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Closure of Gap Junction Channels by Arylaminobenzoates

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ABSTRACT

We determined the effect of flufenamic acid (FFA) and related derivatives on gap junction channel currents, applying the dual whole-cell patch-clamp technique to pairs of N2A neuroblastoma cells transfected with various connexins. FFA reduced gap junction channel currents in a reversible and concentration-dependent manner. Half-maximal concentrations for FFA-induced reduction of junctional conductance in cell pairs coupled by different connexins were similar (20 to 60 μM), indicating that FFA does not greatly discriminate between connexin subtypes. Hill coefficients for blockade were approximately 3, indicating a high degree of cooperativity. Analogs of FFA also reduced junctional conductance with similar potencies, whereas other unrelated chloride channel blockers had no effect. Inhibition of gap junction channels by FFA ($pK_a \sim 3.8$) was increased at low external pH, suggesting that the uncharged form of the drug is important for blockade. The effect of FFA did not seem to be mediated by direct binding of the drug to the pore of the gap junction channel. Internal application of high concentrations of FFA by addition to patch pipettes did not cause inhibition of channel currents. The magnitude of inhibition was neither voltage-dependent nor influenced by the nature of permeant ion. Single-channel recordings indicated that FFA reduced the channel-open probability without modifying the current amplitude and induced slow transitions between open and closed states. We propose that FFA inhibits gap junctions by inducing a conformational change in the protein upon binding to a site that is presumably located within the membrane.

Gap junction channels provide pathways of intercellular communication, allowing the passage of ions and small molecules up to 1 kilodalton in mass or 10 to 14 nm in diameter. In mammalian and other vertebrate cells, gap junction channels are composed of a family of protein molecules known as connexins (Willecke et al., 2002). Gap junction channels formed by various connexins can be closed by a number of factors including pH and phosphorylation by tyrosine kinases and by transjunctional voltage (for review, see Harris, 2001). In addition, a large number of drugs are known to block gap junctional communication (Spray et al., 2002). These include long-chain alcohols (e.g., heptanol and octanol), volatile anesthetics (halothane and ethrane), glycyrrhetinic acid derivatives, oleamide, aminosulfonates (e.g., taurine), tetraalkylammonium ions, and the antimalarial drug quinine (Johnston et al., 1980; Davidson et al., 1986; Burt and Spray, 1989; Bevans and Harris, 1999; Musa et al., 2001; Srinivas et al., 2001). Recently, arylaminobenzoates were shown to reduce the spread of Lucifer yellow through gap junctions composed of Cx43 (Harks et al., 2001) and to inhibit putative hemichannel currents in *Xenopus laevis* oocytes (Zhang et al., 1998; Eskandari et al., 2002).

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The effects of arylaminobenzoates on several ion channels have been well documented. Arylaminobenzoates, diphenylcarboxylate and its derivates, are highly lipophilic molecules that were first systematically studied as blockers of chloride currents (DeStefano et al., 1985). Subsequently, diphenylcarboxylate and its derivatives such as flufenamic acid and niflumic acid were shown to inhibit currents through a wide variety of channel types, including nonselective cation channels, voltage-dependent potassium channels, L-type calcium channels, and chloride channels such as the cystic fibrosis transmembrane conductance regulator (Gögelein et al., 1990; White and Aylwin, 1990; Alton et al., 1991; Richard and Dawson, 1993; Ackerman et al., 1994; Wang et al., 1997; Schultz et al., 1999). In addition, these drugs potentiate the large-conductance Ca^{2+}-activated K^+ channel current (Farrugia et al., 1993; Ottolia and Toro, 1994; Greenwood and Large, 1995). Effects of fenamates on ligand-gated ion channels have also been reported (Woodward et al., 1994).

The mechanism of blockade of these drugs varies for different channel subtypes. Arylaminobenzoates inhibit chloride channels by binding in the permeation pathway in a manner consistent with open channel block (McCarty et al., 1993; McDonough et al., 1994). The mechanism of inhibition of some other channels by arylaminobenzoates is less clear, but it might involve the alteration of channel gating (Lee and

ABBREVIATIONS: FFA, flufenamic acid; MFA, meclofenamic acid; NFA, niflumic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; CFA, clofibric acid; APB, 2-amino-4-phenylbutyric acid; V_j, voltage gradient; g_j, conductance; I_j, junctional current; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; pH_e, external pH.

Wang, 1999). In this study, we characterized the effect of arylaminobenzoates on gap junction channels formed by various connexins at the macroscopic and the single channel level by directly measuring channel currents using the dual whole-cell patch-clamp technique. Our results indicate that block by flufenamic acid is reversible, with both inhibitory potency depending on the concentration and pH of the external solutions. In addition, we determined that these drugs do not cause open-channel block of gap junction channels, but they seem to close junctional channels by affecting gating.

Materials and Methods

Materials. The drugs used in this study were *N*-(3-[trifluoromethyl]phenyl)anthranilic acid (flufenamic acid; FFA), 2-[(2,6-dichloro-3-methylphenyl)amino]benzoic acid (meclofenamic acid; MFA), 2-[3-(trifluoromethyl)anilino]nicotinic acid (niflumic acid; NFA), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), 2-(*p*-chlorophenoxy)-2-methylpropionic acid (clofibric acid; CFA), 2-amino-4-phenylbutyric acid (APB), and 2-aminobenzoic acid (Fig. 3). All drugs used in this study were obtained from Sigma Chemical Co. (St. Louis, MO) and were dissolved in dimethyl sulfoxide as 100 mM stock solutions. Stock solutions were prepared daily.

DNA Construction and Transfection. All electrophysiological experiments described here were performed on N2A cells that were either stably transfected with connexins or transiently cotransfected with connexin and enhanced green fluorescent protein cDNAs in separate vectors as described previously (Srinivas et al., 2001). The connexins used in the study were rCx26, hCx32, rCx43, rCx46, and mCx50 (where r, h, and m refer to rat, human, and mouse cDNAs, respectively). Transiently transfected cells were dissociated at 8 to 12 h after transfection, plated at low density on 1-cm round glass coverslips, and used within 48 h thereafter.

