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TITLE PAGE

2-APB Directly Inhibits Channels Composed of Connexin26 and/or Connexin32*

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RUNNING TITLE PAGE

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Abbreviations:

2-APB, 2-aminoethoxydiphenyl borate; Cx26, connexin26; Cx32, connexin32; Cx26/Cx32, heteromeric Cx26/Cx32 channels; DPBA, diphenylboronic anhydride; DPDM, diphenhydramine; DPTTF, 2,2 diphenyltetrahydrofuran; HA, hemagglutinin epitope; IP_3, inositol triphosphate; OG, *n*-octyl-β-D-glucoside; P_o, channel open probability; TSF, Transport-Specific Fractionation

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ABSTRACT

2-aminoethoxydiphenyl borate (2-APB), a commonly-used blocker of IP_3-induced calcium ion release and of store-operated channels, inhibits gap junction conductance when applied to cultured cells. The character and pharmacology of this inhibition was explored using hemichannels composed of connexin32 (Cx32) and/or connexin26 (Cx26) purified from native sources, and from transfected HeLa cells in which the connexin had a cleavable C-terminal epitope tag, in addition to the corresponding junctional channels. Using reconstituted hemichannels in a liposome-based transport-specific fractionation assay (TSF), 2-APB reversibly inhibited homomeric Cx32 and heteromeric Cx26/Cx32 channels from native tissue, as well as their tagged forms from HeLa cells. The $IC_{50\text{-}TSF}$ of the inhibition was ~47 μM at pH 6.5. 2-APB did not inhibit homomeric tagged Cx26 channels, even after tag cleavage (leaving several amino acids at the carboxy-terminus). Protonated 2-APB is the inhibitory agent, but channel sensitivity to 2-APB also increases as pH is lowered. To help define the chemical requirements for inhibition, the effects of four structural analogs of 2-APB were determined. The inhibitory action of 2-APB was shown to be distinct from that of aminosulfonates. 2-APB and its analogs except phenytoin inhibited dye-coupling through junctional channels formed by all the tagged channel forms except Cx26, consistent with the TSF studies. However 2-APB significantly inhibited dye coupling between cells expressing untagged Cx26, suggesting that an unmodified C-terminus is required for action on Cx26 channels. These results show that protonated 2-APB directly and reversibly inhibits connexin channels composed of Cx26 and/or Cx32, and suggest involvement of the carboxy-terminal domain.

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INTRODUCTION

Connexin channels, which compose most gap junctions in vertebrates, mediate direct intercellular movement of cytoplasmic signaling molecules. There are ~20 isoforms of connexin protein, each of which forms channels with distinct regulatory and permeability properties (Harris, 2001; Willecke et al., 2002). The intercellular signaling mediated by connexin channels is important - every functional deletion of a connexin isoform produces a distinct pathology (Willecke et al., 2002; Wei et al., 2004). Despite the importance of gap junction channels in development, physiology and disease, little is known about *direct* reversible regulation of connexin channel activity by ligands; while many compounds affect junctional coupling when applied to cells, in almost all cases it is unclear or unlikely that the compounds (including most "gap junction blockers") act directly on the connexin protein. The absence of directly-acting reagents has been a substantial handicap to progress in the study of connexin channel structure-function. Identification of such reagents and their molecular modes of action would be of considerable value and utility.

Connexin channels are homo- or hetero-oligomers of isoforms of connexin protein. 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 many cellular processes (Ebihara, 2003; Goodenough and Paul, 2003). Gap junction channels span two plasma membranes and are end-to-end dimers of hemichannels. The intercellular location of gap junction channels presents challenges for detailed biophysical or pharmacological investigation; hemichannels are more accessible for study. Though there are some exceptions, the properties of junctional channels can be

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reasonably well predicted from those of their component hemichannels (*cf*. (Ebihara et al., 1995; Harris, 2001; Beahm and Hall, 2002).

2-APB is a membrane-permeable reagent widely used to block IP_3-induced calcium release and store-operated calcium channels, typically with EC_{50}s in the low micromolar range (Maruyama et al., 1997; Prakriya and Lewis, 2001). At the same concentrations, it also selectively activates or inhibits various TRP channels (Hu et al., 2004). Above 50 μM or 100 μM, 2-APB can affect other channels and transporters (Bilmen et al., 2002; Wang et al., 2002; Xu et al., 2005). Its actions are noncompetitive. Its site of action is unknown; it may act directly on some targets and via upstream regulators on others. It does not appear to act on any channel by direct pore block.

Recently, 2-APB was reported to rapidly and reversibly block junctional conductance when applied to cultured cells with IC_{50} of 5-10 μM without affecting other membrane currents (Harks et al., 2003; Hashitani et al., 2004; Griffith et al., 2005). As for its action on IP3 receptors, this effect was best induced by extracellular application. These properties appear to distinguish 2-APB from the multitude of lipophilic agents widely used to inhibit gap junctions (Harris, 2001; Rozental et al., 2001), which have many nonspecific effects, and suggested the possibility that it acts *directly* on connexin channels.

In this study, the effect of 2-APB on connexin channel activity was characterized using reconstituted connexin hemichannels obtained from native tissues and stably-transfected cells (in which case the connexin had a thrombin-cleavable C-terminal epitope tag; "T"). The effects of 2-APB on dye-coupling of gap junctions formed by the same connexins, tagged and

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untagged, were also determined.The data show that 2-APB directly and reversibly inhibits purified and reconstituted hemichannels composed of homomeric Cx32 and heteromeric Cx26/Cx32, with or without the epitope tag, with inhibitory potency depending on concentration and pH. The epitope-tagged homomeric Cx26 channels (Cx26T) were insensitive in this assay, even after enzymatic tag cleavage (which leaves four additional amino acids, part of the cleavage site, at the C-terminus). Protonated 2-APB is the inhibitory form, but pH also modulates connexin sensitivity to 2-APB. Antagonists of aminosulfonate inhibition of Cx26-containing channels (Tao and Harris, 2004) were without effect on 2-APB inhibition, suggesting action at a distinct modulatory site. Junctional dye coupling studies using cells expressing the same epitope-tagged connexins yielded the same results as the reconstituted hemichannels. However, 2-APB significantly inhibited dye coupling between unmodified Cx26 channels (without the C-terminal tag). The data show that both connexin isoforms are sensitive to 2-APB, but that modification of the very short C-terminus of Cx26 interferes with the inhibition, whereas the same modification of the C-terminus of Cx32 is without effect.

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MATERIALS AND METHODS

Materials - Lipids (egg phosphatidylcholine, bovine brain phosphatidylserine, and lissamine rhodamine B-labeled egg phosphatidylethanolamine) were from Avanti Polar Lipids (Alabaster, IL). *n*-octyl β-D-glucoside (OG) was from Calbiochem (San Diego, CA) or Glycon Biochemicals (Luckenwalde, Germany). Bio-Gel (A-0.5m, 100–200 mesh, exclusion limit 500 kDa) was from Bio-Rad (Hercules, CA). 2-Aminoethoxydiphenylborate (2-APB) was from Calbiochem. Calcein-AM and CM-DiI were from Molecular Probes (Eugene, OR). Cell culture reagents were obtained from Gibco BRL, Life Technologies (Rockville, MD). All other reagents were from Sigma (St. Louis, MO) unless stated otherwise.

