

Fenamates: A Novel Class of Reversible Gap Junction Blockers

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ABSTRACT

The effect of fenamates on gap junctional intercellular communication was investigated in monolayers of normal rat kidney (NRK) fibroblasts and of SKHep1 cells overexpressing the gap junction protein connexin43 (Cx43). Using two different methods to study gap junctional intercellular communication, single electrode voltage-clamp step response measurements and dye microinjection, we show that fenamates are reversible blockers of Cx43-mediated intercellular communication. After adding fenamates to a confluent monolayer of electrically coupled NRK fibroblasts, the voltage step-induced capacitive current transient changed from a transient characteristic for charging multiple coupled cell capacitances to one characteristic for a single cell in isolation. The capacitance of completely uncoupled cells was 19.7 ± 1.0 pF (mean \pm S.E.M.; $n = 11$). Junctional conductance between the patched cell and the surrounding cells in the monolayer changed from $>140.7 \pm 9.6$ nS (mean \pm S.E.M.; $n = 14$) to $<1.4 \pm 0.4$ nS (mean \pm S.E.M.; $n = 11$) after uncoupling. Electrical coupling could be restored to $>51.8 \pm$

4.2 nS (mean \pm S.E.M.; $n = 11$) by washout of the fenamates. Voltage-clamp step response measurements showed that the potency of fenamates in inhibiting electrical coupling decreases in the order meclofenamic acid $>$ niflumic acid $>$ flufenamic acid. The half-maximal concentration determined by dye-coupling experiments was 25 and 40 μ M for meclofenamic acid and flufenamic acid, respectively. Inhibition of gap junctional communication by fenamates did not involve changes in intracellular calcium or pH, and was unrelated to protein kinase C activity or an inhibition of cyclooxygenase activity. Voltage-clamp step response measurements in confluent monolayers of SKHep1 cells that had been stably transfected with Cx43 revealed that fenamates are potent blockers of Cx43-mediated intercellular communication. In conclusion, fenamates represent a novel class of reversible gap junction blockers that can be used to study the role of Cx43-mediated gap junctional intercellular communication in biological processes.

Fenamates belong to the class of *N*-phenylanthranilic acids and are widely used as nonsteroidal anti-inflammatory drugs by their ability to inhibit cyclooxygenases (Brogden, 1986). In addition, fenamates modulate a diversity of ion channels. They have been identified as inhibitors of voltage-gated and ATP-sensitive potassium channels (Grover et al., 1994; Lee and Wang, 1999), voltage-gated calcium channels (Li et al., 1998), calcium-activated chloride channels (White and Aylwin, 1990), and nonselective cation channels (Gögelein et al., 1990). On the other hand, fenamates have been shown to activate calcium-activated and voltage-dependent potassium channels (Farrugia et al., 1993; Ottolia and Toro, 1994). Since we had found that flufenamic and niflumic acid inhib-

ited the propagation of calcium action potentials in monolayers of normal rat kidney (NRK) fibroblasts (de Roos et al., 1997), we investigated whether fenamates could also block gap junctional channels.

Gap junctional intercellular communication (GJIC) is of paramount importance in the regulation of a variety of biological processes. Gap junctional channels allow intercellular diffusion of small (<1 kDa) hydrophilic molecules and ions. This intercellular diffusion of signal molecules regulates a variety of biological processes including embryogenesis, cell proliferation, cardiac function, and propagation of calcium waves (Bruzzone et al., 1996; Kumar and Gilula, 1996). Besides diffusion of small molecules, gap junctions also mediate electrical coupling between cells and allow clusters of cells to behave as an electrical syncytium. Electrical coupling underlies synchronous electrical activity between excitable cells and has been shown to be essential in the propagation of the cardiac action potential (Gros and Jongsma, 1996), the con-

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ABBREVIATIONS: NRK, normal rat kidney; GJIC, gap junctional intercellular communication; Cx43, connexin43; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; MFA, meclofenamic acid; FFA, flufenamic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

traction of smooth muscle (De Mello, 1994) and the coordination of hormone secretion (Stauffer et al., 1993).

Gap junctions are present in the plasma membrane of cells as clusters of tightly packed particles, each of which represents a single channel (Bukauskas et al., 2000). Each channel is formed by the docking of two hemi-channels (connexons) located in apposing cell membranes of neighboring cells. Each hemi-channel consists of six polypeptides called connexins. Connexin43 (Cx43) is the major gap junctional protein identified in fibroblasts (Goldberg and Lau, 1993). GJIC can be regulated by intracellular calcium and pH (Loewenstein, 1981; Spray and Bennett, 1985). In addition, several processes that induce modifications of Cx43, including phosphorylation on serine and threonine residues following activation of PKC (Lampe et al., 2000) and on tyrosine residues upon growth factor receptor activation (Lau et al., 1996), have been shown to modulate GJIC. The contribution of each of these processes to the modulation of GJIC is dependent on the cell type.

We (de Roos et al., 1996) and others (Maldonado et al., 1988) have shown that confluent NRK fibroblasts are electrically well coupled and here we show that fenamates can completely block intercellular communication of not only NRK cells but also of SKHep1 cells overexpressing Cx43. The observed inhibition is reversible and not mediated by changes of intracellular calcium or pH, and was unrelated to PKC activity or an inhibition of cyclooxygenase activity.

Materials and Methods

Cell Culturing. NRKs (clone 49F), SKHep1 human hepatoma cells, and SKHep1 stably transfected with rCx43, designated SKHep1-Cx43 cells (Kwak et al., 1995), were grown in bicarbonate-buffered Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Paisly, UK) supplemented with 10% newborn calf serum (HyClone Laboratories, Logan, UT) under 5% CO₂ at 37°C. Confluent cultures were first made quiescent by subsequent incubation for 1 to 3 days in serum-free medium consisting of a 1:1 mixture of DMEM and Ham's F12 (Life Technologies) supplemented with 30 nM Na₂SeO₃ and 10 µg/ml human transferrin.

Electrophysiology. For patch-clamp experiments cells were perfused with serum-free medium consisting of a 1:1 mixture of bicarbonate-buffered DMEM and Ham's F12, equilibrated with 5% CO₂ to a pH of 7.4. Whole-cell patch-clamp experiments and voltage-clamp step response measurements were carried out as previously described (de Roos et al., 1996) using an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). Current and voltage-clamp protocols and data acquisition were performed using CED software in conjunction with a CED 1401 interface (Cambridge Electronic Design, Cambridge, UK). Data were filtered at 10 kHz and stored on hard disk for subsequent analysis. Briefly, recording of the membrane potential was shortly interrupted by switching to the voltage-clamp mode and the subsequent application of a voltage pulse of +10 mV (duration 180 ms) from a holding potential of -70 mV. The resulting capacitive current transient was analyzed (Bio-Patch software; BioLogic, Claix, France) to obtain the initial peak current I_{pk}, the final steady-state current I_{ss}, and the time constant τ of the initial component of the capacitive transient, representing the charging of the patched cell. This component was in particular recognizable in the later stages of uncoupling. Pipettes were made from borosilicate glass capillaries (GC150-15; Harvard Apparatus LTD, Edenbridge, UK) using a two-stage pipette puller (L/M-3P-A; List Electronic, Darmstadt, Germany). The intracellular pipette solution contained 25 mM NaCl, 120 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10

mM HEPES, 3.5 mM EGTA, pH 7.4, and pipettes had a resistance of 4 to 6 MΩ.