Electrophysiology. Junctional conductance was measured between cell pairs using the dual whole-cell voltage-clamp technique with Axopatch 1C or 1D patch-clamp amplifiers (Axon Instruments, Union City, CA) at room temperature. The solution bathing the cells contained 140 mM NaCl, 5 mM KCl, 2 mM CsCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 5 mM dextrose, 2 mM pyruvate, and 1 mM $BaCl_2$, pH 7.4. Patch electrodes had resistances of 3 to 5 MΩ when filled with internal solution containing 130 mM CsCl, 10 mM EGTA, 0.5 mM $CaCl_2$, 3 mM MgATP, 2 mM Na_2ATP, and 10 mM HEPES, pH 7.2. pH values of external and internal solutions were measured and adjusted before each experiment. Macroscopic and single-channel recordings were filtered at 0.2 to 0.5 kHz and sampled at 1 to 2 kHz. Data were acquired using pClamp software (Axon Instruments) and plotted using Origin 6.0 software (OriginLab Corp, Northampton, MA).

Each cell of a pair was initially held at a common holding potential of 0 mV. To evaluate junctional coupling, 200-ms hyperpolarizing pulses from the holding potential of 0 to −10 mV were applied to one cell to establish a transjunctional voltage gradient (V_j), and junctional current was measured in the second cell (held at 0 mV). To determine the voltage dependence of drug action, 7- to 10-s hyperpolarizing or depolarizing pulses were applied every 20 s to test potentials between −30 and 30 mV. Single-channel currents were investigated in weakly coupled cell pairs (1 to 2 channels) without the use of uncoupling agents by applying −20 mV pulses to one cell of a pair. Gating events were recognized as simultaneously occurring events of equal amplitude and opposite polarity in current traces for both cells in the pair. The channel-open probability was determined from recordings of cell pairs that contained a single active channel and was defined as the absolute fraction of time that was spent in the open state.

Drugs were applied with a gravity-fed perfusion system. Solution exchanges were complete within 30 s. Concentration-response curves for drug-induced uncoupling were determined by exposure of each cell pair to two to three concentrations of each drug. In all experiments, reversibility was assessed by washout of the drug. The magnitude of inhibition caused by drugs is expressed as the fraction of the conductance (g_j) in the absence and presence of the drug, "g_j, % control". Concentrations of drugs ([D]) that caused a half-maximal inhibition (EC_{50}), an index of the potency of a drug, and the Hill coefficients (n_H) of concentration-response relationships were estimated by fitting the data to the equation: g_j, % control = $1/[1 + ([D]/EC_{50})^{n_H}]$. Data are presented as means ± S.E.M.

Results

The effect of FFA on gap junctional conductance of channels formed by Cx50 is illustrated in Fig. 1. Figure 1A shows the effect of external application of 65 and 100 μM FFA on Cx50 gap junction channel currents (I_j). At the lower concentration, FFA decreased I_j by ~60% within 3 min, whereas at the higher concentration, the drug caused near-maximal decrease in Ij within less than 1 min. For both concentrations, washout of drug resulted in complete recovery of the currents within 2 min, i.e., the block was quickly reversible (Fig. 1A, top trace). Figure 1B shows the concentration dependence of FFA-induced inhibition of Cx50 junctional currents determined by the exposure of 4 to 10 cell pairs to each drug concentration. Nonlinear least-squares fit of the individual data points to the Hill equation (see *Materials and Methods*) yielded an EC_{50} value of 47 μM for the FFA-induced inhibition of Cx50 gap junction channels. This EC_{50} value for the effect of FFA is comparable with those found for block of other channels as well as for putative hemichannels formed by Cx50 (Schultz et al., 1999; Eskandari et al., 2002). The Hill coefficient for inhibition of gap junctions was ~3, suggesting that binding is not caused by a simple 1:1 interaction between FFA and individual gap junction channels. A Hill coefficient greater than unity was also observed for the inhibition of putative Cx50 hemichannels expressed in oocytes (Eskandari et al., 2002).

We recently demonstrated that the potency of blockade of gap junctions by quinine was much greater for certain connexin subtypes than for others (Srinivas et al., 2001). To determine whether FFA selectively inhibited gap junctions formed by specific connexin subtypes, we tested the effects of the drug on gap junction channels formed by several other connexins, including Cx26, Cx32, Cx40, Cx43, and Cx46. Figure 2A shows the effect of FFA (30 and 100 μM) on Cx26 gap junction channel currents. FFA at concentrations of 30 and 100 μM reversibly decreased Cx26 junctional currents by ~46 and 100%, respectively, with onset and reversal even more rapid than those seen with Cx50. Although these data suggest that the drug is marginally more potent at inhibiting these channels compared with its effect on Cx50 channels, the effect of FFA on other connexins, however, was similar to that observed for Cx50 channels (Fig. 2B). At the high concentration of 100 μM, FFA blocked Cx32, Cx43, Cx46, and Cx40 currents by 85 to 95% (means ± S.E.M. are 98 ± 2%, $n = 6$, for Cx26; 89 ± 3%, $n = 5$, for Cx32; 88 ± 4%, $n = 3$, for Cx40; 96 ± 2%, $n = 7$, for Cx43; and 86 ± 4%, $n = 4$, for Cx46). These results demonstrate that inhibition of gap junction channels by FFA is only marginally selective among these several different connexins.

We also determined the effects of several other commonly

used chloride-channel blockers on gap junction channels. These included derivatives of FFA such as NPPB, MFA, and NFA as well as unrelated molecules such as clofibric acid and the stilbene derivative DIDS. In addition, the importance of the benzoate moiety and the bulky hydrophobic chain were also investigated by determining the effect of 2-amino-4-phenylbutyric acid and 2-aminobenzoic acid, respectively (compare structures with NPPB in Fig. 3A). The effects of the various derivatives of flufenamic acid on Cx50 junctional currents are summarized in Fig. 3. Application of 30 and 100 μM NPPB caused a decline of 15 ± 3% (n = 4) and 96 ± 4% (n = 4) of the junctional current, respectively, suggesting that the potency of NPPB at causing blockade of gap junctions is not greatly different from that of FFA. Similar results were obtained with MFA (means ± S.E.M.: 7 ± 4, n = 4, at 30 μM and 90 ± 2, n = 4, at 100 μM) (Fig. 3). In the case of NFA, higher concentrations were required to reduce Cx50 junctional currents, with 300 μM and 1 mM inhibiting channels currents by 54 ± 5% (n = 4) and 97 ± 4% (n = 4), respectively. In contrast, the application of high concentration of the unrelated derivative DIDS led to little closure of Cx50 channels. The magnitude of inhibition caused by 100 and 300 μM was 2 ± 1 and 4 ± 2%, respectively. Similarly, clofibric acid caused little reduction of Cx50 junctional currents (n = 4). More importantly, *o*-aminobenzoic acid and 2-amino-4-phenylbutyric acid caused little effect on Cx50 channels, strongly indicating that both the phenyl side chain and the benzoate ring are important for the inhibition of gap junction channel currents.