Purification of untaggd homomeric Cx32 and heteromeric Cx26/Cx32 hemichannels from rodent liver - Connexin was immunopurified from OG solubilized crude plasma membranes from rat or mouse liver using a monoclonal antibody specific for Cx32 (M12.13), as previously described and characterized (Rhee et al., 1996; Bevans et al., 1998). The purification protocol includes high-stringency rinsing with 1M salt to release associated proteins. Previous biochemical and functional studies have shown that hemichannels purified in this manner from rat liver are predominantly homomeric Cx32 and from mouse liver are heteromeric Cx26/Cx32. The heteromeric channels are functionally heterogeneous, presumably because of heterogeneities of isoform stoichiometry and/or arrangement (Bevans et al., 1998; Bevans and Harris, 1999a). The amino acid sequences of rat and mouse Cx32 are identical; the amino acid sequences for Cx26 differ by conservative substitutions at two positions (rat*nn*mouse, Y68H and I215V).

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Cell lines and cell culture - The cell lines expressing tagged connexins were described and characterized previously (Koreen et al., 2004). Stable HeLa cell lines were used, each line expressing one or two connexins under the control of a bidirectional tetracycline-inducible promoter. In the lines expressing a single connexin, the connexin coding sequence was followed by a sequence coding for a thrombin-cleavable C-terminal epitope tag (T; 3.2 kDa) consisting of a hemagglutinin (HA) epitope followed by a 6x(His-Asn) sequence. In the lines expressing two connexins, only one of the connexins was tagged. Thus four cell lines were used: two expressing the homomeric channels Cx26T and Cx32T, and two expressing the heteromeric channels Cx26/Cx32T and Cx26T/Cx32.

The transfected HeLa cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/ml G418 sulfate and 200 μg/ml hygromycin B. Connexin expression prior to purification or dye transfer experiments was induced with 1 μg/ml doxycycline for 48 hours.

A HeLa cell line constitutively expressing untagged Cx26 was a generous gift from Dr. Gina Sosinsky (Elfgang et al., 1995). This cell line was grown at 37 °C in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml puromycin.

Purification of epitope tagged homomeric Cx26, Cx32 and heteromeric Cx26/Cx32 hemichannels from transfected HeLa cells - The purification procedure was that described in (Koreen et al., 2004). Following induction of connexin expression, the cells were solubilized

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using 80 mM OG. The supernatant was incubated with agarose-immobilized anti-HA mouse IgG clone HA-7 (Sigma). Following extensive rinsing, including with 1M salt, the bound protein was eluted by a brief pulse to pH 4.0.

Thrombin cleavage of tagged connexin - The epitope tag was cleaved enzymatically. To 200 µl of purified protein (pH>7), 5.0 units of thrombin (restriction grade; Novagen (Madison; WI)) were added. The reaction was allowed to proceed on ice for 24 h and stopped by diisopropyl fluorophosphate (0.75 mM final concentration) to inactivate the thrombin. Following cleavage, four additional amino acids (LVPR), which are part of the thrombin cleavage site, remained at the C-terminus. The thrombin-cleaved proteins are identified in the text by the suffix "Tc" (*e.g.*, Cx26Tc)

Reconstitution of purified connexin into unilamellar liposomes - Liposome formation and connexin incorporation into unilamellar liposomes followed the protocols described previously (Rhee et al., 1996; Harris and Bevans, 2001). Liposomes were formed by gel filtration of a 1 mg/ml mixture of phosphatidylcholine, phosphatidylserine, and rhodamine-labeled phosphatidylethanolamine at a molar ratio of 2:1:0.03 in urea buffer (see below) and affinity-purified connexin in 80 mM OG. The protein/lipid/detergent mixture was applied to a column of Bio-Gel beads pretreated with sonicated liposomes. Connexin-containing liposomes were collected in the void volume. The protein/lipid ratio intentionally corresponded to an amount of connexin equivalent to less than one hemichannel per liposome so that a significant fraction of the liposomes did not contain functional channels, for use as internal controls in the activity assay (TSF, described below) and to allow accurate detection of changes in channel activity.

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Transport-Specific Fractionation (TSF): What it is, How it works - Channel activity was assessed by Transport-Specific Fractionation (TSF) of the liposomes. TSF has been previously described and characterized (Harris et al., 1989; Harris et al., 1992; Rhee et al., 1996; Harris and Bevans, 2001). It separates liposomes into two populations within a density gradient, based on their permeability to urea and sucrose, which permeate open connexin channels. TSF has proved to be an effective alternative to electrophysiological study of hemichannels in membrane patches. The latter approach is difficult and problematic, and limited to a small subset of connexins (Harris, 2001).

The density gradient is formed from iso-osmolar solutions of urea (459 mM) and sucrose (400 mM). The sucrose solution has the greater density. The gradient is constructed so that the density of lipid is near the bottom. The other components of the solutions are: 10 mM KCl, 10 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, and 3 mM NaN_3.

Unilamellar liposomes are formed in and entrap the less dense, urea-containing solution, and centrifuged through the TSF gradient at 300,000 x *g* for 3 h in a swinging bucket rotor at 37 ºC. With centrifugation, liposomes without functional channels move into the gradient a short distance, buoyed by the entrapped urea buffer, and form a band in the upper part of the gradient. Liposomes with functional channels continuously exchange urea and sucrose through the open connexin pores, equilibrating the aqueous density inside and outside the liposome. As a result, 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

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via rhodamine-PE fluorescence (λ_{ex} 570 nm, λ_{em} 590 nm) making it possible to visualize the bands and to recover them by aspiration. As mentioned above, by design there is on average less than one channel per liposome, so that each TSF gradient has a substantial upper band of liposomes without channels, enabling changes in channel activity to be detected. This also minimizes the correction required for more than one functional channel per liposome (based on Poisson's distribution; see below). 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.

Transport-Specific Fractionation (TSF): TSF dose-response relations - A change in the distribution of liposomes between the upper and lower bands, relative to controls, reflects a change in the fraction of channels that are permeable to the gradient solutes. Such a change can be due to pore block or to a reduction to near zero of channel open probability (P_o). Since equilibration of the internal liposome volume 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 P_o close to zero. However, to achieve this result 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 the channels to mediate molecular signaling between cells and therefore is regarded functionally as eliminating

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biological channel activity. In this way, TSF assessed changes in a key functional property of connexin channels, their ability to mediate molecular flux.

TSF therefore identifies conditions/reagents that dramatically reduce permeability. Because of the requirement that an effect be nearly complete for it to be revealed by this assay, absence of an effect of a reagent in this system does not imply absence of effect on the channels. However, positive findings, such as those reported in this study, reflect truly significant alterations of channel function.

For the same reason, the concentration of a compound that produces channel closure detectible in TSF is greater than that needed to see an effect where reversible activity can be directly observed. Therefore TSF does not give classical Michaelis-Menton binding parameters such as K_m, and does not allow determination of microscopic affinities. It does, however, allow determination of relative affinities (see Bevans and Harris, 1999a, 1999b, Locke et al., 2004, Tao and Harris, 2004). The concentration of a modulator that produces an effect in TSF is an upper limit for the actual half-maximal value.

Data Analysis: Correction for more than one channel per liposome - Previous work indicates that connexin channels distribute among the liposomes in a manner described by Poisson's distribution (Rhee et al., 1996). This means that for a given ratio of functional channels to liposomes (λ), a Poisson distribution accounts for the fraction of the liposomes that have functional channels. λ was estimated from the maximum activity (percent of liposomes with active channels) for a given preparation of liposomes. The distribution of channels in the

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liposome population was calculated from λ by Poisson's distribution, and used to correct for the small fraction of liposomes that contain more than one channel (Bevans and Harris, 1999a). This calculation transforms the fraction of liposomes in the lower TSF band to the fraction of channels that are not inhibited.

