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Regulation of Connexin Channels by pH

DIRECT ACTION OF THE PROTONATED FORM OF TAURINE AND OTHER AMINOSULFONATES*

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Protonated aminosulfonate compounds directly inhibit connexin channel activity. This was demonstrated by pH-dependent connexin channel activity in Good's pH buffers (MES (4-morpholineethanesulfonic acid), HEPES, and TAPS (3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid)) that have an aminosulfonate moiety in common and by the absence of pH-dependent channel activity in pH buffers without an aminosulfonate moiety (maleate, Tris, and bicarbonate). The pH-activity relation was shifted according to the pK_a of each aminosulfonate pH buffer. At constant pH, increased aminosulfonate concentration inhibited channel activity. Taurine, a ubiquitous cytoplasmic aminosulfonic acid, had the same effect at physiological concentrations. These data raise the possibility that effects on connexin channel activity previously attributed to protonation of connexin may be mediated instead by protonation of cytoplasmic regulators, such as taurine. Modulation by aminosulfonates is specific for heteromeric connexin channels containing connexin-26; it does not occur significantly for homomeric connexin-32 channels. The identification of taurine as a cytoplasmic compound that directly interacts with and modulates connexin channel activity is likely to facilitate understanding of cellular modulation of connexin channels and lead to the development of reagents for use in structure-function studies of connexin protein.

Changes in intracellular pH (pH_i)[1] affect gap junction conductance between cells (1). The sensitivity of junctional conductance to changes in pH_i varies with cell type and connexin isoform (2–5). Decrease of pH_i from physiological levels typically produces a decrease in junctional conductance (6–8) and in permeability to large tracers (9, 10). The decrease in junctional conductance is usually reversible with return of pH_i to normal physiological values. The molecular mechanisms that underlie this modulation of connexin channel activity are unclear and may differ among connexin isoforms and cell types. It has been proposed that the modulation is due to direct protonation of connexin (8), changes in ionized calcium concentration (11), and activation of calmodulin (12–14). For connexin-43 and for connexin-32/connexin-38 chimerae, recent work strongly indicates a pH-dependent interaction between segments of the C-terminal domain and the single cytoplasmic loop that inhibits channel activity (3, 15–21).

In this study, we set out to investigate modulation of connexin channel activity as a function of pH, using in a reconstituted system connexin channels immunoaffinity-purified from native tissues. Channel activity was monitored using transport-specific fractionation (TSF) of liposomes into which connexin channels were reconstituted.

Channel activity in this system was affected by changes in pH. To our surprise, the changes in channel activity were accounted for by the direct action of the protonated form of the aminosulfonate compounds used as pH buffers, rather than of proton concentration itself. There is no evidence for the direct action of pH alone on the activity of these connexin channels in this system in the absence of aminosulfonate compounds. The sensitivity to protonated aminosulfonates was connexin isoform-specific. Heteromeric channels containing connexin-26 (Cx26) in addition to connexin-32 (Cx32) were highly sensitive, whereas homomeric Cx32 channels were not. Taken together with recent work by others on the structural basis of pH sensitivity of connexin channels in cells, these data suggest testable hypotheses for cellular and molecular regulation of connexin channel activity. They also provide an opportunity for development of pharmacological and affinity reagents for structure-function studies of connexin channels. This is the first report of a noncovalent modulatory activity of a biological molecule on connexin channels (see Ref. 22). Preliminary reports of this work have appeared in abstract form (23–25).

EXPERIMENTAL PROCEDURES

Materials

Egg phosphatidylcholine, bovine phosphatidylserine, azolectin (soybean L-phosphatidylcholine), and lissamine rhodamine B-labeled phosphatidylethanolamine were purchased from Avanti Polar Lipids. *N*-Octyl-D-glucopyranoside (octylglucoside) was from Calbiochem. Bio-Gel (A-0.5 m; exclusion limit, 500,000 Da) was purchased from Bio-Rad. CNBr-activated Sepharose beads were obtained from Amersham Pharmacia Biotech. Use and care of animals was according to institutional guidelines.

Immunopurification of Connexin Proteins

Connexin was affinity-purified from an octylglucoside-solubilized crude membrane fraction of rat or mouse liver using a monoclonal antibody against Cx32 as described in Refs. 26 and 27, with the modification that 5 mM EGTA was included in the homogenization and phosphate buffers. Rat liver yields homomeric Cx32, and mouse liver yields heteromeric Cx32/Cx26. Homomeric Cx26 channels were not available from a native tissue source; because Cx26 forms heteromeric channels with Cx32, such a source would have to lack Cx32. Homomeric Cx32 can be obtained from rat liver because the Cx26 content of rat liver is very low. There is no wild-type animal for which the converse is true. It was occasionally possible to obtain fractions of connexin from

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[1] The abbreviations used are: pH_i, intracellular pH; Cx, connexin; CT, C-terminal; NT, N-terminal; P_o, channel open probability; TSF, transport-specific fractionation (of liposomes); CL, cytoplasmic loop; MES, 4-morpholineethanesulfonic acid; TAPS, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid.

mouse liver that varied in Cx32/Cx26 ratio by taking advantage of the finding that more Cx26 elutes from the immunobeads (along with Cx32) in the initial fractions than in later fractions. Except where noted, the heteromeric Cx32/Cx26 channels were from pooled elution fractions.

Gel Electrophoresis, Protein Blots, and Immunoblots

Gel electrophoresis, blotting, and staining of blots were carried out as described in Ref. 27.

Antibodies

The monoclonal antibody (M12.13) used in the immunoaffinity purification and for specific staining of Cx32 on Western blots is directed against a cytoplasmic domain of Cx32 (28).

pH Buffers and pK_a Values

TAPS, HEPES, MES, taurine, and maleate were obtained from Sigma. Because all TSF experiments were carried out at 37 °C, the pK_a values for each compound used in calculations of protonated aminosulfonate concentrations were those either measured at 37 °C (TAPS, 6.0; HEPES, 7.1; MES, 8.1 (29)) or calculated from the value at 25 °C and the measured enthalpy of ionization (taurine, 8.78 (30)). Unless otherwise noted, the pK_a values given in the text are those at 37 °C.