To determine how externally applied FFA accesses its binding site, strategies that have been used to investigate the site of block by this drug in chloride channels were applied (Walsh and Wang, 1998; Zhang et al., 2000). Most arylaminobenzoates, including FFA, are weak acids with pK_a values in range of 3 to 5 and therefore can exist in both the membrane-permeable uncharged form and the anionic-charged form, depending on the pH of the external solution. At pH 7.4, the vast majority of the drug is charged, but a small fraction is uncharged and is free to cross cell membranes. To determine whether the uncharged form of the drug is important for inhibition, the effect of FFA was investigated at different external pH values (Fig. 4). Fig.4A shows the effect of 60 to 90 s of exposure to FFA (40 μM) on Cx50 junctional currents at pH_e values of 6.7, 7.4, and 8.0 (where uncharged FFA proportions are calculated as 0.13, 0.03, and 0.007% by

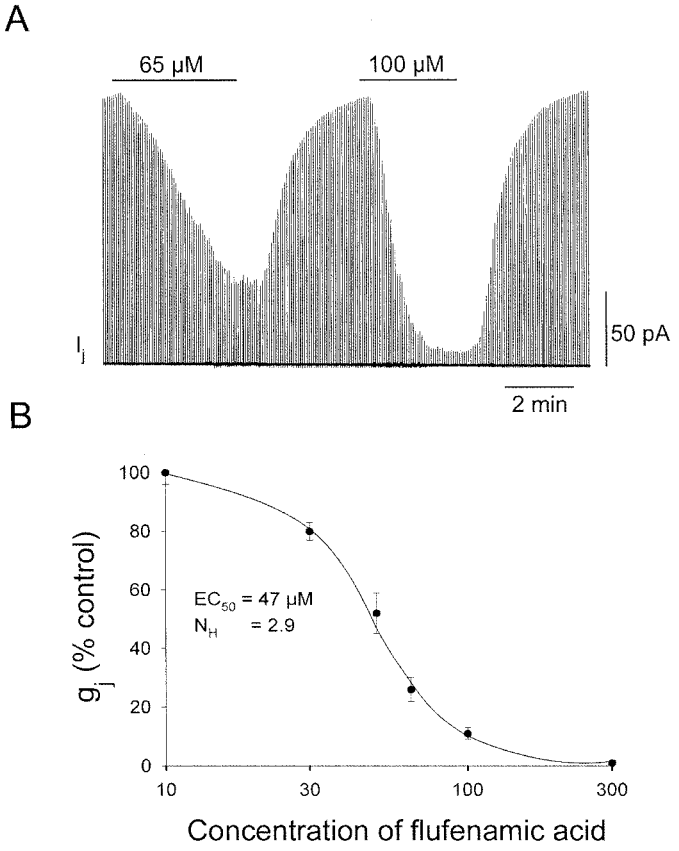

Fig. 1. Flufenamic acid reduces junctional conductance in a reversible, concentration-dependent manner. A, effect of indicated concentrations of FFA on Cx50 junctional currents in response to 200-ms pulses to −10 mV applied to one cell of a pair every 2 s from a holding potential of 0 mV. Application of 65 and 100 μM FFA decreased the Ij of Cx50 by ~60 and 92%, respectively. Washout of the drug resulted in complete recovery of the currents. B, concentration dependence of the effect of FFA on Cx50 gap junction channels. Each point represents the mean ± S.E.M. of g_j (percentage of the initial conductance) values obtained from 4 to 18 cell pairs. The solid line is a fit of the data points to the Hill equation (see *Materials and Methods*). The EC_{50} and Hill slope values (n_H) are indicated.

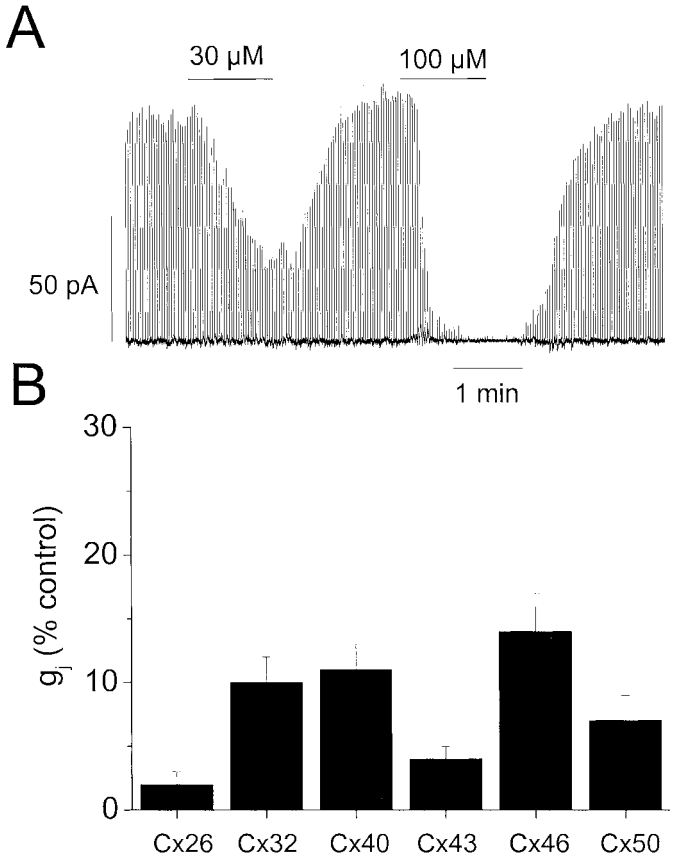

Fig. 2. Flufenamic acid inhibits gap junctions formed by various connexins with marginal differences in potency. A, effect of indicated concentrations of FFA on Cx26 junctional currents in response to 200-ms pulses to −10 mV applied to one cell of a pair every 2 s from a holding potential of 0 mV. Application of 30 and 100 μM FFA decreased the I_j of Cx50 by ~60 and 100%, respectively. Washout of the drug resulted in complete recovery of the currents B, bar graph illustrating that FFA (100 μM) caused near maximal inhibition of Cx26, Cx32, Cx40, Cx43, and Cx46 gap junction channels (percentage of inhibition ranged from 98% for Cx26 to 86% for Cx46). Each bar represents the mean ± S.E.M. of four to six cell pairs.

