J. Membrane Biol. 185, 93–102 (2002)
DOI: 10.1007/s00232-001-0115-0

The Journal of
Membrane
Biology

Inhibition of Gap Junction Hemichannels by Chloride Channel Blockers

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Received: 15 May 2001/Revised: 22 August 2001

Abstract. Electrophysiological methods were used to assess the effect of chloride-channel blockers on the macroscopic and microscopic currents of mouse connexin50 (Cx50) and rat connexin46 (Cx46) hemichannels expressed in *Xenopus laevis* oocytes. Oocytes were voltage-clamped at −50 mV and hemichannel currents (I_{Cx50} or I_{Cx46}) were activated by lowering the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) from 5 mM to 10 μM. Ion-replacement experiments suggested that I_{Cx50} is carried primarily (>95%) by monovalent cations ($P_K : P_{Na} : P_{Cl} = 1.0 : 0.74 : 0.05$). I_{Cx50} was inhibited by 18β-glycyrrhetinic acid (apparent K_i, 2 μM), gadolinium (3 μM), flufenamic acid (3 μM), niflumic acid (11 μM), NPPB (15 μM), diphenyl-2-carboxylate (26 μM), and octanol (177 μM). With the exception of octanol, niflumic acid, and diphenyl-2-carboxylate, the above agents also inhibited I_{Cx46}. Anthracene-9-carboxylate, furosemide, DIDS, SITS, IAA-94, and tamoxifen had no inhibitory effect on either I_{Cx50} or I_{Cx46}. The kinetics of I_{Cx50} inhibition were not altered at widely different $[Ca^{2+}]_o$ (10–500 μM), suggesting that drug-hemichannel interaction does not involve the Ca^{2+} binding site. In excised membrane patches, application of flufenamic acid or octanol to the extracellular surface of Cx50 hemichannels reduced single channel-open probability without altering the single-channel conductance, but application to the cytoplasmic surface had no effect on the channels. We conclude that some chloride-channel blockers inhibit lens-connexin hemichannels by acting on a site accessible only from the extracellular space, and that drug-hemichannel interaction involves a high-affinity site other than the Ca^{2+} binding site.

Key words: Connexins — Hemichannels — Connexin46 — Connexin50 — Pharmacology — Chloride-Channel Blockers

Introduction

Gap junction channels mediate chemical and electrical cell-to-cell information transmission and, therefore, play important roles in development and in physiological as well as pathophysiological states. The cell-to-cell channel complex is often found at very high densities (≈10,000/μm^2) in specialized regions of the plasma membrane, called gap junction plaques, and is composed of two hemichannels, each of which is located in the plasma membrane of adjacent cells (Unger et al., 1999; Unwin & Zampighi, 1980; Yeager, Unger & Falk, 1998). Hemichannels are hexamers of connexin monomers (Unwin & Zampighi, 1980) and in general, their density and functional properties in the plasma membrane are difficult to characterize accurately except in some expression systems (e.g., *Xenopus* oocytes; Zampighi et al., 1999).

The fact that the pool of hemichannels in the plasma membrane has been difficult to characterize has greatly hindered our understanding of the function of connexins in cells. It is thought that, before entering the gap-junction plaque to dock with a partner hemichannel from the opposing cell, hemichannels may be found in nonjunctional plasma membrane and function as large-conductance ion channels (DeVries & Schwartz, 1992; Ebihara, 1996; Eckert et al., 1998; Li et al., 1996; Malchow, Qian & Ripps, 1993; Quist et al., 2000; Zampighi et al., 1999). Hemichannels are activated at low extracellular Ca^{2+}

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concentrations ($[Ca^{2+}]_o$) are inhibited by intracellular acidification and by compounds such as octanol (Li et al., 1996; Zampighi et al., 1999), and are sensitive to the metabolic state of the cell (John et al., 1999; Kondo et al., 2000). That hemichannels provide ionic pathways in the plasma membrane, whose conductance depends on the internal and external environment of the cell, suggests that hemichannels may play as yet unidentified roles in cellular homeostasis. The large conductance and weakly-selective nature of hemichannels suggest the possibility that even a small number in the plasma membrane may contribute significantly to cellular whole-cell conductance.

The physiological importance of hemichannels in nonjunctional regions of the plasma membrane has been difficult to establish due to a lack of specific pharmacological agents that inhibit their activity. The currently available agents, such as heptanol, octanol, and halothane, all have deleterious and nonspecific plasma-membrane effects, which limits their use in cell physiological studies (Verrecchia & Hervé, 1997). In the present study, we describe the inhibitory effect of some chloride-channel blockers on the activity of lens connexin50 (Cx50) and connexin46 (Cx46) hemichannels. Our results suggest that some chloride channel blockers inhibit Cx46 and Cx50 hemichannels with high affinity and may prove useful in examination of the physiological role of connexins in cells.

Materials and Methods

Expression of Connexin Hemichannels in Oocytes

Female *Xenopus laevis* toads were anesthetized in 0.1% tricaine methanesulfonate, small lobes of the ovary were removed, and the animals were sacrificed by an overdose of Nembutal (60 mg) in accordance with the protocol approved by the Chancellor's Animal Protection Committee at UCLA. Stage V–VI oocytes were isolated and injected with cRNA for mouse connexin50 (Cx50) (White et al., 1992) or rat connexin46 (Cx46) (Paul et al., 1991). Oocytes were maintained in Barth's medium (in mM: 88 NaCl, 1 KCl, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 2.4 $NaHCO_3$, 10 HEPES, pH 7.4) at 18°C for 1–3 days until used in experiments (Parent et al., 1992). For Cx46, it was necessary to increase the $CaCl_2$ concentration of Barth's medium to 5 mM in order to inhibit a constitutive Cx46-mediated Na^+ influx into the oocytes, which leads to swelling and death. All experiments were performed at 21 ± 1°C.

