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Gap Junctions between Cells Expressing Connexin 43 or 32 Show Inverse Permselectivity to Adenosine and ATP*

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Gap junctions, composed of proteins from the connexin family, are the only channels that directly connect the cytoplasm of adjacent cells to allow for the intercellular transfer of small hydrophilic molecules. Gap junctional communication is essential for proper development and health in animals and humans. Whereas the study of biological molecules that pass through gap junctions is extremely important, the identification of endogenous transjunctional metabolites is challenging. To help address this problem, we have developed a layered culture system to identify and quantitate the transfer of endogenous molecules that pass between cells through gap junctions. Using these techniques, we have identified several endogenous molecules that showed differential transfer between channels composed of Cx32 *versus* Cx43. For example, adenosine passed about 12-fold better through channels formed by Cx32. In contrast, AMP and ADP passed about 8-fold better, and ATP greater than 300-fold better, through channels formed by Cx43. Thus, addition of phosphate to adenosine appears to shift its relative permeability from channels formed by Cx32 to channels formed by Cx43. This suggests functional consequence because the energy status of a cell could be controlled via connexin expression and channel formation.

Gap junctions are formed by integral membrane proteins, called connexins (commonly abbreviated by Cx[1] followed by their molecular mass in kilodaltons), which have evolved into a family of ~20 members in humans (1). All connexins share the structural motifs of four transmembrane domains, intracellular amino and carboxyl termini, a cytoplasmic loop, and two extracellular loops involved in docking interactions with connexins of adjacent cells (2). These multiple connexins enable selective interactions between family members, differential modes of regulation (3), and the formation of channels with different conductances (4, 5) and permeabilities to ions (6–9), fluorescent dyes (7, 10), and, as we have recently shown, endogenous metabolites (11, 12).

The biological specificity of gap junctional communication is important for events required for homeostasis and interaction between neighboring tissues, such as regions of the heart, lens, or nervous system (1, 13). For example, neuronal cells express predominant levels of Cx43 and are surrounded by supporting Schwann cells that express Cx32. Although the connexins expressed by these cells can be defined, the role of gap junctions in the intimate relationship between cells in such tissues is not thoroughly understood. However, gap junctions are critical for normal system function as evidenced by aberrant connexin expression being associated with specific disorders. For instance, loss of Cx32 may underlie Charcot-Mari-Tooth syndrome (14), while suppression of Cx43 is associated with neoplastic transformation including brain tumorigenesis (15). Knowledge of actual signals mediated by gap junctions may help illuminate the functions of connexins and the consequence of their misregulation in such systems.

We introduce a layered culture system here that can be used to directly study the transfer of endogenous metabolites through gap junctions. The present studies indicate that gap junctions composed of Cx32 and Cx43 allow differential exchange of cellular metabolites that would not necessarily be expected from predictions of pore diameters and charge selectivity. For example, we demonstrate that addition of phosphate can shift the permselectivity of adenosine from channels formed by Cx32 to those formed by Cx43 by over 3 orders of magnitude. Thus, different connexins produce channels with very different properties to selectively transfer signals unique to specific cell types.

MATERIALS AND METHODS

Cell Culture and Isolation of Transjunctional Molecules—C6 glioma cells transfected with Cx32 or Cx43 were maintained as previously described (11, 12, 16). One million cells were plated onto inverted inserts containing porous membranes suitable for cell culture (Costar) as "Receivers" or to each well of a 6-well cluster plate as "Control Receivers." After adhering to the membranes, the inserts containing Receiver cells were turned right side up and plated into wells of 6-well plates. "Donor cells" were metabolically labeled for 3.5 h with 0.2 mCi per ml [35S]Met (Amersham Biosciences SJ5050, 50 mCi/ml) in Met-free Dulbecco's modified Eagle's medium with 10% fetal bovine serum or overnight with 10 μCi/ml glucose (D-[U-14C]glucose, Amersham Biosciences) in medium depleted by three days growth. For fluorescent studies, Donor cells were labeled with calcein and DiI as previously described (11).

After labeling, medium was removed, cells were washed thrice with phosphate-buffered saline, treated with trypsin, and suspended in fresh medium. One million radioactively labeled cells or 200,000 fluorescently labeled cells were added to each insert above Receivers or Control Receivers as Donors, or above no cells as Control Donors, and allowed 3 h to settle onto the membranes and form channels with Receiver cells.