Data Analysis: Normalization of TSF data - For each preparation of connexin, for each experiment the fraction 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 standard errors.

Data Analysis: Curve fitting - The activity-concentration data were fit with a 4-parameter logistic function of the form $f(x) = a/(1+\exp(b\cdot(x-c)))+d$. A Hill equation was not used since TSF does not produce a Michaelis-Menton dose-response curve (see above). For this reason, it would be inappropriate to fit the data with equations based on Michaelis-Menton assumptions. Instead, a smooth logistic function was fit to the data solely to determine the characteristic half-maximal channel activity parameter ($IC_{50\text{-}TSF}$). This parameter represents the empirical effectiveness of a compound as a function of concentration - changes in it reflect differences in the concentration needed to inhibit one-half of the channel population.

"Parachute" dye-coupling assay - This assay for gap junction function was performed as described by Goldberg *et al*. (1995) and Koreen *et al.* (2004). Donor and receiver cells were

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grown to confluence. The donor cells were double-labeled with 5 μM CM-DiI, a membrane dye that does not spread to coupled cells, and 5 μM calcein-AM, which is converted intracellularly into the gap junction-permeable dye calcein. The donor cells were then trypsinized and seeded onto the receiver cells at a 1:150 donor/receiver ratio. The cells were allowed to attach to the monolayer of receiver cells and form gap junctions for 4 h at 37 °C and then examined with a fluorescence microscope. For each experimental condition, the average number of receiver cells containing dye per donor cell was determined and normalized to that of control cultures.

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RESULTS

Homomeric Cx32 channels and heteromeric Cx26/Cx32 channels were immunopurified from rat and mouse liver, respectively, as described and previously characterized (Rhee et al., 1996; Bevans et al., 1998). Homomeric Cx26 channels are not readily obtained from native tissues, so they and additional homomeric Cx32 and heteromeric Cx26/Cx32 channels were obtained from HeLa cells expressing epitope-tagged connexin (see Materials and Methods; (Koreen et al., 2004)). The purification protocols included high-stringency rinsing with 1M salt to release associated proteins. The tagged connexins are designated by a "T" (*e.g.*, Cx26/Cx32T for heteromeric channels composed of Cx26 and Cx32 where the tag is on the Cx32). Where results are the same for tagged and untagged connexins, the "T" is in parentheses (*e.g.*, Cx32(T)). Connexin in which the tag is thrombin-cleaved, leaving four extra amino acids at the C-terminus, is designed by "Tc" (*e.g.*, Cx32Tc).

Hemichannels were reconstituted into unilamellar phospholipid liposomes, and the activities of the reconstituted channels explored by Transport Specific Fractionation (TSF) of the liposomes, described in Materials and Methods, which has been well characterized (Harris et al., 1989; Harris et al., 1992; Rhee et al., 1996; Harris and Bevans, 2001) and used effectively in studies of connexin channel modulation (Rhee et al., 1996; Bevans and Harris, 1999a; Bevans and Harris, 1999b; Kim et al., 1999; Bao et al., 2004; Locke et al., 2004; Tao and Harris, 2004). TSF is essentially an all-or-none assay of per liposome hemichannel activity as reflected by permeability to urea and sucrose. It is of particular utility in study of connexin channels due to the constraints and difficulties of studying them *in situ* or in membrane patches.

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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 fractional change in activity of the population of the channels.

2-APB directly inhibits homomeric Cx32(T) and heteromeric Cx32(T)/26(T) channels but not homomeric Cx26T channels

Fig. 1 illustrates the inhibition of homomeric Cx32 and heteromeric Cx26/Cx32 hemichannels by 2-APB in the TSF system. Fig. 1A shows that the inhibition occurs in a concentration-dependent manner, developing with increasing 2-APB concentration over the range 10-100 μM. The concentrations producing 50% TSF inhibition ($IC_{50\text{-}TSF}$) were 44 μM for heteromeric Cx26/Cx32 and 49 μM for homomeric Cx32. Increasing the concentration of 2-APB to 500 μM did not increase the level of inhibition; maximum effect was achieved at ~100 μM for both connexins. The potency of the inhibition was similar for Cx32 and Cx26/Cx32 channels. The degree of maximal inhibition was typically ~30% and was remarkably consistent across all types of connexin channels that showed sensitivity to 2-APB.

Fig. 1B summarizes the sensitivity of the various types of connexin channels to 2-APB. Untagged (from rodent liver) and tagged (from HeLa cells) channels formed by Cx32 and Cx26/Cx32 were inhibited to the same degree by 2-APB. In all cases, maximal inhibition was

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also achieved by ~100 μM 2-APB. In contrast, channels composed of Cx26T or Cx26Tc were unaffected. From these data we infer that either Cx26 is insensitive to 2-APB, or the presence of the tag, or of the four amino acids remaining at its C-terminus following thrombin cleavage, interfere with 2-APB inhibition. Modifications to the C-terminus of Cx32 do not interfere. The similar degree of inhibition for homomeric and heteromeric channels containing Cx32 suggests that any number of Cx32 monomers in a hemichannel can mediate the full inhibition.

To assess reversibility of 2-APB inhibition, upper band liposomes containing Cx32 or Cx26/Cx32 channels blocked by 100 μM 2-APB were collected from TSF tubes and recentrifuged in TSF gradients containing no 2-APB. If inhibition by 2-APB was irreversible, during the second TSF spin the liposomes would band exclusively at the upper (i.e., blocked) position. In contrast, reversibility was demonstrated by recovery of the same amount of activity that was originally inhibited by the 2-APB, relative to controls subjected to the same procedure without exposure to 2-APB (data not shown).

The inhibitory effect of 2-APB is pH dependent

To assess the effect of pH on the inhibitory action of 2-APB, the degree of inhibition was determined over a range of pH at 100 μM 2-APB in TSF gradients, normalized to activity at pH 6.5 without 2-APB. As previously shown, activity of Cx32 and Cx26/Cx32 hemichannels is insensitive to pH changes over this range in the absence of aminosulfonate pH buffers (Bevans and Harris, 1999; Tao and Harris, 2004). The data shown in Fig. 2A indicate that 2-APB inhibition is pH sensitive, with the inhibition increasing with decreasing pH.

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Protonated 2-APB is inhibitory, but protonation of connexin modulates sensitivity to 2-APB

The pH sensitivity of 2-APB inhibition could be accounted for either by deprotonated 2-APB acting as a channel agonist or protonated 2-APB acting as a channel antagonist. To distinguish these possibilities, channel activity was assessed at the 2-APB pK_a of 9.6 ((Bilmen et al., 2002); verified by us) in 500 μM 2-APB (Fig. 2A, asterisk). Under this condition, 2-APB inhibition was increased. Since the concentration of both the protonated and unprotonated forms of 2-APB were increased equally, the results show that protonated 2-APB is an inhibitory agent.

Note, however, that in this experiment the degree of inhibition at 500 μM 2-APB at pH 9.6 did not reach that obtained at 100 μM 2-APB at pH 6.5, but was only ~75% of that value. This suggested that at pH 9.6 the channels were less sensitive to 2-APB than at pH 6.5.