Reconstitution of Purified Connexin into Unilamellar Phospholipid Liposomes

Liposome formation and protein incorporation followed the protocol of Mimms *et al.* (31) as modified by Harris *et al.* (32) and Rhee *et al.* (26). Liposomes were formed by gel filtration of a 1 mg/ml mixture of PC, PS, and rhodamine-labeled PE at a molar ratio of 2:1:0.03 in urea buffer (see below) containing 80 mM octylglucoside and immunoaffinity-purified connexin. The size of the liposomes was monodisperse with an approximate mean diameter of 900 Å, shown by HPLC gel filtration (33). The protein/lipid ratio of the liposomes was typically 1:60 (w/w), corresponding to an amount of connexin equivalent to $<\sim 1$ hemichannel per liposome (see under "Data Analysis," below). Minor variation in the amount of protein used, the amount damaged in purification, the reconstitution efficiency, and the amount of lipid retained on the column produced variations in the percentage of liposomes containing active channels under standard conditions. The protein/lipid ratios used yielded functional channels in 30–50% of the liposomes.

Transport-specific Fractionation

The procedure used to fractionate liposomes into two populations based on sucrose-permeability is described and fully characterized in Harris *et al.* (32, 34) and Rhee *et al.* (26). The principle of using a density shift to fractionate liposomes was adapted from Goldin and Rhoden (35). Liposomes containing functional channels are separated from liposomes without functional channels by TSF achieved by centrifugation through an isoosmotic density gradient formed by urea and sucrose solutions. Urea buffer contained 10 mM KCl, 10 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, 3 mM sodium azide, and 459 mM urea at pH 7.6. Sucrose buffer was identical except that an osmotically equivalent concentration of sucrose (400 mM) replaced the urea. Osmolality of urea and sucrose buffers was 500 mosmol/kg, and their specific gravities (d_4^{20}) 1.0056 and 1.0511.

An aliquot of liposomes was layered on each 4.4 ml gradient. Gradients were centrifuged at 300,000 × g for 2–3 h in a swinging bucket rotor (Sorvall TST 60.4) at 37 °C. Liposome bands were recovered by aspiration. The distribution of the liposomes in the gradient was calculated from the specific intensity of rhodamine fluorescence (Perkin-Elmer 650–10S or L550B spectrofluorometer; 560 nm excitation; 590 nm emission) and the volume of each collected band.

During the 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 equilibrate their internal solution with the external solution and move to a position in the lower part of the gradient corresponding to the density of the liposome phospholipid membrane.

Equilibration of extraliposomal and intraliposomal osmolytes is rapid (milliseconds for these 900-Å-diameter liposomes). Therefore, even a channel that opens only infrequently for brief times will mediate full exchange of osmolytes and cause liposome movement to the characteristic lower position. Calculations show that the assay is insensitive to large changes in channel P_o down to 0.001.

It is formally possible that a modulatory compound could affect the proportion of liposomes that shift to the lower position by restricting the diameter of the pores, rather than moving P_o close to 0. However, for this to occur, the channels would have to become impermeable to urea and to sucrose (liposomes permeable to urea only move to an intermediate position (36, 37)). Such a change in diameter would effectively eliminate the ability of connexin channels to mediate molecular signaling between cells and therefore can be regarded as a decrease in channel activity.

Data Analysis

Correction for More Than One Channel per Liposome—Previous work with the TSF system suggested that the channels distribute among the liposomes in a manner described by the Poisson distribution (26). This means that for a given protein-lipid ratio (λ) in the liposomes, a Poisson distribution accounts for the fraction of the liposomes that have functional channels. When λ is small, essentially all of the liposomes in which there are channels contain exactly one channel. However, as λ increases, the fraction of liposomes with two or more channels increases. When this is the case, a change in the fraction of liposomes in the lower TSF band does not reflect exactly the change in channel activity (*e.g.* liposomes with two channels will move to the lower band unless *both* channels are closed by a given concentration of ligand). This leads to an underestimate of the inhibitory effect of a test condition, which was corrected in the following manner: 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. This calculation transforms the fraction of permeable liposomes in a population into an index of discrete single channel activity.

Normalization of TSF Data—For each preparation of connexin, the percentage of liposomes in the lower band of TSF data was normalized to the maximum value obtained for that preparation. The maximum value was almost always at the highest pH value for the series. 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 buffer for calculation of means and standard errors.

Curve Fitting—The activity data was fit with a four-parameter logistic function of the form $f(x) = a/(1 + \exp(b^*(x - c))) + d$ using the Marquardt-Levenberg algorithm. A Hill equation was not used because the TSF-pH data does not arise from a titration curve, but rather from the superimposition of responses of an unknown number of channel forms with different properties. For this reason, a smooth function was fit to the data to determine a characteristic half-maximal channel activity parameter.

Modeling of Heterogeneity—Channel activity data was modeled assuming that protonated taurine binds to one or more sites on a connexin hemichannel to inhibit channel activity. The calculations assume: 1) the $pK_{a\ 37\ °C}$ of taurine is 8.78 (30), 2) the Cx32/Cx26 channel population consists of five equal subpopulations each having a different taurine dissociation constant (K_d) or cooperativity (n_{Hill}), and 3) channels with $P_o < 0.001$ are binned as closed by the TSF. At each protonated taurine concentration the fraction of active channels is summed over all the subpopulations. Because the number of subpopulations, the way that the K_d or n_{Hill} values are distributed, and the P_o cutoff value are not known, these calculations only demonstrate the general adequacy of the model rather than provide estimates of binding parameters.

For each subpopulation of channels, a P_o *versus* [protonated taurine] relation was calculated from a standard binding isotherm $K_d^n/(K_d^n + [\text{protonated taurine}]^n)$ where K_d is the dissociation constant and n is n_{Hill} as defined above. The binning of the liposomes into active and inactive populations by the TSF was simulated according to (3) above, giving a step function in the activity$_{\mathrm{TSF}}$ *versus* [protonated taurine] relation for each subpopulation. For different binding parameters the step occurs at different [protonated taurine]. Summing the activity$_{\mathrm{TSF}}$ *versus* [protonated taurine] step functions for each subpopulation produces an aggregate activity$_{\mathrm{TSF}}$ *versus* [protonated taurine] relation that is a series of steps. The binding parameters K_d or n_{Hill} were adjusted so that the relation approximated the TSF data.

RESULTS

Connexin was immunopurified from octylglucoside-solubilized crude plasma membranes from rat and mouse liver using a monoclonal antibody specific for Cx32, as previously described and characterized (26, 27, 38). Earlier biochemical and

functional studies have characterized connexin purified in this way from rat liver as homomeric Cx32 hemichannels, and that from mouse liver as heteromeric Cx32/Cx26 hemichannels. The heteromeric channels are functionally heterogeneous with regard to permeability to large molecules, presumably due to heterogeneities of isoform stoichiometry and/or arrangement (27).

