Biochemical Requirements for Inhibition of Connexin26-containing Channels by Natural and Synthetic Taurine Analogs*

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Previous work has shown that protonated taurine and aminosulfonate pH buffers, including HEPES, can directly and reversibly inhibit connexin channels that contain connexin26 (Cx26) (Bevans, C. G., and Harris, A. L. (1999) J. Biol. Chem. 274, 3711–3719). The structural requirements for this inhibition were explored by studies of the effects of structural analogs of taurine on the activity of Cx26-containing reconstituted hemichannels from native tissue. Several analogs inhibited the channels, with a range of relative affinities and efficacies. Each active compound contains a protonated amine separated from an ionized sulfonate or sulfinate moiety by several methylene groups. The inhibition is eliminated if the sulfonate/sulfinate moiety or the amine is not present. Compounds that contain a protonated amine but lack sulfonate/sulfinate moiety do not inhibit but do competitively block the effect of the active compounds. Compounds that lack the protonated amine do not significantly inhibit or antagonize inhibition. The results suggest involvement of the protonated amine in binding and of the ionized sulfur-containing moiety in effecting the inhibition. The maximal effect of the inhibitory compounds is enhanced when a carboxyl group is linked to the α-carbon. Inhibition but not binding is stereospecific, with L-isomers being inhibitory and the corresponding D-isomers being inactive but able to antagonize inhibition by the L-isomers. Whereas not all connexins are sensitive to aminosulfonates, the well-defined structural requirements described here argue strongly for a highly specific regulatory interaction with some connexins. The finding that cytoplasmic aminosulfonates inhibit connexin channels whereas other cytoplasmic compounds antagonize the inhibition suggests that gap junction channels are regulated by a complex interplay of cytoplasmic ligands.

Connexin channels, which compose most gap junctions in vertebrates, mediate direct intercellular movement of cytoplasmic signaling molecules. There are ~20 isoforms of connexin protein (2), each of which forms channels with distinct regulatory and permeability properties (3). The intercellular signaling mediated by connexin channels is important; every functional deletion of a connexin isoform produces a distinct pathology (2). The pathologies that arise from altered connexin channel function must arise from abnormal molecular movement through connexin channels, whether in modulation, magnitude, or molecular specificity.

Despite the importance of gap junction channels in development, physiology, and disease, little is known about the regulation of connexin channels by cytoplasmic ligands. Identification of endogenous ligands and their modes of action on connexin channels would be of considerable value for understanding intercellular signaling and connexin channel structure function.

Connexin channels are homo- or hetero-oligomers of isoforms of connexin protein (4–8). They have two functional and structural forms. The basic unit is a hexamer, called a “hemichannel” or “connexon.” The hemichannel is the single-membrane form, found in the plasma membrane of many cells and implicated in a variety of cellular processes (9, 10). Gap junction channels are end-to-end dimers of hemichannels. The intercellular location of gap junction channels presents challenges for detailed biophysical investigation; hemichannels are more amenable for study. By and large, the properties of junctional channels are predictable from those of the component hemichannels (3, 11–17).

Whereas many compounds affect junctional channels when applied to cells, in most cases, it is unclear or unlikely that the compounds affect the channels directly. Our previous work showed that the protonated forms of the Good’s pH buffers HEPES, MBS,1 and TAPS can directly and reversibly inhibit channels that contain Cx26 (1). Aside from the effect of pH on the degree of protonation of these compounds, pH changes over the range 6–10 were without effect. This inhibition did not occur for channels composed solely of connexin32 (Cx32). Since the active compounds were all aminosulfonates, the ubiquitous biological aminosulfonate taurine was tested and found to have similar effect at cytoplasmic concentrations.

The present study was undertaken with two goals: to determine the structural basis of the inhibition and to determine whether cytoplasmic aminosulfonates other than taurine affect connexin channels. To these ends, a series of structural analogs of taurine were tested for inhibitory effect, and their relative

1 The abbreviations used are: MES, 4-morpholineethanesulfonic acid; GABA, γ-aminobutyric acid; TAPS, 3-[(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid; Cx, connexin; PS, phosphatidylserine; rho-PE, rhodamine B-labeled phosphatidylethanolamine; l-CSA, l-cysteine sulfonic acid; TSF, transport-specific fractionation; l-CA, l-cysteic acid; MOPS, 4-morpholinepropanesulfonic acid; APSA, 3-amino-1-propane sulfonic acid; l-DPA, l-2,3-diaminopropionic acid; IA, isethionic acid; HPSA, 3-hydroxypropionate sulfonic acid; APA, 2-aminoethanol phosphonic acid; LGIC, ligand-gated ion channel; GlyR, glycine receptor; nACHr, nicotinic acetylcholine receptor; AChBP, acetylcholine-binding protein; CT, C-terminal; NT, N-terminal; CL, cytoplasmic loop; ACES, N-[2-acetamido]-2-aminothanesulfonic acid.
Aminosulfonate Inhibition of Connexin Channels

Effectiveness was determined, using connexin channels purified from native tissue. Analogs found to be inactive were assessed for antagonism of the inhibition produced by taurine. A set of structural rules for the inhibition and its antagonism were defined. We find that to inhibit the channels, a compound must contain a protonated amine separated from an ionized sulfonate or sulfate moiety by two or three methylene groups. The data indicate that the protonated amine is required for binding, and the ionized sulfur-containing moiety is required for efficacy. The inhibition is stereospecific, but binding is not. Because the inhibitory compounds include taurine and cysteic acid, and the competitive antagonists include β-alanine, GABA, glycine, and glutamic acid, it is possible that the site of interaction is similar to the binding sites of these compounds on other channels and receptors. Furthermore, the finding that inhibitors and their antagonists co-exist in cells suggests that cellular control of junctional channels involves a complex interplay among the various metabolic pathways that generate these compounds and the membrane transporters that regulate their cytoplasmic concentrations. Preliminary reports of this work have appeared in abstract form (18, 19).

