membranes and gap-junction channels (GJCs) at intercellular contacting zones. Under physiological conditions, hemichannels have low open probabilities, but their activation under pathological conditions, such as ischemia, induces and/or accelerates cell death. Connexin 46 (Cx46) is a major connexin of the lens, and mutations of this connexin induce cataracts. Here, we report the effects of linoleic acid (LA) on the electrical properties of Cx46 GJCs and hemichannels expressed in *Xenopus laevis* oocytes. LA has a biphasic effect, increasing hemichannel current at 0.1 μM and decreasing it at concentrations of 100 μM or higher. The effects of extracellular and microinjected LA conjugated to coenzyme A (LA-CoA) suggest that the current activation site is accessible from the intracellular but not extracellular compartment, whereas the current inhibitory site is either located in a region of the hemichannel pore inaccessible to intracellular LA-CoA, or requires crossing of LA through an organelle membrane. Experiments with other fatty acids demonstrated that the block of hemichannels depends on the presence of a hydrogenated double bond at position 9 and is directly proportional to the number of double bonds. Experiments in paired oocytes expressing Cx46 showed that LA does not affect GJCs. The block by unsaturated fatty acids reported here opens the possibility that increases in the concentration of these lipids in the lens induce cataract formation by blocking Cx46 hemichannels.

Keywords

Gap junction; Cataract; Frog oocyte; Lens fiber; Cx46; Ion channel

Introduction

A gap-junction channel (GJC) is formed by head-to-head docking of two hemichannels, one from each adjacent cell. These hemichannels, also named connexons, are hexamers of proteins called connexins. The presence of undocked hemichannels at the plasma membrane has been demonstrated in several cell types, including lens fibers [16, 50]. However, their role under physiological or pathological conditions is not well defined, although several reports support the idea that controlled hemichannel opening allows autocrine/paracrine cell communication. For example, it has been demonstrated that hemichannels are involved in the release of signaling molecules such as ATP [54], glutamate [58], NAD^+ [10], and prostaglandin E2 [12], as well as in the uptake of glucose [46]. In pathological conditions such as inhibition of metabolism, hemichannels contribute to cell damage because their opening induces or accelerates cell death due to loss of metabolites and ion gradients, and Ca^{2+} entry [5, 50, 51, 55].

Lacking a vascular system, lens fibers use GJC as a nutritional and excretory pathway, where the flows of ions, water, and metabolites between the cells are determined by their chemical or electrochemical gradients [23, 38]. At least three connexin isoforms are expressed in the lens: Cx43 [6], Cx46 [45], and Cx50 [32], and it has been recently proposed that Cx46 and Cx50 hemichannels play a role in Na^+ and Ca^{2+} influx in lens fibers [16]. The importance of connexins in cataract formation is well documented, with Cx46- and Cx50-knockout mice developing cataracts at early ages, suggesting that connexin function is essential to maintain lens transparency [22, 43]. Increased levels of unsaturated fatty acids in the lens have been shown to induce and/or accelerate the development of cataracts [27, 33], but the mechanism of the damage of lens fibers is unknown. Although generation of radicals and lipid peroxidation products likely plays a role, they are not sufficient to explain the cytotoxicity [see 27]. Linoleic acid (LA), an unsaturated omega-6 fatty acid essential for biosynthesis of arachidonic acid [53], induces cataract formation in human [37] and bovine lens [21, 40]. Although there is no information on lens connexin isoforms, some isoforms are known to be sensitive to fatty acids. For example, oleamide-derived molecules inhibit Cx26 GJC [41].

Since Cx46 is essential for normal lens function, fatty acids are known to modulate connexins, and LA induces cataract formation, we decided to test whether LA alters the function of Cx46, one of the major connexins in the lens. In two-electrode voltage-clamp studies, we found a biphasic effect of LA on Cx46 hemichannels: low concentrations increased Cx46 hemichannel currents, whereas higher concentrations decreased them. We also observed a direct correlation between the number of fatty acid double bonds and the magnitude of the hemichannel current block, and found that a double bond at C9 is essential for hemichannel current inhibition. Unexpectedly, LA at low or high concentration did not affect Cx46 GJC.

