Gap Junctions between Cultured Astrocytes: Immunocytochemical, Molecular, and Electrophysiological Analysis

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The properties of astroglial gap junction channels and the protein that constitutes the channels were characterized by immunocytochemical, molecular biological, and physiological techniques. Comparative immunocytochemical labeling utilizing different antibodies specific for liver connexin 32 and connexin 26 and antibodies to peptides corresponding to carboxy-terminal sequences of the heart gap junction protein (connexin 43) indicates that the predominant gap junction protein in astrocytes is connexin 43. The expression of this connexin in cultured astrocytes was also established by Western and Northern blot analyses. Cultured astrocytes expressed connexin 43 mRNA and did not contain detectable levels of the mRNAs encoding connexin 32 or connexin 26. Further, the cells contained the same primary connexin 43 translation product and the same phosphorylated forms as heart. Finally, electrophysiological recordings under voltage-clamp conditions revealed that astrocyte cell pairs were moderately well coupled, with an average junctional conductance of about 13 nS. Single-channel recordings indicated a unitary junctional conductance of about 50–60 pS, which is of the same order as that found in cultured rat cardiac myocytes, where the channel properties of connexin 43 were first described. Thus, physiological properties of gap junction channels appear to be determined by the connexin expressed, independent of the tissue type.

Gap junctions are plaques of intercellular channels that allow the passage of ions and small molecules between coupled cells (see Bennett and Goodenough, 1978; Bennett and Spray, 1985; Hertzberg and Johnson, 1988). Studies measuring the permeability of gap junctions to dye molecules of various sizes indicate that the maximum channel diameter is about 1.6–2.0 nm (e.g., Flagg-Newton and Loewenstein, 1979), allowing the passage of charged and uncharged molecules with molecular weights below 1 kDa. Thus, gap junction channels permit the exchange of current-carrying ions such as K⁺, second-messenger molecules such as cAMP, Ca, and inositol 1,4,5-triphosphate (IP₃), and

various metabolites. However, gap junctions are impermeable to macromolecules such as large peptides and oligonucleotides. The flow of ions and other small molecules between cells via gap junctions serves different functions in various tissues, depending upon which molecules are most important to the tissue's function.

Recent developments have dramatically extended our understanding of biochemical properties, short- and long-term regulation of function and synthesis, and fundamental biophysical properties of gap junctions (for reviews, see Bennett and Spray, 1985; Hertzberg and Johnson, 1988). Immunological and biochemical studies have indicated that gap junctions belong to a family of homologous but not identical proteins (Gros et al., 1983; Dermietzel et al., 1984; Hertzberg and Skibbens, 1984; Nicholson et al., 1985; Kistler et al., 1988). Full-length cDNAs for three mammalian connexins have been cloned and sequenced (Kumar and Gilula, 1986; Paul, 1986; Beyer et al., 1987; Zhang and Nicholson, 1989; Fishman et al., 1990). Homologous connexins have been identified in the amphibian *Xenopus* (Gimlich et al., 1990). The conductances of the gap junctional membrane and individual gap junction channels have been recorded and shown to be regulated by a variety of physiological and pharmacological stimuli (Spray and Sáez, 1987; Spray and Burt, 1990). Expression of gap junction proteins has been demonstrated to be regulated by hormones, extracellular matrix components, and cell-cycle conditions (Dermietzel et al., 1987; Spray et al., 1987). Control has been localized at both transcriptional and posttranscriptional levels (Azarnia et al., 1981; Flagg-Newton et al., 1981; Sáez et al., 1986, 1989b; Bargiello et al., 1987; Spray et al., 1987; Traub et al., 1987).

Gap junctions between glial cells may act as electrical synapses in the generation of slow electrical fields associated with neural activity (Castellucci and Goldring, 1970; Ransom and Goldring, 1973; Ransom, 1974). They are also believed to create a buffering system that is responsible for the dissipation of focal extracellular K⁺ increases resulting from neural activity (Kuffler and Nicholls, 1966; Orkand et al., 1966; Gardner-Medwin, 1983; Newman, 1986). It has been speculated for decades that both functions depend upon the presence of gap junctions between glial cells (Brightman and Reese, 1969). Gap junctions are commonly observed between astrocytes (Brightman and Reese, 1969; Dermietzel, 1973, 1974; Massa and Mugnaini, 1982, 1985) and between oligodendrocytes (Dermietzel, 1978) and have been reported to occur between astrocytes and oligodendrocytes (Massa and Mugnaini, 1982). Physiological observations complement the morphological data showing extensive electrical coupling of astrocytes *in vitro* (Kettenmann and Ransom, 1988). By combining immunocytochemical, electrophysiological, and

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molecular biological techniques, we were able to obtain substantial evidence that the major gap junction protein in cultured astrocytes is a polypeptide that is likely to be "cardiac" connexin 43.