To distinguish pH effects due to 2-APB protonation and protonation of the connexin, the data from experiments carried out at constant pH and varied 2-APB concentration (Fig. 1A), and at changing pH with constant 2-APB (Fig. 2A), were plotted as functions of the concentration of protonated 2-APB (Fig. 2B).

This figure shows that the inhibitory effect is a function of the concentration of protonated 2-APB (filled symbols; data at pH 6.5 where 2-APB is fully protonated). However, if the only effect of lower pH was to increase the concentration of protonated 2-APB, the two curves would superimpose. Between protonated 2-APB concentrations of 10 μM to 100 μM the relations

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diverge, such that at the same protonated 2-APB concentration the inhibition was less at the higher pHs (open symbols). This reveals that lower pH increases the sensitivity of the channels to protonated 2-APB, presumably due to protonation of the connexin.

The difference between the two data sets in Fig. 2B can give some insight as to the pH dependence of the connexin sensitivity to 2-APB. There is no sensitivity at pH 10, full sensitivity at pH 8 and intermediate sensitivities at the intermediate pHs. To estimate the sensitivities at intermediate pHs, an arbitrary curve was interpolated between the open symbols (dotted line). Based on this interpolation, the half-maximal 2-APB sensitivity$_{TSF}$ occurs at approximately pH 8.8. Because of the nature of TSF measurements (see above and Materials and Methods), this suggests that the actual half-maximal sensitivity of the 2-APB effect to connexin protonation occurs at higher pH.

It may be noted that in Fig. 2A the inhibition at pH 9.6 (at 500 μM 2-APB) was ~75% of that at pH 6.5 (at 100 μM 2-APB) and in Fig. 2 at pH 9.6 the inhibition was ~26% of that at 6.5 (both at 50 μM protonated 2-APB). This suggests that additional factors affect the inhibition at high 2-APB concentrations, including, perhaps, a weak inhibitory effect of unprotonated 2-APB.

For comparison, the pH sensitivity of aminosulfonate inhibition of connexin channels is completely attributable to protonation of the aminosulfonate, and the experiment analogous to that in Fig. 2A resulted in maximal inhibition (see Fig. 4A in (Bevans and Harris, 1999) and Fig 1A of (Tao and Harris, 2004))

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Action of structural analogs of 2-APB

To help to define the pharmacological requirements for the 2-APB inhibition, the effects of several structural analogs of 2-APB were assessed (compounds shown in Table 1). For each compound, the maximal effect and the concentration producing it were determined. The data shown in Table 1 and Fig. 3 show that two of the 2-APB analogs (phenytoin and diphenhydramine (DPDM)) have effects similar to 2-APB, and one, diphenylboronic anhydride (DPBA), has a stronger effect at the same concentration. 2,2 diphenyltetrahydrofuran (DPTTF) has a weaker effect. The compounds have similar effects on Cx32 and on Cx26/Cx32 channels. As for 2-APB, none of the analogs had inhibitory effects on Cx26T or Cx26Tc channels (data not shown).

2-APB inhibition is distinct from inhibition by aminosulfonates

The direct and reversible action at low concentrations and the isoform-specific nature of the inhibition suggest that 2-APB interacts with a specific site in connexin channels. We previously characterized the interaction of protonated aminosulfonates with heteromeric Cx26/Cx32 hemichannels as interacting with a pharmacologically well-defined site (Bevans and Harris, 1999; Tao and Harris, 2004). In that case, a protonated amine was required for binding of the compound to connexin. 2-APB also contains a protonatable amine and the inhibition of connexin is mostly produced by its protonated form. This suggested a potential common mechanism of interaction, at least for the amine-containing compounds.

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To determine whether there is a mechanistic relation between the actions of these two classes of compounds, aminosulfonate (taurine) and a competitive antagonist of aminosulfonate inhibition (β-alanine) were applied along with 2-APB. Fig. 4A shows the effects on heteromeric Cx26/Cx32 channels. The data show that taurine and 2-APB both affect these channels, and to similar degrees (Fig. 4A, bars 1 and 4). When co-applied, the effects are additive (Fig. 4A, bar 6). β-alanine was without effect on 2-APB inhibition (Fig. 4A, bar 5 and Fig. 4B). These data argue strongly that the site of action and mechanisms of the 2-APB effect and the aminosulfonate effect are distinct. Fig. 4C confirms the lack of effect of taurine and β-alanine on Cx32 channels (bars 2 and 3), and that they do not interact with the 2-APB effect on those channels (bars 1, 4 and 5).

Effects of 2-APB on junctional coupling between cultured HeLa cells

The data described above show that 2-APB and several structural analogs block the function of Cx32-containing hemichannels reconstituted into liposomes. To determine whether the compounds have similar effects on junctional channels, experiments were performed to assess the effects of the compounds on dye coupling between cultured cells.

Junctional coupling was assessed by the "parachute" assay for dye coupling as described in Materials and Methods and in (Goldberg et al., 1995; Koreen et al., 2004). Donor cells labeled with the membrane dye DiI and loaded with the junction-permeable dye calcein were seeded onto unlabeled receiver cells. Experiments were carried out using five HeLa cell lines, expressing homomeric Cx32T or Cx26T, heteromeric Cx26/Cx32T or Cx26T/Cx32, or WT

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Cx26 (without C-terminal tag) in the presence of either 2-APB or its analogs. 2-APB is well-known to be freely membrane permeant, and the analogs are at least as hydrophobic (see Table 1). The seeded donor and receiver cells were incubated for four hours, and the number of receiver cells per donor cell containing calcein were counted. Gap junction communication was assessed as the number of receiver cells receiving calcein from a labeled cell, normalized to that for control conditions (without drug).

The data in Fig. 5A show that 2-APB substantially reduces coupling in the cell lines expressing Cx32 (Cx32T, Cx26/Cx32T and Cx26T/Cx32) but was without effect on the Cx26T expressing cells. This is consistent with the results from the hemichannel studies. Dye coupling was not completely inhibited, which is not surprising given the difference in conditions between reconstituted purified protein and junctional channels between cells, that the pH may not be optimal for the inhibition, and that inhibition was not complete in the TSF system either.

Surprisingly, 2-APB significantly inhibited the dye coupling of untagged Cx26 channels (Fig. 5A, bar 3), suggesting the tag and even the four amino acids remaining after thrombin cleavage can block the 2-APB effect on homomeric Cx26 channels.

The 2-APB analogs DPBA, DPDM and DPTTF inhibited the dye coupling of the cells expressing Cx32 (Fig. 5B) with a relative potency similar to that in the reconstituted system. The exception was phenytoin, which, unlike its effect on the reconstituted hemichannels, did not inhibit dye coupling.

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DISCUSSION

These studies show that 2-APB can act directly and reversibly on connexin channels to inhibit their function. 2-APB inhibition of homomeric Cx32 and heteromeric Cx26/Cx32 channels has an $IC_{50\text{-}TSF}$ of ~47 μM at pH 6.5, indicating that the microscopic K_m is somewhat below this value (the IC_{50} in cultured cells is 5-10 μM; Harks et al., 2003; Griffith et al., 2005). The inhibition was unaffected by C-terminal modification of Cx32, and occurred equally for native and heterologously-expressed connexins. 2-APB had no effect on homomeric Cx26 channels modified at the C-terminus with the epitope tag or with four amino acids remaining after tag cleavage. The effects on reconstituted hemichannels were corroborated by effects on dye coupling of junctional channels between HeLa cells. The sensitivity of Cx32T- and Cx26T-containing junctional channels to 2-APB corresponded to that of the reconstituted hemichannels. Surprisingly, 2-APB inhibited junctional dye coupling between cells expressing homomeric Cx26 channels unmodified at their C-terminus, in contrast to the effect on Cx26T and Cx26Tc channels.