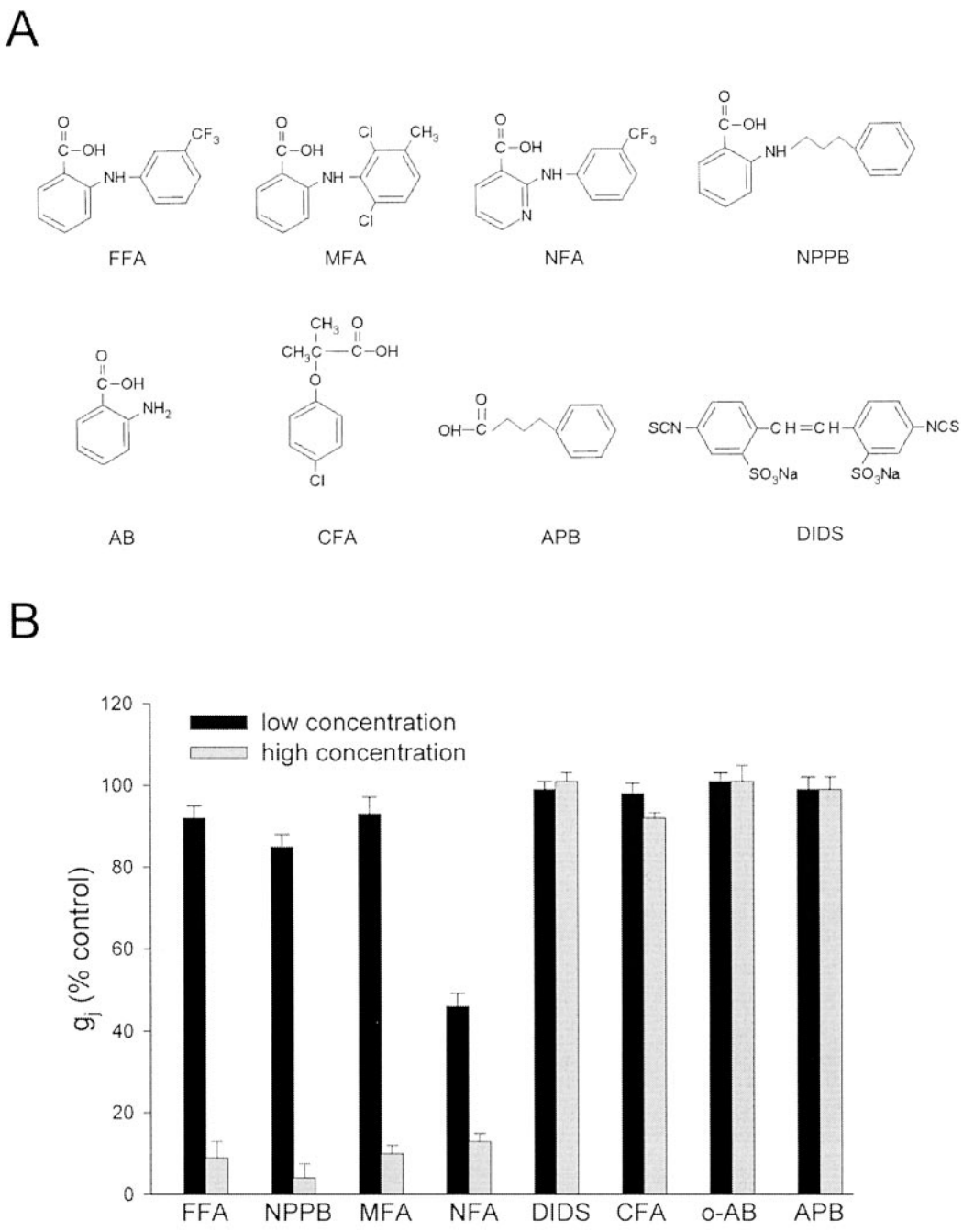

Fig. 3. Effect of various chloride-channel blockers on Cx50 gap junctional channels. A, illustration of structures of FFA, MFA, NFA, NPPB, 2-aminobenzoic acid (AB), CFA, APB, and DIDS tested in this study. B, bar graph illustrating the effect of a low and high concentration of NPPB, MFA, NFA, DIDS, CFA, *o*-aminobenzoic acid (*o*-AB), and APB compared with FFA. Means ± S.E.M. of g_j from four different cell pairs for each drug are shown. The concentrations of NPPB, MFA, and FFA applied were 30 (low) and 100 μM (high). The effect of NFA, DIDS, *o*-AB, and APB were studied at concentrations of 100 and 300 μM. The magnitudes of inhibition obtained by NPPB and MFA were similar to those of FFA. DIDS, *o*-AB, and APB did not cause significant reduction of Cx50 junctional currents.

using the Henderson-Hasselbach equation). Junctional currents were measured initially and during FFA washout in external solution at pH 7.4. Varying pH_e values for 60 s in the absence of FFA caused no significant change in the junctional conductance (Fig. 4B, ■). As illustrated in Fig. 4A, the magnitude of reduction of Cx50 junctional currents by external FFA (40 μM) was maximal at low external pH and minimal at high external pH. The magnitude of block induced by 40 μM FFA was 4 ± 3% at pH 8.0 (n = 7), 41 ± 8% at pH 7.4 (n = 4), and 95 ± 3% at pH 6.7 (n = 4) (Fig. 4B). These results demonstrate that arylaminobenzoate inhibition of the gap junction channel is enhanced by lowering the pH of the external solution.

In the case of chloride channels, arylaminobenzoates access the binding site in the uncharged form but bind to their receptor in the charged form after protonation in the cytoplasmic milieu (McCarty et al., 1993; McDonough et al., 1994; Walsh and Wang, 1998). Evidence that the charged form binds to the receptor and causes open-channel blockade was provided in those studies by 1) potent blockade by drug applied via patch pipettes, 2) voltage-dependence of block, 3) reduction of magnitude of blockade by substitution of chloride by a larger permeant ion, and 4) rapid flicker in the single-channel records in the presence of the drug. We applied each of these criteria to determine whether FFA caused open-channel block of gap junction channels.