Materials and Methods

Cultures of purified astrocytes. Cortex and striatum were dissected from fetal or postnatal rat brains [embryonic day 17 (E17), postpartum day 4/5 (P4/P5)]. Tissues were minced and incubated for 30 min in 0.1% trypsin (Gibco) at 37°C, followed by a 5-min incubation with DNAase I (1 mg/ml; bovine pancreas; Sigma). Following removal of the enzyme solutions, tissues were dissociated in medium by trituration with a small-bore Pasteur pipette and subsequent passage through a 50- μ m nylon mesh. Cells were mounted on poly-D-lysine coated glass coverslips or plastic tissue-culture dishes or flasks (Nunc). Cultures were grown in a medium consisting of 45% minimal essential medium, 45% Ham's F12, 10% fetal calf serum, penicillin (50 μ g/ml), streptomycin (50 μ g/ml), and glutamine (final concentration, 2 mM), buffered with 21 mM bicarbonate. Cultures were maintained in a 5% CO₂, 95% air atmosphere at 37°C at nearly 100% relative humidity.

Immunofluorescence. Immunofluorescence was carried out on the plated astrocytes at different time intervals. With cell densities given above, isolated or paired astrocytes were obtained between 3 and 12 hr after plating. Subconfluent cultures were obtained between the second and third week of culture; confluence was usually achieved after 4 weeks of cultivation.

Processing for immunofluorescence was performed as described (Dermietzel et al., 1984). In brief, cells were fixed in absolute ethanol at -20°C for 30 min, washed with Dulbecco's phosphate-buffered saline (PBS), and incubated in PBS, supplemented with 0.1% albumin in order to block nonspecific labeling. Incubation with primary antibodies was performed for 60 min at room temperature, followed by three washes in PBS and subsequent incubation with secondary anti-IgGs coupled to fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC; Sigma). Double immunofluorescence was performed in the same manner: the primary antibodies were added simultaneously, followed by the secondary antibodies.

Immunogold labeling. Immunogold labeling was performed on London Resin (LR)-White embedded ultrathin sections. Fixation was done by superfusion with 2% paraformaldehyde plus 0.1% glutaraldehyde in PBS (pH, 7.4) for 5 min, followed by 2% paraformaldehyde for 1 hr. The cultures were then rinsed in PBS at 4°C overnight, dehydrated in a graded series of ethanol concentrations, and immersed in 100% LR-White at 4°C for 12 hr. After reincubation of the samples in fresh LR-White, the samples were encapsulated in gelatin and resin and polymerized by irradiation using a black light source (UV light, 282 nm) at 4°C. Suitable consistency of the blocks was obtained after 3–4 d of polymerization. Ultrathin sections were sliced by means of a Reichert Scientific Instruments Ultratome (Buffalo, NY), mounted on Formvar/carbon-coated Cu electron microscopical grids, and further processed for immunolabeling. Labeling with the primary antibody was achieved by using a 10- μ l droplet of polyclonal anti-connexin 43 antiserum (obtained from E. Beyer, Washington University, St. Louis, MO) or affinity-purified anti-connexin 26 or anti-connexin 32 (5 μ g/ml) at room temperature for 15 min. Affinity-purified goat anti-rabbit IgG coupled to colloidal gold (5–6 nm; Janssen Pharmaceutica, Beerse, Belgium) was used as a secondary marker. The goat anti-rabbit IgG gold solution exhibited minimal background labeling at a dilution of 1:20. Staining of the sections was obtained with a solution of 5% uranyl acetate in ethanol. Electron microscopy was performed using a Phillips electron microscope Model EM 400 fitted with a goniometer stage.