Methods

Chemicals

Linoleic acid, palmitic acid, linoleoyl-CoA, and calphostin C were purchased from Sigma-Aldrich (Schnellendorf, Germany). Oleic acid, arachidonic acid, 9-thiastearic acid, and 9-

nitrooleate were purchased from Cayman (Ann Arbor, Michigan, USA), and BAPTA-AM from Calbiochem (San Diego, California, USA).

Plasmid engineering, cRNA preparation, and injection into *Xenopus laevis* oocytes

The plasmid pSP64T-Cx46 containing rat Cx46 DNA was obtained from Dr. Lisa Ebihara (Rosalind Franklin University) [45]. Oocytes were injected with 12.5 ng of antisense Cx38 oligonucleotide alone or in combination with 25 ng of cRNA coding for Cx46. After cRNA injection, oocytes were maintained in Barth's solution (88 mM NaCl, 1 mM KCl, 5 mM CaCl₂, 0.8 mM MgCl₂, and 10 mM HEPES/NaOH; pH 7.4, supplemented with 0.1 mg/ml gentamycin and 20 U/ml penicillin–streptomycin each) for 24–48 h, to allow for a good expression level. Additional details have been published [2].

Electrophysiological recordings and calculations

Whole-cell hemichannel currents were measured as described [47]. Briefly, oocytes were placed in a 1-ml recording chamber and superfused with ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, and 5 mM HEPES/NaOH; pH 7.4) at room temperature. Glass microelectrodes were filled with 3 M KCl and had tip resistances of 0.5–1.5 MΩ when immersed in ND96. For data acquisition and analysis, we used a voltage-clamp amplifier (OC-725C, Warner Instruments, Hamden, CT) with pCLAMP 10 and a Digidata 1440A A/D Board (Molecular Devices, Foster City, CA). Currents were measured following 15-s rectangular voltage pulses, ranging from –50 to +60 mV, in 10-mV steps, with a holding potential of –60 mV and 10-s intervals between pulses. Current–voltage (*I*–*V*) relationships were calculated from the peak current values. Currents through GJCs were measured in paired oocytes. Briefly, both cells of the pair were clamped at –40 mV, and junctional currents were measured after changing the cell-membrane voltage of one cell to values between –140 and +60 mV (20-mV steps, 15-s intervals between pulses), while holding constant the voltage of the other cell (used as reference). The current supplied to the cell clamped at –40 mV is equal in amplitude, but opposite in sign, to the transjunctional current. To evaluate the effects of fatty acids, 500 μl of lipids dissolved in ND96 was carefully added to the 1-ml recording chamber, to final concentrations ranging from 0.1 to 1,000 μM. Unless otherwise indicated, the recordings were performed after 5 min of incubation with the fatty acids

Statistical analysis

Results are expressed as means ± SEM, and *n* refers to the number of independent experiments. For statistical analysis, each treatment was compared to its respective control, and significance was determined using a one-way ANOVA or paired Student's *t* test, as appropriate. Differences were considered significant at *P*<0.05.

Results

Linoleic acid affects Cx46 hemichannel currents

As previously described [47, 48], membrane depolarization of oocytes expressing rat Cx46 hemichannels induces slow outward currents that do not show inactivation (Fig. 1a). The maximum current at +60 mV was 5.5±0.3 μA (*P*<0.001, *n*=10). Upon returning to the holding potential of –60 mV, small tail currents were observed (Fig. 1a). Addition of LA to the bath solution produced a biphasic response. At 0.1 μM LA, the currents increased ~15% (Fig. 1d), whereas at concentrations equal to or greater than 100 μM LA, both outward and tail currents were significantly reduced (Fig. 1b). With 100 μM LA, the current was reduced by ~40% (Fig. 1e), with >90% of the inhibition within 2 min of exposure (not shown). The inhibitory effect of LA was sustained for at least 30 min (Fig. 2a). To study the reversibility