Determining Electrical Coupling. We have used two simple equations allowing an estimation of the gap junctional conductance G01 between the patched cell (#0) and the cells in the surrounding cell ring (#1) (see *Appendix*). G01 was assessed by measuring parameters from the overshooting current response to voltage-clamp steps of 10 mV (Fig. 1B) and then filling in these parameters in one of the two equations under *Appendix*. The first equation, which may only be used for G01 < Gser (R01 > Rser), is as follows:

$$G01 = I_{sh} \cdot G_{ser} / (I_{pk} - I_{sh}) \quad (1)$$

with Gser, the series conductance (I_{pk}/10 mV) between the patch pipette and the interior of cell #0; I_{pk}, the peak of the current transient after cancellation of the fast capacitive current charging the pipette capacitance or obtained by back extrapolation to $t = 0$ from the current transient after the fast transient; and I_{sh}, the peak of the current component charging cell ring #1 and visible as a shoulder in the foot of the C_{m0} charging current. The second equation is more general and practical and has the same shape:

$$G01x = I_{ss} \cdot G_{ser} / (I_{pk} - I_{ss}) \quad (2)$$

with G01x, the (under)estimated value of G01, and I_{ss}, the final steady-state current of the current transient, i.e., after completion of charging all the membrane capacitances of the successive cell rings around cell #0 (set at 180 ms in our recordings). Equation 2 is used in all cases, i.e., for measuring uncoupling and recoupling kinetics and for dose-response curves, while eq. 1 is only used for checking G01x values under conditions that R01 > Rser.

Fluorescence Measurements. Dye-coupling of cells was determined by microinjection of a 10% lucifer yellow (Molecular Probes, Eugene, OR) solution in 0.33 M LiCl into a single monolayer cell by means of a glass capillary tip using a vertical microinjection system (Olympus IMT-2; Olympus, Tokyo, Japan) as previously described (de Haan et al., 1994). About 20 different monolayer cells per dish were injected (one per 5 s) and the number of fluorescent cells was counted 10 min after injection. Only the five injections per dish that caused most substantial dye transfer were used for further analysis.

For ratio fluorometric measurements of intracellular calcium concentrations, cells grown to confluence on glass coverslips were made quiescent and subsequently loaded with 2 µM Fura-2-AM (Molecular Probes) for 30 min, after which they were washed for 20 min in serum-free medium, as previously described (de Roos et al., 1997). Fluorescence measurements were performed on a SPF-500 spectrofluorometer (Aminco, Silver Spring, MD) at 37°C. Excitation wavelengths were 340 and 380 nm and emission was detected at 492 nm. Background fluorescence was determined after addition of 2 µM ionomycin and 2 mM MnCl₂, which caused complete quenching of Fura-2 fluorescence. Ratio values of the background-corrected fluorescence intensities at 340- and 380-nm excitation were used as a measure for the cytoplasmic calcium concentration.

Fluorimetric intracellular pH measurements were carried out as previously described (van Zoelen and Tertoolen, 1991). Briefly, cells grown to confluence on glass coverslips were made quiescent and subsequently loaded with 10 µM 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester (Molecular Probes) for 45 min at 37°C, after which they were washed with a 1:1 mixture of DMEM and Ham's F12 supplemented with 5 mM bis tris propane. Excitation was performed at 506 nm and emission was detected at 532 nm. Calibration of fluorescence intensity to intracellular pH was done in the presence of 5 µM nigericin.

Chemicals. Meclofenamic acid, niflumic acid, flufenamic acid, tolfenamic acid, indomethacin, flurbiprofen, and nigericin were from Sigma (St. Louis, MO).

Numerical data are represented as mean ± S.E.M. throughout this article.