Monoclonal and polyclonal antibodies. Six polyclonal antibodies were used in the studies presented here. Two of these were directed to rat liver gap junctional proteins, connexins 26 and 32. The specificity of the anti-liver gap junction antibodies has been documented previously (Dermietzel et al., 1984; Traub et al., 1989); each recognizes its own antigen in hepatocytes without any cross-reactivity with the other antigens. Four site-specific antibodies were used to determine presence and localization of connexin 43. These were prepared by immunization of rabbits with polypeptides corresponding to carboxyl-terminal regions of connexin 43, which is believed to be on the cytoplasmic portion of the molecule (Yancey et al., 1989). The antigenic polypeptides were amino acid positions 252–271 ("Petunia"; characterized in Beyer et al., 1989), 241–260 ("16A"), 283–298 ("17B"), and 346–360 ("18A"). These

anti-connexin 43 antibodies have been proven to react specifically with gap junctions in the intercalated disks of heart muscle cells through immunofluorescence and electron microscopic immunocytochemistry (Beyer et al., 1989; Yamamoto et al., 1990). GFAP immunoreactivity was evaluated by means of a monoclonal anti-GFAP antibody (Dianova, Hamburg).

Electrophysiology. Cell pairs were obtained by dissociating fresh, pure populations of confluent glial cultures using a trypsin-EDTA protocol (see above). These cells remained round in form for as long as 24 hr after treatment, quickly adhering to plastic Petri dishes. Each cell from a pair was penetrated with brief, strong suction after a high-resistance seal was obtained against the cell membrane using a polished patch pipette (3–5 M Ω ; filled with 120 mM K glutamate, 10 mM KCl, 10 mM EGTA, 1 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES at pH 7.2, with or without 2–5 mM MgATP). Cells were voltage clamped at holding potentials of -60 mV, and command steps were presented alternately to each cell of the pair. Junctional conductance (g_j) was measured as the current evoked in one cell by the voltage step in the other, divided by the amplitude of the voltage step delivered to the other cell (Spray et al., 1981, 1986a,b; Spray and Bennett, 1985). Intracellular injection of Lucifer yellow (5% in 150 mM LiCl, introduced by hyperpolarizing current pulses or by brief overcompensation of the capacity neutralization circuit) was used to assay dye coupling between astrocytes. Injected fields were photographed 1 min after the injection was initiated by exposing Kodak TMAX 400 film for 30 sec.

Western and Northern blots. For Western blots, astrocyte cultures were scraped, pelleted, and washed with PBS prior to lysis by sonication and solubilization in SDS-containing buffer. Rinse and solubilization buffers also contained a protease inhibitor, phenylmethylsulfonyl fluoride (2 mM), and phosphatase inhibitors, sodium pyrophosphate (20 mM) and sodium fluoride (100 mM). Adult rat hearts and brains were homogenized using a polytron prior to solubilization in SDS. Proteins (50 μ g) were resolved by electrophoresis on 10% polyacrylamide gels and electroblotted to sheets of nitrocellulose paper (Hertzberg et al., 1988). Blots were then blocked in 5% dry milk and probed with an anti-connexin 43 peptide (amino acid positions 346–360) raised to a peptide-BSA conjugate in a rabbit. Antibody binding was visualized by incubating the blot with ¹²⁵I-Protein A and autoradiography. Intensities of bands on the autoradiograph were determined using a Molecular Dynamics scanning densitometer.

RNA was isolated from cultured astrocytes by minor modifications of published methods (Chomczynski and Sacchi, 1987). Briefly, the cells were washed and scraped in 4 M guanidinium thiocyanate containing 25 mM sodium citrate (pH, 7), 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. After phenol/chloroform extraction, the RNA was twice precipitated in ethanol, dried, and resuspended in water. PolyA⁺ RNA was isolated by oligo-dT-cellulose chromatography by the method of Maniatis et al. (1982).