of the effect, we exposed oocytes for 5 min to 100 μM LA and then washed them for 5 min with ND96 without LA. The current, reduced 45% by LA, recovered to $\sim 90\%$ of the control value after washing (Fig. 2b). In four of the 12 experiments, the hemichannel current after wash actually increased to values similar to those elicited by 0.1 μM LA (not shown). The simplest explanation for this phenomenon and the dose–response effect in Fig. 1e is that LA has at least two mechanisms of action, one with higher affinity (that increases hemichannel currents) and other with lower affinity (that decreases hemichannel currents). Fits to the Boltzmann equation of the current data before and after 0.1 or 100 μM LA did not show changes in the voltage dependency of Cx46 hemichannels, suggesting that LA modifies the open probability and/or the hemichannel conductance. In summary, LA has a biphasic effect, with a current increase at lower concentration and a decrease at higher concentrations. The inhibitory effect is complete in approximately 2 min and is reversible.

The inhibitory LA effect does not depend on an increase in intracellular free- $[\text{Ca}^{2+}]$ or PKC-mediated phosphorylation

Since LA can increase intracellular $[\text{Ca}^{2+}]$ [17] and also activate protein kinase C [31], we tested the effects of the Ca^{2+} chelator BAPTA-AM (100 μM) and the PKC inhibitor calphostin C (1 μM) on the LA effect. Oocytes were pre-incubated for 1 h with one of these agents, briefly washed, exposed to 100 μM LA for 5 min, and then Cx46 hemichannel currents were recorded. Neither BAPTA-AM nor calphostin C by themselves affected Cx46 hemichannel currents (data not shown, $n=6$), nor reduced the inhibitory effect of 100 μM LA (Fig. 3). If anything, the LA effect was increased at the more positive clamping voltages. We did not examine further the increase in the response to LA, but one possibility is that BAPTA and calphostin C block the stimulatory effect elicited by lower LA concentrations (see Fig. 1, panels d and e). Nevertheless, the data strongly suggest that intracellular $[\text{Ca}^{2+}]$ or PKC-mediated phosphorylation is not necessary for the LA inhibitory effect.

Unsaturated fatty acids other than linoleic acid also inhibit Cx46 hemichannels

Oleamide and certain derivatives need specific chemical features, i.e., number and positions of double bonds, to affect GJC expressed in rat astrocytes [7]. Therefore, we tested the effects of fatty acids with different number of double bonds on Cx46 hemichannel currents. We used 100 μM of each fatty acid and compared their effects to that of LA (18 carbons, two double bonds) at the same concentration. The choice of 100 μM is based on concentrations of free C18:2 fatty acids of 50–100 μM reported in human plasma [20, 28, 30]. The best Cx46 hemichannel inhibitor was arachidonic acid (AA) which is a polyunsaturated fatty acid with four double bonds and 20 carbons. The current reduction with AA was $>70\%$ (Fig. 4a). Oleic acid (OA), with one double bond and 18 carbons, reduced the Cx46 current by 20% (Fig. 4a). Palmitic acid (PA), with 16 carbons and no double bonds, had no effect on the current (Fig. 4a). These results suggest that the inhibition by fatty acids depends on the presence of double bonds and seems to be proportional to the degree of unsaturation.

A common characteristic between OA, LA, and AA is that all of them have a double bond at position 9, which seems to be important in the GJC inhibition by oleamide [7]. To test the importance of the double bond at position 9, we determined the effects of 100 μM of 9-thiastearic acid (9-Th, $\text{C}_{17}\text{H}_{34}\text{O}_2\text{S}$) and 9-nitrooleate (9-NO, $\text{C}_{18}\text{H}_{33}\text{NO}_4$). The former has a sulfur atom at position 9, and the latter has a NO_2 at position 9, with the double bond not fully hydrogenated. The Cx46 hemichannel current was not affected by 5-min incubation with either of these fatty acids (Fig. 4b). These results suggest that an unblocked double bond at carbon 9 is essential for the effect of fatty acids on Cx46 hemichannels.