Electrophysiology and Experimental Solutions

The two-microelectrode voltage-clamp technique was used for the recording of whole-cell macroscopic hemichannel currents (Loo et al., 1993; Zampighi et al., 1999). In the experimental recording chamber, oocytes were initially perfused with a NaCl buffer containing (in mM):100 NaCl, 2 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.5. The high extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was needed to maintain the hemichannels in the closed state (Zampighi et al., 1999). Hemichannel currents (I_{Cx50} or I_{Cx46}) were evoked by lowering the extracellular Ca^{2+} concentration, and inhibition was achieved by adding the blockers (*see* below) to the bathing medium. The calcium concentration was adjusted by adding appropriate amounts of Ca^{2+} and ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) (Bers, Patton & Nuccitelli, 1994), and the final free Ca^{2+} concentration was confirmed by using a Ca^{2+}-selective electrode (Corning). In Cl^--free solutions, chloride was replaced with either gluconate or 2-(N-morpholino)ethanesulfonic acid (MES). In Na^+-free solutions, sodium was replaced with choline or tetraethylammonium (TEA). In the experiment of Fig. 1*C*, NaCl was isosmotically replaced with mannitol, and the resulting junction potentials of 1–10 mV were corrected. To acidify the intracellular compartment, acetate was added to the bathing medium (50 mM equimolar replacement with Na-acetate in the NaCl buffer; pH 7.5) (Zampighi et al., 1999). In all experiments, the reference electrode was connected to the experimental oocyte chamber via an agar bridge (3% agar in 3 M KCl). To obtain current-voltage (*I-V*) relations, the pulse protocol (pCLAMP, Axon Instruments, Foster City, CA) consisted of 1000-msec voltage steps from a holding potential of −50 mV to a series of test voltages from +50 to −150 mV in 20-mV steps. Currents were low-pass filtered at 500 Hz, and sampled at 1 kHz. The reversal potential (V_{rev}) was determined from the I_{Cx50}-V plot obtained 10 msec after the onset of the voltage pulse (Zampighi et al., 1999). To determine I_{Cx50} at each test voltage, the endogenous currents measured at 5 mM $[Ca^{2+}]_o$ were subtracted from those obtained at 10 μM $[Ca^{2+}]_o$. For continuous holding current measurements, currents were low-pass filtered at 20 Hz, and sampled at 1 Hz.

For the patch-clamp studies (Hamill et al., 1981), oocytes were placed in a chamber bathed in a buffer containing (in mM): 100 NaCl, 2 KCl, 5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.5. The vitelline membrane was manually removed. Patch pipettes (1–3 MΩ) were pulled from borosilicate glass. Recordings were carried out by using an Axopatch 1B amplifier equipped with an IHS-1 integrating headstage (Axon Instruments). Currents were low-pass filtered at 2 kHz and sampled at 10 kHz. In cell-attached and excised patches (inside-out or outside-out), the solution facing the extracellular membrane surface contained (in mM): 100 NaCl, 2 KC1, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 5 EGTA, pH 7.5, and the solution facing the cytoplasmic membrane surface contained (in mM): 100 K-aspartate, 10 NaCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, 5 EGTA, pH 7.2. In inside-out patches of Fig. 4*C*, the cytoplasmic solution at pH 6.0 was buffered with MES. In all current records, negative current denotes an inward current (movement of positive charge from the extracellular surface of the membrane to the cytoplasmic surface).

Blockers of Gap Junction Channels and Chloride Channels

Stock solutions of the inhibitors were prepared in ethanol or dimethyl sulfoxide (DMSO), and were then diluted in the low-Ca^{2+} NaCl buffer. In control experiments, neither ethanol nor DMSO had an inhibitory effect on connexin hemichannels at concentrations resulting from dilution of the vehicle. Anthracene-9-carboxylate (A9C), 4,4′-diisothiocyanato-stilbene-2,2′-disulfonate (DIDS), flufenamic acid (FFA), niflumic acid (NFA), gadolinium chloride, 18β-glycyrrhetinic acid (18β-Gly), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), 1-octanol, 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulfonate (SITS), and tamoxifen ([Z]-1-[*p*-dimethylaminoethoxyphenyl]-1,2-diphenyl-l-butene) were purchased from Sigma (St. Louis, MO). Diphenylamine-2-