Northern blot analyses were performed as described in our previous publications (e.g., Spiegel et al., 1990). Single-stranded antisense RNA probes were synthesized with SP6 or T7 polymerase. A full-length rat liver cDNA encoding connexin 32 was obtained from Dr. D. P. Paul (Harvard University Medical Center). A full-length rat heart cDNA encoding connexin 43 was obtained from Dr. E. Beyer (Washington University School of Medicine, St. Louis, MO). A full-length rat liver cDNA encoding connexin 26 was obtained from Dr. B. J. Nicholson (State University of New York, Buffalo, NY). Northern blots were prehybridized for 6–24 hr at 65°C in 50% formamide, 1 M NaCl, 1% SDS, and 10% dextran sulfate. Blots were hybridized at 65°C for 6–24 hr in prehybridization solution to which 0.1 mg/ml denatured calf thymus DNA and 5 $\times$ 10⁶ dpm of the probe had been added. Blots were then extensively washed at 65°C and exposed to Kodak XAR-5 film at -90°C. Autoradiographic signals were quantitated by densitometric analysis using a Hoefer model GS 300 Transmittance/Reflectance Scanning Densitometer interfaced with a Hewlett-Packard 3392A Integrator.

Results

Purity of cultured astrocytes

Prescreening of cultured astrocytes proved essential for each experimental series. A careful examination was necessary in order to prevent contamination by leptomeningeal cells, which have recently been shown to coexpress not only cardiac connexin 43 but also a connexin homologous to the liver connexin 26

(Dermietzel et al., 1989). The purity of astrocyte cultures was therefore consistently controlled by anti-GFAP immunostaining. Only cultures of the highest purity (>95%) were utilized for further experimentation. A representative culture stained with anti-GFAP is shown in Figure 1A.

Localization of connexin 43 immunoreactivity in GFAP-positive cells

Freshly plated cells (<12 hr) revealed vesicular intracytoplasmic staining with antibodies prepared against connexin 43 (Fig. 1B, C). In subconfluent and confluent cultures (Fig. 1D, E), immunoreactivity was preferentially prevalent along contiguous plasma membrane sites. Intracytoplasmic staining in the form of vesicular structures, however, remained abundant and was even found to increase after prolonged culturing (>4 weeks). The plasma membrane-associated stain occurred in punctate or threadlike assemblies along the cell periphery. By phase-contrast microscopy, most of these points were localized at margins of abutting cells (Fig. 1D, E).

Double immunofluorescence using a monoclonal antibody to GFAP and the anti-connexin 43 antibody demonstrated connexin immunoreactivity in anti-GFAP positive cells (Fig. 2A, B). No immunoreactivity was observed when antibodies to liver connexin 32 and connexin 26 were applied to the astrocytes at any time during cultivation (not shown).

In order to determine whether diverse connexin 43 antibodies also produced similar staining of cell appositional membranes, cultured confluent astrocytes were stained with three antibodies prepared against polypeptides corresponding to unique epitopes in the carboxyl terminal domain (Fig. 3A, B, D, E, G, H). Whereas each of these antibodies revealed punctate outlining of cells, in no case was staining observed with preimmune serum (Fig. 3C, F, I).

Immunogold labeling of connexin 43-positive sites

Ultrathin sections of gap junction domains showed significant gold labeling after incubation with anti-connexin 43 (Fig. 3C). No labeling was encountered following the use of anti-connexin 32 or anti-connexin 26. In addition to plasmalemma-associating staining, intracytoplasmic double membrane-bound structures were frequently observed (Fig. 3D); these structures are believed to represent endocytosed gap junctions (Risinger and Larsen, 1981; Mazet et al., 1985).

Western and Northern blots

On Western blots of astrocyte homogenates, two distinct bands were observed: a faint lower band with an apparent molecular weight of approximately 41 kDa, and a higher-molecular-weight band, resolved as a doublet, of approximately 43 kDa (Fig. 4, lane C). Similar profiles were observed for heart and brain homogenates (Fig. 4, lanes A, B, respectively), although the relative abundance of the 43- and 41-kDa material differed in these samples. It has been demonstrated that the 43-kDa protein bands arise from phosphorylation of the 41-kDa band (Crow et al., 1990; E. L. Hertzberg, R. Corpina, C. Roy, M. J. Dougherty, and J. A. Kessler, unpublished observations). Densitometric analysis of these profiles indicated that the brain and astrocytes had, respectively, approximately 300% and 180% of the immunoreactive connexin 43 present in heart (41 kDa and 43 kDa combined). The relative amount of the 41 kDa form of connexin 43, as a percent of total immunoreactivity, was about 11% for

the astrocyte sample, as compared to 57% for the brain and 15% for the heart homogenates.