2-APB acts on cellular targets involved in calcium/IP3 signaling, so its previously reported effects on junctional coupling could have been due to downstream effects. However, the effects reported here on purified, reconstituted channels establish that the inhibition occurs by direct interaction of 2-APB with connexin protein. Therefore, while 2-APB may affect other aspects of cellular physiology, there is an unambiguous direct effect at low micromolar concentrations on connexin channels themselves. Demonstration of such direct action is absent for almost all other compounds used as connexin channel blockers.

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Even though the connexin preps are highly pure, as previously characterized (*cf.* Harris et al., 1992; Rhee et al., 1996), it is theoretically possible that ABP acts on a modulatory protein that remains closely associated with connexin throughout the purification and reconstitution. However, this is unlikely because purification involves extensive high-stringency rinsing with 1M salt, and also because a modulatory protein would have to be present in substantial stoichiometric ratio with the connexin, which is not seen in blots stained for total protein.

The inhibition was markedly enhanced at low pH. It was therefore necessary to distinguish between effects due to protonated 2-APB versus the effects of lower pH on the response of the connexin channels to this ligand. We found that both factors were involved - protonated 2-APB is an inhibitory ligand, and the sensitivity of connexin to protonated 2-APB is enhanced at lower pH. Interestingly, 2-APB inhibition of the SERCA pump is similarly enhanced by a pH change from 7.2 to 6.0, also attributed to pH effect on the target protein (Bilmen et al., 2002).

To help define the structure-activity requirements of 2-APB inhibition, four structural analogs were tested. DPBA had greater effect than 2-APB. DPBA also is a better inhibitor of intracellular calcium release than is 2-APB (Dobrydneva and Blackmore, 2001). Comparison of the structures suggests that the diphenylboronic moiety may be involved and the ethanolamine moiety of 2-APB and its charge are not required. The enhanced inhibition might be attributable to the presence of two diphenylic groups, which could interact more effectively with connexin, or increase access via enhanced lipid solubility.

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The crystal structure of 2-APB shows that the ethanolamine moiety can coordinate with the boron to form a boroaxozolidine ring (Rettig and Trotter, 1976). It is thought that such a five-membered ring structure, unmodified, is an important component of the pharmacophore by which 2-APB inhibits calcium release from internal stores (Dobrydneva and Blackmore, 2001). DPDM cannot form such a ring structure, and phenytoin has a highly modified five-membered ring, yet both are almost as effective regarding connexin channels as 2-APB. Therefore the structural requirements differ from those for inhibition of Ca release.

Both DPDM and phenytoin have a biphenyl moiety, attached to a tetrahedral carbon of a five membered ring in phenytoin, and attached to a tertiary carbon in DPDM. Neither compound contains boron, so it, *per se*, is not required. Phenytoin contains two secondary amines and DPDM a tertiary amine, compared with the primary amine of 2-APB and the absence of an amine in DPBA. Phenytoin is fairly polar because of the acidic protons of the five-membered ring. None of these differences seem to be crucial for action on connexin channels, since phenytoin and DPDM have similar effects.

DPTTF was a relatively weak inhibitor, even though its structure is the closest to the internally coordinated form of 2-APB, with a tetrahedral carbon linking the two phenyl groups (as in phenytoin) instead of boron.

A conservative interpretation is that the structure of two phenyl groups attached to boron, tetrahedral carbon or oxygen atom is necessary for inhibition of connexin, and that amines may enhance the inhibition. Net charge does not appear to be an important factor, as at physiological

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pH the charges are +1 for 2-APB (pK_a 9.6; (Bilmen et al., 2002), +1 for DPDM (pK_a 9.62; (Barbas et al., 2000), +2 for phenytoin (pK_a 8.3; (Philip et al., 1918)) and 0 for DPBA.

These analogs inhibited junctional coupling in HeLa cells with roughly the same order of effectiveness as in the reconstitution system, with the exception of phenytoin. It is unclear why phenytoin was without effect on the cells. Its octanol-water partition coefficient is approximately the same as that of 2-APB (2.5 and 2.6, respectively; (Valko et al., 2001); NAID/OI Chemical Compound Database (http://chemdb.niaid.nih.gov)). Phenytoin does have a greater polar character, with two amines. Due to its polar nature it binds strongly to serum proteins, and this effect increases as pH is raised (Melten et al., 1986) so its effective concentration in the cultures may be reduced through binding to serum proteins in the culture medium.

It is notable that the two compounds with the greatest hydrophobic character, DPBA and DPTTF, lacking ionizable groups, were the most and least effective, respectively. The differences in their structures suggests the importance of the chemical character of the B–O linkage is key for the effect of DPBA.

The effect of 2-APB on homomeric Cx32 channels indicated that modulation by 2-APB differs from that of aminosulfonate. The fact that β-alanine, which competitively antagonizes taurine inhibition, has no effect on 2-APB action, and that co-application of aminosulfonate (taurine) with 2-APB results in additive effects, support the inference that the site of action and mechanism of 2-APB are different from those of aminosulfonates.

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To identify potential sites of 2-APB action, it would be helpful to know whether it acts from the cytoplasmic or extracellular side of the connexin channel. This cannot be determined from these studies, since 2-APB is membrane permeable, and it and its analogs are likely to be permeable through connexin channels (all the compounds are smaller than maltose, a disaccharide that permeates these channels (Bevans et al., 1998)) and thus would have rapid access to the both sides of the channel. Harks et al. (2003) suggested that electrical uncoupling by 2-APB was due to action at the extracellular side of connexins. However, the data presented here demonstrate that phenytoin, a polar analog of 2-APB, can inhibit the activity of connexin hemichannels in the TSF assay but not the dye coupling of gap junctions in HeLa cells. If the lack of effect of phenytoin is due to relative membrane impermeability rather than binding to serum proteins (noted above), this would suggest that 2-APB and its analogs inhibit connexin channels by acting at the intracellular side of connexin.

In HeLa cells, 2-APB does not inhibit the dye coupling of gap junctions composed of Cx26T, but significantly decreases that of untagged Cx26 channels. Because the intracellular environments are the same, the simplest explanation is that 2-APB can inhibit gap junction channels composed of homomeric Cx26, but that the effect is blocked by the C-terminal epitope tag or the four amino acids that remain after tag cleavage. These results suggest that the C-terminal domain of Cx26 is involved in the modulation of connexin channels by 2-APB. The C-terminal domain of Cx26 is very short (~10 amino acids). The fact that modification at the C-terminus of Cx32, which has a much longer C-terminal domain than Cx26, is without effect suggests that it is not the C-terminus itself, but rather the segment of the C-terminal domain closest to the membrane that is involved in 2-APB inhibition. The influence of modifications of

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the C-terminal domain on sensitivity to 2-APB suggests a cytoplasmic site of action, in contrast to the suggestion of Harks et al., 2003. In addition, chemical modulation of connexin channels is widely believed to involve interactions and flexibility of the cytoplasmic domains of the protein (Muller et al., 2002; Delmar et al., 2004; Liu et al., 2006). Also, the effects on junctional channels argue against direct interaction of 2-APB with the extracellular aspects of the hemichannels.