To isolate metabolites, medium was aspirated, the cells were harvested into microcentrifuge tubes in cold phosphate-buffered saline, centrifuged for 1 min, aspirated, frozen in dry ice, and stored at −70 °C. Potential transjunctional molecules were obtained as previously described (11). Briefly, each cell pellet was lysed in 112.5 μl of lysis

[1] The abbreviations used are: Cx, connexins; HPLC, high pressure liquid chromatography; Anova, analysis of variance; Voa, apparent Vo.

solution (10 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) for 20 min at 4 °C, diluted with 634.5 μl 10 mM Tris, pH 8.0, and 10 mM EDTA, pH 8.0, sequentially filtered through 50- and 3-kDa Centricon filters, and frozen at −70 °C. Aliquots of all filtrates, retentates, and medium from the top and bottom chambers of the inserts in the plates were examined by scintillation counting (11).

Components in the lysates were then resolved by HPLC and thin layer chromatography as previously described (11). Briefly, filtrates were resolved through a C18 column with a water:acetonitrile:trifluoroacetic acid gradient from 99.9:0:0.1 to 79.9:20:0.1 over 20 min at 0.6 ml/min and 37 °C. Material eluting between 6–10 min and 10–20 min was pooled, and aliquots were counted while the remainder was resolved though an Aminex HPX-87H column (Bio-Rad) with isocratic elution using 5 mM H_2SO_4 at 0.35 ml/min and 40 °C. Aliquots were scintillation-counted or examined by thin layer chromatography on silica gel plates (Whatman LK6DF) resolved by ascending chromatography in 0.6% ammonium hydroxide/70% isopropanol and examined by exposure to Storage Phosphor Screens, which were scanned with a Molecular Dynamics PhosphorImager equipped with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Molecules were identified by comigration with standards on C18 and aminex HPLC columns and thin layer chromatography plates (11, 16, 17).

Western Immunoblotting—C6 cells expressing Cx32 or Cx43 were rinsed in phosphate-buffered saline and lysed on ice in Laemmli sample buffer supplemented with 50 mM NaF, 500 μM Na_3VO_4, 1× complete protease inhibitor mixture (Roche Molecular Biochemicals), and 2 mM phenylmethylsulfonyl fluoride. Following sonication to shear DNA, protein (20 μg per lane) from each cell type were separated on 10% SDS-polyacrylamide gels. Protein was transferred to nitrocellulose (Nitrobind Nitrocellulose, Micron Separations, Inc.), blocked, and incubated with a monoclonal antibody to Cx32 (SD4) (18) or to Cx43 (Cx43NT1-antipeptide antibody prepared against amino acids 1–20 of Cx43) followed by peroxidase-conjugated goat anti-mouse (Jackson Immuno-Research Laboratories, Inc). Peroxidase detection was done using SuperSignal West Pico Chemiluminescent Substrate (Pierce) followed by exposure to Kodak Biomax MR film. Coomassie staining was used to verify equal loading of samples.

Biophysical Characterization of Channels—The dual whole-cell voltage clamp technique (19) was applied on cell pairs to measure their junctional conductance (Gj). Data on total conductance was obtained from cell pairs 4–6 h after plating. Access to the cytoplasm was achieved using brief negative-pressure pulses after a gigaohm seal was formed between polished glass micropipettes (3–5 megohms) and the cell membranes. Micropipettes were filled with a patch solution containing 130 mM CsCl₂, 0.5 mM CaCl₂, 10 mM EGTA, and 10 mM Hepes at pH 7.2. During recording, cells were kept at room temperature in a solution containing 130 mM NaCl, 7.0 mM CsCl₂, 2.0 mM CaCl₂, 0.6 nM MgCl₂, and 10 nM Hepes at pH 7.4.

Channels formed by Cx32 or Cx43 were distinguished from each other by comparing the apparent voltage at which half-maximal conductance is reached in transjunctional voltage-sensitive junctions at steady state of inactivation (Voa) obtained for each cell line during voltage ramps applied at different time/voltage rates (see Veenstra, Ref. 20). To obtain Voa values, voltage ramps of both polarities from 0 to 100 mV at 500 ms/mV were applied on one cell of pairs expressing either Cx43 or Cx32. The value 500 ms/mV was determined after testing progressively faster ramp rates until Voa for Cx43 was not different from the steady state values reported (21). These ramps were applied using software and recording systems by HEKA (Lambrecht, Germany). The corresponding digitized current values obtained were divided by the voltage ramp values that resulted in the Gj/V_m relations shown in Fig. 5.

Each transjunctional conductance that resulted from each positive and negative ramp was normalized to a 10-mV hyperpolarizing prepulse. Best fit for the data was performed using a Boltzmann relation through Origin software (Microcal, Northampton, MA). To avoid series resistance interference, we only considered those experiments where the initial junctional conductance was smaller than 15 nanoSiemens.