Northern blot analysis of astrocytic RNA using a cDNA for heart connexin 43 demonstrated a band at about 3.0 kDa, which was identical in size to mRNA detected in heart (Fig. 5, leftmost lanes). By contrast, Northern analyses using probes for connexin 32 and connexin 26 failed to demonstrate the corresponding mRNAs in astrocytic RNA but did label appropriate bands in liver RNA (Fig. 5, center and rightmost lanes).

Electrical and dye coupling between pairs of astrocytes

Pairs of astrocytes viewed 2 hr (Fig. 6A, B) or 5 hr (Fig. 6C, D) after dissociation from confluent cultures exhibited strong coupling with Lucifer yellow CH. Within 1 min after dye injection into one cell of a pair, dye was detected in the other cell, often approaching equilibrium where the recipient cell was nearly as brightly fluorescent as the injected one (Fig. 6B).

In order to quantify the degree of electrical coupling between astrocytic pairs, each cell was voltage clamped to a common holding potential (usually -60 mV); voltage pulses were then delivered alternately to each cell. The current flowing through the junctional membrane is equal to the magnitude of current introduced into one cell in order to compensate for current passing into the cell through the junctional membrane from the command pulse to the other cell (Fig. 7, inset). We recorded junctional currents in 44 astrocytic cell pairs (Fig. 7) in which nonjunctional and seal conductances were less than 1 nS. Mean junctional conductance in these cell pairs was calculated to be 12.79 ± 11.4 nS ($\pm$ SD).

In order to evaluate the sensitivity of junctional conductance to transjunctional voltage, prolonged voltage pulses of ± 50 mV were applied to one cell while the other cell's voltage was held constant at 0 mV. Even when transjunctional voltage pulses as long as 10 sec in duration were applied, the junctional current remained constant (to within 90% of the initial value; not illustrated). At higher transjunctional voltages, junctional current relaxed during the step, but this indication of weak voltage dependence was not systematically pursued. Compared to some other systems, astrocyte gap junctions thus display less sensitivity to transjunctional voltage.

Sensitivity of junctional conductance in astrocytes to gap junction inhibitors

In order to determine whether junctional conductance between pairs of astrocytes can be reduced by treatments that affect gap junctions in other tissues (for review, see Spray and Burt, 1990), cell pairs were exposed to 1.5 mM heptanol (Fig. 8A), to elevated CO_2 (Fig. 8B), or to 2.0 mM halothane (Fig. 9A). Junctional conductance recorded between cell pairs was reversibly reduced by each of these treatments; the minimal level of coupling achieved following brief exposure to these agents was 5 pS or less.

In order to determine the magnitude of the unitary conductance of single gap junction channels between astrocytes, recordings were made with high gains in cell pairs in which junctional conductance was initially low. As g_j was further reduced by exposure to halothane, the reduction in conductance was associated with steplike declines of about 50–60 pS (Fig. 9A, B). Upon rinsing, g_j s resumed normal values; this recovery was associated with incremental changes in conductance similar to those obtained upon uncoupling. The conductance increments and decrements satisfy the primary requirement for gap junction

Figure 2. *A* and *B*, Double-immunolabeled astrocytes showing anti-connexin 43 immunoreactivity (*A*) and anti-GFAP immunoreactivity (*B*) in identical sets of cells. *C*, Immunogold-labeled gap junction between two astrocytes (*A*₁, *A*₂). *D*, Annular (presumably endocytosed) gap junctions labeled with anti-connexin 43 immunogold. Magnification: *A* and *B*, 700 $\times$; *C* and *D*, 80,000 $\times$.

channels: they occurred synchronously as equal and opposite events in the two cells' current traces.

In three cell pairs (including the pair illustrated in Fig. 9*A,B*), we were able to measure current steps corresponding to more than 100 transitions between open and closed states of the gap junction channels. The relative frequencies of unitary conductances of various amplitudes are shown in Figure 9*C*. More than 50% of the unitary events corresponded to 55-pS channels, and an additional 40% were measured as 50 or 60 pS. Similar unitary

conductance values were calculated from three other experiments where smaller numbers of transitions were measured (not illustrated).