The direct 2-APB effect on connexin channels described here is likely to both facilitate development of pharmacological tools for study of the molecular modulatory mechanisms of connexin channels and lead to great understanding of the cellular mechanisms understanding of the cellular mechanisms of intercellular communication.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. 2-APB inhibits connexin hemichannel activity. A. Inhibition of connexin channels purified from rodent liver as a function of 2-APB concentration. Homomeric Cx32 channels are from rat liver, and heteromeric Cx26/Cx32 channels from mouse liver, as described in text. These definitions apply to subsequent figure legends. Data points are means ±S.E. for five protein preparations. *B.* Inhibition of connexin channels purified from native tissues and from transfected HeLa cells. *Cx26T*: homomeric Cx26 with a C-terminal epitope tag. *Cx26Tc*: Cx26T channels in which the epitope tag was cleaved by thrombin, leaving four additional amino acids at the C-terminus. Bars are means ±S.E. for five protein preparations. Channel activity for each protein purification was normalized to the maximum values within each data set. The smooth curves in this and subsequent figures are fits to the data of a four-parameter logistic function ($f(x) = a/(1+\exp(b\cdot(x-c)))+d$), as described in Methods.

Figure 2. Inhibition by 2-APB is pH sensitive. A. Inhibition of Cx32 channels (filled circles) and Cx26/Cx32 channels (open squares) induced by 100 μM 2-APB over a range of pH. The asterisk is the inhibition by 500 μM 2-APB at its pK_a (pH 9.6). *B.* The protonated form of 2-APB produces the inhibitory effect on Cx32 and protonated connexin modulates the sensitivity. Inhibition is plotted as a function of the concentration of protonated 2-APB, using two sets of data - one in which 2-APB was varied at constant pH 6.4 (open circles, data from Fig. 1A) and one in which the concentration of 2-APB was constant and the pH varied (filled squares, data from Fig. 2A). The difference between the two relations indicate that protonation of connexin enhances the sensitivity of 2-APB. *From left to right,* the filled squares correspond to 100 μM 2-

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APB data at pH 11, 10, 9 and 8, with the points at pH 7 and pH 6 superimposed at far right (*i.e.*, for which all 2-APB is protonated). Data points are means ±S.E. for five protein preparations.

Figure 3. Relative maximal inhibition of Cx32 channel activity by 2-APB and its structural analogs. Data are shown for the lowest concentration of each compound that produced its maximal effect. Inhibitory effect of each compound was normalized to the inhibition produced by 100 μM 2-APB. Experiments were carried out at pH 6.5. The bar graphs are means ±S.E. from six to ten protein preparations. Structures of the compounds are given in Table 1.

Figure 4. 2-APB inhibition differs from that aminosulfonates. A. Taurine (10 mM) and 2-APB (100 μM) both inhibit heteromeric Cx26/Cx32 hemichannels, but the effects are additive. 10 mM β-alanine reduces taurine-induced inhibition but not 2-APB-induced inhibition. *B.* Inhibition-concentration relations for 2-APB alone and with 10 mM β-alanine shows that inhibition by 100 μM 2-APB is unchanged by coapplication with β-alanine. *C.* Inhibition of Cx32 induced by 2-APB is not affected 10 mM by taurine or 10 mM β-alanine. All compounds tested at pH 6.5. The data points are means ±S.E. from five to seven protein preparations.

Figure 5. Effects of 2-APB and analogs on dye-coupling of gap junctions in transfected HeLa cells. Degree of dye coupling was assessed using the "parachute" method as described in the text. *A.* Effects of 2-APB (100 μM) on dye coupling through gap junctions composed of different connexins. The data show that 2-APB significantly reduced due coupling in between cells with the same connexin-specificity seen in reconstituted hemichannels. Cx26T channels

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were unaffected. In addition, untagged Cx26 junctional channels were inhibited by 2-APB. *B.* Effects of 2-APB and its analogs on dye coupling of Cx32T channels. The effects of the analogs are consistent with the findings in reconstituted hemichannels, with the exception of phenytoin, which did not significantly inhibit the junctional channels. Gap junctional communication was assessed as the average number of receiver cells containing calcein from each donor cell, normalized to controls without exposure to 2-APB or analogs. The bar graphs are means ±S.E. from four to six dishes for each condition.

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TABLES

Table 1. 2-APB analogs that inhibits connexin channels. The values for inhibition are means normalized to that for 2-APB, from the data graphed in Fig. 3. Charge given is that at pH 6.5. Log P refers to the octanol:water partition coefficient.

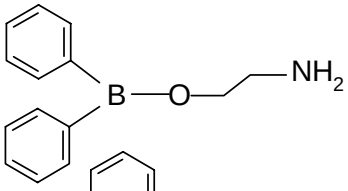
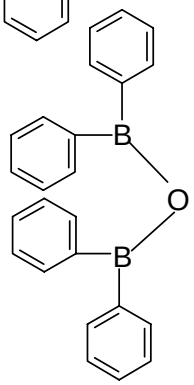
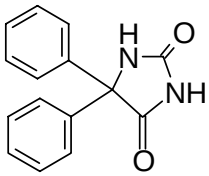
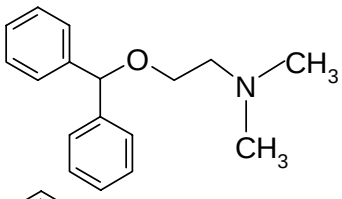
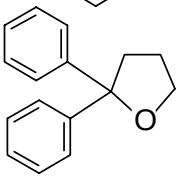
	Compound	Maximal inhibition relative to 2-APB		
		Cx32	Cx32/Cx26	
	2-aminoethoxydiphenyl borate (2-APB)	1.0	1.0	pKa 9.6 charge +1 log P 2.1-2.6
	diphenylboronic anhydride (DPBA)	1.5	1.3	no pKa weak dipole highly hydrophobic
	phenytoin	0.86	0.89	pKa 8.1-8.3 charge +2 log P 2.4-2.5
	diphenhydramine DPDM	0.87	0.85	pKa 9.62 charge +1 log P 3.1-3.5
	2,2-diphenyltetrahydrofuran (DPTTF)	0.4	0.2	no pKa perhaps weak dipole highly hydrophobic