Quantitation of Data—Equal numbers of cells were used as Donors, Receivers, or Control Receivers. Quantitative analysis was performed on a known number of cells. The transfer of radioactive material between cells was calculated by measuring the radioactivity from Donor or Receiver cells by scintillation counting or PhosphorImager analysis as described above. The radioactivity from Receivers or Control Receivers was divided by that of Donors and multiplied by 100 to obtain the percent that traveled from Donors to Receivers or Control Receivers, respectively.

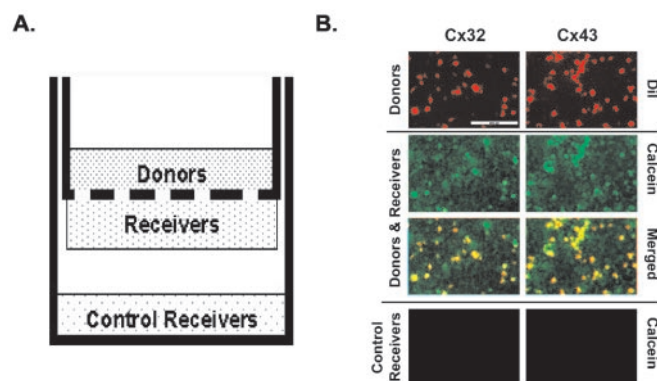The number of channels per cell pair was calculated by dividing the

A.

B.

FIG. 1. **Transfer of fluorescent dye between cells in a layered culture system.** *A*, diagram illustrating labeled Donor cells on a porous membrane directly over Receiver cells and Control Receivers that are 1 mm below the membrane. *B*, Donor cells were labeled with DiI and calcein and plated over non-labeled Receiver cells to visualize gap junctional communication between cells transfected with Cx32 or Cx43 as indicated. Thus, Donors could be distinguished by the presence of DiI. Communication was evidenced by the transfer of the dye calcein, but not DiI, from Donors to Receivers, which were seen together at the focal point of the membrane. Consistent with movement through gap junctions, calcein did not enter Control Receivers, which were distinguished from Donors and Receivers because they were seen at a focal point 1 mm below the membrane. Detection of calcein, DiI, and merged signals are shown in *green*, *red*, and *yellow*, respectively.

total conductance by the unitary conductance exhibited by each cell type (80 pS for Cx32 and 120 pS for Cx43). The percent of the molecule that traveled from a Donor cell to a Receiver cell was measured by dividing the radioactivity of a metabolite in a Donor cell by its radioactivity from a Receiver cell and multiplying by 100. The relative transfer of the compound between cells transfected with Cx32 or Cx43 was calculated by dividing the percent transfer of a molecule through between cells transfected with one channel type (*e.g.* Cx32) by that transferring between cells transfected with the other channel type (*e.g.* Cx43). The permselectivity of a compound to Cx32 or Cx43 on a per-channel basis was calculated by dividing the relative transfer of the compound between cells by the ratio of the number of active channels expressed by those cells.

RESULTS

A Layered Culture System to Investigate Gap Junctional Communication—We have recently established techniques to investigate the transfer of endogenous metabolites through gap junctions made from different connexins. This protocol utilizes fluorescence-activated cell sorting to isolate radioactive metabolites that transfer through gap junctions from metabolically labeled Donor cells to Receiver cells (11, 12, 16, 17). However, the cell populations analyzed by this method are interspersed with each other, as opposed to organized layers that may be formed *in vivo*. We have utilized a layered culture system to overcome this problem. The technique utilizes a porous membrane to keep two cell populations physically separate from each other, while allowing gap junctions to form through the pores. As shown in Fig. 1*A*, this system forms three distinct cell layers. Donor cells and Receiver cells are able to contact and form gap junctions with each other through the pores in the membrane. However, the pores, which are 3 μm in diameter, are small enough to block the cells, which are about 20 μm in diameter, from actually migrating through to the other side of the membrane within the time frame of the assay (about 2 h). The system also includes a layer of Control Receiver cells that are placed 1 mm below the membrane, thus preventing direct cell contact or gap junction formation with the Donors or Receivers.