Discussion

Astrocytes form an electrical syncytium

Astrocytes possess an elaborate system of intercellular communication mediated by gap junctions (Brightman and Reese, 1969; Dermietzel, 1974; Massa and Mugnaini, 1982) and ex-

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Figure 1. Localization of gap junction protein in astrocytes. *A*, Distribution of anti-GFAP immunoreactivity in confluent astrocytes (>3 weeks in culture). *B*, Anti-connexin 43 immunoreactivity in freshly plated cells (<12 hr) showing abundant vesicular staining (arrows). *C*, Phase-contrast micrograph corresponding to *B*. *D*, Anti-connexin 43 immunoreactivity in confluent (>3 weeks) astrocytes. Note that in addition to some intracytoplasmic staining (asterisks), the fluorescence is confined to plasmalemmal areas (arrows). *E*, Phase-contrast micrograph corresponding to *D*. Antibody used in *B* and *D* (Petunia; Beyer et al., 1987) was prepared against an epitope overlapping with that used to prepare antibody illustrated in Figure 2*A*. For these experiments, cells were permeabilized in 70% EtOH for 30 min at -20°C and incubated in 1:200 dilution of primary antibody, and then in FITC-coupled secondary antibody. Magnification, 700 $\times$.

Figure 3. Antibodies prepared against three separate connexin 43 epitopes demonstrate immunostaining consistent with the presence of gap junctions between cultured astrocytes. *A* and *B*, *D* and *E*, and *G* show results obtained with antibodies 16A, 17B, and 18A, respectively. Phase-contrast photographs of the confluent cultures are shown in *A*, *D*, and *G*; immunofluorescence photographs are shown in *B*, *E*, and *H*, and immunofluorescence photographs of the permeabilized cells are shown in *C*, *F*, and *I*. For these experiments, cells were permeabilized with 70% EtOH at -20° for 20 min and treated with 1:1000 dilution of primary anti-connexin 43 antibody (or preimmune control serum), then with FITC-labeled secondary antibody.

hibit electrical and dye coupling *in vivo*. The development of procedures for isolating the two classes of macroglia and maintaining them in culture for a prolonged period has permitted an analysis of questions concerning cell-to-cell interaction that were previously not tractable (Kettenmann et al., 1983; Kettenmann, 1985; Ransom and Carlini, 1986). Electrophysiological studies show that cultured astrocytes retain their basic physiological properties and, as a unit, form a highly coupled electrical and biochemical syncytium (Fischer and Kettenmann, 1985; Kettenmann and Ransom, 1988).

The unique topological arrangement of astrocytes *in vivo*, constituting a physical link between the neurons and the perivascular compartment, provides a shunt between excitable nervous tissue and blood vessels as part of the potassium homeostatic mechanism (Gardner-Medwin, 1983; Newman, 1986, 1987). In this manner, gap junctions, consisting of transmembranous channels that link individual astrocytes together, create a complex, electrically coupled network (Kettenmann and Ransom, 1988). Our application of molecular techniques to astrocytic gap junctions provides new insights into the molecular composition of these channels and contributes to a better understanding of their functional properties.

Evidence that the gap junction protein in astrocytes is cardiac connexin 43.

Our immunofluorescence data indicate that astrocytes express the same connexin *in vitro* as has been detected in *in vivo* samples of cryostat-sectioned brains (Dermietzel et al., 1989; Yamamoto et al., 1990). In addition to showing significant immunolabeling of astrocytic gap junctions with site-specific antibodies directed to the cardiac gap junction protein connexin 43, immunoblots demonstrated comigration of an astrocytic protein with cardiac and leptomeningeal connexin 43. Furthermore, Northern blots of polyA⁺ mRNA from cultured astrocytes using cDNA encoding cardiac connexin 43 (Beyer et al., 1987) showed hybridization to a 3.0-kilobase band that was similar in size (and also similar in abundance) to connexin 43 mRNA in heart samples. The high level of binding indicated the presence of a considerable number of mRNA copies of connexin 43 in cultured astrocytes.

Functional relationship between astrocytic and cardiac connexins

The findings presented above allow the discussion of the electrophysiological and pharmacological properties of the astrocytic gap junction channels on a comparative basis. We found that the unitary conductance of single astrocytic channels was on the order of 50–60 pS, similar to that found in neonatal rat heart cells and other cell types that express connexin 43 (Burt and Spray, 1988a; Rook et al., 1988; Rudisli and Weingart, 1989; Fishman et al., 1990; Spray and Burt, 1990). Heptanol as well as halothane led to significant and reversible reduction of astrocytic coupling as found in cardiac cells (Burt and Spray, 1989).