EXPERIMENTAL PROCEDURES

Materials

Egg phosphatidylcholine, bovine phosphatidylserine (PS), and lissamine rhodamine B-labeled phosphatidylethanolamine (rheo-PE) were from Avanti Polar Lipids. N-Octyl-β-D-glucopyranoside was from Calbiochem and Glycon Biosciences. Bio-Gel (A-0.5m, exclusion limit 500,000 Da) was from Bio-Rad. CNBr-activated Sepharose beads were from Amersham Biosciences. Taurine and its analogs were from Sigma. Use and care of animals were according to institutional guidelines.

Immunopurification of Connexin

Heteromeric Cx26/Cx32 and homomeric Cx32 hemichannels were affinity-purified from an N-octyl-β-D-glucopyranoside-solubilized crude membrane fraction of mouse or rat liver, respectively, using a monoclonal antibody against Cx32 as previously described and characterized (1, 6, 20).

Antibodies

The monoclonal antibody (M12.13) used in the immunosaffinity purification and for specific staining of Cx32 on Western blots is directed against a cytoplasmic domain of Cx32 (21, 22).

pKₐ Values

Since experiments were carried out at 37 °C, the pKₐ values used to calculate the concentration of protonated aminosulfonate at a given pH were those measured at 37 °C: taurine, 8.8; l-cysteic acid, 8.1; HEPES, 7.3; TAPS, 8.1; ACES, 6.5; l-homocysteic acid (l-HCA), 8.8; l-cysteine sulfinic acid (l-CSA), 8.7; hyotaurine, 9.6.

Reconstitution of Purified Connexin into Unilamellar Phospholipid Liposomes

Liposome formation and protein incorporation followed the protocol of Mimms et al. (23) as modified by Harris et al. (24) and Rhee et al. (20). Liposomes were formed by gel filtration of a mixture of phosphatidylcholine, PS, and rho-PE at a molar ratio of 2:1:0.03 in urea buffer (see below) containing 80 mM N-octyl-β-D-glucopyranoside and immunosaffinity-purified connexin. The size of the liposomes was monodisperse with mean diameter of ~900 Å (25). The protein/lipid ratio of the liposomes was typically 1:60 (w/w), corresponding to an amount of connexin equivalent to ~1 hemichannel/liposome (see “Data Analysis”). The protein/lipid ratios used yielded functional channels in 20–50% (mean 37%) of the liposomes, corresponding to a mean of ~0.5 functional hemichannels/liposome.

Transport-specific Fractionation (TSF)

What It Is and How It Works—Channel activity was assessed by TSF of the liposomes. TSF has been previously described and characterized (20, 24, 26, 27). It separates liposomes into distinct populations within a density gradient based on their permeability to urea and sucrose, which permeate open connexin channels. The density gradient is formed from iso-osmolar solutions in which the major component is either urea (459 mOsm) or sucrose (400 mOsm). The sucrose solution has a greater density than the urea solution, and the gradient is constructed so that the density of lipid is near the bottom. The other components of the solutions are as follows: 10 mM KCl, 10 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, and 3 mM NaH₂PO₄. Osmolality of urea and sucrose buffers is 500 mOsm/kg, and their specific gravities (D₄₀°) are 1.0056 and 1.0511, respectively.

Unilamellar liposomes are formed in and entrapped the (less dense) urea-containing solution and were centrifuged through the TSF gradient at 300,000 × g for 3 h in a swinging bucket rotor at 37 °C. With centrifugation, liposomes without functional channels move into the gradient a short distance, being buoyed by the (lighter) entrapped urea buffer and form a band in the upper part of the gradient. Liposomes with functional channels continuously exchange solutes through the open connexin pores, equilibrating the aqueous density inside and outside the liposome. As this occurs, the phospholipid membrane becomes the determinant of liposome density, and the liposomes move to a position in the lower part of the gradient. The positions of liposomes are monitored via rheo-PE fluorescence (λₑ 570 nm, λₑ 590 nm), making it possible to visualize and to recover the liposome bands by aspiration. The distribution of the liposomes between the two bands in the gradient is calculated from the specific intensity of rhodamine fluorescence and the volume of each collected band.

A change in the distribution of liposomes between the upper and lower bands, relative to control, reflects a change in the fraction of channels that are permeable to the gradient solutes. A reduction of this fraction can be due to pore block or to a reduction in open channel pore open probability (Pₒ). Since solution exchange is rapid for these 900-Å liposomes, even a channel that opens only infrequently for brief times will mediate sufficient exchange of solutes to cause liposome movement to the characteristic lower position. TSF is therefore essentially an all-or-none assay for channel function per liposome.

A modulatory compound could reduce the proportion of liposomes that shift to the lower position by restricting the diameter of the pores, rather than moving Pₒ close to zero. However, in this case, the restriction would have to render the channels impermeable to urea and to sucrose. Such a change in diameter would effectively eliminate the ability of connexin channels to mediate molecular signaling between cells and therefore is regarded functionally as eliminating channel activity, with regard to molecular signaling.

TSF therefore identifies conditions/reagents that dramatically reduce channel function. Absence of an effect of a reagent in this system does not imply absence of effect on the channels. But positive findings, such as those reported in this study, reflect truly significant alterations of channel function.

Understanding TSF Dose-response Relations—TSF fractionates liposomes according to whether the channels they contain have Pₒ less than a very low value. Consequently, the concentration of a compound that produces channel closure detectable in TSF is much greater than that needed to see an effect in biochemical studies, corresponding to the concentration needed to induce Pₒ very close to 0. Therefore, TSF does not give classical Michaelis-Menten binding parameters such as Kᵣ and does not allow determination of actual affinities. By the same token, the concentration of a modulator that produces an effect in TSF is a far upper limit for the real half-maximal value.

For simple inhibition of a homogeneous channel population, TSF yields a step change in the activity-concentration relation, with the step occurring where Pₒ is very close to 0. The step in the activity-concentration relation occurs at the concentration at which all channels in the population are effectively closed by the inhibitor.