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Fig. 1

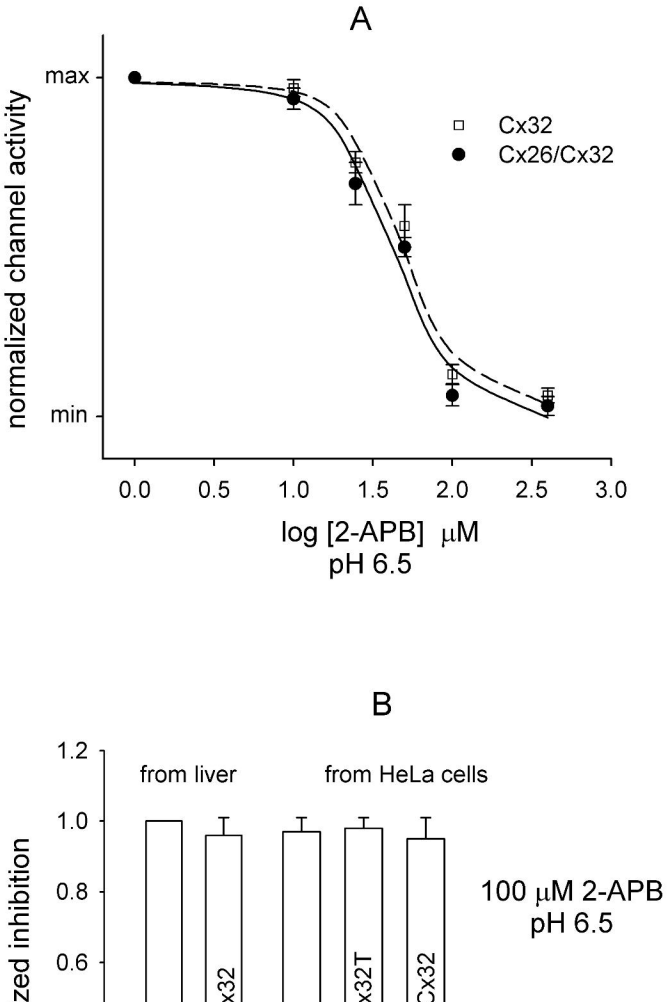

A
max
min
normalized channel activity
Cx32
Cx26/Cx32
0.0
0.5
1.0
1.5
2.0
2.5
3.0
log [2-APB] μM
pH 6.5
B
normalized inhibition
1.2
1.0
0.8
0.6
0.4
0.2
0.0
from liver
from HeLa cells
Cx32
Cx26/Cx32
Cx32T
Cx26/Cx32T
Cx26T/Cx32
Cx26T
Cx26Tc
100 μM 2-APB
pH 6.5

Fig. 2

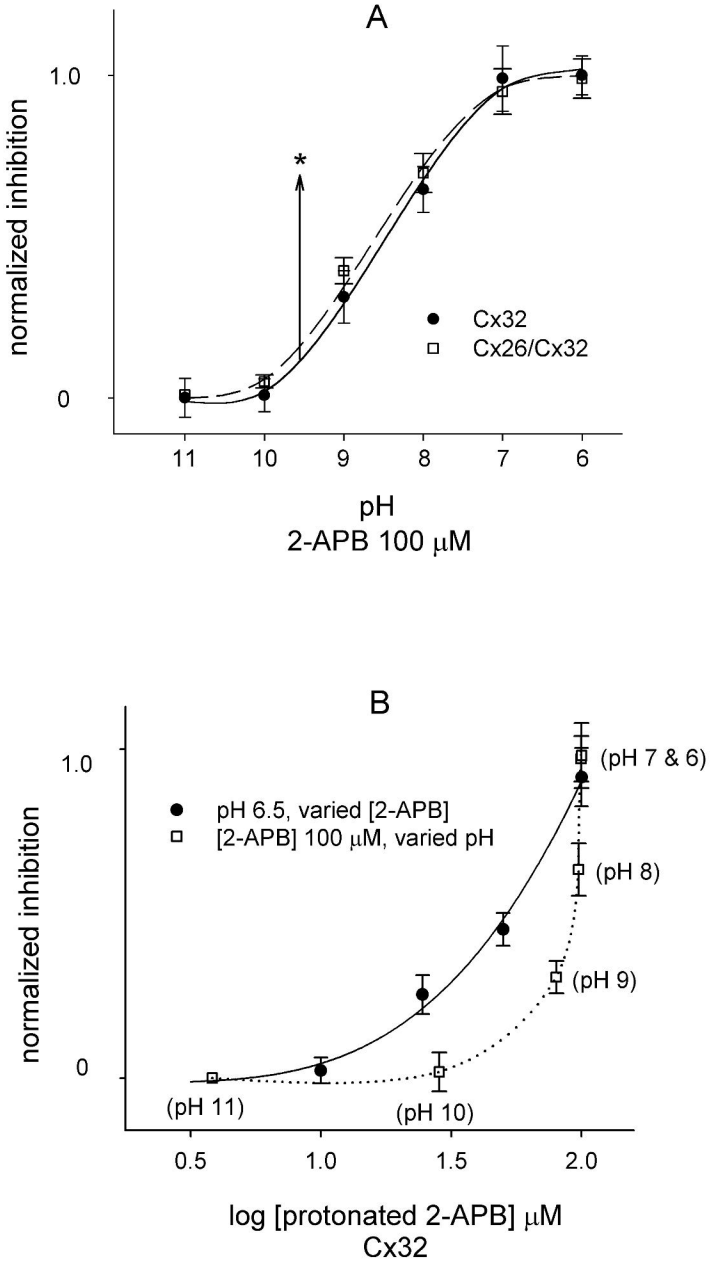

A
1.0
0
normalized inhibition
*
Cx32
Cx26/Cx32
11
10
9
8
7
6
pH
2-APB 100 μM
B
1.0
0
normalized inhibition
pH 6.5, varied [2-APB]
[2-APB] 100 μM, varied pH
(pH 7 & 6)
(pH 8)
(pH 9)
(pH 11)
(pH 10)
0.5
1.0
1.5
2.0
log [protonated 2-APB] μM
Cx32

Fig. 3

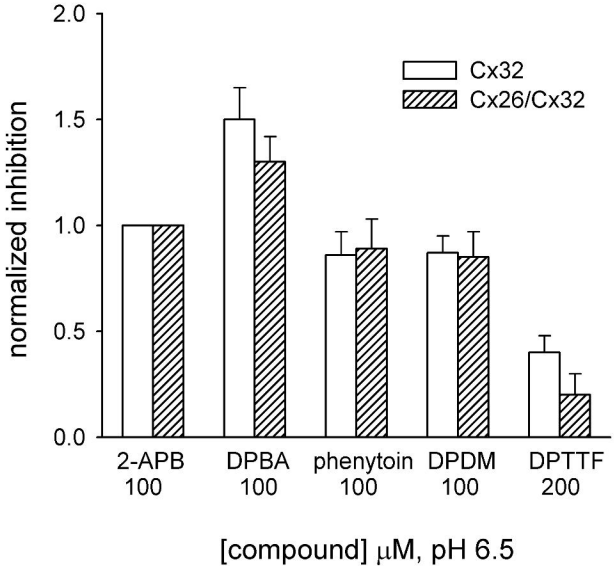

2.0
1.5
1.0
0.5
0.0
normalized inhibition
Cx32
Cx26/Cx32
2-APB
100
DPBA
100
phenytoin
100
DPDM
100
DPTTF
200
[compound] μM, pH 6.5