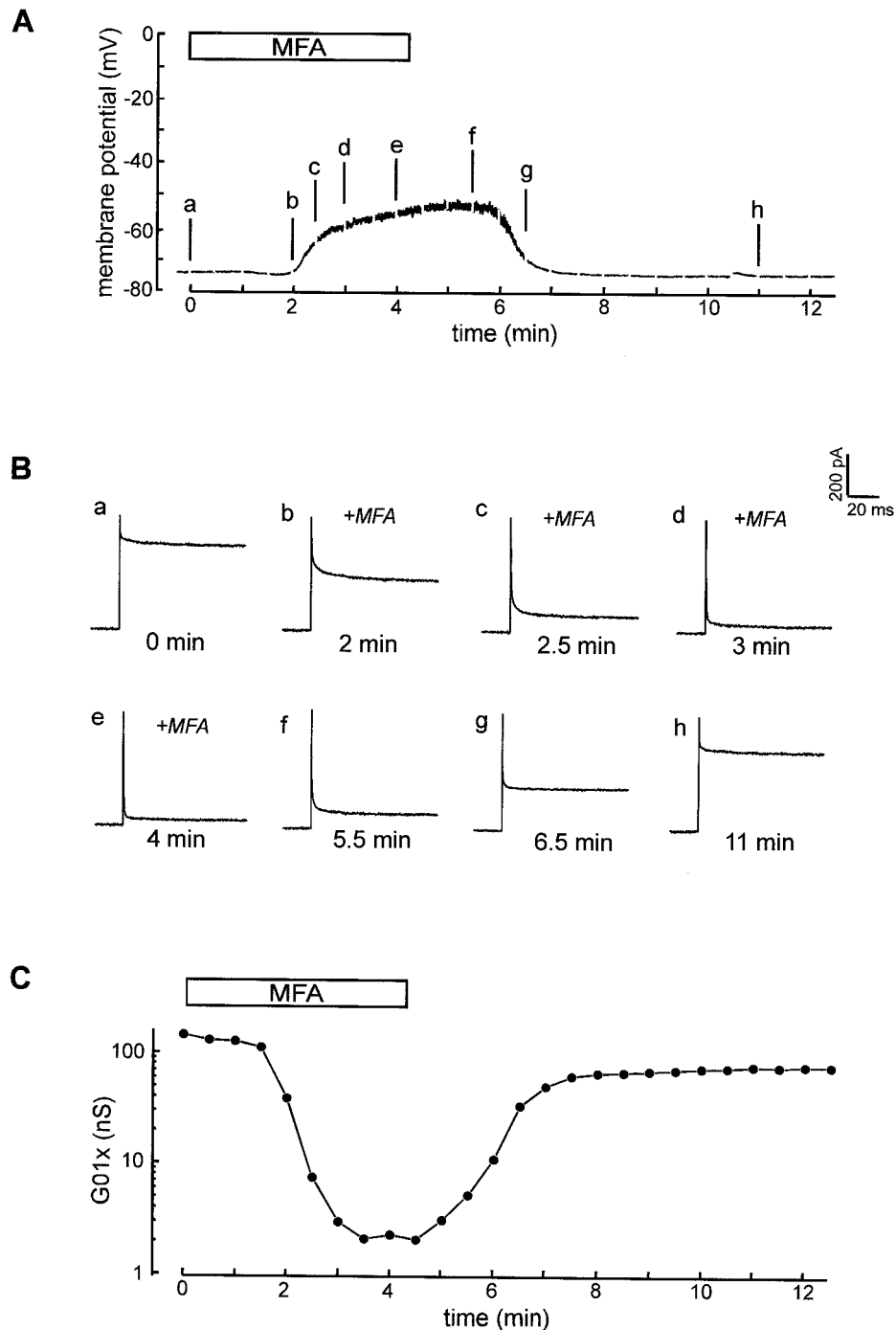


Fig. 1. A, membrane potential of a confluent monolayer of NRK cells was measured and addition of 100 μ M MFA is indicated by the bar. The membrane potential recording shows short interruptions to the voltage-clamp mode. B, capacitive current transients, evoked by application of a short voltage-clamp step (10 mV), at the indicated time points (a–h). C, corresponding calculated gap junctional conductance (G01x) as a function of time. Addition of 100 μ M MFA is indicated by the bar.

Results

Previously, we showed that capacitance measurements can be used to study changes in GJIC in small clusters of NRK cells (de Roos et al., 1996). Here we added meclofenamic acid, flufenamic acid, niflumic acid, and tolufenamic acid to a confluent monolayer of NRK cells and investigated their effect on the membrane potential and capacitive current transient at different time points after their addition. Meclofenamic acid (100 μ M) depolarized the membrane of monolayer NRK

fibroblasts (Fig. 1A) and the average depolarization was 25.7 ± 2.8 mV ($n = 33$). The depolarization could be restored to the resting membrane potential by washout of the fenamate. Flufenamic acid, niflumic acid, and tolufenamic acid induced comparable reversible depolarizations in monolayers of NRK fibroblasts of 23.2 ± 4.6 , 25.9 ± 2.6 , and 24.8 ± 3.7 mV (all $n = 10$), respectively.

Application of a voltage-clamp step to a single NRK fibroblast evokes a characteristic current transient that is deter-

mined by the series resistance (R_{ser}) and the membrane capacitance and conductance of the individual cell (de Roos et al., 1996). In confluent monolayers of NRK fibroblasts, however, membrane capacitance and conductance of the surrounding cells also contribute to this current transient, since these cells are electrically well coupled. This is reflected by a slow and multiexponential initial decay of the induced current and a subsequent large prolonged steady-state current (Fig. 1B, a). Application of 100 μ M meclofenamic acid to a monolayer of NRK fibroblasts caused an acceleration of the decay of the induced current and reduced the level of the prolonged steady-state current. This effect of meclofenamic acid was already observed under conditions that the membrane potential was still unchanged (Fig. 1B, b). In this typical example, after 4 min the steady-state current reached a minimal value. The evoked current transient could be fitted by a single exponential, indicating a complete block of intercellular communication, since more than one exponential is needed to fit the transient during a partial block of intercellular communication (de Roos et al., 1996). The capacitance calculated from this transient was 23.7 pF (Fig. 1B, e). The capacitance of cells completely uncoupled by meclofenamic acid was 19.7 ± 1.0 pF ($n = 11$), which is of the same order of magnitude as previously measured in single isolated NRK cells (de Roos et al., 1996). Electrical coupling could be largely restored by washout of the fenamate (Fig. 1B, f–h). These results demonstrate that fenamates can reversibly block electrical coupling in NRK fibroblasts and that blocking is not mediated by changes in the membrane potential.

The time course of the uncoupling could be followed by calculating the estimated gap junctional conductance between the patched cell and the surrounding cell ring (G_{01x}) from each capacitive current transient (Fig. 1C). G_{01x} in the coupled monolayer was 140.7 ± 9.6 nS ($n = 14$) and was reduced after addition of 100 μ M meclofenamic acid to 1.4 ± 0.4 nS ($n = 11$) within 6.0 ± 0.4 min ($n = 11$). Since the single cell conductance is close to 1 nS (de Roos et al., 1996), this indicates that the patched cell is completely uncoupled from the surrounding cells. Although G_{01x} could not completely be restored, partial recovery to 51.8 ± 4.2 nS ($n = 11$) was reached within 7.2 ± 1.4 min ($n = 11$).

To compare the potency of different fenamates, the concentration dependence of their effects on electrical coupling has been determined. To obtain dose-response curves, G_{01x} was calculated 15 to 20 min after addition of the fenamates when the capacitive current transient had reached a steady-state (Fig. 2). Although G_{01x} reflects only an estimation of the gap junctional coupling, the dose-response curves clearly show that the potency of the fenamates in blocking electrical coupling decreases in the order meclofenamic acid > niflumic acid > flufenamic acid.

Fluorescent dye transfer of lucifer yellow was used to investigate whether fenamates, in addition to electrical coupling, can also affect dye coupling. Lucifer yellow was injected into a single cell of a confluent monolayer of NRK fibroblasts, and intercellular diffusion of the dye showed that these cells were extensively dye coupled (Fig. 3, top). Pretreatment for 5 min with 100 μ M meclofenamic acid completely prevented this diffusion of the injected dye to the neighboring cells (Fig. 3, bottom). Dye transfer was also completely inhibited when flufenamic acid was used at a concentration of 250 μ M. These results demonstrate that

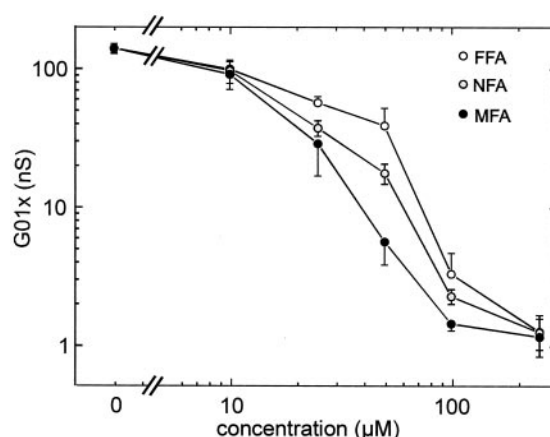


Fig. 2. Dose-response curve of the inhibition of electrical coupling by fenamates. The calculated gap junctional conductance (G_{01x}) as a function of the concentration FFA, niflumic acid (NFA), and MFA is shown. G_{01x} was determined 15 to 20 min after addition of fenamates when the capacitive current transient had reached a steady state (mean \pm S.E.M., $n = 4$; control, $n = 14$).