A graded TSF activity-concentration relation occurs when there is heterogeneity of ligand-channel interactions. In the present work, this arises from the known structural heterogeneity of Cx26/Cx32 heteromeric channels purified from native tissue heterogeneity with regard to connexin concentration (1, 6). Multiple pore and arrangement (1, 6). Multiple pore and arrangement (1, 6). Multiple pore and arrangement (1, 6). Multiple pore and arrangement (1, 6). Multiple pore and arrangement (1, 6).
Data Analysis

Correction for More than One Channel per Liposome—Previous work with the TSF system suggested that connexin channels distribute among the liposomes in a manner described by Poisson’s distribution (20). This means that for a given ratio of functional channels to lipid (λ) in the liposomes, a Poisson distribution accounts for the fraction of the liposomes that have functional channels. λ was estimated from the maximum activity (percentage of liposomes with active channels) for a given preparation of liposomes. Using the Poisson distribution, this λ was used to calculate the distribution of channels in the liposome population, which was used to compensate for the error introduced by some of the liposomes containing more than one channel (1). This calculation transforms the fraction of liposomes in the lower TSF band to the fraction of channels that are not inhibited.

Normalization of TSF Data—For each preparation of connexin, the percentage of liposomes in the lower TSF band was normalized to the maximum value obtained for that preparation. This enabled comparison of modulatory effects across reconstitutions that produced different amounts of channel activity (fractions of liposomes with functional channels). Where several preparations were used, normalized data sets were combined for each condition for calculation of means and S.E. values.

Curve Fitting—The activity-concentration data were fit with a four-parameter logistic function of the form

\[ f(x) = \frac{a}{1 + \exp(b(x - c)) + d} \]

using the Marquardt-Levenberg algorithm. A Hill equation was not used, since TSF does not produce a binding/effect curve but in this case gives a superimposition of behaviors of an unknown number of channel forms with different properties (see above). A smooth function was fit to the data solely to determine the characteristic half-maximal channel activity parameter. This parameter is not a \( K_a \), but does represent the relative effectiveness of a compound as a function of concentration; changes in it reflect differences in the concentration needed to fully inhibit one-half of the (heterogeneous) channel population and is used as an indicator of relative affinity (IC_{50-TSF}).

RESULTS

Heteromeric Cx26/Cx32 channels were obtained from mouse liver as previously described and characterized (6, 20). This heteromeric channel population is functionally heterogeneous with regard to permeability to large molecules (6) and sensitivity to modulatory ligand (1), indicating that it is composed of channels that are heterogeneous in the stoichiometry and/or arrangement of Cx26 and Cx32 isoforms. It does not contain homomeric Cx26 or Cx32 channels (6). The data shown are averages from at least five purifications to control for differences in range or distribution of stoichiometries and isoform arrangements in the channel population from preparation to preparation.

Hemichannels were reconstituted into unilamellar phospholipid liposomes. The activities of the reconstituted channels were explored by TSF, which has been well characterized and used effectively in studies of connexin channel permeability and modulation (1, 6, 20, 24, 26, 28–35). The technique is described under “Experimental Procedures.” In brief, when centrifuged in an appropriate isosmolar density gradient, solute exchange through open channels causes liposomes to become more dense and move to a position deep in the gradient. Liposomes without open channels remain in the upper part of the gradient. Any significant \( P_e \) results in sufficient osmolyte exchange to cause the change in density. TSF is therefore an essentially all-or-none assay of per liposome channel activity. TSF is of particular utility in study of connexin channels due to the constraints of studying connexin channels in situ and their apparent refractoriness to functional reconstitution in planar bilayers (3). It also directly assesses their molecular permeability, the key functional property of connexin channels (except in neurons).

The effects of test compounds on channel activity were assessed by exposing connexin-containing liposomes to the compounds during a TSF centrifugation. The change in distribution of liposomes between the upper and lower positions, relative to a control gradient without the compound, is a quantitative measure of the aggregate fractional change in activity of the population of the channels.

Protonated Taurine Inhibits Cx26/Cx32 Heteromeric Channels

Fig. 1 illustrates the inhibition of Cx26-containing channels by protonated taurine, as established in previous work (1). Fig. 1A shows the change in channel activity of Cx26-containing channels in 10 mM taurine over a wide range of pH and insensitivity of homomeric Cx32 channels under the same conditions. The decline in activity with decreased pH could be accounted for either by deprotonated taurine acting as a channel agonist or protonated taurine acting as a channel antagonist. To distinguish these possibilities, channel activity was assessed at the taurine \( pK_a \) of 8.8 in 50 mM taurine. At the higher concentration, channel activity was reduced to that at 10 mM taurine at low pH (i.e. when it is fully protonated) (Fig. 1A, asterisk). This suggests that the protonated form of taurine is...
the channel inhibitor, since in this experiment, the concentrations of both the protonated and deprotonated species were increased equally.

This point is made more rigorously by an experiment in which the concentration of protonated taurine was controlled by changing the concentration of total taurine while keeping pH constant at 6.5. Normalized inhibition of connexin channel activity is plotted as a function of protonated taurine concentration at constant pH in Fig. 1B. If the channel activity was being controlled by pH, there would be no change in activity. Furthermore, these data superimpose with those from Fig. 1A (in which total taurine was constant and pH changed). Therefore, the channel activity is a simple function of protonated taurine concentration, independent of changes in total taurine concentration or pH over these ranges.

### Inhibition of Connexin Channels by Aminosulfonate Structural Analogs of Taurine

A series of aminosulfonate analogs of taurine were tested for effect at pH 6.0 (to maximize protonation) by including them in the TSF solutions. The concentration producing maximal effect was determined for each compound and was the concentration used unless otherwise indicated. The selection of analogs to test was based on three overlapping criteria: cytoplasmic analogs that may be involved in biological regulation of connexin channels, biological analogs for which there is detailed information about binding sites on other proteins, and synthetic analogs that may help to define the biochemical structural requirements for the inhibition.