Initially, FFA was added at a high concentration to patch pipettes and the magnitude of inhibition was measured (Fig. 5). Application of high concentrations of FFA (1 mM) caused little or no reduction of Cx50 channel currents even after 10

min of dialysis of drug into cells (mean ± S.E.M. of inhibition is 2 ± 0.1%, $n = 5$). However, extracellular application of a 10-fold lower concentration of the drug to the same cell pair caused a marked decrease in the current (mean ± S.E.M.: 88 ± 3%, $n = 5$) that was fully reversible upon removal of external FFA. Application of the drug at a higher internal pH of 8.0, which would favor higher concentrations of the charged form of the drug in the patch pipette, also caused little reduction of the junctional current (mean decrease after 10 min ~5 ± 3%, $n = 7$), whereas external application of 100 μM FFA again decreased the current significantly by 82 ± 5%, suggesting that the binding site for FFA (at least for the charged form) is not intracellular. Not surprisingly, the inhibition of gap junction channels by FFA was not voltage-dependent. Voltage dependence of block was assessed by measuring the magnitude of steady-state block induced by external FFA at voltages between −30 and +30 mV, in which the inherent closure of Cx50 channels induced by transjunctional voltage is minimal (Srinivas et al., 1999). The normalized values of junctional conductance (G_j) in the absence (■) and presence (●) of externally applied 50 μM FFA are plotted at various transjunctional voltage gradients (Fig. 6C). The steady-state inhibition induced by FFA (50 μM) was similar at all voltages. The magnitude of inhibition at V_j of 10 and 30 mV were 44 ± 3% ($n = 3$) and 42 ± 4% ($n = 3$), respectively.

Permeant ions are known to modulate the potency of open-channel blockers by competing with the drug at its binding site. Block of chloride channels was shown to be significantly reduced when chloride concentrations in the pore were lowered and when larger permeant ions were used as charge carriers (McDonough et al., 1994). To determine whether permeant ions affected the reduction of gap junction channel currents by FFA, chloride was replaced with the larger glutamate ion. As illustrated in Fig. 6, the magnitude of inhibition of Cx50 channels by FFA with glutamate as the primary anion was similar to that observed in the previous experiments with Cl^- internal solution. The magnitudes of inhibition caused by 30 and 100 μM were 12 ± 3% ($n = 3$) and 93 ± 6% ($n = 4$), respectively, values that are close to those observed when chloride was used as the primary anion, indicating that block by FFA is not influenced by the nature of the permeant ion.

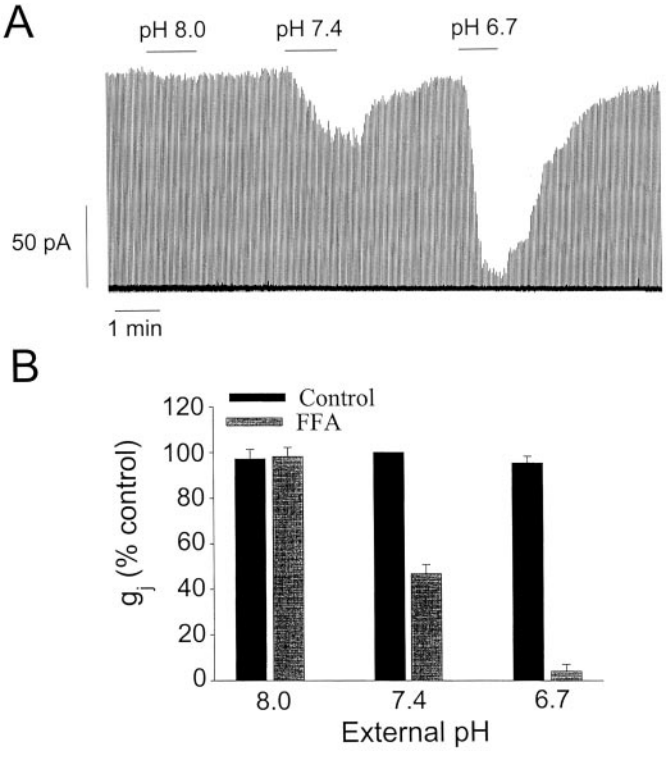

Fig. 4. External pH affects the potency of flufenamic acid. A, the magnitude of inhibition of Cx50 gap junction channels by 40 μM flufenamic acid ($pK_a \sim 4.6$) at pH_e values of 8.0, 7.4, and 6.7 is illustrated. The magnitude of inhibition by flufenamic acid is markedly increased at low external pH. The pH of the pipette solution throughout the recording was 7.2. B, summary of experiments illustrated in A showing the effect of 40 μM FFA at various external pH values. Mean ± S.E.M. of g_j (percentage of the initial conductance at pH 7.4) values in the presence of flufenamic acid (▨) at external pH values of 6.7, 7.4, and 8.0 are shown. A 90-s exposure to solutions at external pH values of 7.0 and 8.2 in the absence of FFA did not cause a significant change in g_j (percentage of control at pH 7.4; ■).

Block at the Single-Channel Level. Single-channel studies were undertaken to investigate further the mechanism of channel closure of gap junction channels by FFA. The effect of FFA (50 and 100 μM) on a Cx50 single channel at a V_j of 30 mV is shown in Fig. 7. At this voltage, Cx50 channels are open 80 to 90% of the time with occasional transitions from an open state of 210 pS to a subconductance state of 40 pS, as has been reported previously (Fig. 6) (control: Srinivas et al., 1999). These open-substrate transitions are typically observed in response to transjunctional voltage gradients and are usually "fast" with increase and decay times of ~1 to 2 ms (Bukauskas and Peracchia, 1997; Verselis et al., 2000; Harris, 2001) (Fig. 8). External application of FFA (50 and 100 μM) caused a reduction in open-channel time. At 50 μM FFA, channels exhibited transitions between the open and closed state in the entire duration of the recording, whereas at 100 μM FFA, the channels were rarely open. The open probability of the channel was reduced from 0.85 in control solution to 0.5 at 50 μM FFA and to 0.1 at 100 μM FFA, values that are very close to the magnitudes of inhibition of g_j by these FFA concentrations obtained in macroscopic measurements (Fig. 1B). Similar results were obtained in two other experiments.

Even though the channel activity was reduced for prolonged intervals, the single-channel conductance was not appreciably modified. Amplitude histograms of the recordings in the absence and presence of FFA are illustrated to the right of each recording. In control recordings, histograms had three peaks at 0, 1.2, and 6.3 pA corresponding to the closed state, subconductance state (40 pS), and open state (210 pS), respectively (Srinivas et al., 1999). In the presence of 50 μM FFA, histograms had one peak at 6.3 pA corresponding to the open state (210 pS) and a second peak corresponding to the closed state (0 pA). Similarly, at 100 μm FFA, two peaks at 0 and 6.2 pA corresponding to open and closed states were observed. Similar results were obtained from three such experiments (mean ± S.E.M. values: 207 ± 12, 199 ± 10, and 203 ± 8 pS for control, 50 μM, and 100 μM FFA, respectively, verifying the lack of significant reduction in unitary conductance during channel blockade by FFA).