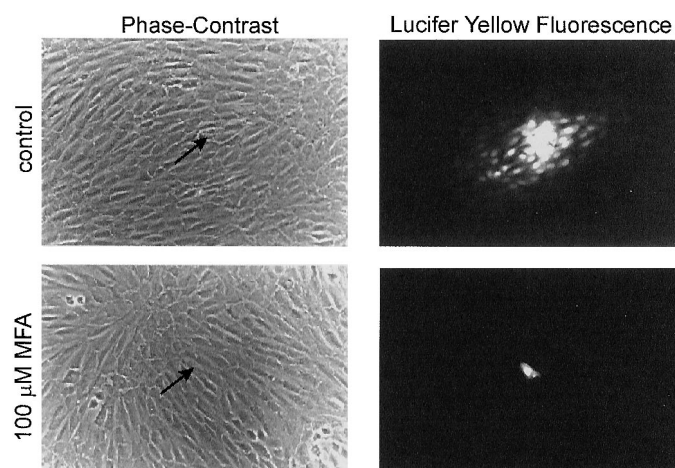


Fig. 3. Dye coupling in NRK fibroblasts. Lucifer yellow was injected into one single cell of the monolayer and intercellular diffusion of the dye was monitored 10 to 15 min after injection. Phase contrast and lucifer yellow fluorescence in control cells (top) and after pretreatment with 100 μ M MFA for 5 min (bottom). Arrows indicate the injected cell.

fenamates can also completely block dye coupling in NRK cells.

To quantify the reduction of intercellular communication by fenamates, the number of cells to which lucifer yellow could be transferred was determined 10 min after injection into a monolayer NRK cell. For meclofenamic acid (MFA), complete block of dye coupling was achieved at a concentration of 100 μ M, while block was half-maximal at 25 μ M (Fig. 4). Flufenamic acid (FFA) completely blocked dye coupling at 250 μ M. The inhibition of dye coupling by flufenamic acid was half-maximal at 40 μ M (Fig. 4). In agreement with their effects on electrical coupling, dye-coupling experiments show that meclofenamic acid is a more potent blocker of GJIC than flufenamic acid.

Next, we investigated whether the block of intercellular communication by fenamates could result from changes in the levels of the intracellular calcium concentration or pH, which are physiological modulators of gap junctions (Spray and Bennett, 1985). However, exposure of the cells to 250 μ M meclofenamic acid for 10 min did not change the intracellular

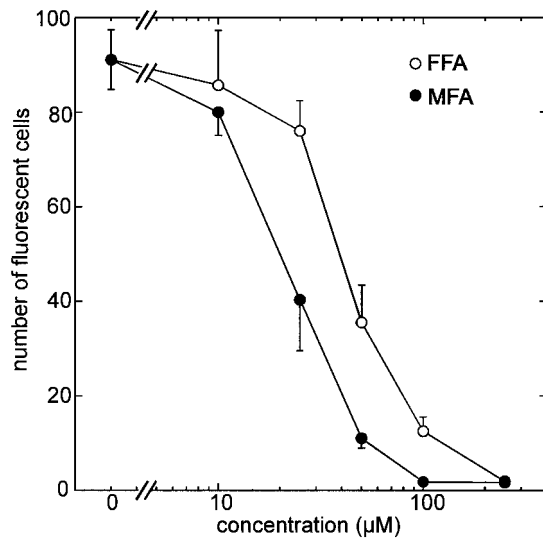


Fig. 4. Dose-response curve of the inhibition of intercellular communication by fenamates. Number of dye-coupled cells as a function of the concentration of MFA and FFA. Number of dye-coupled cells was determined 10 to 15 min after dye injection into a single cell (mean \pm S.E.M., $n = 4$; control, $n = 10$).

calcium concentration significantly. Fluorescence (340 nm/380 nm) values of intracellular Fura-2 before and after exposure were 0.44 ± 0.01 and 0.43 ± 0.01 ($n = 10$), respectively. Furthermore, the intracellular pH was unaffected under these conditions. The basal intracellular pH was 7.06 ± 0.04 and after adding meclofenamic acid the pH was 7.01 ± 0.05 ($n = 8$). These data demonstrate that inhibition of GJIC by fenamates is mediated neither by changes in intracellular calcium nor pH.

PKC has been described as a regulator of GJIC (Lampe et al., 2000) and therefore we investigated whether block of GJIC by fenamates is mediated by activation of PKC. In a previous study, we investigated modulation of gap junctions by activation of PKC using the phorbol ester TPA (de Roos et al., 1996) and found that TPA caused a partial block of GJIC. In the present study we have found that after prolonged (24-h) pretreatment of NRK fibroblasts with TPA, which causes the complete down-regulation of PKC activity in these cells, electrical coupling was completely restored and could not be blocked anymore by 100 ng/ml TPA. However, in these PKC down-regulated NRK fibroblasts electrical coupling could still be blocked by fenamates. After addition of meclofenamic acid and niflumic acid (250 μ M) to these cells for 10 min, the voltage step-induced current transients could be fitted by single exponentials and the calculated capacitance of the cells was 20.1 ± 1.2 and 18.7 ± 2.6 pF ($n = 7$), respectively. This excludes a role for PKC in the inhibition of GJIC by fenamates.

Whether the impairment of GJIC by fenamates could be directly related to an inhibition of cyclooxygenase activity in the NRK cells was investigated by application of two other potent cyclooxygenase inhibitors, indomethacin and flurbiprofen. Pretreatment of NRK fibroblasts for 3 h with 1 μ M of either of these inhibitors has been shown to cause a complete inhibition of cellular cyclooxygenase activity (Lahaye et al., 1994). We have found in the present study, however, that pretreatment of the cells for 3 h with either indomethacin or flurbiprofen did not affect their electrical coupling at all.

G01x before and after the pretreatment with these inhibitors was 155.3 ± 12.8 nS ($n = 8$) and 153.9 ± 16.4 nS ($n = 8$), respectively. These results indicate that the impairment of GJIC by fenamates is unrelated to an inhibition of cyclooxygenase activity.

Since GJIC in NRK fibroblasts is mainly mediated by Cx43 (Li et al., 1996) we investigated whether impairment of intercellular communication can be attributed to a direct effect of fenamates on Cx43 function. To address that issue we added meclofenamic acid, flufenamic acid, and niflumic acid to SKHep1 cells and SKHep1 cells that had been stably transfected with Cx43 (SKHep1-Cx43 cells). Monolayers of SKHep1 as well as SKHep1-Cx43 cells showed a membrane potential around -35 mV and application of 250 μ M meclofenamic acid reversibly depolarized the membrane with 10.3 ± 2.5 mV ($n = 7$) in SKHep1 cells and 11.4 ± 2.4 mV ($n = 7$) in SKHep1-Cx43 cells. Voltage-clamp step response experiments showed that monolayers of wild-type SKHep1 cells, which do not express Cx43 and endogenously express only low levels of Cx45 (Moreno et al., 1995), were less well coupled than NRK fibroblasts (Fig. 5A, control). The capacitance derived from the initial fast current transient was 16.9 ± 0.3 pF ($n = 7$), which is in the range of that of single cells in isolation. Thus, this capacitance is that of the patched cell in the monolayer. The corresponding G01x was 10.3 ± 0.3 nS ($n = 7$) and was reduced to 2.9 ± 0.2 nS ($n = 7$) after application of 250 μ M meclofenamic acid to these cells (Fig. 5A, 8 min). The capacitance of the patched cell was 17.1 ± 0.4 pF ($n = 7$). G01x could partially be recovered to 8.2 ± 0.3 nS ($n = 7$) by washout of meclofenamic acid, whereas the initial fast capacitive transient remained unchanged (Fig. 5A, washout). Thus, 250 μ M meclofenamic acid at least partly blocked intercellular coupling mediated by Cx45. SKHep1-Cx43 cells showed larger capacitive current transients, indicating that these cells were better electrically coupled (Fig. 5B, control). G01x was 60.6 ± 4.3 nS ($n = 7$) for the coupled monolayer and was reduced to 2.7 ± 0.2 nS ($n = 7$) after adding 250 μ M meclofenamic acid (Fig. 5B, 6 min). The capacitance calculated from the initial fast transient was 17.5 ± 0.4 pF ($n = 7$), also indicating that the cells were largely uncoupled under these conditions. Electrical coupling could be restored by washout of meclofenamic acid and G01x was partially recovered to 23.0 ± 1.9 nS ($n = 7$) (Fig. 5B, washout). Flufenamic acid and niflumic acid were also able to