Dye Transfer through Gap Junctions in a Layered Culture System—As shown in Fig. 1*B*, calcein readily traveled from Donor cells transfected with Cx32 or Cx43 to homotypic Re-
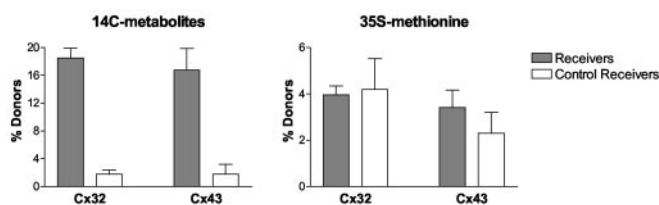
FIG. 2. **Transfer of endogenous metabolites between cells in a layered culture system.** Donor cells were labeled with [14C]glucose or [35S]methionine to analyze the flow of metabolites to Receiver cells. Data are shown as the percent of the radioactivity of Donor cells found in Receivers (mean ± S.E., $n = 3$) or Control Receivers (mean + max, $n = 2$) (transfer to equilibrium would result in 100%). A significant amount of metabolites derived from glucose traveled from Donors to Receivers, but not Control Receivers ($p < 0.0005$ by ANOVA). In contrast, methionine traveled equally well to both Receivers and Control Receivers ($p > 0.6$ by ANOVA). Thus, many metabolites derived from glucose required cell junctions to transfer between cells, while methionine did not.

ceiver cells on the other side of the membrane. This dye did not travel to Control Receivers, consistent with its movement through gap junctions. Consistent with its lipophilic properties, DiI did not transfer from Donors to Receivers (11, 22). Donors and Receivers were separately released from both sides of the membrane with trypsin and analyzed to confirm that no cells passed through the pores to the other side (data not shown).

Transfer of Metabolites through Gap Junctions in a Layered Culture System—To investigate the transfer of endogenous metabolites between these cells, Donors were metabolically labeled with radioactive glucose before being plated above non-labeled Receivers. The amount of transfer was quantitated as the percent radioactivity of a Control Donor cell that traveled to a Receiver or Control Receiver. As shown in Fig. 2, molecules metabolically derived from glucose transferred about 9-fold better from Donor cells to Receivers than to Control Receivers ($p < 0.0005$ by ANOVA), indicating that cell contact was needed for the intercellular transfer of many metabolites. This is consistent with a requirement for gap junctions to transfer these molecules.

Donor cells were metabolically labeled with radioactive methionine to investigate the transfer of amphipathic molecules between cells in this system (Fig. 2). In contrast to the hydrophilic metabolites derived from glucose, methionine freely diffused from Donor cells to enter Control Receivers and Receivers with equal efficiency ($p > 0.6$ by ANOVA). Therefore, this procedure was able to differentiate between the transfer of molecules derived from glucose that required gap junctions to move between cells, and molecules such as methionine which did not.

Identification and Quantitation of the Transfer of Endogenous Metabolites—After isolation, transjunctional metabolites were resolved by HPLC and thin layer chromatography to identify and quantitate the transfer of specific intercellular signals. We have previously been able to identify transjunctional glucose, ADP, ATP, glutamate, and glutathione in Receiver cells that were separated from Donor cells by cell sorting (11). In addition to these, as shown in Fig. 3A, the increased sensitivity afforded by the layered culture system enabled the detection of several other transjunctional metabolites including adenosine, AMP, and three other metabolites that we have yet to identify.