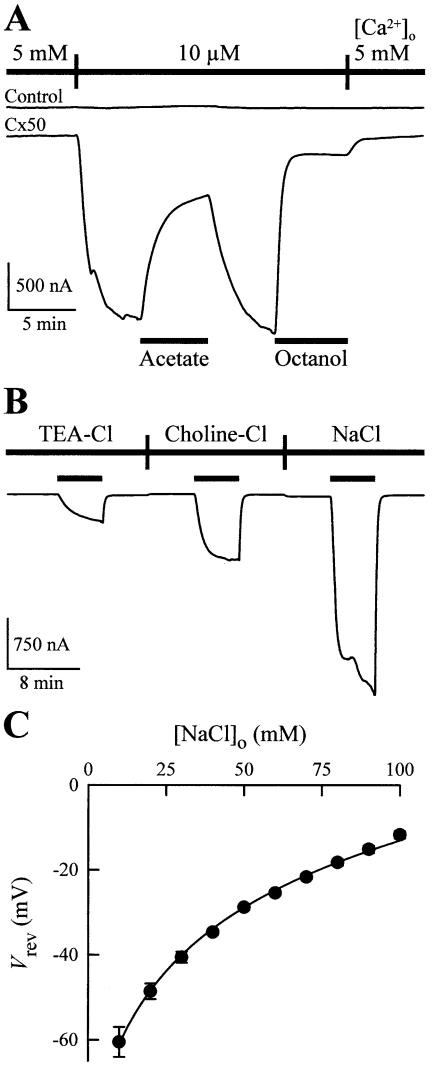


Fig. 1. The Cx50 hemichannel is cation selective. (*A*) Current traces from a control (*upper trace*) and an oocyte expressing Cx50 (*lower trace*) maintained at a holding potential of −50 mV. In the Cx50-expressing oocyte, lowering $[Ca^{2+}]_o$ from 5 mM to 10 μM (*top bar*) evoked an inward current (I_{Cx50}). I_{Cx50} was reversibly inhibited by intracellular acidification (addition of acetate to the bathing medium) or by addition of octanol (1 mM) to the bath (*bottom bars*). No such currents were activated at low $[Ca^{2+}]_o$ in control oocytes. Baseline holding current in 5 mM $[Ca^{2+}]_o$ was 20 nA for the control and 60 nA for the Cx50-expressing oocyte. (*B*) I_{Cx50} is primarily carried by monovalent cations. The major cation (100 mM) of the bathing medium was changed while $[Cl^-]_o$ was maintained at 104 mM (*upper bar*). As $[Ca^{2+}]_o$ was lowered from 5 mM to 10 μM (*lower bars*), the magnitude of I_{Cx50} depended on the major extracellular cation. (*C*) Reversal potential (V_{rev}) of I_{Cx50} as a function of $[NaCl]_o$ in the bathing medium. The smooth curve is the fit of the data to the Goldman-Hodgkin-Katz equation yielding the relative permeability ratios $P_K : P_{Na} : P_{Cl} = 1 : 0.74 : 0.05$ for I_{Cx50} ($n = 3$).

carboxylate (DPC; also known as *N*-phenylanthranilic acid) was purchased from Fluka (Milwaukee, WI). R(+)-[(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-l-oxo-lH-inden-5-yl)-oxy]acetic acid (IAA-94) was purchased from Research Biochemicals International.

Data Analysis

Steady-state whole-cell currents (I_{Cx50} and I_{Cx46}) evoked at a given $[Ca^{2+}]_o$ were obtained as the magnitude of departure from the baseline current at 5 mM $[Ca^{2+}]_o$. In the presence of various blocker concentrations, currents were fitted to Equation 1:

$$\frac{I}{I_0} = \frac{(K_i)^{n'}}{(K_i)^{n'} + [B]^{n'}} \quad (1)$$

where I_0 is the current evoked by lowering $[Ca^{2+}]_o$, from 5 mM to a given concentration in the absence of blocker, I is the current in the presence of a given concentration of blocker (B), K_i is the apparent half-inhibition constant, and n' is a pseudo Hill coefficient. Unless otherwise indicated, results obtained from experiments on individual oocytes are presented, but all experiments were repeated on at least three oocytes from different donor toads. Data fits were performed by using Sigma Plot (SPSS Inc.). Errors are reported as standard error of the estimate obtained from the fit, or as standard error of the mean obtained from data from several oocytes.

Cx50 single-hemichannel data were analyzed by constructing all-points amplitude histograms of recordings from patches containing single channels, and from uninterrupted records of at least five minutes in duration. The mean single-channel current and the open probability (P_o) were determined by fitting the histograms to Gaussian functions. At each pipette voltage (V_p), the mean current was plotted, and the slope of the current versus V_p curve yielded the single-hemichannel conductance (*see* Fig. 4*A* and *B*).

Results

Hemichannel Sensitivity to Octanol and Intracellular pH

Connexin hemichannel function is characterized by sensitivity to extracellular Ca^{2+} concentration, intracellular acidification, and compounds such as octanol, heptanol, and halothane (Fig. 1*A*) (DeVries & Schwartz, 1992; Ebihara & Steiner, 1993; Ebihara, 1996; Li et al., 1996; Quist et al., 2000; Trexler et al., 1999; Zampighi et al., 1999). When oocytes expressing Cx50 are incubated in 5 mM $[Ca^{2+}]_o$, Cx50 hemichannels are closed and the whole-cell conductance is not different from that of control oocytes (Zampighi et al., 1999). As $[Ca^{2+}]_o$ is reduced, hemichannels become activated, resulting in a large inward current (Fig. 1*A*). The current activated at low $[Ca^{2+}]_o$ is mediated by hemichannels because (*i*) this current can be inhibited by octanol (Eskandari & Zampighi, 2000; Zampighi et al., 1999) and other gap junction-uncoupling agents (*see* below), and (*ii*) we can identify Cx50 hemichannels in the plasma membrane of oocytes (Eskandari et al., 1998; Eskandari & Zampighi, 2000; Zampighi et al., 1999), and we have shown previously that the Ca^{2+}-sensitive whole-cell current in Cx50-expressing oocytes is directly proportional to the total number of hemichannels in the oocyte plasma membrane (Zampighi et al., 1999). We refer to this current as I_{Cx50}. Currents that are acti-