The activities of channels formed by Cx32 and Cx32/Cx26 were explored and compared by TSF of liposomes. TSF has been well characterized (32, 34, 36) and effectively used in channel permeability studies (26, 27, 39, 40). In brief, connexin channels are incorporated into the membranes of unilamellar liposomes. When centrifuged in an appropriate isoosmolar density gradient, solute exchange through active channels causes liposomes to become more dense and move to a position deep in the gradient. Liposomes without active channels remain in the upper part of the gradient. Any significant channel open probability (P_o) results in sufficient osmolyte exchange to cause the change in density. Because the change in liposome density can result from brief channel openings, only when P_o changes above or below a very low value are changes in channel activity detected by TSF. TSF is therefore an essentially all-or-none assay of per-liposome channel activity. The technique is more fully described under "Experimental Procedures."

The effects of test compounds on channel activity were assessed by exposing connexin-containing liposomes to the compounds during a TSF spin. 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 fractional change in activity of the population of the channels.

Apparent pH Dependence of Activity of Heteromeric Cx32/Cx26 Channels—To assess the effects of pH on immunopurified, reconstituted connexin channels, activity was assessed in TSF gradients in which the pH was adjusted by addition of strong acid or base. In these initial studies, the reference condition was pH 7.6 in 10 mM HEPES. Activity was assessed at pH 5 and pH 9 and normalized to that at the reference condition. Experiments were carried out using homomeric Cx32, and two fractions of a population of heteromeric Cx32/Cx26 channels with different ratios of Cx32 to Cx26. The Cx32/Cx26 channels were sensitive to pH changes over this range, whereas the homomeric Cx32 channels were essentially insensitive (Fig. 1). Furthermore, the Cx32/Cx26 channel population with the greater proportion of Cx26 was more sensitive than that with less Cx26. Thus, pH sensitivity correlated with Cx26 content. These results suggest that Cx26 is directly responsible for the sensitivity to pH or indirectly confers pH sensitivity on the heteromeric connexin channels. The pH-induced loss of activity with decreased pH in this system is fully reversible, as it is in cells.

Aminosulfonate pH Buffers Directly Modulate Heteromeric Cx32/Cx26 Channels—In the experiment shown in Fig. 1, the one-half maximal effect was near the pK_a of HEPES, the pH buffer in the solutions. To test whether the pH sensitivity was buffer-specific, other pH buffers were used in place of HEPES in the TSF solutions, all at 10 mM. In MES and TAPS, the Cx32/Cx26 channels showed pH effects similar to those seen in HEPES. However, the pH range over which channel activity declined was different for each pH buffer (Fig. 2, *filled symbols*). A smooth function was fit to each data set to define half-maximum values for activity in each buffer. It was found that the half-maximum activity value in each buffer was displaced toward the pK_a of that buffer (see Fig. 3). Half-maximum values for MES, HEPES, and TAPS were at pH 6.1, 7.3, and 8.5, respectively, close to the corresponding buffer pK_a values of 6.0, 7.3, and 8.1 (29, 30). (All TSF experiments were carried out at 37 °C, and all pK_a values given are at that temperature.)

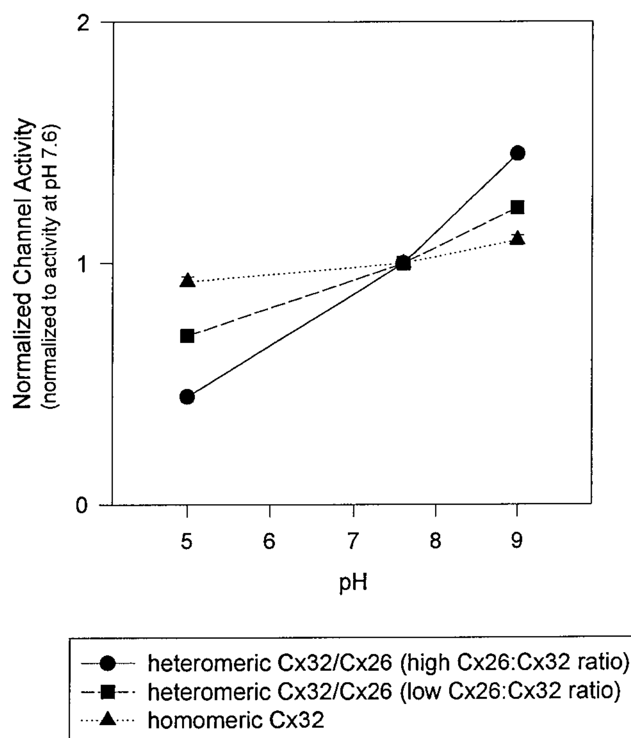

FIG. 1. **Channels containing Cx26 are pH-sensitive in HEPES.** Activity of channels containing various ratios of Cx26 to Cx32 over a range of pHs were compared using the TSF system. Channel activities were normalized to that at pH 7.6. Homomeric Cx32 channels were nearly insensitive to changes in pH over the range 5–9. Heteromeric Cx32/Cx26 channels were much more sensitive, with the sensitivity correlating with Cx26 content. Western blots showed the predominance of Cx32 in the low Cx26:Cx32 sample and the predominance of Cx26 in the high Cx26:Cx32 sample.

Furthermore, pH buffers that were not in this chemical family, such as maleate and Tris (Fig. 2, *open symbols*) and bicarbonate (not shown), elicited no change in channel activity over the same pH range. The pH buffers in which activity was pH-dependent were all Good buffers (41) and are all aminosulfonate compounds (Fig. 3). Chemical precursors of the Good buffers include the non-aminosulfonates 2-bromoethanesulfonate, isethionate, and 2-propanesulfonate. These compounds, and the amino acid glycine, were tested and were without effect.

The data in Figs. 1 and 2 strongly suggest that the observed changes in connexin channel activity are not due to protonation of connexin, but to the action of protonated aminosulfonate compounds on connexin channels that contain Cx26.

Protonated Aminosulfonates Are Connexin Channel Inhibitors—The data in Fig. 2 could be accounted for if either the deprotonated aminosulfonate acts as a channel agonist or protonated aminosulfonate acts as a channel antagonist.

To help distinguish these possibilities, channel activity was assessed at pH 7.6 in 50 mM rather than 10 mM HEPES. At the higher concentration, channel activity was reduced to the level of activity at 10 mM HEPES at low pH (*i.e.* when it is fully protonated) (Fig. 2, *asterisk*). This indicates that the protonated (zwitterionic) form of aminosulfonate buffers acts as a channel antagonist, since in this experiment the concentrations of both the protonated and deprotonated species were increased equally. This point is made more rigorously in the following experiment.