The transfer of radioactive molecules from Donor cells to Receiver cells was measured to compare the transfer of each metabolite between cells expressing Cx32 or Cx43. As shown in Fig. 3B, AMP, ADP, ATP, glutathione, glutamate, and two metabolites not yet identified were shared more efficiently by
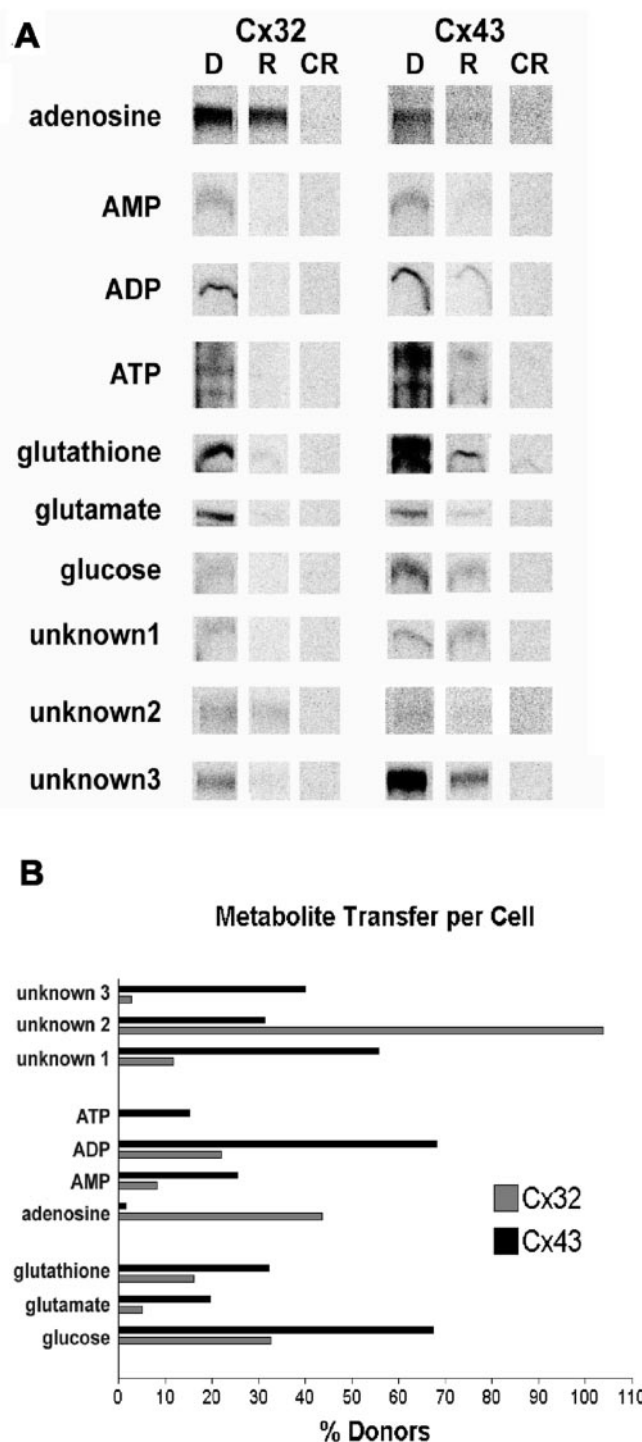

FIG. 3. **Analysis of transjunctional metabolites by thin layer chromatography.** *A*, molecules were resolved by HPLC and thin layer chromatography to visualize specific transjunctional metabolites that passed between Cx32 or Cx43 transfectants. Molecules from 10,000 co-cultured Donor, Receiver, and Control Receiver cells were visualized by PhosphorImager analysis of thin layer plates to compare their transfer between Cx32 and Cx43 transfectants; transfer to equilibrium would result in equivalent signals between Donors and Receivers. *B*, data were quantitated as the percent radioactivity of each molecule from a Donor cell that traveled to a Receiver cell. Addition experiments showed results consistent with this data.

Cx43 transfectants. This is in agreement with our previous findings of metabolites traveling preferentially between Cx43 transfectants (11). However, in extending this analysis to additional metabolites, these data show that adenosine and one
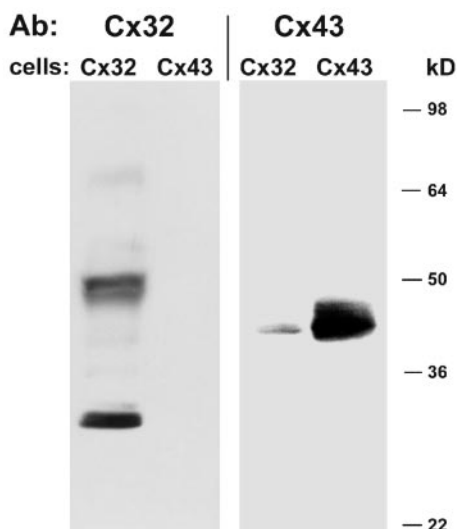
FIG. 4. **Detection of Cx32 and Cx43 in cells by Western blot analysis.** Protein was examined by antiserum specific for Cx32 or Cx43 from cell lysates as indicated. Cx32 or Cx43 are predominantly expressed by cells transfected with Cx32 or Cx43, respectively. Positions of molecular mass markers in kilodaltons are given at the *right hand side* of the figure.

unidentified molecule were shared better by cells transfected with Cx32.

Results from Western blot analysis confirmed appropriate connexin expression in these cells as shown in Fig. 4. Cx32 was not detected in the Cx43 transfectants, while Cx32 transfectants displayed a Cx32 immunoreactive band at the appropriate molecular weight, as well as apparent dimers migrating at twice the molecular weight of the monomeric protein (18). Cx43 transfectants expressed robust levels of Cx43, while only slight levels of endogenous Cx43 were detected in cells transfected with Cx32. Thus, Cx32 or Cx43 appeared to be predominantly expressed in cells transfected with Cx32 or Cx43, respectively.