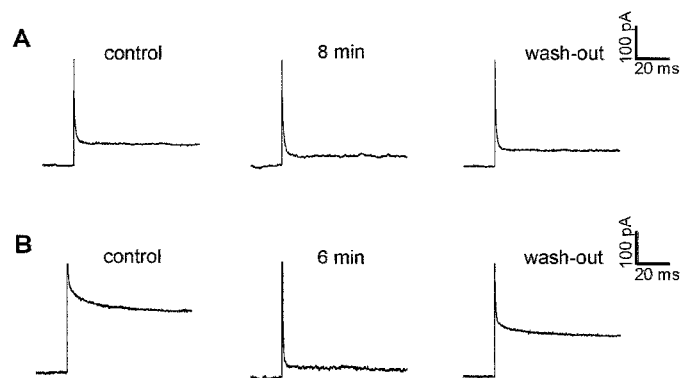


Fig. 5. Effect of 250 μ M MFA on the capacitive current transient, evoked by a short voltage-clamp step (10 mV), in confluent monolayers of SKHep1 cells (A) and SKHep1-Cx43 cells (B). Data shown are representative traces of at least four experiments.

block electrical coupling in these cells when added at a concentration of 250 μM . After uncoupling with flufenamic acid G01x was $2.7 \pm 0.4 \text{ nS}$ ($n = 3$) and the calculated capacitance was $19.0 \pm 0.8 \text{ pF}$ ($n = 3$). Niflumic acid reduced G01x to $2.9 \pm 0.2 \text{ nS}$ ($n = 3$) and the capacitance to $17.9 \pm 1.1 \text{ pF}$ ($n = 3$). These results demonstrate that fenamates are reversible blockers of Cx43-mediated intercellular communication.

Discussion

In the present study we show that fenamates represent a novel class of reversible blockers of Cx43-mediated GJIC. The effects of fenamates on GJIC were assessed by using an analysis of single electrode voltage-clamp step responses, which is based on a simplified electric circuit of the patched cell with the surrounding monolayer.

Gap junctions have been shown to be essential for the direct diffusion of, for example, calcium and small signal molecules such as inositol triphosphate between neighboring cells (Giaume and Venance, 1998) and for their electrical coupling. A variety of biological processes, including cellular growth, propagation of calcium waves, and cardiac function is regulated by GJIC (Yamasaki and Naus, 1996; Kumar and Gilula, 1996). Impaired GJIC has been reported in several diseases, including cardiovascular diseases (Jongsma and Wilders, 2000) and tumorigenesis (Zhang et al., 1998; Laird et al., 1999; Yamasaki et al., 1999). Although in some cells a decrease in GJIC correlates with tumor growth (Hotz-Wagenblatt and Shalloway, 1993), phenotypic transformation of NRK cells is accompanied by an increase in intercellular communication (van Zoelen and Tertoolen, 1991).

GJIC can be inhibited by physiological agents that initiate complex signaling pathways, resulting in activation of kinases, phosphatases, and interacting proteins (Hossain and Boynton, 2000) or changes in levels of intracellular calcium and pH (Bruzzone et al., 1996). Furthermore, different classes of chemicals have been shown to block GJIC. Some of them affect the conformation of the connexins by disturbing the bulk membrane fluidity or the membrane protein interface. Examples of chemicals that block GJIC by changing the lipid structure around connexins include alcohols such as heptanol and octanol (Johnston et al., 1980), halothane (Burt and Spray, 1989), and fatty acids such as oleamides (Lerner, 1997), arachidonamide (Boger et al., 1999), and anandamide (Venance et al., 1995). Phorbol esters block GJIC by phosphorylation of connexin residues (Lampe, 1994), whereas glycerethinic acid derivatives block by dephosphorylation of connexin43 (Guan et al., 1996). For selective inhibition of gap junctions, synthetic oligopeptides that interact with the external loop of connexins have been developed (Kwak and Jongsma, 1999).

Fenamates are widely used as inhibitors of cyclooxygenase activity (Brogren, 1986). Their inhibitory effect on gap junctions, however, is not related to their ability to inhibit cyclooxygenase activity, since indomethacin and flurbiprofen, two potent cyclooxygenase inhibitors, did not affect the electrical coupling of the cells at all.

Previously, we found that blocking GJIC with the phorbol ester TPA resulted in a depolarized membrane (de Roos et al., 1996). Furthermore, halothane, another gap junction blocker, has been reported to have effects on the resting membrane potential in skeletal muscle cells (Sauviat et al.,

2000). Complete uncoupling by fenamates was also accompanied by depolarizations of the membrane of variable magnitude. Uncoupling of the NRK cells by fenamates, however, already started when the membrane potential was still unchanged (Fig. 1, A, b and B, b). Moreover, complete uncoupling was sometimes observed under conditions that the membrane potential was unaffected. These results exclude that membrane depolarization caused the uncoupling by fenamates.

Complete block of GJIC by fenamates was sometimes accompanied with increased fluctuations of the membrane potential (Fig. 1, A, e and B, e). Most likely, electrical coupling of the monolayer cells caused fluctuations in the membrane potential to be averaged by a mechanism of channel-sharing (Atwater et al., 1983; Sherman et al., 1988), resulting in a stable membrane potential. So, the increased fluctuations of the membrane potential after application of fenamates may reflect the loss of channel sharing by blocking GJIC. However, since fenamates have been reported to block anionic as well as cationic channels at various concentrations from 10 to 100 μM (Gögelein et al., 1990; White and Aylwin, 1990; Grover et al., 1994; Li et al., 1998; Lee and Wang, 1999), additional effects of fenamates on plasma membrane ion channels may also have contributed to the increased membrane potential fluctuations and the depolarization of the uncoupled cell.

Fenamates did not affect intracellular calcium and pH, two physiological modulators of gap junctions. In addition, fenamates could still block GJIC in PKC down-regulated cells, excluding a role for PKC. Although a role for other second messenger pathways cannot be excluded, it is more likely that the reversible block of GJIC by fenamates results from either a direct interaction with connexins or an indirect action through perturbations in the bulk membrane fluidity or the membrane protein interface that would affect the conformation of the connexins.