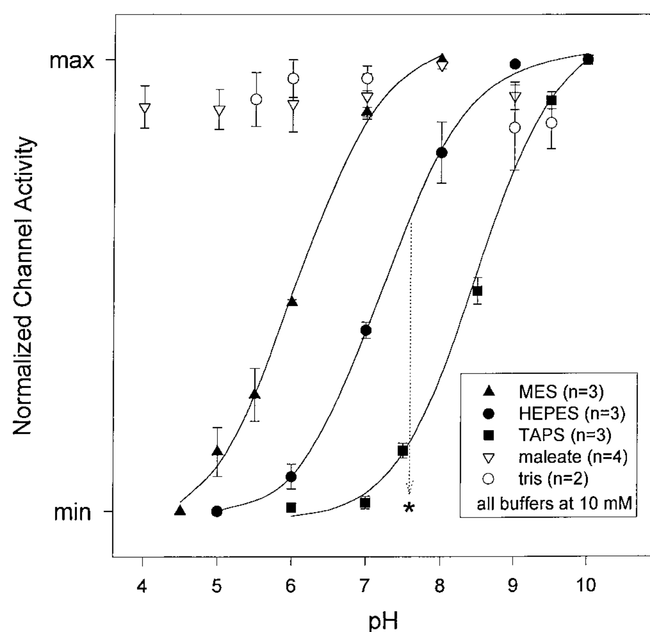

FIG. 2. **Connexin channel activity as a function of pH and pH buffer.** Cx32/Cx26 channel activity was determined over a range of pH in five buffer systems. All buffers were at 10 mM. Four parameter logistic fits show that for the aminosulfonate buffers, the half-maximum values for connexin channel activity were influenced by the pK_a value of each buffer (MES pK_a = 6.0; HEPES pK_a = 7.1; TAPS pK_a = 8.1). There is no such trend, and no obvious pH sensitivity, for the pH buffers that are not aminosulfonates. When the concentration of HEPES was increased to 50 mM but pH kept at 7.6, the channel activity decreased to the same minimal levels achieved at 10 mM HEPES and lower pH (*asterisk*). This indicates that the protonated form of HEPES acts as an inhibitor of channel activity. Channel activities for each protein purification (*n* in *inset legend*) were normalized to the maximum values within each data set. Average activity for data in this figure was 43%, corresponding to ~0.6 functional channels per liposome (λ). Of the functional channels, ~60% were sensitive to aminosulfonates. *Bars* are S.E.

Protonated Taurine Directly Modulates Connexin Channels—The amino acid taurine (2-aminoethanesulfonic acid), a chemical precursor of the Good buffers, is an aminosulfonate and also can function as a pH buffer. Activity of Cx32/Cx26 channels was assessed in 10 mM taurine solutions adjusted to pH values from 7 to 10.5 (Fig. 4*A*, *open circles*). A smooth function fit to the data (*curved line*) shows that the half-maximum value for activity modulation by taurine approximates the pK_a value for taurine, consistent with the findings for the other aminosulfonate buffers (Fig. 2). When taurine concentration was increased to 50 mM at pH 9.1, the channel activity was reduced to the same level as at fully protonated 10 mM taurine (Fig. 4*A*, *asterisk*), as was found for the parallel experiment using HEPES.

Channel activity is plotted as a function of protonated taurine concentration in Fig. 4*B*. The concentration of protonated taurine can be controlled by taurine concentration as well as by pH. Therefore, experiments were carried out at constant pH (at pH 8 and at pH 6), but at taurine levels such that the resulting concentrations of protonated taurine spanned the range achieved by changing the pH of 10 mM taurine from 7 to 10.5 (*i.e.* the same range as in Fig. 4*A*). From the Henderson-Hasselbalch equation, this concentration range of protonated taurine is 9.84 mM at pH 7 to 0.19 mM at pH 10.5. pH values of 6 and 8 were chosen to represent extremes of physiological intracellular pH.

At both pH 6 and pH 8, Cx32/Cx26 channel activity was essentially the same as that at the same protonated taurine concentrations when taurine concentration was constant and pH was varied. Protonated taurine concentration, and not pH, regulates heteromeric Cx32/Cx26 connexin channel activity.

Cx32/Cx26 Population of Channels Is Heterogeneous in Affinity for Taurine and/or Cooperativity of Taurine Effect—Due to the all-or-none nature of liposome fractionation in the TSF assay, it is difficult to derive binding parameters from these studies. Classical binding studies are equilibrium or kinetic measurements, whereas this assay is neither: liposomes are binned by TSF according to whether the channel(s) they contain has a P_o value greater than a threshold level. For this reason, under a given condition, a homogeneous population of channels should either remain entirely at the upper position or move entirely to the lower position, producing a step change in the activity-[ligand] relation at the ligand concentration at which P_o becomes detectable by the TSF.

This is clearly not the case; intermediate values (*e.g.* partial effects at intermediate protonated taurine concentrations) are seen. This could arise from heterogeneity in the properties of the channels. The Cx32/Cx26 channel population is known to be heterogeneous with regard to molecular selectivity (27). This has been attributed to channels in this population being composed of several isoform stoichiometries and/or arrangements. This same structural heterogeneity could give rise to heterogeneities in pharmacological sensitivity as well. The summed activity contributions of several populations of channels with distinct ligand sensitivities would produce the intermediate channel activity data points seen.

The heterogeneity of the Cx32/Cx26 channels with regard to aminosulfonate sensitivity was qualitatively verified by experiments utilizing different fractions eluted from the immunobeads during purification. We observed that more Cx26 elutes (along with Cx32) in the initial fractions from the immunobeads than in later fractions. With care, it was sometimes possible to collect two fractions with substantially different Cx32:Cx26 ratios (the data in Fig. 1 was obtained in this manner). Using such fractions, it was established that Cx26 content correlated with a rightward shift along the pH axis (indicating greater sensitivity to protonated aminosulfonate), as well as a greater fraction of the channels being pH-sensitive. Therefore, the sigmoid relation between pH and TSF channel activity in Figs. 2 and 4*A* is not a titration curve, but rather the superposition of responses of several channel forms with different properties. (For this reason, it is inappropriate to fit the pH data with the Hill equation, as it suggests erroneous notions of the underlying mechanisms. Instead, a smooth logistic function was fit to the data solely to determine a characteristic half-maximal channel activity parameter.)

To show that simple heterogeneities in binding parameters can account for the findings, the channel activity data was modeled assuming that the Cx32/Cx26 channel population was composed of several subpopulations, each of which had a distinct dose-response relation for inhibition by protonated taurine. The data could be accounted for by the subpopulations differing in either affinity for taurine (K_d) or cooperativity of taurine effect (n_{Hill}) (see under "Experimental Procedures" for details). The calculations are for an arbitrarily simple set of assumptions and demonstrate only the general adequacy of the model, suggesting possible ranges of K_d or n_{Hill} for the given assumptions. The calculations shown are for five equal subpopulations of channels, each corresponding to a different Cx32/Cx26 stoichiometry (5:1, 4:2, 3:3, 2:4, 1:5; homomeric Cx32 and Cx26 channels are not present (27)).