#### Aminosulfonates Directly Inhibit Cx26/Cx32 Heteromeric Channels—
The data shown in Fig. 2 show that several aminosulfonates in addition to taurine inhibit connexin channel activity. The rank order of maximal inhibitory effect was as follows: L-cysteic acid (L-CA) ~ ACES ~ L-HCA ~ taurine ~ TAPS ~ L-CSA ~ MOPS > HEPES > hypotaurine > 3-amino-1-propane sulfonic acid (APSA). (~ indicates that the difference in inhibition is not significant compared with next compound; > indicates that the difference is significant, by t test). As for taurine, these compounds had no effect on Cx32 channels (data not shown). Table I shows the structures of these inhibitory compounds. They all share a common structural motif: a protonatable amine separated from an ionized sulfur-containing moiety by two or three methylene groups.

#### Protonated Aminosulfonate Is the Inhibitory Form—
Three representative aminosulfonates were characterized with regard to whether it was the protonated form that was inhibitory, as it was for taurine. The open bars in Fig. 3 show that inhibition by L-CA, ACES, and TAPS at 10 mM is pH-dependent; lower pH enhances inhibition. In each case, the middle open bar is at the approximate $pK_a$ of the compound.

As for taurine in Fig. 1A, inhibition was also assessed at the $pK_a$ at 50 mM aminosulfonate (hatched bars). At the higher concentration, channel activity was reduced to the level of activity at 10 mM at low pH. This indicates that the protonated form of each aminosulfonate is the inhibitory species.

#### Protonated Aminosulfonates Inhibit Connexin Channels in a Concentration-dependent Manner and with Different Apparent Affinities—
To compare the effects of the different aminosulfonates, inhibition versus concentration curves were obtained (Fig. 4). The displacements of the relations relative to each other reflect the relative affinities of the aminosulfonates for the connexin channels. As outlined under “Experimental Procedures,” these relations are not Hill plots; their shape and position reflect the combined all-or-none TSF response of each of several populations of heteromeric channels, not the fractional occupancy of a binding site (for a detailed analysis, see Ref. 1). The data for each curve are aggregates for at least five different purifications, so differences in range or distribution of channel stoichiometries from preparation to preparation are averaged out. The displacement of the curves from one another therefore reflects differences in aggregate relative affinities of the amionosulfonates.
Aminosulfonate Inhibition of Connexin Channels

**Fig. 3.** The protonated form of the aminosulfonates produces the inhibitory effect. For each compound at 10 mM, the degree of channel inhibition was determined at the pKₐ, and values above and below the pKₐ (open bars). The data show that for each compound, the inhibition increases as the pH is decreased. The hatched bars represent inhibition at the pKₐ but for a 50 mM concentration of the compound. The increased inhibition at the higher concentration at the pKₐ shows that the protonated form is the inhibitor.

Taurine Structural Analogs Lacking either the Protonatable Amine or the Ionized Sulfur-Containing Group Do Not Inhibit the Channels

To determine the structural requirements for the inhibition, a series of analogs of aminosulfonates were tested, in which either the protonatable amine or the sulfur-containing group was replaced by other groups. The data in Fig. 5 show that analogs that lack either of these groups are without substantial effect on the channels as assessed by TSF. The structures and abbreviations of these compounds are shown in Table II.

The structural changes that drastically reduce the inhibition fall into two categories: 1) replacement of the sulfonate/sulfinate group with a carboxyl group (β-alanine, GABA, glycine, DPA, and L-glutamic acid) or by a phosphoryl group (APA), and 2) replacement of the protonatable amine by a hydroxyl group (IA, HPSA) or a thiol group (MSA).

The data suggest that both moieties (the protonatable amine and the sulfonate/sulfinate group) are required for the inhibitory effect on Cx32/Cx26 channels. Possible exceptions are MSA (amine → thiol) and APA (sulfate → phosphoryl), which show ~20 and 17% inhibition relative to taurine, respectively.

Competitive Block of the Effect of Aminosulfonates by Structural Analogs

These aminosulfonate analogs did not significantly inhibit the channels, but it was possible that they could nevertheless interfere with the binding of the active compounds. To explore this possibility, the effects of the inactive analogs on inhibition produced by taurine were examined (both taurine and the analogs at 10 mM). Fig. 6A shows that these inactive compounds fell into two categories of degree of interference with taurine-induced inhibition. The values for degree of block of taurine inhibition are calculated as the unblocked fraction for the compound alone divided by the unblocked fraction for the compound plus taurine. The more effective group and the percentage reduction of taurine inhibition produced by each compound were as follows: glutamic acid, 83%; GABA, 79%; L-DPA, 76%; APA, 71%; β-alanine, 65%; glycine, 56%. These compounds all contain a protonatable amine but lack a sulfonate/sulfinate group.

The second group had much less effect on taurine inhibition: HPSA, 35%; MSA, 33%; IA, 19%. These compounds all lack the protonatable amine but do contain a sulfonate/sulfinate group.

To further characterize the interaction between the effects of taurine and its antagonists, inhibition versus taurine concentration data were obtained in the absence and presence of β-alanine, L-glutamic acid, or GABA. Fig. 6B shows that each compound shifted the curve to the right without change in shape or in the maximal inhibition achieved. This shift indicates that these compounds, which lack the ionized sulfur-containing group, antagonize taurine inhibition in a competitive manner, consistent with binding at the same site. Furthermore, the data suggest that the affinities of the compounds for the site increase in the order β-alanine > L-glutamic acid > GABA. This ranking
correlates with the degree of antagonism with maximal taurine inhibition shown in Fig. 6A, as expected.

Taken together, these data suggest that the amine-containing analogs can displace taurine from its binding site on the channels.