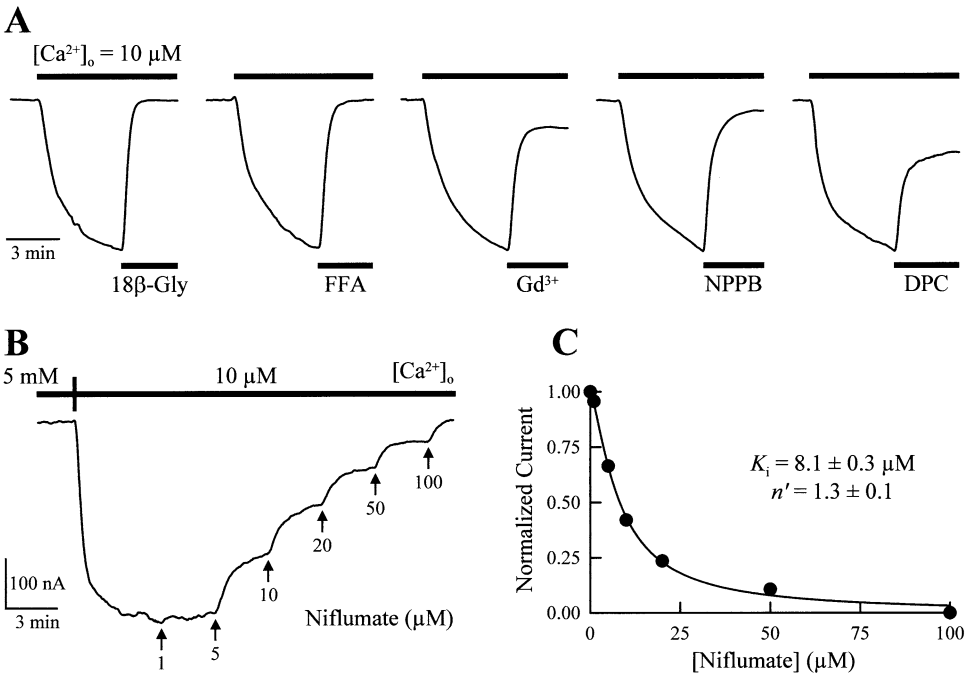


Fig. 2. Blockade of Cx50 hemichannel current I_{Cx50}. (*A*) I_{Cx50} was blocked by a variety of agents. The oocytes were maintained at −50 mV, I_{Cx50} was evoked by lowering $[Ca^{2+}]_o$ from 5 mM to 10 μM (*top bars*), and inhibitors were added to the bath (*bottom bars*). Each drug was applied to a different oocyte from the same batch and, for clarity of comparison, the currents (1200–1500 nA) were normalized. Blocker concentrations were as follows (in μM): 50 18β-Gly, 100 FFA, 10 gadolinium, 100 NPPB, and 100 DPC. Other chloride channel blockers that were tested but did not lead to a noticeable inhibition of I_{Cx50} include: Furosemide, IAA-94, SITS, DIDS, A9C, and tamoxifen (*not shown*; *see* Table 1). (*B*) Concentration-dependent inhibition of I_{Cx50} by niflumic acid, I_{Cx50} was activated at 10 μM $[Ca^{2+}]_o$ (*top bar*) and niflumic acid was added to the bath at increasing concentrations (*arrows*). No desensitization of the inhibitory effect was seen upon prolonged exposure of hemichannels to individual concentrations of the inhibitors (*not shown*). (*C*) At any given concentration of niflumic acid, the current was normalized to the maximum current in the absence of niflumic acid, and the data *(filled circles)* were fitted to Equation 1 (smooth line). The data reported in Table *2* were obtained in this fashion.

vated at low $[Ca^{2+}]_o$ were never observed in our control oocytes (Fig. 1*A*), even at nominal 0 mM Ca^{2+} and Mg^{2+} and 10 mM EGTA in the extracellular bathing medium (*not shown*). This suggests that the endogenous *Xenopus* connexin38 is not expressed at a significant level in our cells.[1]

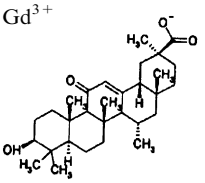
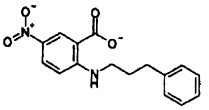
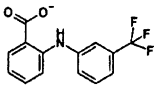
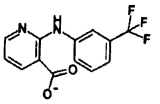
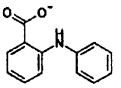
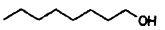
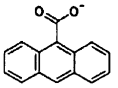
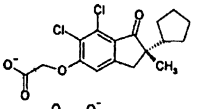
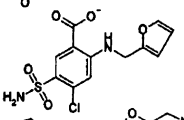
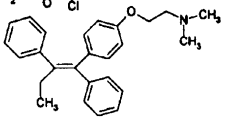
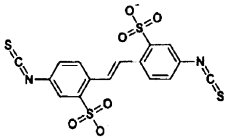
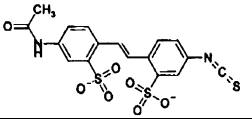
I_{Cx50} is Primarily a Cationic Current

At low $[Ca^{2+}]_o$ (10 μM) and a holding potential of −50 mV, the magnitude of I_{Cx50} was dependent on the major cation in the bathing medium; as the cation was changed from TEA to choline to Na^+, increasingly larger inward currents resulted (Fig. 1*B*). The reversal potentials (V_{rev}) in NaCl, choline-Cl, and TEA-Cl were −12 ± 1 mV (n = 6), −30 ± 2 mV (n = 3), and −38 ± 2 mV (n = 3), respectively (*not shown*). Using the Goldman-Hodgkin-Katz (GHK) equation, $P_{Choline}/P_{Na}$ was ≈0.50, and P_{TEA}/P_{Na} was ≈0.36. The effect of the major extracellular anion was examined by replacing NaCl with Na-gluconate or Na-MES. The resulting V_{rev} values in Na-gluconate and Na-MES were not significantly different from those obtained in NaCl; −15 ± 2 mV (n = 3), and −14 ± 2 mV (n = 3), respectively (*not shown*).