Anti-inflammatory therapy is often accompanied by unwanted side effects, including gastrointestinal toxicity. However, nonsteroidal anti-inflammatory drugs have also been described to prevent colon cancer and Alzheimer's disease (Rich et al., 1995; Sheng et al., 1997). Whether there is a link between these unwanted and/or beneficial effects of nonsteroidal anti-inflammatory drugs and the (partial) block of gap junctions by fenamates requires further investigation. It should, however, be mentioned that the concentration needed to inhibit cyclooxygenase is much lower than that required to block GJIC. For example, the IC_{50} of meclofenamic acid is about 0.05 μM in inhibiting cyclooxygenase (Kalgutkar et al., 2000) and about 25 μM in blocking GJIC (Fig. 4). Nevertheless, the present finding that fenamates are able to block GJIC not only in NRK fibroblasts but also in Cx43-overexpressing SKHep-1 cells strongly indicates that fenamates can be used as a pharmacological tool to reversibly block Cx43-mediated intercellular communication. Whether fenamates can also be used as blockers of intercellular communication mediated by other connexins remains to be investigated.

Acknowledgments

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Appendix: Assessment of Fenamate-Induced Electrical Uncoupling of NRK Cells in a Monolayer with Use of an Electrical Equivalent Circuit.

In the whole-cell voltage-clamp configuration we consider the patched cell in the middle of an NRK cell monolayer as a central cell (#0), surrounded by concentric rings (#1, 2, 3, etc.) of cells (Fig. 6A). Cell #0 is coupled to the voltage source E by G_{ser} (or R_{ser}), the series conductance (or resistance) associated with the pipette-cell connection. Each ring is considered as one isopotential compartment with the summed membrane conductance (G_{m1} , G_{m2} , etc.) and capacitance (C_{m1} , C_{m2} , etc.) of all its cells. Furthermore, each ring is electri-

cally coupled to its inner and outer neighbor ring. Thus, cell #0 is coupled to ring #1 by G_{01} (or R_{01}), ring #1 is coupled to ring #2 by G_{12} (or R_{12}), etc. With increasing ring diameter, ring conductance and capacitance increase with the number of cells in the rings, e.g., $G_{m1} \sim 6G_{m0}$ and $C_{m1} \sim 6C_{m0}$ for 6 NRK cells in ring #1 (as often found). Coupling conductance between the rings also increases with ring diameter. Membrane and coupling conductance are assumed to be voltage-independent for the voltage steps applied (+10 mV from -70 mV).

In such an electrical equivalent circuit, which is essentially the same as described by Siegenbeek van Heukelom et al. (1970), the current response upon a voltage-clamp step may

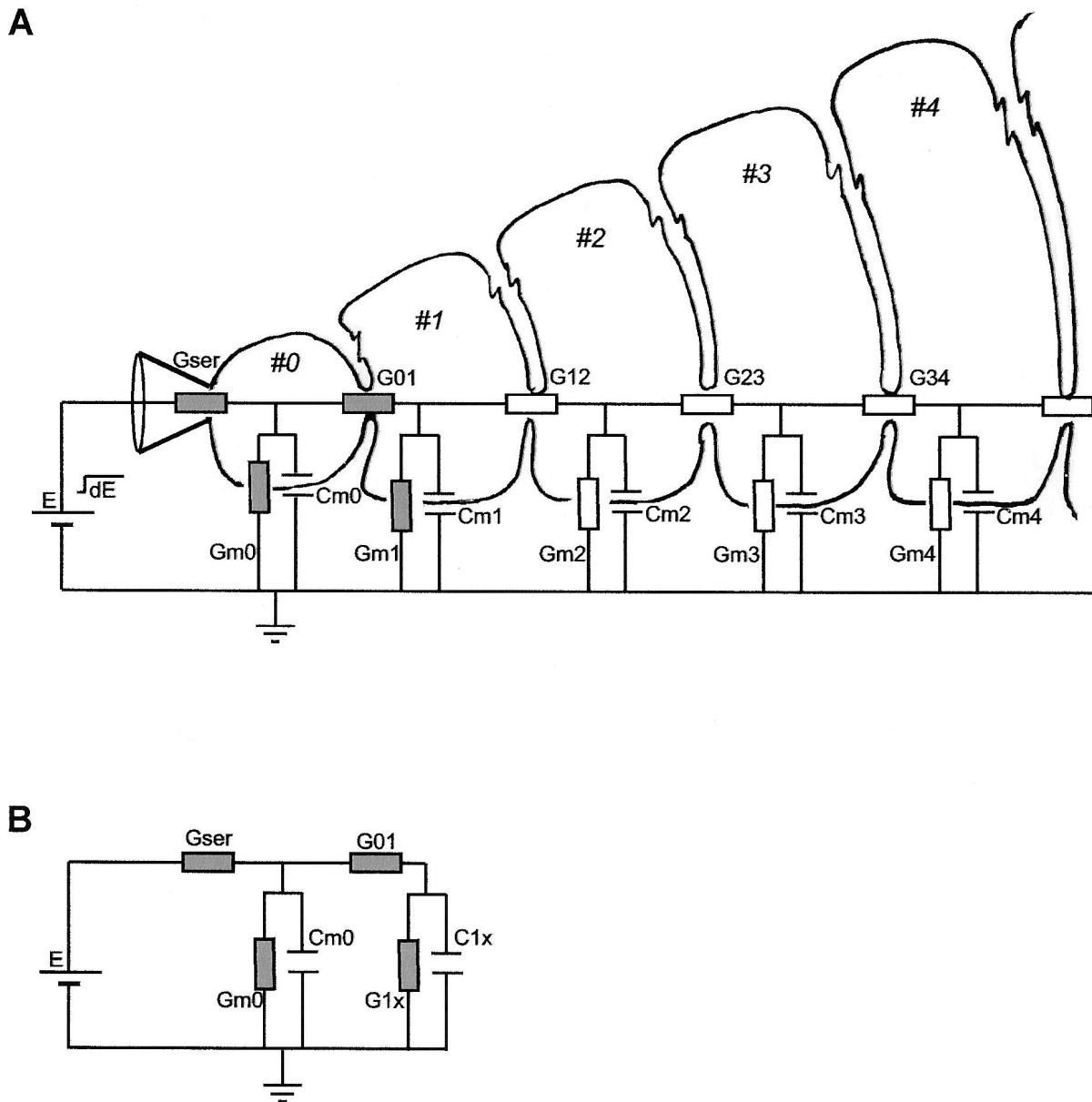


Fig. 6. Electrical equivalent circuit of a voltage-clamp step response measurement on a patched whole-cell in a confluent monolayer of NRK cells. The voltage clamp is represented by the voltage source E , which provides voltage steps dE through the patch pipette (at the left). A, circuit, drawn in a (compressed) concentric ring representation of the monolayer around the patched cell (#0). This reduces the two-dimensional monolayer to a one-dimensional cable-like circuit. Pipette capacitance charging components are omitted in the circuit. For further explanation, see Appendix. B, simplified monolayer circuit, in which G_{1x} represents the inverse of the total exit resistance (R_{1x}) for current leaving the interior of cell ring #1 through whatever path (junctional or membrane resistance). The equivalent monolayer resistance R_{1x} from ring #1 is paired with an equivalent monolayer capacitance C_{1x} .