Fig. 5*A* illustrates calculations in which each subpopulation has a constant K_d but different n_{Hill} constrained to be between 1 and 5. A K_d that gives a good fit for these assumptions is 3.2 mM. Fig. 5*B* illustrates calculations in which each subpopula-

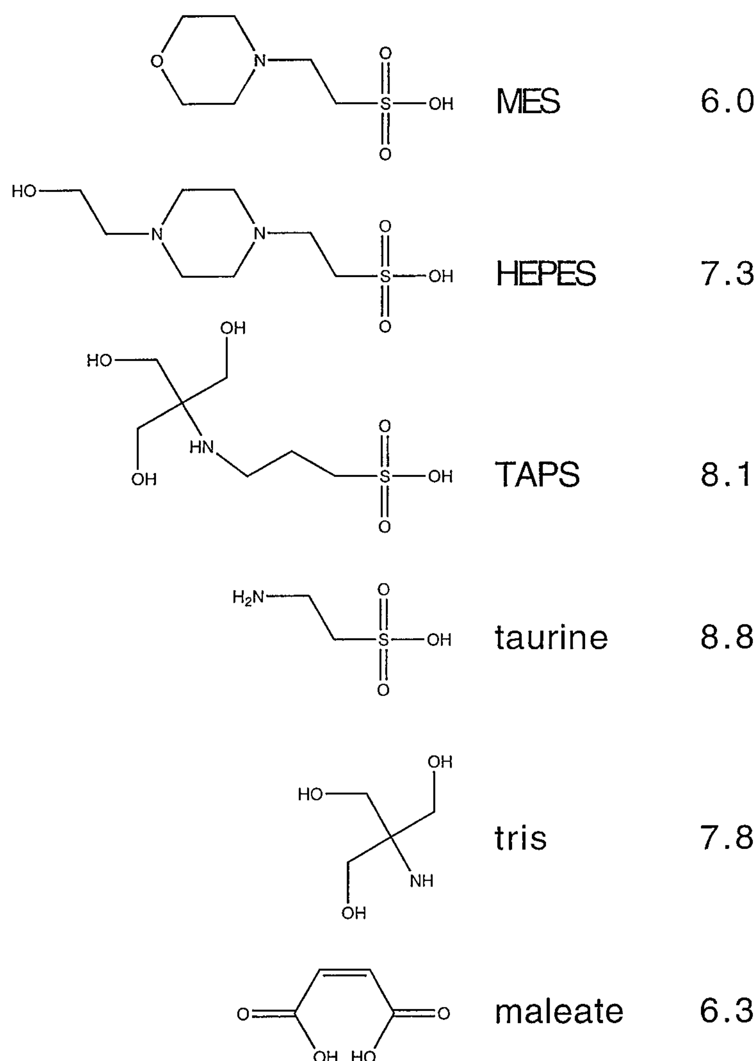

FIG. 3. **Chemical structures of the pH buffers.** MES, HEPES, and TAPS are commonly used Good buffers (41). They share the common structural motif of a protonatable amine moiety that is separated from an ionized sulfonate moiety by two or three methylene groups. This functional motif derives from the naturally occurring precursor compound, the β amino acid taurine.

tion has a different K_d and no cooperativity ($n_{\mathrm{Hill}} = 1$). Values of K_d for the illustrated fit to the data range between 1 and 10 μM. The TSF activity data can be accounted for by either scheme; at present, one cannot distinguish between these two pharmacological heterogeneities.

When Connexin Channels from Rat Liver Show Aminosulfonate Sensitivity, It Is because They Contain Cx26—On rare occasions, a preparation of immunopurified connexin from rat liver showed a substantial sensitivity to the aminosulfonate compounds. This correlated with the presence of detectable amounts of copurified Cx26 on immunostained Western blots (Cx26 is not typically present in such preparations). The copurification of Cx26 with Cx32 from rat liver occurred rarely. It is not known what influences the levels of Cx26 that copurify in these preparations; it may result from variations in the physiological state of the tissue or animal. This result suggests that heteromeric Cx32/Cx26 channels can exist in rat liver. It also indicates that the modulation of channel properties by aminosulfonates that occurs for connexin from mouse liver can also occur in rat liver when Cx26 levels are increased, as may occur following hepatic trauma (42, 43).

DISCUSSION

The data presented here demonstrate a direct modulatory effect of protonated aminosulfonates on connexin channel activity. The significance of this finding is 2-fold: 1) it identifies for the first time a class of cytoplasmic compounds that directly and reversibly regulates connexin channel activity, and 2) it suggests a mechanism by which changes in pH_i can affect connexin channel activity. The active compounds all share a common structural motif: a protonatable amine moiety separated from an ionized sulfonate moiety by two or three methylene groups. It is possible that compounds containing other ionized sulfur-containing moieties (*i.e.*, sulfinyl groups) would also be effective. The sensitivity to aminosulfonate depends on isoform composition of the channels, and requires that Cx26 be present.

The data suggest that at least some of the effects on connexin channels that have been attributed to direct action of low pH_i are mediated instead by protonated aminosulfonates. The data provide no evidence for an effect on connexin channel activity due to protonation of connexin. It is possible that pH has direct effects not revealed in this experimental system and that it can act directly on other connexins. On the basis of this data one cannot assert that taurine is a biological regulator of connexin channels (other cytoplasmic aminosulfonates may have higher affinities and therefore act in different pH ranges), only that it is present, and protonated, in cells at levels sufficient to have an effect.