Closer inspection of recordings in the presence of FFA revealed that the drug induced "slow" transitions between the open and closed states and not flickery transitions that are characteristic of open-channel block (Fig. 8). The recordings in Fig. 8 were obtained by applying short (16-s) pulses in

the absence and presence of FFA (50 μM). The junctional current (I_j) and the current in the pulsed cell (I_2) are both illustrated in this figure to demonstrate that slow events occur simultaneously in both cells' current recordings and are thus junctional in origin. In the absence of FFA, channels exhibited fast transitions between the fully open and subconductance states. Slow transitions to the fully closed state were only rarely observed (arrowhead, control). Application of FFA led to an increase in slow transitions between the open and closed states, although fast transitions were also occasionally observed (arrowhead, FFA). Increase and/or decay times of these slow transitions varied between 8 and 100 ms (Fig. 9). Such slow closures to the fully closed state detected here have been observed in response to certain drugs and uncoupling agents (e.g., low pH, heptanol, halothane, and quinine) and have been attributed to a gating mechanism (the so-called chemical gate) (Bukauskas and Peracchia, 1997; Verselis et al., 2000; Harris, 2001). The presence of slow transitions induced by FFA reflects an action of the drug on this gating mechanism.

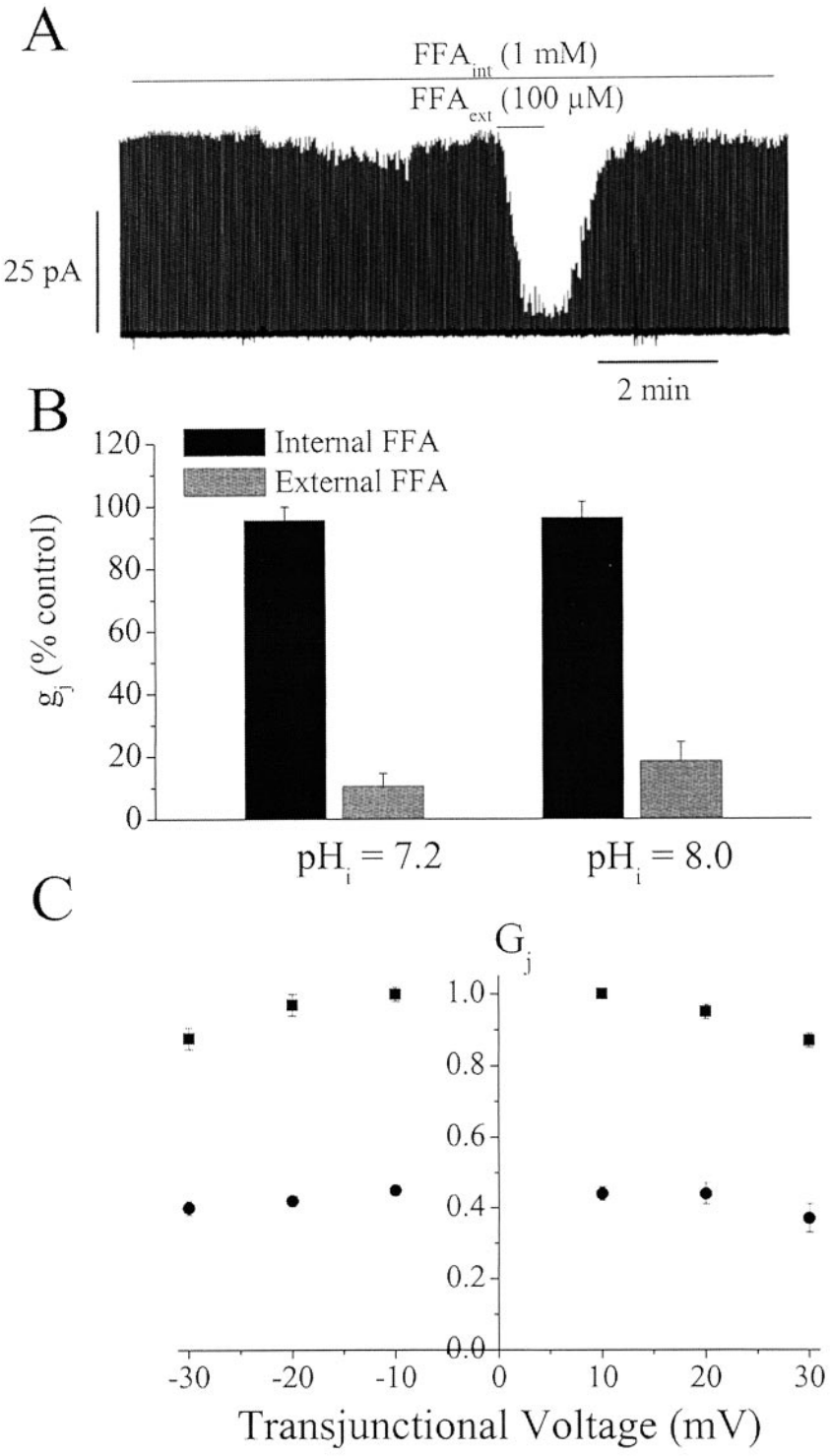

Fig. 5. The effect of flufenamic acid is not mediated by the binding of the drug in the pore. A, internal FFA (FFA_{int}) added to both patch pipettes, pH 7.2, had no effect on Cx50 junctional currents. In contrast, external application of a 10-fold lower concentration to the same cell pair (FFA_{ext}) markedly reduced junctional currents. B, mean ± S.E.M. of g_j values in the presence of internal and externally applied flufenamic acid when patch pipettes contained solutions at pH 7.2 and 8.0 are shown. At both intracellular pH values, internal application of FFA had little effect on Cx50 channels, whereas external application of 10-fold lower concentrations markedly reduced junctional conductance. C, block by FFA is not voltage dependent. Mean ± S.E.M. of normalized steady-state junctional conductance (g_j) in the absence (■) and presence of 50 μM FFA (●) are shown in response to 8-s pulses to various transjunctional voltages.