be viewed as the successive charging of cell #0 through Rser, ring #1 through Rser + R01, ring #2 through Rser + R01 + R12, etc. This monolayer circuit allowed us to derive a simple equation to calculate whole-cell gap junctional conductance of cell #0 to ring #1 (G01) under conditions that $\tau_1 (= [Rser + R01] \cdot Cm1) \gg \tau_0 (= Rser \cdot Cm0)$. Under these conditions, which exist during the development of uncoupling ($R01 > Rser$, $Cm1 \sim 6Cm0$), Cm0 and subsequent Cm1 charging are more or less independent (cf. Ypey and DeFelice, 2000). Therefore, the beginning of the charging current of ring #1 can be seen as a shoulder current (Ish, i.e., the peak of the Cm1 charging current) in the foot of the charging current of cell #0. According to Ohm's law we can write for the peak of the current transient (Ipk) upon voltage steps dE the equation $Ipk = dE/Rser$ and for the shoulder current $Ish = dE/(Rser + R01)$. Combining both equations results in the following:

$$G01 = Ish \cdot Gser / (Ipk - Ish) \quad (1)$$

For $R01 < Rser$, G01 calculation from eq. 1 may become less reliable because the charging components of cell #0 and ring #1 become much less independent and Ish less recognizable. Furthermore, under favorable conditions ($R01 > Rser$) back extrapolation of the current transient to $t = 0$ may cause errors because this transient is not single-exponential due to the subsequent slower charging of the more peripheral rings.

Because of the limitations mentioned we derived another simple, but more robust and general although less precise, steady-state equation for the estimation of G01. It is based on a simplification of the electrical equivalent circuit of the monolayer to a two-compartment circuit (Fig. 6B), in which ring #1 represents the whole monolayer around cell #0 with an equivalent membrane resistance R1x (or G1x), the exit DC resistance from ring #1 to ground for the entire current entering ring #1 through R01. Circuit analysis shows that $Rm1/R12$ (the equivalent resistance of Rm1 and Rm2 in parallel) $< R1x < Rm1$. Parallel to R1x one may imagine an equivalent membrane capacitance C1x giving a best possible (although not perfect) fit to the time course of the current transient upon the voltage step, but the precise C1x value is not of importance for the estimation of G01. Ohm's law allows us to write for the peak of the current transient (Ipk) $Ipk = dE/Rser$ and for the steady-state current (Iss), reached after completion of the charging process of all capacitances of the monolayer involved, $Iss = dE/(Rser + R01 + R1x)$, assuming that $Rm0 \gg R01$ (as is the case under control conditions and during uncoupling until coupling becomes weak). Combining both equations results in the following:

$$G01x = Iss \cdot Gser / (Ipk - Iss) \quad (2)$$

with $G01x = 1/(R01 + R1x)$. This equation implies that G01x, called here the estimated coupling conductance between cell #0 and ring #1, is always smaller than the real coupling conductance (G01). Using eq. 1 we found for not strongly uncoupled cells with a clear Ish (Fig. 1B, a–c, f–h) that the (G01/G01x) ratio was 1.8 ± 0.3 ($n = 6$) meaning that $G1x \sim G01$. Under conditions of complete uncoupling ($R01 \gg Rm0$), eq. 2 has become the expression for Gm0. Thus, when eq. 2 provides single cell conductance values $\sim Gm0$ for the apparent G01x, as in the present study, one may conclude that $G01x < Gm0$. In conclusion, although eq. 2 does not provide

exact G01 values, it is useful for G01 estimations of the right order of magnitude, for monitoring drug-induced uncoupling and for comparing relative potencies of uncoupling drugs.

References

- Atwater I, Rosario L and Rojas E (1983) Properties of the Ca-activated K^+ channel in pancreatic beta-cells. *Cell Calcium* **4**:451–461.
- Boger DL, Sato H, Lerner AE, Guan X and Gilula NB (1999) Arachidonic acid amide inhibitors of gap junction cell-cell communication. *Bioorg Med Chem Lett* **9**:1151–1154.
- Brogden RN (1986) Non-steroidal anti-inflammatory analgesics other than salicylates. *Drugs* **32** (Suppl 4):27–45.
- Bruzzzone R, White TW and Paul DL (1996) Connections with connexins: the molecular basis of direct intercellular signaling. *Eur J Biochem* **238**:1–27.
- Bukauskas FF, Jordan K, Bukauskiene A, Bennett MV, Lampe PD, Laird DW and Verselis VK (2000) Clustering of connexin 43-enhanced green fluorescent protein gap junction channels and functional coupling in living cells. *Proc Natl Acad Sci USA* **97**:2556–2561.
- Burt JM and Spray DC (1989) Volatile anesthetics block intercellular communication between neonatal rat myocardial cells. *Circ Res* **65**:829–837.
- de Haan LH, Bosselaers I, Jongen WM, Zwijsen RM and Koeman JH (1994) Effect of lipids and aldehydes on gap-junctional intercellular communication between human smooth muscle cells. *Carcinogenesis* **15**:253–256.
- De Mello WC (1994) Gap junctional communication in excitable tissues; the heart as a paradigm. *Prog Biophys Mol Biol* **61**:1–35.
- de Roos AD, van Zoelen EJ and Theuvsen AP (1996) Determination of gap junctional intercellular communication by capacitance measurements. *Pfluegers Arch* **431**:556–563.
- de Roos AD, Willems PH, Peters PH, van Zoelen EJ and Theuvsen AP (1997) Synchronized calcium spiking resulting from spontaneous calcium action potentials in monolayers of NRK fibroblasts. *Cell Calcium* **22**:195–207.
- Farrugia G, Rae JL, Sarr MG and Szurszewski JH (1993) Potassium current in circular smooth muscle of human jejunum activated by fenamates. *Am J Physiol* **265**:G873–G879.
- Giaume C and Venance L (1998) Intercellular calcium signaling and gap junctional communication in astrocytes. *Glia* **24**:50–64.
- Gögelein H, Dahlem D, Englert HC and Lang HJ (1990) Flufenamic acid, mefenamic acid and niflumic acid inhibit single nonselective cation channels in the rat exocrine pancreas. *FEBS Lett* **268**:79–82.
- Goldberg GS and Lau AF (1993) Dynamics of connexin43 phosphorylation in pp60v-src-transformed cells. *Biochem J* **295**:735–742.
- Gros DB and Jongsma HJ (1996) Connexins in mammalian heart function. *Bioessays* **18**:719–730.
- Grover GJ, D'Alonzo AJ, Sleph PG, Dzwonczyk S, Hess TA and Darbenzio RB (1994) The cardioprotective and electrophysiological effects of cromakalim are attenuated by meclofenamate through a cyclooxygenase-independent mechanism. *J Pharmacol Exp Ther* **269**:536–540.
- Guan X, Wilson S, Schlender KK and Ruch RJ (1996) Gap-junction disassembly and connexin 43 dephosphorylation induced by 18 beta-glycyrrhetic acid. *Mol Carcinog* **16**:157–164.
- Hossain MZ and Boynton AL (2000) Regulation of Cx43 gap junctions: the gatekeeper and the password. Science's STKE: http://www.stke.org/cgi/content/full/OC_sigtrans;2000/54/pe1.
- Hotz-Wagenblatt A and Shalloway D (1993) Gap junctional communication and neoplastic transformation. *Crit Rev Oncog* **4**:541–558.
- Johnston MF, Simon SA and Ramon F (1980) Interaction of anaesthetics with electrical synapses. *Nature (Lond)* **286**:498–500.
- Jongsma HJ and Wilders R (2000) Gap junctions in cardiovascular disease. *Circ Res* **86**:1193–1197.
- Kumar NM and Gilula NB (1996) The gap junction communication channel. *Cell* **84**:381–388.
- Kalgtutkar AS, Crews BC, Rowlinson SW, Marnett AB, Kozak KR, Rimmel RP and Marnett LJ (2000) Biochemically based design of cyclooxygenase-2 (COX-2) inhibitors: facile conversion of nonsteroidal antiinflammatory drugs to potent and highly selective COX-2 inhibitors. *Proc Natl Acad Sci USA* **97**:925–930.
- Kwak BR, Hermans MM, De Jonge HR, Lohmann SM, Jongsma HJ and Chanson M (1995) Differential regulation of distinct types of gap junction channels by similar phosphorylating conditions. *Mol Biol Cell* **6**:1707–1719.
- Kwak BR and Jongsma HJ (1999) Selective inhibition of gap junction channel activity by synthetic peptides. *J Physiol (Lond)* **516**:679–685.
- Lahaye DHTP, Afink GB, Bleijs DA, Van Alewijk DCGJ and Van Zoelen EJJ (1994) Effect of bradykinin on loss of density-dependent growth inhibition of normal rat kidney cells. *Cell Mol Biol* **40**:717–721.
- Laird DW, Pistouris P, Batist G, Alpert L, Huynh HT, Carystinos GD and Alaoui-Jamali MA (1999) Deficiency of connexin43 gap junctions is an independent marker for breast tumors. *Cancer Res* **59**:4104–4110.
- Lampe PD (1994) Analyzing phorbol ester effects on gap junctional communication: a dramatic inhibition of assembly. *J Cell Biol* **127**:1895–1905.
- Lampe PD, TenBroek EM, Burt JM, Kurata WE, Johnson RG and Lau AF (2000) Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication. *J Cell Biol* **149**:1503–1512.
- Lau AF, Kurata WE, Kanemitsu MY, Loo LW, Warn-Cramer BJ, Eckhart W and Lampe PD (1996) Regulation of connexin43 function by activated tyrosine protein kinases. *J Bioenerg Biomembr* **28**:359–368.
- Lee YT and Wang Q (1999) Inhibition of hKv2.1, a major human neuronal voltage-gated K^+ channel, by meclofenamic acid. *Eur J Pharmacol* **378**:349–356.
- Lerner RA (1997) A hypothesis about the endogenous analogue of general anesthesia. *Proc Natl Acad Sci USA* **94**:13375–13377.