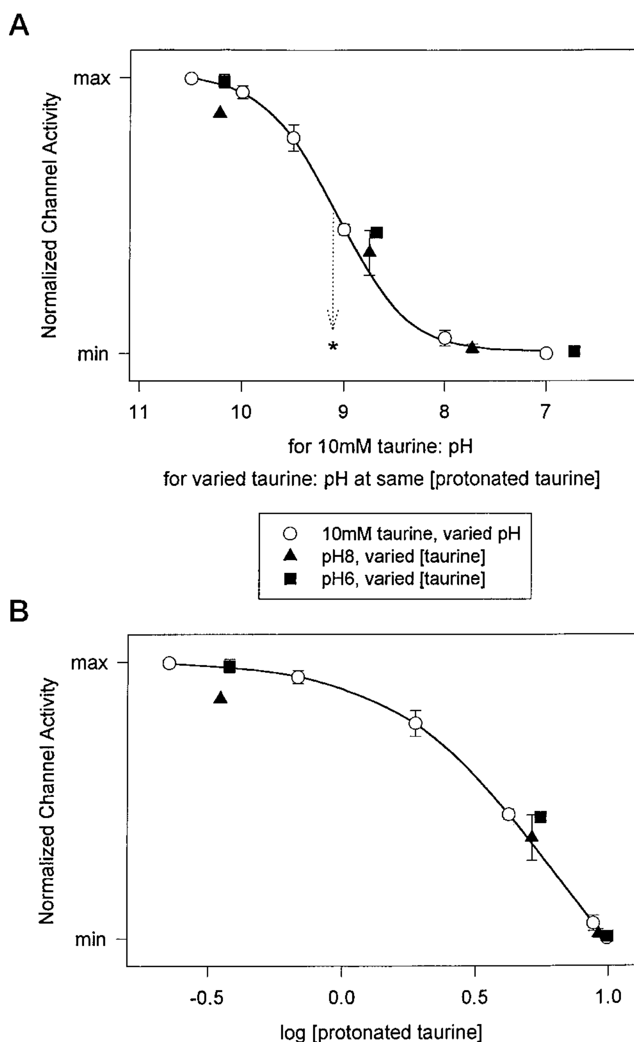

FIG. 4. **Protonated taurine modulates connexin channel activity.** Activity of Cx32/Cx26 channels was determined in 10 mM taurine over a range of pH (*open circles*). Channel activity was also determined at constant pH (pH 6, *squares*; pH 8, *triangles*) but at taurine concentrations that resulted in levels of protonated taurine that spanned the same range as when pH of 10 mM taurine was varied. The channel activity was the same whether the concentration of protonated taurine was controlled by changes in pH (*open symbols*) or by changes in taurine concentration (*solid symbols*). *Bars* are S.E. for six protein preparations. In *A,* the data are plotted as function of pH. The *asterisk* is the activity at 50 mM taurine at pH 9.1. The data obtained at constant pH (*squares* and *triangles*) are plotted at the pH that corresponds to the appropriate protonated taurine concentration. In *B,* the data are plotted as a function of protonated taurine. Both curves are four-parameter logistic functions fit to the open symbols. The changes in channel activity are fully accounted for by changes in protonated taurine concentration alone.

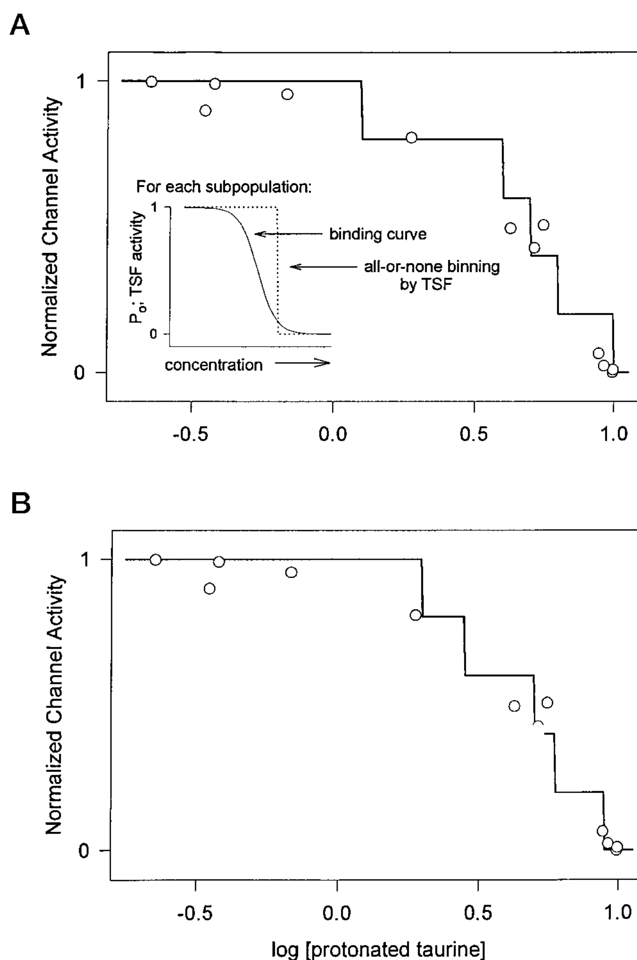

FIG. 5. **Modeling of the channel activity.** The relationship between protonated taurine concentration and channel activity in the Cx32/Cx26 channel population was modeled as described under "Experimental Procedures." Calculations are for five equal subpopulations that differ in either K_d or n_{Hill}. The *open symbols* are the means of the taurine data presented in Fig. 4. *A,* calculations for subpopulations with constant K_d and different n_{Hill}s, with $1 \leq n_{\text{Hill}} \leq 5$. Fit to the data (*solid line*) is for n_{Hill} values of 2.4, 2.8, 3, 4, and 5 and K_d of 3.2 mM. *B,* calculations for subpopulations with $n_{\text{Hill}} = 1$ and different K_d values. Fit to the data (*solid line*) is for K_d values of 1.3, 4.0, 5.0, 6.3, and 10 μM. The calculations only demonstrate that the TSF activity data can be accounted for by the model described in the text rather than providing estimates of binding data. The particular values for n_{Hill} and K_d used to generate these curves are not unique, and they only suggest possible ranges. The *inset* in *A* shows how the behavior of each subpopulation was modeled. The solid curve is a binding isotherm $K_d^{\text{nHill}}/(K_d^{\text{nHill}} + [\text{protonated taurine}]^{\text{nHill}}$ representing the relation between channel activity and protonated taurine concentration. The *dotted line* shows how the smooth relation is transformed by the TSF into a step function (threshold P_o is 0.1 in this illustration). For the five subpopulations modeled here, the step functions of the subpopulations are summed.

Site(s) of Aminosulfonate Action—Because on the basis of size it is likely that taurine can permeate connexin channels, sites accessible from either the inside or outside of a liposome would be accessible by external taurine, regardless of the orientation of the protein in the membrane.

Taurine binds to many proteins, the most prominent of which are the inhibitory glycine receptor (44–46) and amino acid transporters (47). Although the structure of the taurine binding site on the glycine receptor is not known, the most important sequence elements have been defined (48). The site is thought to be formed by at least two noncontiguous segments. The amino acid segments are: (*a*) an aromatic-small-aromatic sequence (159–161; loop-2) common to the ligand/gated ion channel family, and (*b*) a cationic-X-aromatic-X-small sequence (200–204; loop-3). Inspection of the amino acid sequences of Cx32 and Cx26 shows that they have four loop-2-like motifs and three loop-3-like motifs (connexin sequence alignments and numbering are from Ref. 49). All are in the putative transmembrane and extracellular domains. However, Cx26 contains an additional loop-2-like motif (YLF, 212–214) in its small C-terminal cytoplasmic domain that Cx32 does not. Also, one of the loop-3-like motifs of Cx26 (K·W·T, 22–26) partially extends into the cytoplasmic N-terminal (NT) domain, unlike that of Cx32.