In addition to Western blot analysis, analysis of voltage gating illustrated expression of appropriate channels in Cx32 and Cx43 transfectants. Vo has been previously reported to be similar for Cx43 and Cx32 (between ± 55 to ± 65 mV, depending on the expression system used (21, 23). However, an alternative approach to distinguish between these two connexins takes advantage of their differences in voltage-gating kinetics during inactivation. According to Revilla *et al.* (24), at 100 mV, the time constant of inactivation will be 100 ms and 2 s for Cx43 and Cx32, respectively. Therefore, the distinction between connexins can be readily accomplished by comparing the Voa obtained for each cell line during voltage ramps applied at different voltage/time rates (see Veenstra, Ref. 20). As shown in Fig. 5A, gap junction channels between Cx43 transfectants responded to a 500-ms/mV ramp of both polarities with Voa values of 62.9 ± 0.8 mV (mean $\pm$ S.E., $n = 10$), resembling previous results from long pulses of increasing voltage (*e.g.* Moreno *et al.* (21)). In Fig. 5A an example of $+62$ and -63 mV is shown where G_{min}, or the minimal voltage recorded at high voltages, corresponded to 40 and 39% of the initial conductance. In contrast, as shown in Fig. 5B, Voa values for cell pairs transfected with Cx32 were substantially larger at 91.2 ± 2.4 mV (mean $\pm$ S.E., $n = 9$). The slower time constant of inactivation compared with the time constant of the applied ramp consistently shifted Voa toward larger values in Cx32 cells, indicating that most of their functional channels were composed of Cx32.

Determination of total and unitary conductances between cells allowed us to compare the relative abilities of each of these
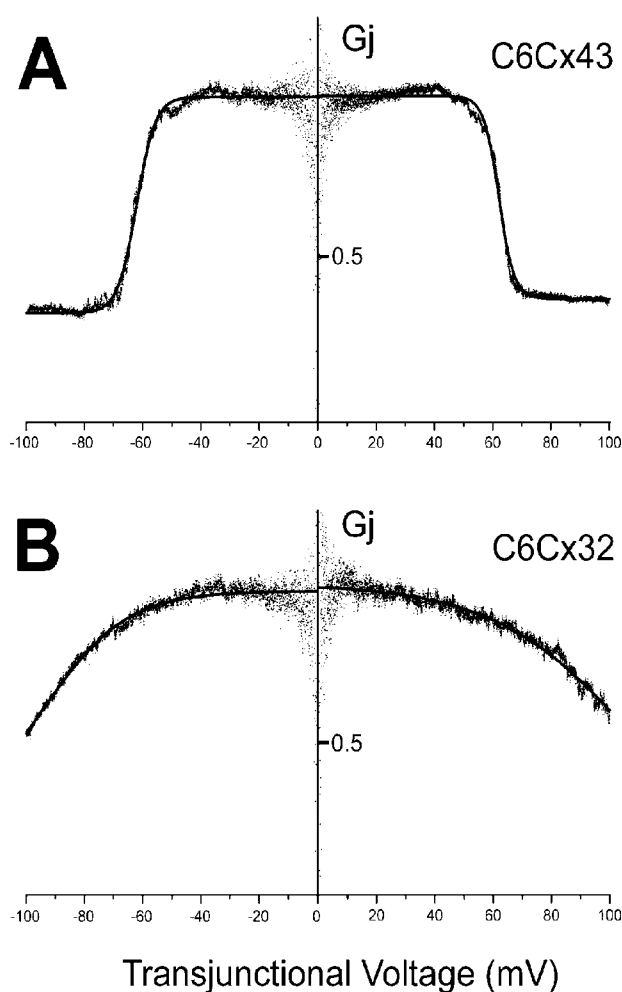

FIG. 5. **Voltage gating of Cx32 and Cx43 in C6 transfected cells during a voltage ramp protocol.** *A*, all points represent the normalized Gj calculated from one experiment and obtained after dividing the digitized junctional current-time curve by the transjunctional voltage ramp applied at both polarities from 0 to 100 mV in one of the cells of a pair expressing Cx43. The initial holding potential for both cells was 0 mV. The continuous line indicates the best fit for a Boltzmann relation. Each half was adjusted independently. *B*, all points represent digitized currents as in *A*, but for a pair of C6 cells expressing Cx32. Note that the curves do not reach a lower plateau (voltage independent conductance) as in Cx43-expressing cells and that the steepness of the curve has been substantially reduced. For both curves, data was normalized using a 200-ms and 10-mV hyperpolarizing pre-pulse.