The cation/anion selectivity of the Cx50 hemichannel was further examined by measuring V_{rev} at 10 μM $[Ca^{2+}]_o$ as $[NaCl]_o$ was reduced from 100 to 10 mM while the total osmolarity was maintained by addition of mannitol. V_{rev} plotted as a function of $[NaCl]_o$ could be adequately described by the GHK equation (Fig. 1*C*). Assuming oocyte intracellular activities for K^+, Na^+ and Cl^- to be 120 mM (Dascal, 1989; Stampe et al., 1998), 6 mM (Sciortino & Romero, 1999), and 33 mM (Katayama & Widdicombe, 1991), respectively, the fit of the data to the GHK equation predicted (P_K = 1.0) a P_{Na}/P_K of 0.74 ± 0.01, and a P_{Cl}/P_K of 0.05 ± 0.01 (n = 3). These data suggest that monovalent cations are the major carriers of I_{Cx50}. Divalent cations have no effect on the reversal potential of I_{Cx50} (Zampighi et al., 1999).

[1]Some groups have reported endogenous currents in *Xenopus* oocytes, which are activated at low extracellular $[Ca^{2+}]$ (Arellano, Woodward & Miledi, 1995; Ebihara, 1996; Reifarth et al., 1997; Weber et al., 1995; Zhang, McBride & Hamill, 1998). The discrepancy between the results obtained from oocytes of different groups is not clear (*see* Zampighi et al., 1999).

Table 1. Connexin46 and Connexin50 Hemichannel Inhibition by chloride channel and gap junction channel blockers

Compound	Structure	Inhibition	
		Cx46	Cx50
Gadolinium[a]	Gd^{3+}	Yes	Yes
18β-glycyrrhetinic acid		Yes	Yes
NPPB		Yes	Yes
Flufenamic acid		Yes	Yes
Niflumic acid		No	Yes
Diphenylamine-2-carboxylate		No	Yes
Octanol		No	Yes
Anthracene-9-carboxylate		No	No
IAA-94		No	No
Furosemide		No	No
Tamoxifen		No	No
DIDS		No	No
SITS		No	No

[a] The trivalent form of gadolinium is assumed to be the predominant form. However, it is not known whether this is the form that interacts with the hemichannels.

Pharmacological Inhibition of Connexin50

Table 1 shows a list of the chloride channel and gap junction channel inhibitors that were examined. I_{Cx50} was inhibited by a variety of these agents (Fig. 2*A*). Octanol (1 mM) and 18β-glycyrrhetinic acid (18β-Gly; 100 μM), two agents known to inhibit many connexin gap junction channels (Johnston, Simon & Ramón, 1980; Spray & Burt, 1990; Yamamoto et al., 1998), also inhibited the Cx50 hemichannel (Figs. 1*A* and

Table 2. Kinetics of connexin50 hemichannel inhibition by chloride channel and gap junction channel blockers

Inhibitor	Apparent K_i (μM)	Pseudo Hill Coefficient (n')	Maximal Inhibition (%)
18β-glycyrrhetinic acid	2.4 ± 0.6	1.3 ± 0.2	95 ± 1
Gadolinium	3.3 ± 1.4	0.8 ± 0.1	79 ± 10
Flufenamic acid	3.4 ± 0.6	1.3 ± 0.1	99 ± 1
Niflumic acid	11 ± 1	1.8 ± 0.2	100 ± 2
NPPB	15 ± 4	2.3 ± 0.2	100 ± 8
Diphenylamine-2-carboxylate	26 ± 8	1.4 ± 0.1	99 ± 3
1-Octanol	177 ± 4	2.4 ± 0.1	88 ± 1

Inhibition was carried out at −50 mV and at 10 μM $[Ca^{2+}]_o$. Data are reported as mean ± SE from at least three oocytes.

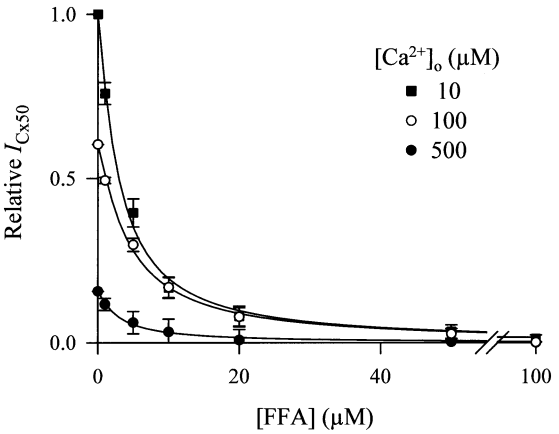


Fig. 3. Kinetics of hemichannel inhibition at different $[Ca^{2+}]_o$. Inhibition of I_{Cx50} by flufenamic acid (FFA) was carried out at 500 μM *(filled circles)*, 100 μM *(open circles)*, and 10 μM (*filled squares*) $[Ca^{2+}]_o$. The holding potential was −50 mV. For comparison, I_{Cx50} values were normalized with respect to the maximum value obtained at 10 μM $[Ca^{2+}]_o$ in the absence of FFA. In the absence of FFA, raw current values ranged from ∼−300 nA at 500 μM $[Ca^{2+}]_o$ to ∼−3000 nA at 10 μM $[Ca^{2+}]_o$. Data points represent the mean ± SE from at least four oocytes. The smooth lines represent the fit of the data to Equation 1. The resulting kinetic parameters were: At 10, 100, and 500 μM $[Ca^{2+}]_o$ the K_i for flufenamic acid was (in μM) 3.4 ± 0.6, 4.1 ± 0.4, and 3.0 ± 0.4, respectively. The corresponding pseudo Hill coefficients were 1.3 ± 0.1, 1.2 ± 0.1, and 1.2 ± 0.1.