Impermeable LA has no effects on hemichannel currents

Fatty acids are present in the inner and outer leaflets of the membrane bilayer because of fast flip-flop of the protonated species [29]. To test whether LA incorporation into the membrane inner leaflet is necessary for the fatty acid inhibitory effect, we studied the hemichannel response to LA attached to coenzyme A (LA-CoA, MW 1,029). LA-CoA is inserted in the outer leaflet of the plasma membrane, but as other CoA-linked fatty acids, it is not expected to flip to the internal monolayer [9]. The addition of 100 μM LA-CoA to the bath solution for 5 min had no effect on Cx46 hemichannel currents (Fig. 5a). However, when LA-CoA was microinjected into the oocytes to a final estimated concentration of ca. 250 μM , it did not decrease, but increased Cx46 hemichannel currents (Fig. 5b). In contrast, a decrease was observed when LA was micro-injected to reach ca. 250 μM (Fig. 5b).

LA does not affect Cx46 GJC currents

The data presented earlier showed that LA at 0.1 μM activates and at 100 μM inhibits Cx46 hemichannel currents. Here, we tested the effects of both concentrations on GJC currents in paired oocytes expressing Cx46. Under control conditions, Cx46 GJC exhibit currents of about 300 nA (283 ± 63 nA at $V_j -100$ mV, and 361 ± 36 nA at $V_j \pm 100$ mV), with a clear relaxation at V_j over 80 mV (Fig. 6a). A 5-min exposure to 0.1 or 100 μM LA did not affect the GJC currents (data at +60 mV are shown in Fig. 6b). These results indicate that LA, at the same concentration and exposure time that affects hemichannel currents, does not alter the electrical properties of GJC.

Discussion

Mice expressing certain Cx46 mutants [44] and a Cx46 knockout [22] develop cataracts, clearly pointing to an essential role of Cx46 in maintaining lens transparency. Cataracts are frequent in diabetes [8, 56], and their incidence increases with age [34]; however, few studies have correlated cataract formation with potential alterations in Cx46 function induced by aging or diabetes. The studies available focused mostly on Cx46 modifications due to increases in free radicals [4, 47] and changes in phosphorylation state [19], but there are no studies on the effects of fatty acids on lens connexins, even though high concentrations of unsaturated fatty acids are toxic to human lens epithelial cells [27, 37]. In the present studies, we show the effect of physiological levels of unsaturated fatty acids on Cx46 hemichannels and GJCs. We focused our work on LA because it is present at the plasma membrane of lens epithelial cells and elevations of its concentration in the lens produce or accelerate cataract formation [37]. LA had a biphasic effect, increasing Cx46-hemichannel currents at low concentrations and decreasing the currents at higher concentrations. This is consistent with a high-affinity stimulatory effect and a low-affinity inhibitory effect. Dual effects of arachidonic acid on plasma-membrane Ca^{2+} -ATPase [42] and of oleic acid on gap-junction coupling in A7r5 cells [25] have also been reported.

In order to investigate the mechanism of action of LA, we evaluated the roles of increases in intracellular free- $[\text{Ca}^{2+}]$ and stimulation of PKC, two known responses to LA in other systems [17, 31]. We found no involvement of either signaling pathway in the hemichannel inhibitory response to LA. Modulation of BKCa^{2+} channels [13], voltage-gated K^+ channels [18], TRP channels [39], and voltage-gated Ca^{2+} channels [49] by fatty acids has been reported, and in most cases, it has been suggested that there is a direct interaction between lipid and ion channel [14, 15, 49]. For example, arachidonic acid appears to bind to Thr250 and Val275, in the pore of Ca^{2+} -activated K^+ channels [24].