- Li H, Liu TF, Lazrak A, Peracchia C, Goldberg GS, Lampe PD and Johnson RG (1996) Properties and regulation of gap junctional hemichannels in the plasma membranes of cultured cells. *J Cell Biol* **134**:1019–1030.
- Li L, Vapaatalo H, Vaali K, Paakkari I and Kankaanranta H (1998) Fenamates inhibit contraction of guinea-pig isolated bronchus in vitro independent of prostanoïd synthesis inhibition. *Life Sci* **62**:PL289–PL294.
- Loewenstein WR (1981) Junctional intercellular communication: the cell-to-cell membrane channel. *Physiol Rev* **61**:829–913.
- Maldonado PE, Rose B and Loewenstein WR (1988) Growth factors modulate junctional cell-to-cell communication. *J Membr Biol* **106**:203–210.
- Moreno AP, Laing JG, Beyer EC and Spray DC (1995) Properties of gap junction channels formed of connexin 45 endogenously expressed in human hepatoma (SKHep1) cells. *Am J Physiol* **268**:C356–C365.
- Ottolia M and Toro L (1994) Potentiation of large conductance KCa channels by niflumic, flufenamic, and mefenamic acids. *Biophys J* **67**:2272–2279.
- Rich JB, Rasmusson DX, Folstein MF, Carson KA, Kawas C and Brandt J (1995) nonsteroidal anti-inflammatory drugs in Alzheimer's disease. *Neurology* **45**:51–55.
- Sauviat MP, Frizelle HP, Descorps-Declere A and Mazoit JX (2000) Effects of halothane on the membrane potential in skeletal muscle of the frog. *Br J Pharmacol* **130**:619–624.
- Sheng H, Shao J, Kirkland SC, Isakson P, Coffey RJ, Morrow J, Beauchamp RD and DuBois RN (1997) Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. *J Clin Invest* **99**:2254–2259.
- Sherman A, Rinzel J and Keizer J (1988) Emergence of organized bursting in clusters of pancreatic beta-cells by channel sharing. *Biophys J* **54**:411–425.
- Siegenbeek van Heukelom J, Denier van der Gon JJ and Prop FJA (1970) Epithelial monolayers: a study object for cell communication. *Biochim Biophys Acta* **211**:98–101.
- Spray DC and Bennett MV (1985) Physiology and pharmacology of gap junctions. *Annu Rev Physiol* **47**:281–303.
- Stauffer PL, Zhao H, Luby-Phelps K, Moss RL, Star RA and Muallem S (1993) Gap junction communication modulates $[Ca^{2+}]_i$ oscillations and enzyme secretion in pancreatic acini. *J Biol Chem* **268**:19769–19775.
- van Zoelen EJ and Tertoolen LG (1991) Transforming growth factor-beta enhances the extent of intercellular communication between normal rat kidney cells. *J Biol Chem* **266**:12075–12081.
- Venance L, Piomelli D, Glowinski J and Giaume C (1995) Inhibition by anandamide of gap junctions and intercellular calcium signalling in striatal astrocytes. *Nature (Lond)* **376**:590–594.
- White MM and Aylwin M (1990) Niflumic and flufenamic acids are potent reversible blockers of Ca^{2+} -activated Cl^- channels in *Xenopus* oocytes. *Mol Pharmacol* **37**:720–724.
- Yamasaki H and Naus CC (1996) Role of connexin genes in growth control. *Carcinogenesis* **17**:1199–1213.
- Yamasaki H, Omori Y, Krutovskikh V, Zhu W, Mironov N, Yamakage K and Mesnil M (1999) Connexins in tumour suppression and cancer therapy. *Novartis Found Symp* **219**:241–254, 254–260.
- Ypéy DL and DeFelice LJ (2000) The patch-clamp technique: a theoretical and practical introduction using simple electrical equivalent circuits, in *Channels, Receptors & Transporters* (DeFelice LJ ed) volume of the Biophysics Textbook On Line (BTOL) of the Biophysical Society: <http://www.biophysics.org/biophys/society/btol/>.
- Zhang ZQ, Zhang W, Wang NQ, Bani-Yaghoub M, Lin ZX and Naus CC (1998) Suppression of tumorigenicity of human lung carcinoma cells after transfection with connexin43. *Carcinogenesis* **19**:1889–1894.

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