2*A*; Table 2). In addition, five chloride-channel blockers (FFA, NFA, NPPB, DPC, and gadolinium) were found to be potent inhibitors of I_{Cx50} (Fig. 2; Table 2). Inhibition was rapid and limited only by the speed of the perfusion system. In all cases, inhibition was reversible, however, in some cases (octanol, FFA, NFA, and NPPB), a long wash-out time (5–20 min.) was required for full recovery of activity (*not shown*). Inhibition was saturable and the apparent half-inhibition constant (K_i) was determined by fitting the data to Equation 1 (Fig. 2*B* and *C*; Table 2). The interaction of some of the blockers with hemichannels appeared to be cooperative as indicated by the pseudo Hill coefficient obtained from the fit; however, there was a large variability (0.8–2.4) for this parameter among the blockers (Table 2).

Kinetics of Connexin50 Hemichannel Inhibition

To test whether binding of the chloride-channel blockers to hemichannels involves the Ca^{2+} binding site, we tested the kinetics of I_{Cx50} blockade by FFA at three different $[Ca^{2+}]_o$ (Fig. 3). In the absence of FFA, the magnitude of I_{Cx50} varied from ∼−300 nA at 500 μM $[Ca^{2+}]_o$ to ∼−3000 nA at 10 μM $[Ca^{2+}]_o$ (the relative current values at 10, 100, and 500 μM $[Ca^{2+}]_o$, were 1 : 0.60 : 0.16) ($n = 4$; Fig. 3). Flufenamic-acid inhibition of I_{Cx50} was complete at all three $[Ca^{2+}]_o$, and neither the apparent K_i, nor the pseudo Hill coefficient for FFA changed significantly as $[Ca^{2+}]_o$ was varied 50-fold from 10–500 μM ($P > 0.5$; *see* legend to Fig. 3). These results suggest that FFA acts at a site different from the Ca^{2+} binding site. Furthermore, Ca^{2+} gating of Cx50 hemichannels does not seem to alter accessibility of the blocker binding site.

Sidedness of Inhibitor Action

To gain insight into the site and mechanism of blocker action, Cx50 single-channel currents were examined in cell-attached and excised inside-out and outside-out patches. Cx50 single-channel conductance (γ_{Cx50}) was determined in cell-attached patches under ionic conditions, which closely resembled those in whole-cell measurements (Fig. 4*A* and *B*). γ_{Cx50} was 31 ± 1 pS and the reversal potential (V_{rev}) was −11 ± 2 mV ($n = 6$ patches from 3 oocytes), similar to the V_{rev} obtained in whole-cell measurements with Na^+ as the main extracellular cation (−12 ± 1 mV; *see* Fig. 1*C*). The single-channel conductance obtained here for Cx50 is significantly smaller than those reported for Cx46 (300 pS; Trexler et al., 1996; Valiunas & Weingart, 2000), Cx50 (350 pS; Valiunas & Weingart, 2000), and Cx56 (180 pS; Ebihara et al, 1999) hemichannels. It is also significantly smaller than the single-channel conductance for the Cx50 cell-to-cell channel (220 pS; Srinivas et at., 1999). Different experimental conditions may be responsible for this discrepancy, and experiments are currently under way to examine this further.

In excised inside-out patches, Cx50 hemichannel activity could be completely abolished by changing

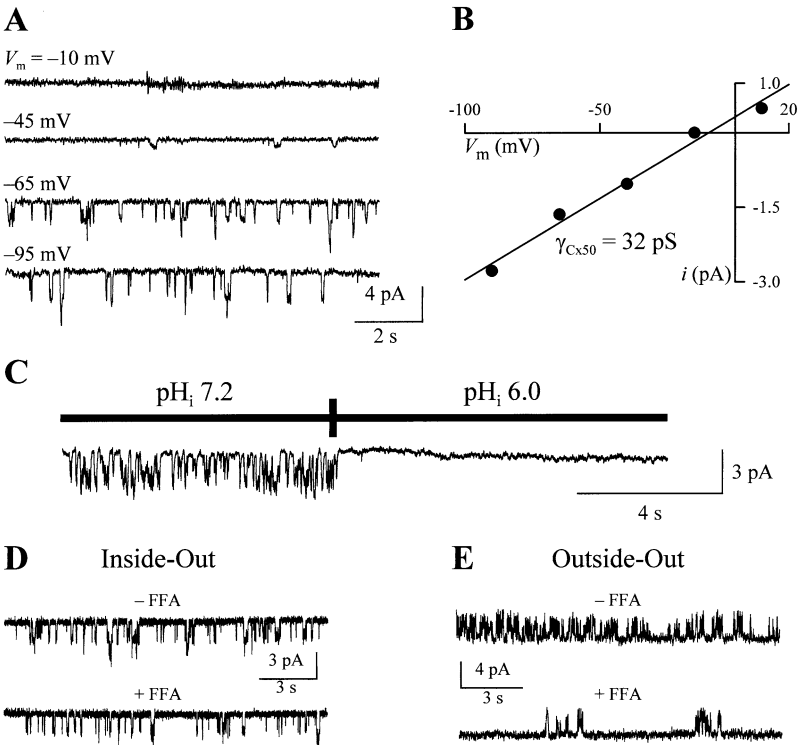


Fig. 4. Connexin50 single-hemichannel currents. (*A*) Single-channel recording in the cell-attached configuration. Records were obtained at different pipette potentials (*V*). In this oocyte, immediately before the cell-attached patch was obtained, the membrane potential (V_{cell}) was –40 mV. The potential across the patch membrane was determined by using $V_m = V_{cell} - V_p$. (*B*) Current-voltage relationship for the Cx50 single-channel currents from the same cell-attached patch as that in panel *A*. γ_{Cx50} was 31 ± 1 pS ($n = 6$ patches). (*C*) In excised inside-out patches, exposure of the cytoplasmic surface of Cx50 hemichannels to low pH (6.0) completely, but reversibly (*not shown*), abolished single-channel activity ($V_p = +90$ mV). Similar observations were made in five patches. (*D*) In excised inside-out patches, Cx50 hemichannel activity was not altered significantly by application of flufenamic acid (100 μM) to the cytoplasmic surface of Cx50 ($V_p = +50$ mV). Similar observations were made in eight patches. (*E*) In excised outside-out patches, exposure of the Cx50 external surface to flufenamic acid (100 μM) led to a significant inhibition of hemichannel activity (single-channel open probability decreased from ≈0.7 to ≈0.1) ($V_p = -80$ mV). Similar observations were made in four patches.