To determine whether the amine group of the antagonists needed to be protonated for the antagonism to occur, the effect of APA (pKₐ 6.3) on inhibition by L-CA (pKₐ 8.8) was assessed at pH 8.0. At this pH, the L-CA is 83% protonated, whereas only 2% of the APA is. Fig. 7 shows that there is no APA antagonism under these conditions and confirms antagonism when APA is protonated (at pH 6.0, at which APA is 67% protonated). This result shows that the competitive antagonism requires the amine moiety to be protonated and confirms the role of this moiety in binding.

**Stereoisomer Specificity of Inhibition and Binding**

Some of the taurine analogs tested contain an optically active α-carbon (L-CA, L-HCA, L-CSA, and L-glutamic acid). These compounds have an asymmetric center at the α-carbon that can exist in two forms, designated L and D, which are mirror images of each other. As described above, the L form of these analogs inhibits connexin channels or antagonizes the taurine inhibition. To determine whether optical isomers have different effects on connexin channels, we compared the effects of L and D forms of the aminosulfonate HCA (the only one for which a D form is commercially available) and the L and D forms of glutamic acid (an inactive, but competitive compound). Fig. 8

**Table II**

**Taurine analogs that do not significantly inhibit Cx26/Cx32 channels**

The values for inhibition are means normalized to that for taurine and are from the data graphed in Fig. 6. The values for relative block of taurine inhibition are calculated as the unblocked fraction for the compound + taurine divided by the unblocked fraction for the compound alone.

| Compound                  | pKₐ at 37°C | Maximal Inhibition relative to taurine | Relative block of taurine inhibition (%) |
|---------------------------|------------|---------------------------------------|----------------------------------------|
| H₂N-COOH                  | L-glutamic acid | 9.7                                   | 0.10                                   |
| H₂N-COOH                  | GABA        | 9.6                                   | 0.20                                   |
| H₂N-COOH                  | L-2,3-diaminopropionic acid (L-DPA) | 10.2                                  | 0.12                                   |
| H₂N-COOH                  | 2-aminomethyl phosphonic acid (APA) | 6.3                                   | 0.17                                   |
| H₂N-COOH                  | β-alanine    | 10.2                                  | 0.44                                   |
| H₂N-COOH                  | glycine     | 9.6                                   | 0.08                                   |
| HO-SO₂H                   | 3-hydroxypropionate sulfonic acid (HPSA) | 8.5                                   | 0.08                                   |
| HS-SO₂H                   | 2-isocyanoethane sulfonic acid (MSA) | 8.2                                   | 0.20                                   |
| HO-SO₂H                   | isethionic acid (IA) | 9.9                                   | 0.05                                   |

**Fig. 5. Effects of aminosulfonate analogs on connexin channel activity.** All compounds were tested at a 30 mM concentration of their protonated form. The inhibitory effect was normalized to inhibition by 10 mM protonated taurine. The bar graphs are means ± S.E. from 5–10 protein preparations.

**Fig. 6. Antagonism of taurine-induced inhibition by aminosulfonate analogs.** A, coapplication of aminosulfonate analogs with taurine. Each compound was applied alone at 10 mM (open bars) and with 10 mM taurine (hatched bars). Compounds in which the amine is retained (β-alanine, L-glutamic acid, APA, GABA, glycine, DPA) significantly antagonized taurine-induced inhibition. Compounds in which the amine was replaced by another moiety (HPSA, MSA, and IA) antagonized taurine-induced inhibition to a much lesser degree. The inhibitory effect of each compound was normalized to the inhibition by 10 mM taurine. The bar graphs are means ± S.E. from five protein preparations. B, competitive inhibition of taurine-induced inhibition by β-alanine, L-glutamic acid, and GABA. The dose-response relations for protonated taurine alone (open circles) and with 5 mM protonated of the analogs (filled symbols) are shown. The rightward shifts of the relations indicate competitive inhibition, with affinity increasing in the order β-alanine > L-glutamic acid > GABA. Data points are means ± S.E. of five protein preparations. Curves were generated as before.
shows that whereas L-HCA inhibits the activities of connexin channels, D-HCA and L- and D-glutamic acid do not. The absence of activity by the D-glutamic acid was not surprising, since L-glutamic acid was without effect. However the absence of effect of D-HCA shows that the efficacy of this compound is stereospecific.

The ability of D-glutamic acid to antagonize the effects of L-CA was determined. Fig. 9A shows that D-glutamic acid antagonizes the effect of L-CA to roughly the same degree as does L-glutamic acid. Thus, in contrast to efficacy, the antagonism, and therefore the apparent binding site, is not stereospecific. This point is strikingly illustrated in Fig. 9B, in which D-HCA is shown to antagonize the inhibition produced by its optical isomer L-HCA.

Fig. 9C confirms the competitive nature of the interaction between the L- and D-isomers of HCA. The inhibition versus L-HCA concentration relations were obtained in the absence and presence of 5 mM D-HCA. The addition of the D-isomer shifted the curve to the right without change in shape or in maximal inhibition.

These data suggest that the D and the L forms of aminosulfonates interact at the same binding site. However, the efficacy of the bound molecule in inhibiting the channel is specific for the L-isomer. One may speculate that the lack of inhibitory effect by D-isomers is because the group responsible for efficacy (the sulfonate group) cannot interact at the effector site because of the way its spatial position differs from that in the L-form.

DISCUSSION

Structural Determinants of Aminosulfonate Inhibition of Heteromeric Cx26/Cx32 Channels—The present study investigates the effects of structural analogs of taurine on the activity of Cx26/Cx32 channels purified from native tissue. The results show that both a protonated amine and an ionized sulfur-containing moiety are required for the inhibition. Elimination of either moiety essentially eliminates the effect.

Structural analogs containing a protonated amine but not an...
ionized sulfur-containing moiety have little effect on connexin channels but do competitively antagonize taurine inhibition of the channels, shifting the inhibition-taurine concentration curve to the right without change in maximal value or slope. The implication is that these compounds directly displace taurine from its binding site, reducing its effective affinity. The amine of the antagonists must be protonated to be effective. On the other hand, analogs without the amine have no effect on the channels and do not significantly antagonize the inhibition even if they contain a sulfonate/sulfinate moiety. These results indicate that the sulfonate/sulfinate moiety is the functional group responsible for the inhibition of connexin channel activity, and the protonated amine is necessary for binding.