metabolites to transfer through gap junction channels composed of Cx32 or Cx43. The mean total conductances between Cx32 transfectants and Cx43 transfectants were 33.8 nanoSiemens (S.E. = 4.0, $n = 13$) and 19.4 nanoSiemens (S.E. = 3.9, $n = 5$), while, as shown in Fig. 6, Cx32 and Cx43 transfectants displayed prominent unitary conductances of 80 and 130 pS, respectively. Values for total conductance were divided by the unitary conductance to calculate the number of functional channels expressed by each cell type. Based on these data, cells transfected with Cx32 or Cx43 expressed an average of 422 ± 50 or 162 ± 33 functional channels per coupled cell pair, respectively. This information was combined with comparisons of the intercellular transfer of specific metabolites shown in Fig. 3B to calculate their relative abilities to pass through channels formed by Cx32 or Cx43. As shown in Fig. 7, consistent with our previous data that utilized cell sorting (11), glucose, glutamate, and glutathione traveled ~5–10-fold more efficiently through channels formed by Cx43 than Cx32. The new layered culture system allowed us to reconfirm these molecules with
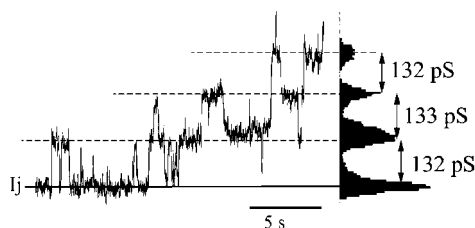
Cx32
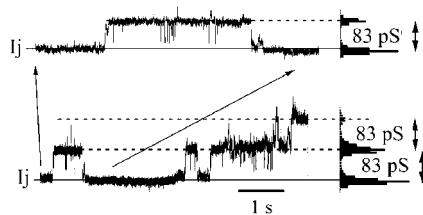
Cx43

FIG. 6. **Unitary conductances between C6 cells transfected with Cx32 or Cx43.** Representative current traces of unitary conductances displayed by pairs of cells transfected with Cx32 or Cx43 are shown. Cx32 or Cx43 transfectants produced channels with unitary conductances of about 80 pS or 130 pS, respectively.
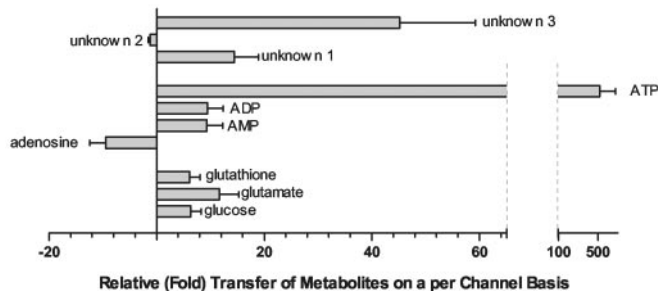

FIG. 7. **Relative transfer of specific metabolites through gap junctions formed by Cx32 or Cx43 on a per channel basis.** The relative permeability of a channel formed by Cx32 or Cx43 to a specific metabolite was calculated as percent of the radioactive molecule that traveled from a Donor cell to a Receiver cell divided by the ratio of the number of active channels expressed by each cell type as described under "Materials and Methods" and "Results." Data is shown as the mean + the highest estimated value based on the mean ± S.E. of channel numbers per cell pair.

much higher signals and to find two additional molecules that transferred about 10- or 40-fold more efficiently through channels formed by Cx43. In addition, the increased sensitivity of this technique showed that adenosine and an unidentified molecule traveled about 12- and 1.5-fold better, respectively, through channels formed by Cx32. Thus, not all metabolites prefer to travel through Cx43 channels.

Interestingly, the addition of phosphate to adenosine shifted its relative permeability from channels formed by Cx32 to channels formed by Cx43. While adenosine traveled about 12-fold more efficiently through channels formed by Cx32, Cx43 mediated the transfer of AMP and ADP an average of about 8-fold better and ATP greater than 300-fold better than Cx32. These data suggest that channels formed by Cx32 or Cx43 may
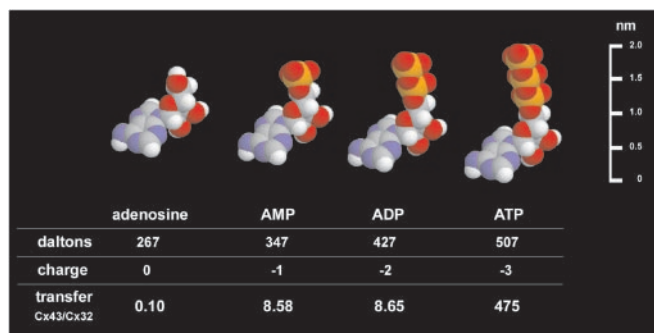