Polyunsaturated fatty acids increase BK channel activity by shifting the voltage dependence of the open probability [15]. In this study, the magnitudes of the effects of OA, LA, and AA

were in the same order as our inhibitory results on Cx46 hemichannels. *Cis*-unsaturated fatty acids disorder the bilayer interior and order the head-group region [31], and therefore, the effects on membrane proteins could be mediated by changes in the biophysical properties of the phospholipid membrane. Several facts argue against this interpretation of our results. In a study using 100 μM concentrations, the oil/water partition coefficients were $\text{PA} > \text{OA} > \text{LA} > \text{AA}$ [1], i.e., the opposite sequence to their inhibition of hemichannel conductance. In addition, in the BK channel study quoted above [15], there was no correlation between membrane fluidity and BK channel activation in response to the fatty acids. In sum, our results do not support an effect of LA and other unsaturated fatty acids mediated by changes in the properties of the plasma membrane. Nevertheless, additional experiments will be needed to determine whether the effects of fatty acids on Cx46 are due to direct binding to the hemichannels or are mediated by more complex signaling mechanisms, as suggested by the LA-CoA results discussed below. In addition, since Cx46 and Cx50 are the main lens connexin [35] and they form heteromeric hemichannels [26], further studies will be needed to determine whether hemichannels containing Cx50 are also affected by unsaturated fatty acids.

The absence of response to extracellular LA-CoA, not expected to flip across to the internal monolayer and therefore not to cross lipid bilayers [9], suggests that fatty acids need to access the inner leaflet of the plasma membrane and/or enter the intracellular compartment to affect hemichannels. Since the intracellular injection of sufficient LA-CoA to reach inhibitory concentrations produces an increase in Cx46 hemichannel currents, it is obvious that the mechanisms of stimulation and inhibition by LA are distinct. The current-activation site is accessible from the intracellular compartment to both LA and LA-CoA, whereas the inhibitory site is only accessible to LA, suggesting that it is located in a region of the hemichannel pore inaccessible to LA-CoA or that the signaling mechanism involved requires crossing of LA through an organelle membrane. Understanding of the complex mechanisms of the fatty acid effects on hemichannels will require additional studies.

Hemichannels are affected by changes in membrane phospholipid composition [36]. GJCs formed by Cx26 or Cx32 associate preferentially with certain phospholipids; phosphatidylcholine does not associate with Cx26 hemi-channels, whereas phosphatidylserine does not associate with Cx32 hemichannels [11, 36]. In contrast, phosphatidylcholine and phosphatidyl serine are associated with both isoforms in junctional plaques [36]. The concept of specific lipid–connexin interaction is also supported by Boger and co-workers [7], who found that molecules derived from the fatty acid primary amide oleamide affect GJCs when they contain 16–24 carbon atoms, a hydrophobic methyl terminus, a polarized carboxy-terminal and a *cis* double bond at position 9. In agreement with these observations, our results with fatty acids containing varied number of double bonds, with and without an unsaturated *cis* double bond at position 9, support the idea that the inhibitory effect of the fatty acids requires the unsaturated *cis* double bond at position 9 and is increased by the degree of unsaturation. As mentioned above, some phospholipids interact differentially with hemichannel or GJCs formed by Cx26 and Cx32 [36]. Our results showing that LA affects hemichannels, but not the GJCs formed by Cx46, extend previous studies to a lens connexin and show differences in functional responses between GJCs and hemichannels. We have not determined whether the selective effect on hemichannels vs. GJCs is due to differences in affinity for LA of hemichannels and GJCs. However, since LA is present on both leaflets of the plasma membrane, the absence of effect on GJCs may be due to structural differences between hemichannels and GJCs.

Pathophysiological significance

Unsaturated fatty acids modulate a wide range of cellular processes [52], and in the lens they induce or accelerate cataract formation by unknown mechanisms [21, 27, 33, 37, 40]. The

cytotoxic effects of fatty acids on lens fibers depend on unsaturated fatty acid uptake by the cells and increase with the fatty acid-to-albumin ratio [27, 57]. In aqueous humor, the concentration of albumin is very low compared to that in plasma [27], and increases in the fatty acid-to-albumin ratio occur in a number of conditions associated with cataracts, including diabetes, lipid disorders, nephritic syndrome, and liver diseases [see 27]. Under these conditions, unsaturated fatty acid damage of the lens fibers can occur, particularly in the elderly, where there is a significant loss of the blood-aqueous humor barrier [see 27].