Correlation between differences in maximal effect (efficacy) and apparent relative affinity with the structural changes allows inferences about the structural bases of efficacy and of binding. The rank order of maximal inhibition is as follows: 1-L-CA > ACES > 1-HCA > (taurine > TAPS > ACES > 1-CSA) > MOPS > (HEPES > hypotaurine) > APSA.

The rank order of apparent affinity (highest to lowest) is as follows: 1-L-CA > (ACES > 1-HCA) > TAPS > (taurine > 1-CSA) > MOPS > hypotaurine > APSA.

Replacement of the sulfur-containing group by a carboxyl or phosphoryl group (taurine → β-alanine; APSA → GABA; L-HCA → L-glutamatic acid; taurine → APA) eliminates the inhibitory effect, but the resulting compounds competitively antagonize the inhibition by the active compounds. The analogs DPA and glycine, for which we did not identify/test corresponding compounds with an ionized sulfur-containing group, had the same property. The simplest explanation is that the compounds without the sulfur-containing group can bind at the same site as the active compounds and that the sulfur-containing group is required for the inhibition. The 17% inhibition produced by APA may imply that a phosphoryl group can weakly effect channel inhibition.

Replacement of the amine by a hydroxyl or thiol group (taurine → IA; hypotaurine → MSA; APSA → HPSA) essentially eliminated the inhibition, but these compounds also did not significantly antagonize the effect of the active compounds. Together with the data on elimination of the sulfonate/sulfinate moiety, this identifies the protonatable amine as required for binding. The 20% inhibition produced by MSA may imply that a thiol group can contribute to weak binding.

The only difference between taurine and hypotaurine, and between 1-L-CA and 1-L-CSA, is the substitution of a sulfonate group by a sulfinate group. In both cases, the substitution reduces the maximal inhibition. This indicates that the less electronnegative sulfinate group can mediate inhibition, but it is less efficacious than the sulfonate group. The lesser effective affinity of hypotaurine relative to taurine suggests that the sulfonate-containing moiety can also contribute to binding (although it is unable to mediate binding on its own, shown by lack of effect of compounds that contain it but not a protonatable amine: e.g., IA, MSA, and HPSA).

The data also suggest that the presence of a carboxyl group linked to the α-carbon atom enhances efficacy; two of the most efficacious compounds have this substitution. 1-L-CA is so modified from taurine, and 1-L-HCA is so modified from APSA, and in both cases the modified compound has greater maximal effect. Also, 1-L-CSA is so modified from hypotaurine and has enhanced relative efficacy. The negative charge of the carboxyl may play a role in the enhanced efficacy of these compounds. However, note that ACES has equal efficacy to carboxyl-substituted 1-L-CA and 1-L-HCA, suggesting that a carbonyl substituted at this position can achieve the same effect, but without having a negative charge (however, an analog of ACES lacking the carboxyl was not identified, so this difference may be due to the other aspects of the ACES structure). The fact that 1-L-CA, 1-L-HCA, and 1-L-CSA have greater affinity than taurine, APSA, and hypotaurine, respectively (Fig. 4), suggests that a carboxyl at this position enhances binding as well. On the other hand, 1-L-glutamic acid is so modified from GABA, yet the ability to block the taurine effect is unaltered.

The number of methylene groups that separate the amine and the sulfur-containing moiety also influence the interaction, but not in a systematic way. Increasing the number of methylene groups from two to three appears to decrease the efficacy in one case (taurine → APSA) and have no effect in another (1-L-CA → 1-HCA). For the nonactive competitors, increasing the number of methylene groups (from one to two to three for glycine → β-alanine → GABA or from two to three for IA → HPSA) increases the apparent affinity (Fig. 6, A and B, and Table II).

In those cases in which stereoisomers were available, both D- and L-isomers were able to interact with the binding site, but only the L-isomer was able to effect inhibition of the channels. The most dramatic demonstration of this is that D-HCA was able to antagonize inhibition by its optical isomer, L-HCA. This suggests that the site at which the ionized sulfur-containing group acts is in a specific spatial location relative to the other substitutions to the asymmetric carbon, specifically the carboxyl moiety; the sulfur-containing group of only the l-isomer of HCA can access the key effector site when a carboxyl group is attached to the α-carbon (see Fig. 10). This suggests that the carboxyl group restricts the orientation of the bound aminosulfonate in addition to enhancing its affinity.

It is striking that substitutions that improve efficacy also improve apparent affinity. This suggests that the classical distinction between the two processes may not be completely appropriate for this interaction. The implication is that the energetics of interaction at the effector site (and necessarily the processes to which they are coupled) contribute to the effective affinity. Formally, it could not be otherwise (36), but the extent of the coupling is unusual, indicating a substantial contribution to binding energy due to interactions at the effector site.

There was no obvious correlation between the net molecular charge of the compounds and their effects, either inhibitory or as antagonists of inhibition. At the pH of the experiments (6.0), some of the inhibitory compounds have no net charge (taurine, TAPS, HEPES, hypotaurine, and APSA), some have a net negative charge (1-L-CA, 1-HCA, and 1-L-CSA), and some are partially protonated, either having a net negative charge or being uncharged (ACES, MOPS). Of the antagonists, some have no net charge (GABA, APA, β-alanine, glycine), one has a net negative charge (1-L-glutamic acid), and one has a net positive charge (1-L-DPA). The factors that seem to matter are the ionized sulfur-containing moiety and the protonation of the amine, not the overall charge of the molecule. Therefore, the data do not suggest a role of charge screening in the effects of these compounds.