the pH of the bathing solution (cytoplasmic surface of Cx50) from 7.2 to pH 6.0 (Fig. 4*C*), thus confirming the sidedness of the patch and sensitivity of the channels to acidification of the internal compartment (Trexler et al., 1999). In inside-out patches, addition of FFA (100 μM, Fig. 4*D*) or octanol (1 mM, *not shown*) to the bath (cytoplasmic surface of Cx50 hemichannels) had no effect on either the single-channel open probability or the unitary currents. However, in outside-out patches, addition of FFA (100 μM) to the bath (extracellular surface of Cx50 hemichannels), significantly reduced single-channel open probability (P_o decreased from ≈0.7 to ≈0.1) without altering the unitary currents (Fig. 4*E*). Octanol (1 mM) exhibited similar effects (*not shown*). The channels described above in Cx50-expressing oocytes are not seen in our control cells (*not shown*). In addition, channels whose activity was abolished by low cytoplasmic pH were not observed in over 30 patches of the plasma membrane of control oocytes.

Pharmacological Inhibition of Connexin46

To test the generality of hemichannel inhibition by chloride channel blockers, we examined the effect of the above blockers on I_{Cx46}, another connexin isoform expressed in the mammalian lens (Fig. 5). I_{Cx46} was activated by lowering the $[Ca^{2+}]_o$ from 5 mM to 10 μM, and the blockers were added to the bath as described above. With the exception of octanol (1 mM), all agents were tested at 250 μM. The extent of I_{Cx46} inhibition was compared to that of I_{Cx50} at the same blocker concentration (Fig. 5). Flufenamic acid, 18β-glycyrrhetinic acid, gadolinium, and NPPB inhibited I_{Cx46} (Fig. 5). The extent of inhibition was similar to that for I_{Cx50}. Octanol, niflumic acid, and DPC did not inhibit I_{Cx46}.

Other Pharmacological Agents

Several other chloride-channel blockers were found not to lead to a noticeable inhibition of either I_{Cx46} or I_{Cx50} (at 100 μM); these were A9C, furosemide, IAA-94, DIDS, SITS, and tamoxifen (Table 1). In addition, compounds known to block membrane proteins involved in Na^+ transport such as amiloride, phlorizin, and phosphonoformic acid did not inhibit I_{Cx50} or I_{Cx46} (all tested at 100 μM; *not shown*).

Discussion

Direct cell-to-cell communication is made possible by gap junction channels composed of two hemichannels in the plasma membrane of adjacent cells. The formation of these cell-to-cell channels requires the presence of hemichannels in the plasma membrane of adjacent cells, and it is possible that there is a pool of functional hemichannels in the plasma membrane before their entry into gap junction plaques (Laird, 1996; Zampighi et al., 1999). That hemichannels can behave as large-conductance ion channels, and are permeable to large molecules of up to a 1 kDa (DeVries & Schwartz, 1992; Li et al., 1996; Paul et al.,

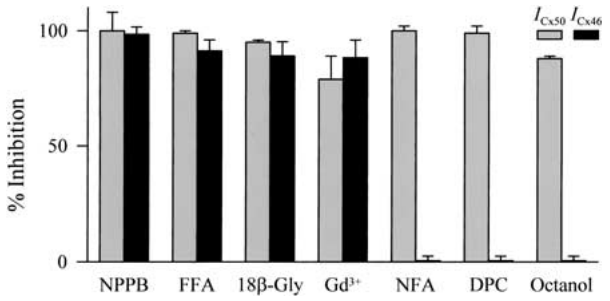


Fig 5. Comparison of Cx46 and Cx50 inhibition. Macroscopic I_{Cx50} or I_{Cx46} was activated at 10 μM $[Ca^{2+}]_o$ and the inhibitors (1 mM for octanol; 250 μM for others) were added to the oocyte bath. For each agent, the extent of I_{Cx50} and I_{Cx46} inhibition is plotted. The holding potential was −50 mV. Grey bars represent I_{Cx50} inhibition and black bars represent I_{Cx46} inhibition. Notice that NFA, DPC, and octanol do not inhibit I_{Cx46}. Data are reported as mean ± se from at least three oocytes.

1991; Pfahnl & Dahl, 1998; Trexler et al., 1996) suggests that even a small number in the plasma membrane may be sufficient to make a significant contribution to the ionic conductance and permeability of the cell plasma membrane. The importance of gap junction channels in growth and development is well-established (Simon & Goodenough, 1998), but the contribution of hemichannels to the normal physiology of the cell is still controversial. Availability of pharmacological agents that inhibit hemichannel activity with high affinity will enhance our ability to examine the potential role of these molecules in cell function.

Here we report that a number of chloride-channel blockers inhibit connexin46 and connexin50 hemichannels with high affinity. Since these are anion channel blockers, we examined the selectivity of the currents under study. Selectivity experiments for I_{Cx50} suggested that >95% of the current mediated by Cx50 hemichannels is carried by monovalent cations. This is consistent with the selectivity known for Cx46 (Ebihara & Steiner, 1993; Trexler et al., 1996) and Cx56 (Ebihara et al., 1999) hemichannels, and that for gap junction channels (*see* Veenstra, 1996). Importantly, 100% of I_{Cx46} and I_{Cx50} is blocked by chloride-channel blockers such as FFA and NPPB. In addition, 18β-glycyrrhetinic acid, an inhibitor of gap junction channels (Yamamoto et al., 1998), as well as hemichannels (Quist et al., 2000) also inhibited this current. Thus, the chloride channel blockers used here are potent inhibitors of Cx46 and Cx50 hemichannels.