The block of Cx46 hemichannel currents by LA and other unsaturated fatty acids at concentrations found in the lens, without affecting Cx46 GJCs, may contribute to the induction of cataracts by: (a) reducing the uptake of physiological molecules from the extracellular space, as has been proposed for astrocytes [46], (b) reducing the release of signaling molecules, as it has been shown in many cell types [50], and/or (c) diminishing cell-to-cell communication. It has been proposed that the activity of Cx46 hemichannels mediates GJC formation [3], and therefore, reduced hemichannel activity may decrease the number of GJCs.

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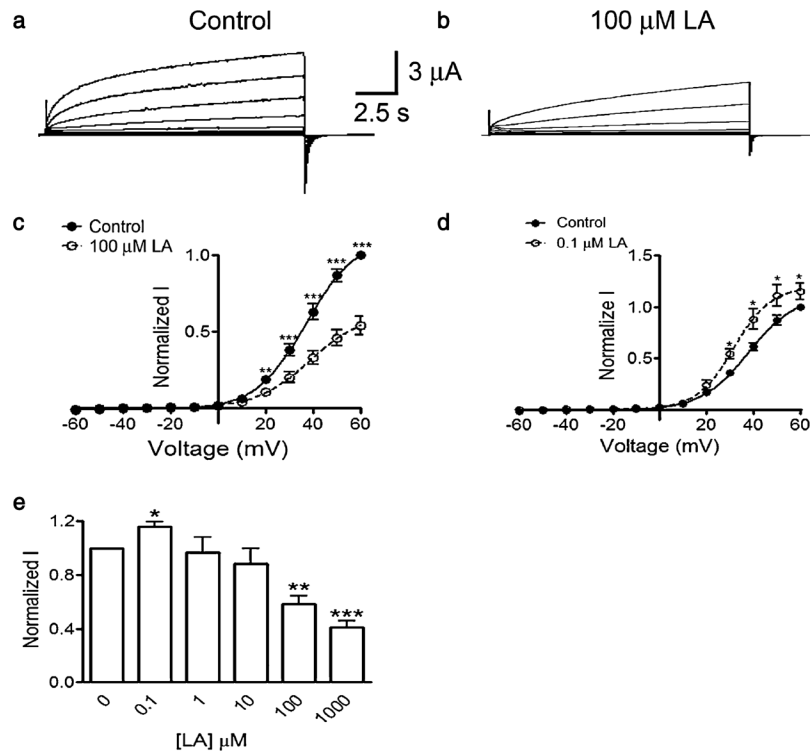


Fig. 1. Cx46 hemichannels are inhibited by linoleic acid. **a** Representative whole-cell current records from *Xenopus* oocytes injected with Cx46 cRNA under control conditions. Oocytes were depolarized from -60 to $+60$ mV in steps of 10 mV for 15 s. At the end of each depolarizing step, the membrane was clamped to -60 mV for additional 10 s. **b** Same as **a** but after a 5-min exposure to 100 μM linoleic acid (LA). **c** Average effect of 100 μM LA ($n=10$). **d** Average effect of 0.1 μM LA ($n=10$). Currents were normalized to that under control conditions, at $+60$ mV, in the absence of LA. The symbols ** and *** correspond to $P<0.01$ and $P<0.001$ compared to the corresponding control values. **e** LA concentration dependence of Cx46 hemichannel currents. Results from ten oocytes were obtained at $+60$ mV and were normalized as described for **c**. The symbols *, **, and *** correspond to $P<0.05$, $P<0.01$, and $P<0.001$, respectively, compared to control condition, in the absence of LA. Average data in **c–e** are means \pm SEM