Fig. 4

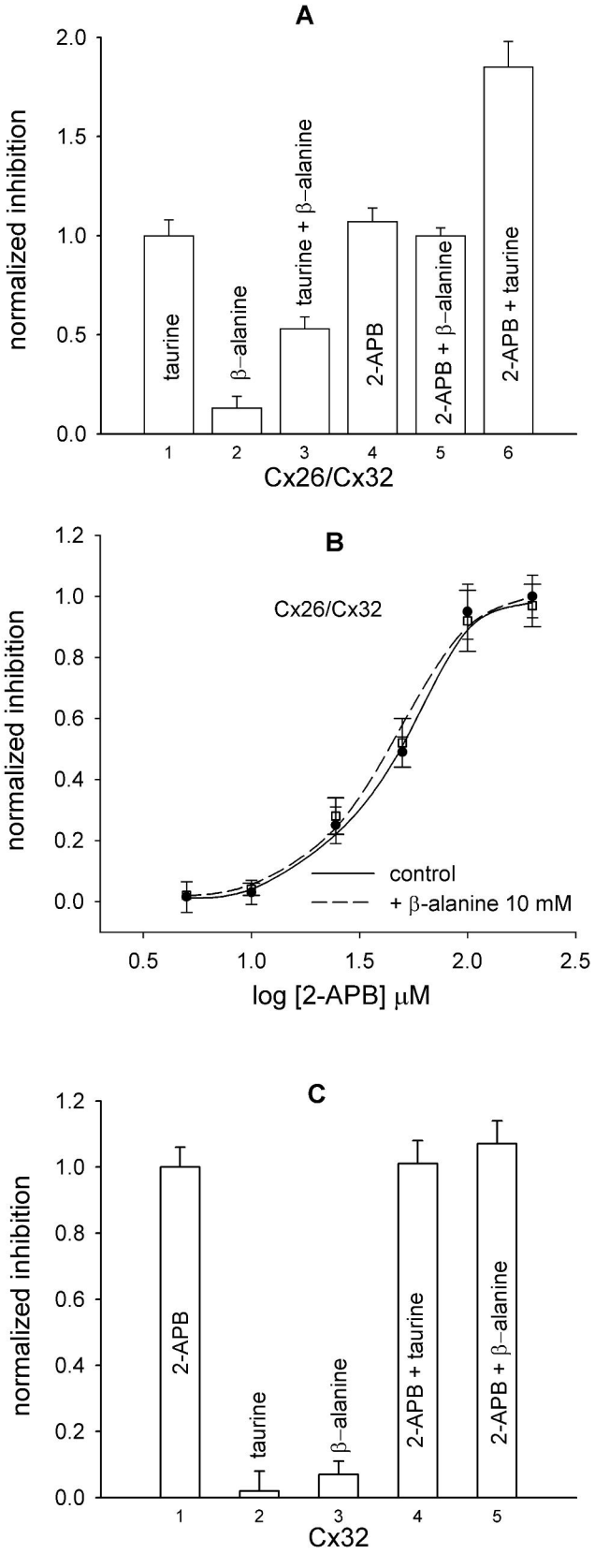
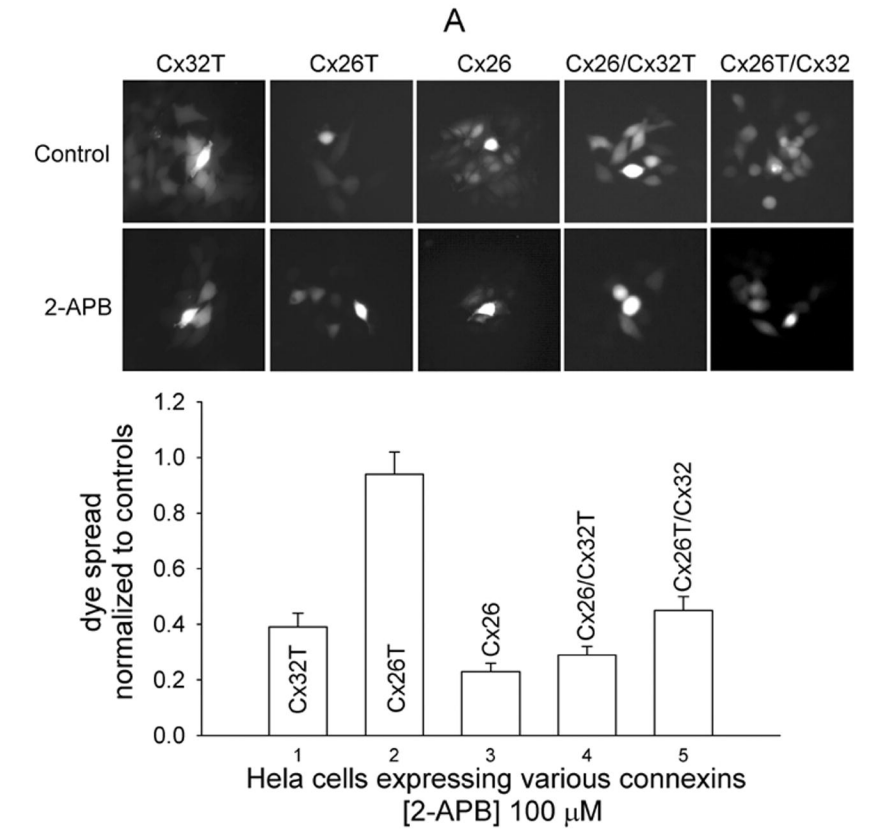
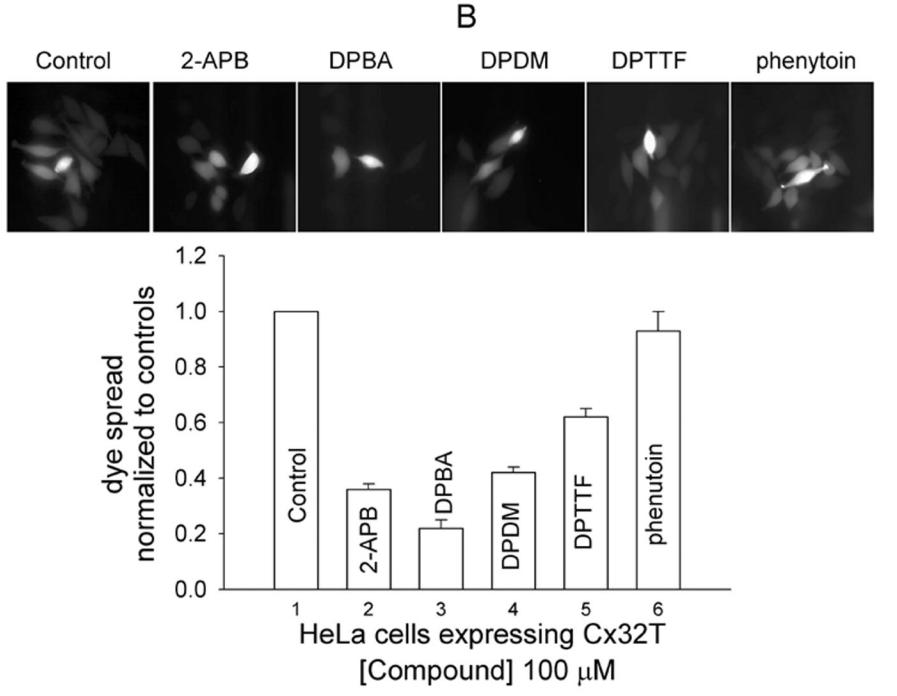

A
normalized inhibition
2.0
1.5
1.0
0.5
0.0
taurine
β–alanine
taurine + β–alanine
2-APB
2-APB + β–alanine
2-APB + taurine
1
2
3
4
5
6
Cx26/Cx32
B
normalized inhibition
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cx26/Cx32
control
+ β-alanine 10 mM
0.5
1.0
1.5
2.0
2.5
log [2-APB] μM
C
normalized inhibition
1.2
1.0
0.8
0.6
0.4
0.2
0.0
2-APB
taurine
β–alanine
2-APB + taurine
2-APB + β–alanine
1
2
3
4
5
Cx32

Fig. 5


A
Cx32T
Cx26T
Cx26
Cx26/Cx32T
Cx26T/Cx32
Control
2-APB
dye spread
normalized to controls
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Cx32T
Cx26T
Cx26
Cx26/Cx32T
Cx26T/Cx32
1
2
3
4
5
Hela cells expressing various connexins
[2-APB] 100 μM


B
Control
2-APB
DPBA
DPDM
DPTTF
phenytoin
dye spread
normalized to controls
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
2-APB
DPBA
DPDM
DPTTF
phenutoin
1
2
3
4
5
6
HeLa cells expressing Cx32T
[Compound] 100 μM