Site of Aminosulfonate Action

Specific Chemical Interactions—Inspecting the list of compounds that either cause inhibition or competitively block it, one is struck by the presence of many compounds (taurine, GABA, 1-glutamic acid, β-alanine, and glycine) that interact with the superfamily of pentameric ligand-gated ion channels (LGICs), which includes the inhibitory glycine receptor (GlyR) as well as the nicotinic acetylcholine receptor (nAChR) and serotonin and GABA receptors (37).

The pharmacological profile of binding or efficacy for the tested compounds on connexin channels does not match that...
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for any individual member of the LGIC family. Nevertheless, the LGIC binding site may be informative in identifying key interactions. Much is known about the homologous ligand binding sites of the LGICs, particularly since the crystal structure of an acetylcholine-binding protein (AChBP) was solved (38). The common features include a conserved tertiary structure of an acetylcholine-binding protein (AChBP) was solved (38).

These results suggest a common mode of interaction for taurine and its structural analogs in connexins and in members of the LGIC family: the positively charged amine of the aminosulfonates and their competitive analogs interacting via a cation-π interaction with aromatic amino acid side chains. This view is consistent with our data that independently identify the charged amine as the primary determinant of binding.

Additional clues to the nature of aminosulfonate binding may be obtained from examination of protein crystal structures in which aminosulfonates are bound at enzymatic active sites or at ligand binding sites. These cases fall into three categories: those in which it is clear that both the amino and sulfonate groups participate in the interactions and those in which either the amino or the sulfonate moieties do so.

In the first category is the binding of MES at the active site of a lactamase (40) and HEPES at a nonsubstrate modulatory site of a glutathione S-transferase (41). There were several instances of specific interaction involving the sulfonate group but where interactions involving the amine are difficult to distinguish from those dominated by the hydrophobic nature of the piperazine ring of HEPES. These include binding of HEPES to the active site of a tautomerase (42) and binding of HEPES and MES to the substrate binding site of a protein-tyrosine phosphatases (43–45). It may be that these interactions involving the amine are in fact mediated by the cation-π mechanism.

Structures in which the primary interaction is with the sulfonate group include binding of MES at the phosphate binding site of indole-3-glycerol phosphate synthase (46) and at the active site of dialkyl decarboxylase (47), PIPES at the binding site of bile salt-stimulated lipase (48), and HEPES at the active site of avian sarcoma virus integrase (49), near the active site of carbonic anhydrase (50) and at the active site of the mannoside receptor (51). The only structure at which the primary identifiable interaction is via the amino group is in the AChBP structure, as described above (38).

Case-by-case consideration of the specific interactions in the above examples leads to the following generalization. Where there are direct interactions with amino acid side chains, the sulfonate group tends to interact with the nitrogen of Lys, Arg, and Asn, and the charged amino group tends to interact with hydrophobic/aromatic side chains.

Sequence Localization—To identify potential positions in the connexin amino acid sequence involved in the binding, it would be helpful to know whether the aminosulfonates act from the cytoplasmic or extracellular side of the connexin channel. In the reconstitution system used in this work, protease protection studies show that the connexins reconstitute in the liposome membrane with their cytoplasmic domains outward (35). However, the present studies do not allow determination with certainty whether the aminosulfonates act from the outside or the inside of the liposomes, or from both sides, for the simple reason that taurine and the other aminosulfonates are likely to be permeable through the channels (all are smaller than maltose, a disaccharide known to permeate these channels (6)). When taurine is entrapped in the liposomes during formation or activity is assessed by TSF in its absence, the inhibition is 30% relative to application from the outside (data not shown), which is inconclusive. Cellular work indicates that the effects of pH on connexins are from the cytoplasmic side (52), to which
are exposed the N- and C-terminal domains and a central cytoplasmic loop of connexin.

The AChBP structure and information about sequence determinants of taurine binding to other members of the LGIC superfamily provide some clues regarding localization of the binding site in the connexin sequence. Some of this information was summarized in an earlier report (1), but new information about the LGIC binding site is included below. In nAChRs, the two ligand-binding sites are located at interfaces between two different subunits, with loop domains being contributed from both. It is intriguing to speculate that the aminosulfonate binding site in connexins similarly involves contributions from more than one connexin monomer.

The ligand binding site of the LGICs, confirmed by the AChBP structure, is formed by several discontinuous loop domains (one subunit contributing loops A–C and the other subunit loops D–F). The sequences within these domains show some variability, but the character of the residues in them (size, hydrophobicity, and charge) are consistent across members of the family.

Of the LGICs, the site for taurine binding has been best characterized for the GlyR (53, 54). For the GlyR, in loop A, Asn102 and Glu103 (small-basic) contribute to the agonist binding site for taurine and glycine. In loop B, residues Phe159, Gly160, and Tyr161 (aromatic-small-aromatic) are important for agonist discrimination and antagonist binding and are common to all of the LGICs. Loop C residues Lys200, Tyr202, and Thr204 (cationic-X-aromatic-X-small) are involved in both agonist and antagonist binding.

The amino acid sequences of Cx32 and Cx26 have in common four loop B motifs and three loop C motifs in putative transmembrane and extracellular domains. The differences between the two connexins are as follows: 1) Cx32 has a loop A motif (Asn246-Glu247) in its C-terminal (CT) domain, and Cx26 has a loop A motif in its cytoplasmic loop (CL) (Asn113-Glu114), and 2) Cx26 contains an additional loop B motif (Tyr212-Leu213-Phe214) in its small CT that Cx32 does not. Also, one of the loop C motifs of Cx26 (Lys225-X-Trp226-X-Thr227) partially extends into the cytoplasmic N-terminal domain, unlike that of Cx32. If these loops form the binding pocket for aminosulfonates and their analogs, they contain the elements found to characterize aminosulfonate binding in the crystal structures mentioned above (a preponderance of aromatic plus Lys and Asn residues).