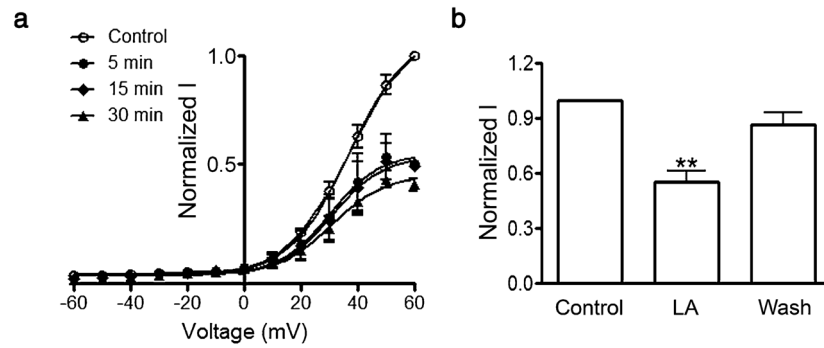


Fig. 2.

The linoleic acid effect is sustained and reversible. **a** Rapid and stable current block by 100 μ M LA. Oocytes were exposed to 100 μ M LA for 5, 15, or 30 min, and hemichannel currents were measured. Data were normalized and expressed as described in the legend to Fig. 1c. The *solid lines* are the fits of the Boltzmann equation to the data ($n=3$ each). **b** Reversibility of the inhibition by LA. Oocytes expressing Cx46 hemichannels ($n=12$) were exposed to LA for 5 min (100 μ M LA), and hemichannel currents were recorded. Currents were measured again after washing without LA for 5 min (*Wash*). Currents were normalized to control values. A $P<0.001$ compared to control is denoted by **

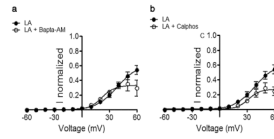
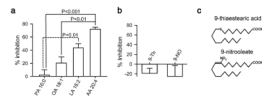


Fig. 3.

The effect of linoleic acid is not due to PKC- or Ca^{2+} -activated pathways. **a** Response to LA in oocytes incubated with 100 μM BAPTA-AM for 1 h. Immediately after removing the Ca^{2+} chelator, the response to a 5-min exposure to 100 μM LA was recorded and compared to control data from oocytes not treated with BAPTA-AM. **b** Response to 100 μM LA in oocytes incubated with 1 μM calphostin C (*Calphos*) for 1 h. Immediately after removing the PKC blocker, the response to a 5-min exposure to 100 μM LA was recorded and compared to control data from oocytes not treated with calphostin C. See legend to Fig. 1 for additional details

**Fig. 4.**

Inhibition of Cx46 hemichannel currents by other unsaturated fatty acids. **a** Dependence of the inhibitory effect on the number of unsaturated bonds. The effects of exposure to palmitic acid (PA, $n=5$), oleic acid (OA, $n=5$), linoleic acid (LA, $n=6$), or arachidonic acid (AA, $n=6$) for 5 min at 100 μM were determined. Protocol as described for Fig. 1d. Data are presented as means \pm SEM. The labeling of the bars shows, for each fatty acid, the number of carbons followed by the number of double bonds; e.g., AA 20:4 for arachidonic acid, with 20 carbons and four double bonds. **b** Importance of the double bond at position 9. The experimental protocol was identical to that in **a**, but the fatty acids used were 9-thiastearic acid (9-Th) and 9-nitrooleate (9-NO). No statistically significant effects on the Cx46 hemichannel current were detected ($n=7$ for each compound). **c** Chemical structures of 9-thiastearic acid and 9-nitrooleate