These similarities are not strong. However, if they are significant, they suggest the possibility that both connexins may interact with taurine in the pore and that the C-terminal (CT) region of Cx26, but not Cx32, may interact with taurine, per-

haps in concert with other domains. If the latter is the case, a taurine binding site could be composed of the CT domain (providing a loop-2-like motif) and the region of the transition between the NT domain and first transmembrane domain mentioned above (providing a loop-3-like motif). The NT-first transmembrane domain transition is thought to be at the mouth of the pore (50). The CT domain of Cx32 does not contain any of the motifs, which, in this hypothesis, is the reason it is unresponsive to aminosulfonate. Intriguingly, the CT domain of connexin-43 (which is required for its pH sensitivity and can confer some pH sensitivity on Cx32 (17)) contains two loop-2-like motifs, one of which is precisely aligned with that of Cx26 (YVF, 230–232) and the other of which has been positively identified as essential for pH-modulation (YAY, 265–267) (18).

It is thus possible that aminosulfonates regulate connexin channels by occupying a binding site composed of a part of the CT domain and another domain, perhaps at the NT-first transmembrane domain boundary. The effect on channel activity could be due to occupancy of the site or to the conformational changes caused by coordination of disparate parts of the connexin molecule. These possibilities may be tested directly by molecular biological approaches.

The amino acid sequences of several high affinity taurine transporters have been determined, but the residues that interact with taurine have not yet been identified (51–55). The affinities of these transporters for taurine range from 4 to 40 μM, and there are several regions of potentially significant homology with connexins. An invertebrate odorant receptor has been recently shown to have two high affinity binding sites for taurine (K_d values of 18 pM and 6 μM), but the amino acid sequence has not yet been determined (56).

Nature of Aminosulfonate/Connexin Interaction—The calculations in Fig. 5 show how connexin channels with (*a*) single binding sites of different affinities ($n_{\text{Hill}} = 1$; K_d variable), or (*b*) multiple binding sites of identical affinity but different degrees of cooperativity (n_{Hill} variable; K_d constant), could lead to pH-dependent connexin channel activities consistent with the data. A key element is the functional heterogeneity of the heteromeric channel population, which has been established and seems to arise from variation in isoform stoichiometry and/or arrangement (27). The possibility of different subunit isoform stoichiometries or arrangements suggests a basis for heterogeneity of ligand response. It also suggests that cellular control of isoform composition could control the responsiveness to aminosulfonates and changes in pH_i.

The relative potency of the aminosulfonates in affecting connexin channels is indicated by the relation between the activity curves and the pK_a values. For all the aminosulfonates except TAPS, the pH at which the effect was half-maximal in 10 mM buffer was at or below the corresponding buffer pK_a. For TAPS, the half-maximal pH was a full 0.4 pH unit basic relative to the pK_a. Thus, a lower concentration of protonated TAPS than the other compounds achieved the same effect. Inspection of the molecular structures shows that TAPS is the only compound tested with three methylene groups between the protonatable amine and the ionized sulfonate moiety. It is possible that this structural difference contributes to the increased efficacy.

Why Are Some Heteromeric Cx32/Cx26 Channels Not Aminosulfonate-sensitive?—Not all Cx32/Cx26 channels show sensitivity to protonated aminosulfonates over the range of concentrations tested. When protonated aminosulfonate concentration was increased to ~25 mM (experiments using 50 mM HEPES or taurine near their pK_a values), the inhibition was no greater than that at 10 mM (experiments using 10 mM pH buffers at low pH). A possible explanation is that a minimum number of Cx26 monomers must be present in the hexameric channel for the channel to be sensitive to aminosulfonates.

The apparent aminosulfonate-insensitive component could arise in other ways as well. If the model based on several K_d values applies, the apparently aminosulfonate-insensitive channels could represent a population of channels with substantially lower affinity for aminosulfonates. If the model based on several cooperativities applies, the insensitive channels could correspond to a population of channels with low n_{Hill} or negative cooperativity. In either case, if the protonated aminosulfonate concentration were to be increased substantially beyond that used in this study, a greater fraction of the channels would be expected to be inhibited.

Heterogeneity of binding parameters is most likely to arise from the structural heterogeneity known to exist in this population of channels but could additionally arise from differences in phosphorylation states of Cx32 (Cx26 is not a phosphoprotein), or possibly from proteolytic degradation of a domain responsible for mediating the action of aminosulfonate binding.

Relation to Apparent pH Sensitivity of Connexin Channels in Cells—Cytoplasmic acidification is known to inhibit cell-cell coupling (5). The molecular basis for this is not well understood, but recent work in the paired *Xenopus* oocyte expression system suggests a molecular mechanism for pH sensitivity of connexin channels. A proposed model for the pH sensitivity of Cx43 (4) is consistent with the aminosulfonate interaction with heteromeric Cx32/Cx26 channels described here. It involves a low pH-facilitated interaction of the cytoplasmic Cx43 CT domain with a receptor domain elsewhere in the connexin molecule that causes the channel to close. His^{95}, which is in the cytoplasmic loop (CL) of nearly all connexins, is proposed to be part of the receptor (16). There could be a receptor in each connexin monomer (*i.e.* six per hemichannel) or a single receptor could be formed by parts of all six monomers.

In *Xenopus*, Cx32 channels are only minimally pH-sensitive (3, 19), consistent with our data. From studies of chimerae of Cx32 and Cx38 (which is more pH-sensitive than Cx32), a model of pH sensitivity has been proposed (12) that is mechanistically similar to that proposed for Cx43, though it differs in the details. It involves a receptor domain in the N-terminal half of the CL that interacts competitively in a pH-sensitive manner either with the C-terminal half of the CL to close the channel or with a proximal region of the CT domain, which does not close the channel. A Cx43 CT peptide can enhance the degree of pH sensitivity of Cx32 channels, suggesting conservation of receptor structure across connexin isoforms (17). However, Cx32 is in a different subclass of connexins than Cx43 and Cx38 (57), so the molecular mechanisms of pH sensitivity may differ.

In both models, protonation favors reflexive interactions between noncontiguous domains of connexin molecules: a region of the CT domain interacts with a receptor region of the CL in a pH-dependent manner to close the channels. Based on our data, and the consensus view from the *Xenopus* system, we propose two possible mechanisms.