These considerations suggest that taurine and its analogs may interact with the cytoplasmic CL, CT, and perhaps NT regions of Cx26 but not of Cx32. A Cx26-specific taurine binding site could be composed of the CT domain (providing a loop B motif), the CL (providing a loop A motif), and the region of the transition between the NT domain and first transmembrane domain mentioned above (providing a loop C motif). Intriguingly, this region is thought to be at the mouth of the pore (55).

It is also intriguing to note that in Cx32, a E102G mutation makes the channels highly pH-sensitive (56). This creates a loop A motif in the CL (Gly102-Lys103) where one did not previously exist. Also, the CT domain of Cx43 (which is required for its pH sensitivity and can confer some pH sensitivity on Cx32 (57)) contains a loop B motif that is absent in Cx32 that has been identified as essential for pH modulation (Tyr240-Phe241-Ala245-Tyr247) (58).

It is thus possible that aminosulfonates regulate connexin channels by occupying a binding site composed of a part of the CT domain and one or more other domains, perhaps the CL and NT-first transmembrane domain boundary. The effect on channel activity could be directly due to occupancy of the site or to conformational changes caused by coordination of these disparate parts of the connexin molecule.

To summarize, the taurine binding pocket on GlyR mainly consists of aromatic residues. We suggest that the charged amines of the aminosulfonates and their competing analogs bind to connexin at a site that includes the identified LGIC loop motifs via cation-π interactions with aromatic residues and that additional interaction between the charged sulfur-containing moiety and Lys and/or Arg residues in these loops are involved in effecting the inhibition.

Regulation of Connexins by Aminosulfonates—This report demonstrates and characterizes a high degree of chemical specificity for the interactions of aminosulfonates with channels that contain Cx26. The relation between the pH sensitivity of this interaction and the well documented mechanism of pH sensitivity derived from studies of Cx43 studied in oocytes (59–61) is discussed at length in previous work (1, 3). The mechanism proposed from the Cx43 studies posits a pH-dependent interaction between segments of the CT domain and the CL that inhibits channel activity. This mechanism has obtained strong support from combined surface plasmon resonance and NMR studies of pH-dependent conformational changes and interactions between peptides that correspond to these regions (62, 63). On the face of it, this mechanism cannot apply to Cx26 channels, since the CT of Cx26 is relatively short (17 residues) and does not include the regions or sequences of the Cx43 CT on which the pH sensitivity of Cx43 has been shown to depend. Nevertheless, Cx26 channels have been shown to be quite pH-sensitive in Xenopus oocytes (60).

Despite the apparent contradictions, there are two reasonable ways to account for and reconcile these two mechanisms. One is that either protonated aminosulfonate or competent CT (i.e. that of Cx43 or Cx38 but not Cx32) can bind to the receptor domain to close the channel. The other is that to close the channel, a ternary complex must form between all three elements: protonated aminosulfonate, competent CT, and receptor. In cells, cytoplasmic aminosulfonates could be involved. See Ref. 1 or 3 for a more complete discussion.

Alternatively, mechanisms of pH sensitivity may not be common across all connexins (61); truncation of the CT of Cx37, mCx50, Cx45 (60), Cx32 (64, 65), or Cx46 (66) has little effect on pH sensitivity assessed in oocytes. For these connexins, pH dependence may require soluble factors, such as aminosulfonates or pH-dependent kinase activity (67) or direct protonation of other domains, as for Cx46 (68). In addition, there is evidence that pH regulation may differ among species orthologs and in different expression systems (69, 70).

Implications for Cellular Regulation of Connexin Channels—The new information regarding cellular regulation of connexin channels is that 1) cytoplasmic compounds can inhibit some connexin channels (taurine, hypotaurine, L-cysteic acid, L-homocysteic acid, and L-cysteine sulfenic acid), some with greater efficacy than taurine, and 2) several related cytoplasmic compounds can antagonize this inhibition (β-alanine, glycine, GABA, and L-glutamic acid). The finding that inhibitors and their antagonists co-exist in cells has implications for the dynamics of cellular regulation of connexin channels. It suggests that control of junctional channels involves a complex interplay among the various metabolic pathways that generate these compounds, and the activity of their plasma membrane transporters.

Taurine is found in many mammalian tissues and can achieve relatively high cellular concentrations (71, 72). At physiological pH, essentially all taurine is protonated, so connexin channel modulation by taurine would have to be effected by changes in taurine concentration. Cytoplasmic taurine levels are dynamically regulated by transporters (73–77). All of the other cytoplasmic aminosulfonates we have tested would
also be fully protonated at cellular pH, but there may be others with pK_a values in the physiological range, allowing their action on connexin channels to be regulated by changes in pH in addition to changes in concentration. The concentrations of most of the antagonists we identify are known to be dynamically regulated as well.

The interaction among these compounds in cells may be part of the regulation by which cells regulate their connexin channels. In addition, the structure-function analysis using these ligands may lead to implementation of pharmacological and biochemical strategies for exploration of regulatory binding sites and allosteroy in connexin channels.

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Additions and Corrections

Vol. 279 (2004) 8999–9007

Electrophysiological characterization of the human Na+/nucleoside cotransporter 1 (hCNT1) and role of adenosine on hCNT1 function.

Ignacio M. Larráyoz, Francisco Javier Casado, Marçal Pastor-Anglada, and M. Pilar Lostao

Page 9005, Fig. 6A: An incorrect scale is plotted. The correct version is shown below:

A

\[
\begin{align*}
\text{Na}^+ \text{ buffer} \\
0.25 \text{ mM } ^3\text{H-Uridine} \\
Q = 8.4 \times 10^{-5} \text{ Coulombs}
\end{align*}
\]

FIG. 6A

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Biochemical requirements for inhibition of connexin26-containing channels by natural and synthetic taurine analogs.

Liang Tao and Andrew L. Harris

Page 38548: There are two errors in the next to last paragraph in the left-hand column:

Line 3 should read “. . . effect on Cx26/Cx32 channels.”

Line 4 should read “. . . and APA (sulfonate → phosphoryl) . . .”

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.