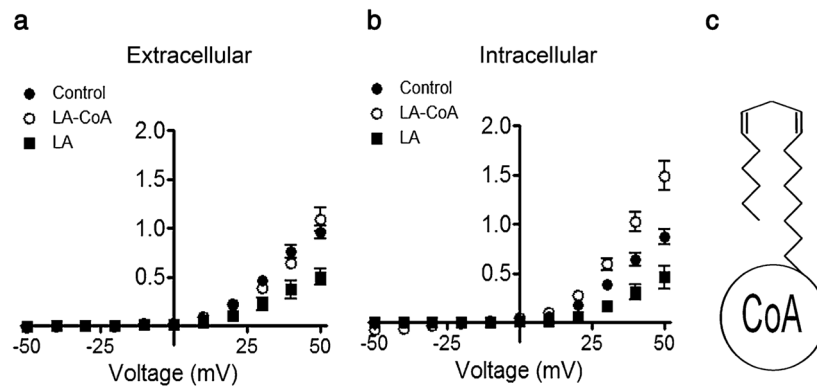


Fig. 5. Microinjection of linoleic acid-CoA increases Cx46 hemichannel currents. **a** Current–voltage relationship of Cx46 hemichannel currents under control conditions and after a 5-min exposure to 100 μ M linoleic acid attached to coenzyme A (*LA-CoA*) or *LA* alone added to the extracellular solution ($n=8$ for each group). **b** Current–voltage relationship of hemichannel currents under control conditions and 5 min after microinjection of *LA-CoA* ($n=15$) or *LA* ($n=14$) to reach final concentrations of ca. 250 μ M. Intracellular concentrations were estimated assuming a 1- μ l oocyte volume. Data are presented as means \pm SEM of currents normalized to the control value at +60 mV

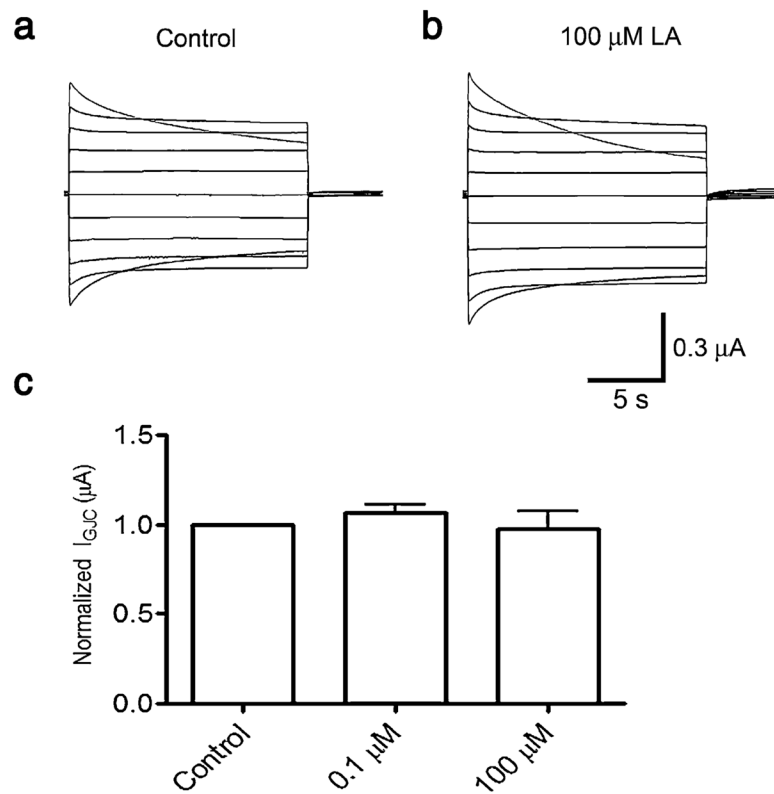


Fig. 6. Linoleic acid does not affect GJC currents. **a** Representative transjunctional current records from paired oocytes under control conditions. Both cells of the pair were clamped at -40 mV, and junctional currents were measured after changing the cell-membrane voltage of one cell to values between -140 and $+60$ mV, in 20 -mV steps. **b** Lack of effect of LA on transjunctional conductance. GJC currents measured at $+60$ mV in the presence of 0.1 or 100 μ M LA were normalized to the current at $+60$ mV under control conditions. No differences were observed when 0.1 μ M or 100 μ M LA were used. Data are means \pm SEM ($n=6$ for each concentration used)