One mechanism is that either protonated aminosulfonate or competent CT domain (*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 complex must form between all three elements: protonated aminosulfonate, competent CT domain, and receptor. Each hypothesis is consistent with the available data and has distinct consequences and predictions (Fig. 6).

In cells, the first mechanism involves a competition between the available CT domains and the available cytoplasmic protonated aminosulfonate. Interaction between the CT domain and receptor would be pH-sensitive. An ineffective Cx32 CT domain would account for the relative pH insensitivity of homomeric

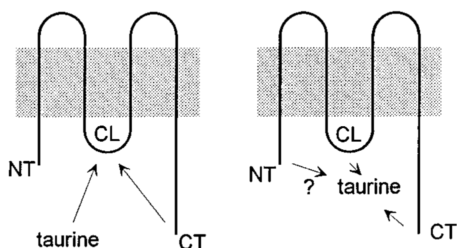

FIG. 6. **Two mechanisms for aminosulfonate action.** Two mechanisms for aminosulfonate action are diagrammed. *Left,* aminosulfonate (*e.g.* taurine) and C-terminal region of connexin (*CT*) independently interact with a receptor domain in the CL to effect channel closure. *Right,* aminosulfonate and the CT domain form a complex that interacts with other connexin domains (*e.g.* the CL and/or the N-terminal region (*NT*)) to effect channel closure.

Cx32 channels, because it would behave as a competitive blocker in binding to the receptor but not effecting channel closure. The increased pH sensitivity with Cx26 content that we see would be due to the absence of a significant CT domain on Cx26; with increased Cx26 in Cx32/Cx26 hexameric hemichannels, the number of Cx32 CT domains present decreases, and access to the receptor increases, permitting aminosulfonates in the solution (or cytoplasm) to interact with the receptor and effect closure. In this view, Cx26 subunits would either effectively decrease the local Cx32 CT domain "blocker" concentration, and/or act as "spacers" to alleviate steric crowding by Cx32 CT domain.

For the second mechanism, pH-dependent channel closure requires both aminosulfonate and competent CT domain. The aminosulfonate could interact directly with both other elements, or could bind to one to enable binding of the resulting two-element complex to the remaining element. In this case, the pH insensitivity of homomeric Cx32 would arise from an inability of the Cx32 CT domain to form this complex, rather than from steric crowding or occupancy of the receptor as above. In cells, pH sensitivity of Cx32 is enhanced by co-expression of the Cx43 CT domain because it provides a competent CT domain that can now effectively interact with both the receptor and cytoplasmic aminosulfonate. For this mechanism to account for the pH sensitivity of the Cx32/Cx26 channels in our studies, one must postulate that the small Cx26 CT domain can participate in this complex formation (perhaps via the loop-2-like motif mentioned above).

Both hypotheses predict that because Cx26 lacks a bulky CT domain, channels formed by homomeric Cx26 in cells should be pH-sensitive (*i.e.* allow freer access to the receptor by cytoplasmic modulators). The published Delmar model, which relies exclusively on a full CT domain for pH sensitivity, if applied to Cx32 and Cx26, predicts that homomeric Cx26 would be pH-insensitive. Recent unpublished work from the Delmar group has established that homomeric Cx26 channels are in fact highly pH-sensitive,[2] as our models predict.

Functional interaction between aminosulfonate and the CT domain does not require that they act at the same site: the possibility of homotropic or heterotropic cooperative linkages allows for interaction of the effects of the ligands without requiring competition for the same binding site. For either mechanism, "pH regulation" of connexin channels between cells would be modified by application of exogenous aminosulfonates.

In cells, CT domain-truncated Cx32 is not as pH-sensitive as Cx26 (58). This suggests that for these two isoforms the properties of the receptor domains differ, or the short CT domain of Cx26 plays a role that the Cx32 CT domain cannot. If the former, the Cx32 CT domain could be effective at a Cx43 or Cx26 receptor.

It is possible that the CT domain interacts with the connexin pore in a manner analogous to that of N-type inactivation of potassium channels, in which a single bound CT "particle" occludes the pore, interacting with receptor regions from several subunits (59). For potassium channels, one particle-receptor complex is sufficient to block the channel. It is unlikely that a single taurine could serve the same steric function in the wide connexin pore. Therefore, for the analogy to hold, taurine molecules would bind to several subunits and collectively occlude the pore. This would be consistent with $n_{\text{Hill}} > 1$ and inconsistent with multiple affinities. On the other hand, a single taurine molecule could coordinate or permit binding of one or more CT domains to occlude the pore, consistent with $n_{\text{Hill}} = 1$ and multiple affinities.

pH-mediated Sensitivity to Aminosulfonate Compounds in Cells—The demonstration that the naturally occurring amino acid taurine directly modulates connexin channel activity is intriguing. Taurine has diverse biological functions in mammalian tissues. It is found at relatively high cellular concentrations, up to 50 mM (25, 60). Cellular modulation of taurine concentration could directly modulate the activity of connexin channels (at physiological pH, essentially all taurine is protonated).

Presently, there is no evidence that taurine is an endogenous modulator of connexin channels in cells. Other cytoplasmic aminosulfonates may be more effective and therefore act over different ranges of pH. A host of cytoplasmic compounds have structures that include the motif common to the active agents. These include taurochloric acid, cysteic acid, homocysteic acid, tauropine, taurocyamine, *N*-phosphotaurocyamine, *N*-phosphohypotaurocyamine, taurolithocholate, homotaurine, and 5-glutamyl-taurine. Closely related compounds include cysteinesulfinic acid, homocysteinesulfinic acid, homohypotaurine, and 3-sulfinoalanine. The efficacies of these compounds in inhibiting connexin channels are unknown.

There is evidence for changes in pH_i under physiological conditions that could affect the protonation of aminosulfonates with pK_a values near resting pH_i. For example, with neuronal activity, glial pH_i can undergo substantial alkalization (up to 0.4 pH unit), followed by a rebound acidification (up to 0.2 pH unit from resting levels) (61) as the extracellular pH is regulated. This could produce substantial changes in the level of a protonated modulator, which would presumably enhance glial-glial coupling and spatial buffering during the acute phase of neuronal activity. pH sensitivity of glial and neuronal gap junctions in the nervous system is well established (10), and it is known that in astrocytic gap junctions, Cx26 and Cx43, but not the pH-insensitive Cx32, are present (62).

The direct aminosulfonate effect on connexin channels described here is likely to both facilitate development of pharmacological tools for study of connexin channels and lead to greater understanding of the cellular mechanisms of intercellular communication.

[2] M. Delmar, unpublished observations.

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