Discussion

Previous studies indicated that FFA blocked dye transfer between mammalian cells expressing Cx43 (Harks et al., 2001) and also inhibited putative hemichannel currents expressed in oocytes (Zhang et al., 1998; Eskandari et al., 2002). We extended these observations by directly measuring the effects of FFA and related analogs on junctional currents using the dual whole-cell patch-clamp technique. Closure of junctional channels by these drugs was rapid and quickly reversible, concentration-dependent, and exhibited only marginal selectivity with regard to connexin subtype. The efficacy of inhibition was markedly enhanced at low external pH values and decreased at high external pH values, indicating that the uncharged form of the drug is important for the effect of FFA. The results of other experiments, illustrated in Figs. 6 through 8, suggested that the effect of FFA is not caused by the binding of the molecule within the pore, but rather by the binding of the drug to a modulatory site, presumably located within the membrane, that induces channel closure. A Hill coefficient of ~3.0 suggests that binding of more than one molecule of the drug is required to effect channel closure.

Arylaminobenzoates affect a number of processes within the cell. Thus, it is possible that inhibition of gap junction channel currents is caused by an indirect action of the drug. However, the rapid onset and reversibility suggests that the effects of these drugs are most likely mediated by a direct action of the drug on the channel and are not caused by indirect effects such as lowering the intracellular pH and/or increasing intracellular calcium, factors which have also been shown to reduce coupling between cells. Internal solutions contained 10 mM HEPES and 10 mM EGTA, which should be sufficient to buffer small internal pH changes expected to occur when exposing cells to submillimolar concentrations of weak acids. In addition, increasing the concentration of HEPES or substituting EGTA with BAPTA had little effect on the potency of inhibition (data not shown). These results are consistent with those obtained by Harks et al. (2001), who demonstrated that the effects of the drug are not caused by changes in pH, calcium, protein kinase C, or cyclooxygenase activity.

Preliminary structure-activity studies also support the hypothesis that gap junction channels contain a distinct receptor for arylaminobenzoates. Analogs of flufenamic acid, such

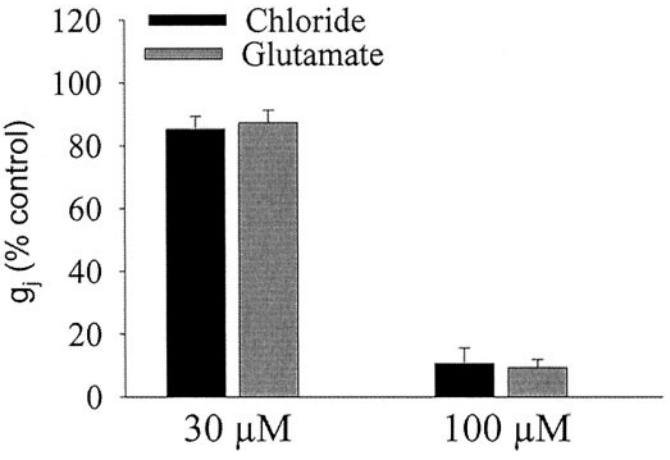

Fig. 6. The effect of flufenamic acid is not dependent on the nature of the permeant ion. Bar graph showing the effect of 30 and 100 μM FFA applied externally when chloride was replaced by glutamate in the internal solution. The magnitude of reduction of Cx50 junctional currents at these concentrations was not significantly different between the two internal solutions. Values are mean ± S.E.M. from four cell pairs under each condition.

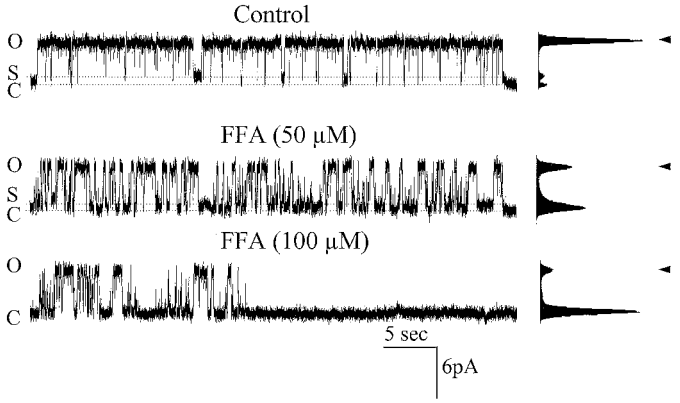

Fig. 7. FFA reduces the open probability of single Cx50 channels. 50-s recordings of Cx50 channels at a V_j of 30 mV applied to one cell of a pair in the absence and presence of FFA (50 and 100 μM, respectively) is illustrated. In the absence of FFA, Cx50 channels are predominantly open (O) at this V_j and exhibit occasional transitions to either the closed (C) or subconductance state (S). Application of 50 and 100 μM FFA to the same cell pair caused a reduction in the open probability of the channels from 0.85 in control to 0.5 and 0.1 at 50 and 100 μM FFA, respectively. Amplitude histograms shown to the right of each trace indicate that the conductance of the open state was not affected in the presence of 50 and 100 μM FFA. Arrowheads in all histograms indicate the peaks corresponding to the open state of 6.3 pA (in control), 6.3 pA (in 50 μM FFA), and 6.1 pA (in 100 μM FFA). The peak at 0 pA for the histogram of the control recording predominantly reflects the amplitude of the baseline current before the application of pulse.

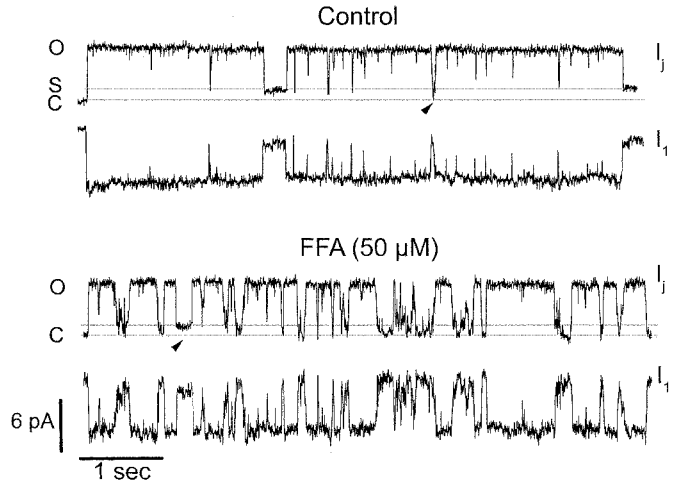

Fig. 8. Flufenamic acid induces slow transitions between open and closed states of the Cx50 gap junction channel. Segments of 16-s duration recordings of a single-channel current in the absence (control) and presence of 50 μM FFA at a V_j of 30 mV is illustrated. In the absence of FFA, Cx50 channels predominantly exhibited rapid transitions between the open (O) and subconductance state (S). Slow transitions between open and closed (C) states are rare but occasionally observed (arrowhead, control). Cx50 single-channel events during closure of Cx50 channels by FFA primarily exhibited slow transitions between the open and closed states. Fast transitions between the open and subconductance states were also occasionally observed (arrowhead, FFA). Simultaneously occurring events of equal amplitude and opposite polarity in current traces for both cells in the pair are shown. The upper traces represent the junctional current (I_j), whereas the lower traces represent the current in the pulsed cell (I_1).