FIG. 8. **Effects of phosphorylation on adenosine structural properties and connexin permselectivity.** Least energy conformation structures of adenosine, AMP, ADP, and ATP in an aqueous solution of neutral pH are shown along with molecular masses in daltons, net charge, and permselectivity given as the average of the fold preferential transfer by Cx43 over Cx32 shown in Fig. 7. Carbon, nitrogen, oxygen, phosphorous, and hydrogen are shown in *gray*, *blue*, *red*, *orange*, and *white*, respectively (*scale bar* = nm).

serve specific roles via the selective transfer of certain molecules. In this case, Cx43 would equilibrate metabolic energy in the form of ATP throughout a coupled cell population much more efficiently than Cx32.

DISCUSSION

This report introduces a novel strategy to identify and quantitate the transfer of specific transjunctional metabolites. We isolated 10 metabolites of glucose as transjunctional molecules. The isolation of a specific transjunctional compound from Receivers does not necessarily indicate that it traveled through a channel. For example, transjunctional glutamate may have contributed to the presence of radioactive glutathione and dephosphorylation of transjunctional ATP may have contributed to ADP, AMP, and adenosine in Receiver cells. In any case, the biological consequences of gap junctional communication may rely on the fate of transferred molecules as well as the actual transjunctional molecule itself. Because our data indicate that homomeric channels formed from Cx32 and Cx43 show very different conductive properties for endogenous molecules, they suggest that the two do not transfer the same panel of transjunctional molecules and likely play distinct roles.

Bevans and Harris (25) have reported the existence of high affinity binding sites for cGMP and cAMP that affect permeability through hemichannels composed of Cx32 and Cx26. These interactions seem specific because other nucleotides including AMP, ADP, ATP, cTMP, and cCMP had no effect. In addition, cGMP transferred more readily through hemichannels formed by Cx32 than heteromeric channels formed by Cx32 and Cx26 (26). However, the channels used for these studies consisted of connexins incorporated into liposomes. Therefore, this previous work assayed the permeability of hemichannels, or connexons, as opposed to complete gap junctions. Nonetheless, the data demonstrate sensitive interactions between specific connexins and endogenous metabolites. We have more recently confirmed this scenario by documenting the preferential transfer of specific metabolites through channels composed of Cx32 and Cx43 (11).

In contrast to previous investigations, this study presents an approach designed to examine communication through gap junctions between organized cell layers that may resemble the order of tissues that exist *in vivo*. In addition to identifying several transjunctional metabolites, these results indicate that that phosphorylation of adenosine alters its permselectivity from channels formed by Cx32 to those of Cx43. Consistent with other reports, we have found that size and charge alone do

not exclusively dictate the relative abilities of unrelated molecules to pass through channels made from different connexins (10, 11, 26).

As shown in Fig. 8, adenosine, AMP, ADP, and ATP are likely to exhibit a similar overall shape. Increasing phosphorylation of adenosine would increase both the size and negative charge of the molecule (ATP is 240 Daltons larger and has three more negative charges than adenosine). A simplistic interpretation would be that Cx32 forms channels that restrict the passage of larger and more negatively charged molecules. However, the relative permselectivities of Cx32 and Cx43 may not be completely dictated by the formula weight or net charge of the permeant. For example, some significantly larger and more negatively charged compounds than ATP, such as calcein with a molecular weight of 623 and charge of -5, transfer equally well through gap junctions formed by either connexin (11). Nonetheless, the contrasting permeabilities of adenosine, AMP, ADP, and ATP through channels formed by Cx32 or Cx43 indicate that charge probably in combination with molecular shape may regulate the permselectivity of closely related molecular species, in this case metabolic currency in the form of adenosine and ATP.

Reduction of connexin expression often accompanies cell transformation. In the case of C6 glioma cells, the major endogenous gap junction protein, Cx43, is reduced compared with normal glial cells or astrocytes (15). Restoration of gap junctional communication by transfection with Cx43 induces contact growth inhibition of C6 cells, while Cx32 does not (12). Although a contribution by low basal levels of endogenous Cx43 that persists in these cells (as shown in Fig. 4) can not be excluded, the addition of Cx32 significantly altered the permselectivity of the channels to natural metabolites. Therefore, this system illustrates how the differential transfer of molecules through gap junctions between cells may have functional significance. Indeed, heteromeric and heterotypic connexin combinations may produce channels with stunning complexity to selectively transfer signals that underlie many processes in specific cell types.

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