We used single-channel recordings from excised patches to examine the sidedness of inhibitor action. The channels examined in membrane patches represent Cx50 hemichannels because: (*i*) they were sensitive to low cytoplasmic pH, and this pH sensitivity is similar to that of Cx46 hemichannels (Trexler et al., 1999); (*ii*) the channels were sensitive to FFA and octanol only when applied to the extracellular surface of hemichannels, similar to the sensitivity of the whole-cell I_{Cx50} to these agents; (*iii*) under similar ionic conditions, the reversal potential of the unitary currents in cell-attached patches was similar to that for the Cx50 whole-cell currents (≈−10 mV); and (*iv*) channels whose activity is abolished by low pH have not been observed in over 30 patches of the plasma membrane of control oocytes. Altogether, the data suggest that Cx50 hemichannels are sensitive to cytoplasmic pH, however, the site of chloride channel blocker action is accessible only from the extracellular surface.

While all of the inhibitors used are hydrophobic molecules, three observations suggest that their effects on hemichannels do not result from non-specific alterations of the membrane domain immediately surrounding the hemichannels: (*i*) Although octanol, NFA, and DPC inhibited the Cx50 hemichannels, when used at the same concentration they did not inhibit Cx46 hemichannels; (*ii*) octanol and FFA inhibited the Cx50 hemichannel only when applied to the extracellular face of the hemichannel, however, they were without effect when applied to the cytoplasmic surface; and (*iii*) neither octanol, 18β-glycyrrhetinic acid, nor any of the chloride-channel blockers used here altered the activity of several ion-coupled cotransporters studied by this group, such as the intestinal Na^+/glucose cotransporter (*not shown*). Therefore, the data are suggestive of a specific and high-affinity interaction of the inhibitors with Cx46 and Cx50 hemichannels. Inhibition is insensitive to the extracellular $[Ca^{2+}]_o$, suggesting that the anionic inhibitors act at a site different from the Ca^{2+} binding site. However, the site of gadolinium interaction with the hemichannels may be different from that of the other inhibitors examined.

It is interesting that inhibition of gap junction hemichannels by some chloride-channel blockers has been observed in cells isolated from different tissues. Zhang et al. found that the endogenous hemichannel of *Xenopus laevis* oocytes (Cx38) is blocked by FFA and NFA (1998).[2] As described above, not all groups observe these endogenous hemichannels in control or water-injected oocytes (*see* Fig. 1*A* and Zampighi et al., 1999), and the difference may be due to different oocyte isolation and maintenance procedures. In addition, cells isolated from the amphibian urinary bladder express functional hemichannels that are blocked by gadolinium (Vanoye, Vergara & Reuss, 1999). Therefore, the observations reported here, together with those reported in the literature, suggest that inhibition of connexin hemichannels by some chloride channel blockers may be common among

[2]In the study of Zhang et al. (1998), Cx38 hemichannels were also reported to be sensitive to amiloride. Cx50 and Cx46 hemichannels are not sensitive to amiloride (see Results).

the members of this family of proteins. Future experiments should examine the ability of these blockers to inhibit other connexin hemichannel isoforms.

It is well established that inhibitors of anion channels are not specific and frequently act on more than one class of channels (Weber, 1999); e.g., CFTR (McCarty, 2000), members of the ClC family (e.g., Schmieder et al., 1998), Ca^{2+}-activated chloride channels (Frings, Reuter & Kleene, 2000), volume-regulated anion channels (Nilius et al., 1997), non-selective cation channels (Koch & Korbmacher, 2000), and even some Ca^{2+} channels (Doughty, Miller & Langton, 1998; Oba, 1997). Although the high sensitivity of connexin hemichannels to these blockers is similar to those observed for other membrane proteins, the blocker selectivity differs significantly from that of other membrane proteins. Thus, our study suggests that connexin hemichannels must now be added to the list of molecules affected by the wide and diverging class of compounds categorized as "chloride channel blockers". These blockers may help explore the function of hemichannels in cells that naturally overexpress them, e.g., hepatocytes, or neurons of the supraoptic nucleus that express hemichannels but exhibit no functional cell-to-cell coupling or gap junctions (Micevych & Abelson, 1991).

In summary, we have shown that flufenamic acid, niflumic acid, NPPB, and diphenyl-2-carboxylate are potent inhibitors of Cx50 hemichannels. Flufenamic acid and NPPB also inhibit Cx46 hemichannels. In excised-patch experiments, drug application to the extracellular (but not cytoplasmic) surface of hemichannels led to channel inhibition. Therefore, the site of drug action is extracellular. Further, the site of drug action is most likely different from the Ca^{2+} binding site, as the kinetics of blockade were independent of $[Ca^{2+}]_o$. The high degree of homology among the members of the connexin family points to the possibility that this inhibitory effect may be common to other members of the family as well. At present, it is not clear whether the chloride-channel blockers that inhibit Cx46 and Cx50 hemichannels also inhibit cell-to-cell channels formed by these connexins. Additional experiments are needed to explore this possibility.

We thank Elsa Gallardo for technical assistance, Dr. David L. Paul for providing the connexin clones, and Dr. Daniel F. Stiffler for the use of his calcium-selective electrode. This study was supported by U.S. National Institutes of Health Grants DK-19567 and DK-44602 (to E.M.W.), and EY-04110 (to G.A.Z.). This work was completed while S.E. was supported by a postdoctoral fellowship from a U.S. National Institutes of Health Cellular Neurobiology Training Grant (T32 NS-07101-22).

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