as NPPB and meclofenamic acid, inhibited gap junction channels with potencies similar to those observed for FFA. Niflumic acid was less potent at inhibiting gap junction channels, but complete inhibition was achieved at concentrations of greater than 300 μM. In contrast, unrelated analogs such as clofibric acid and the stilbene derivative DIDS had little or no effect on gap junction channels. The potency of arylaminobenzoates at inhibiting gap junctions corresponds with their octanol/water partition coefficients (P), with the least potent blockers having low logP values.

Structure-activity studies of chloride-channel blockers defined four elements critical for arylaminobenzoate blockade: 1) the anionic carboxylate group, 2) the negative partial charge in *meta* (or *para*) position to the carboxylate group, 3) the amino "bridge" carrying a positive partial charge, and 4) the bulky hydrophobic side chain (Walsh and Wang, 1998; Walsh et al., 1999). Compounds that lacked the benzoate ring and/or those that decreased the acidity of this ring caused a marked attenuation in the potency. Although we did not conduct elaborate structure-activity studies, our results similarly indicate that a benzoate group and a bulky hydrophobic side chain are important determinants of inhibition. Compounds that lack the benzoate ring (APB) and/or the bulky phenyl ring (e.g., *o*-aminobenzoic acid) did not cause significant inhibition of gap junction channels at submillimolar concentrations. Similarly, compounds such as clofibric acid that contain substantial modification in this part of the molecule did not block gap junction channels at 1 mM or lower, whereas niflumic acid, which contains a nicotinic acid moiety instead of a benzoate ring, was less potent compared with FFA, MFA, and NPPB. However, additional studies are clearly essential to determine the importance of other regions of the molecule.

The effects of FFA were dependent on the external pH. External pH is known to markedly affect ion channel block by tertiary amine drugs such as local anesthetics and antimalarials as well as weak acids such as arylaminobenzoates by altering the ratio of charged and the membrane-permeable uncharged forms (Hille, 2001). We showed that the inhibitory effect of FFA, which has a pK_a between 3 and 4, is increased at low external pH and markedly decreased at low external pH. These results indicate that extracellularly applied FFA reaches its blocking site by permeating across the membrane in its uncharged form and strongly indicate that the uncharged form of the drug is the active species.

Inhibition of gap junction channels by flufenamic acid does not seem to be mediated by binding of the anionic form of the drug in the pore of the gap junction channel, as has been reported to be the mode of action on the cystic fibrosis transmembrane conductance regulator channel (McCarty et al., 1993). Several lines of converging evidence suggest that an alternative mechanism is involved. Internal application of the drug via patch pipettes did not cause inhibition of gap junction channels even with 10-fold higher concentration and after sufficient time was allowed for the diffusion of the drug to intracellular compartment (Srinivas et al., 2001, show experiments using other uncouplers). Although it is possible that the drug diffuses out of the cell before it reaches the binding site, the use of high concentrations would be expected to cause a moderate amount of blockade (DeCoursey, 1995). In addition, application of the drug at high internal pH, where the majority of the drug is charged and therefore would be expected to diffuse out of the cell more slowly than at pH 7.2, did not cause any detectable reduction by internally applied drug. Moreover, the recovery of g_j in response to extracellularly applied FFA while internal FFA concentration remained high provides evidence that the binding site is not accessible from the cytoplasm. The inhibitory action of FFA was not affected by the nature of the permeant ion. If the effect were mediated by binding within the pore, it should be influenced by the concentration and the nature of the permeant ion. In the case of chloride channels, the potency of these drugs was markedly reduced when the concentration of chloride ions in the pore was increased or when chloride was substituted with thiocyanate ions (McDonough et al., 1994). Although we did not systematically vary the concentration of chloride in our internal solutions, we conducted experiments wherein the magnitude of inhibition was assessed when chloride was replaced by glutamate and determined no significant change in the degree of reduction of channel currents caused by 30 and 100 μM FFA.

Finally, single-channel currents measured in the presence of FFA provided additional evidence that the effect of the drug is not caused by open channel block. A hallmark of open channel block is the rapid flickering of single-channel current between open and closed states as a result of binding and unbinding of the drug to a site in the pore of the channel (Hille, 2001). If the binding and unbinding rates of the blocker are fast, the single-channel current amplitude will seem to be reduced (Hille, 2001). In our experiments, however, single-channel currents in the presence of FFA did not exhibit rapid flickering between open and closed states, and single-channel current amplitudes were not altered by the presence of the drug.

The predominant effect of FFA on single channels was a reduction in the open probability of the channel and an increase the incidence of slow gating transitions between open and closed states. In these respects, FFA is similar to almost all other gap junction channel blockers and inhibitors studied, including low pH, halothane, *n*-alkanols, and quinine (Harris, 2001). Most of these uncoupling agents elicit closure by binding to regions outside the pore. For example, low pH affects coupling by protonation of histidine residues in the cytoplasmic loop (Stergiopoulos et al., 1999; Duffy et al., 2002), whereas *n*-alkanols have been reported to exert their effects by altering the fluidity of cholesterol-rich domains surrounding the connexin protein (Bastiaanse et al., 1993). Slow transitions elicited by these uncoupling agents have been attributed to a distinct form of closure that presumably involves conformational rearrangements of the channel (Trexler et al., 1996). Whether FFA and its analogs similarly induce channel closure by this same mechanism is difficult to determine experimentally. Our results nevertheless indicate that FFA and its analogs seem to function as allosteric modifiers of channel gating, presumably by binding directly to the channel. Because these drugs are highly lipophilic molecules, it seems most likely that they cause closure by partitioning into the plasma membrane in their uncharged forms and inducing a conformational change in the channel protein. An alternate possibility is that these drugs act by altering bulk membrane fluidity surrounding the channel protein, as has been proposed for other gap junction channel inhibitors. Additional studies are required to address this issue.

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