The relative voltage independence of the astrocytic gap junction channels resembles that of cardiac gap junctions (Burt and Spray, 1988b). This property of the astrocytic gap junction substantiates the function of the astrocytic electrical network as a "spatial buffer" for potassium ions when the extracellular potassium level is elevated by neuronal activity (Orkand et al., 1966; Newman, 1987). Uniform depolarization of glial cells resulting from a local increase of extracellular potassium would

Figure 4. Identification of connexin 43 from astrocyte cultures. Lane A, immunoblot from heart homogenate; lane B, brain homogenate; lane C, astrocyte homogenate. Homogenates were resolved by 10% SDS-PAGE, transferred to nitrocellulose, and probed with an antibody specific for amino acids 346–360 of the connexin 43 sequence. Arrows indicate mobilities as determined from *M_r* markers.

thus not affect junctional conductance and would not impede current flow that is necessary for the redistribution of potassium in every direction (Kettenmann and Ransom, 1988).

Immediate and long-lasting uncoupling of astrocytes *in vitro* by cytoplasmic acidification via lactic acid has been previously described (Anders, 1988). Our data show a clear decrease of junctional coupling between astrocytes after exposure to an elevated CO₂ level in the bathing medium. A similar effect also occurs in cardiac cells when intracellular acidosis is induced by CO₂ superfusion (Burt and Spray, 1988b). It has been suggested that, under pathological conditions, intracytoplasmic acidification of astrocytes may impair their regulation of the CNS microenvironment (Anders, 1988).

Probable involvement of astrocytic gap junctions in signal processing of the CNS

Of special relevance to our observations is the finding that some neurotransmitters and second messengers are capable of modulating junctional coupling. cAMP has been reported to promote junctional coupling in sympathetic neurons (Kessler et al., 1985)

and hepatocytes (Sáez et al., 1989b). Neurotransmitter effects on junctional coupling between retinal horizontal cells have been reported (Piccolino et al., 1984; Lasater, 1987); furthermore, norepinephrine has been shown to modulate the coupling strength between pinealocytes (Sáez et al., 1989b). Recently, Burt and Spray (1988a) showed that ionotropic agents can modulate coupling in cardiac cells.

Although there is no direct evidence of a regulatory effect of second messengers and/or neurotransmitters on glial coupling, the recent discovery of glutamate-activated ion channels in type 1 (Sontheimer et al., 1988) and type 2 astrocytes (Urowicz et al., 1989) raises the important issue of neuronal–glial interaction. As has been suggested by Urowicz et al. (1989), the activation of glial–glutamate receptors could alter local concentrations of sodium and/or potassium, especially at sites of nonsynaptic neurotransmitter release, for example, at the node

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Figure 5. Northern blot analysis of connexin 43 (*cx43*) in cultured astrocytes. RNA isolated from cultured astrocytes (A), heart (H), or liver (L) was examined for content of connexins 43, 32, and mRNA. Ten micrograms of RNA were loaded onto each lane. The lower and upper arrows represent the positions of 18S and 28S RNA, respectively.

Figure 6. Dye coupling between pairs of astrocytes in culture. On the left are phase-contrast micrographs of astrocytes at 2 hr (A) and 5 hr (C) after dissociation. On the right (B, D) are corresponding fluorescence micrographs taken with FITC barrier and excitation filters at 1 min after injection of Lucifer yellow into the cell to the left in the pair (asterisks, stars).

Figure 7. Strength of electrical coupling between pairs of astrocytes. The inset shows a typical recording from an astrocytic cell pair under voltage-clamp conditions. Voltage command steps (V_1 , V_2) were delivered alternately to each cell, and corresponding currents (I_1 , I_2) were recorded. Upwardly directed responses in the current records represent current through junctional membranes (I_j); junctional conductance, g_j , is calculated as $-I_j/V$. Junctional conductances recorded between the 44 pairs of astrocytes used in these experiments are shown in the histogram. The mean g_j value was 12.8 ± 1.75 nS ($\pm$ SE).

of Ranvier (Marraro et al., 1989). Alternatively, glutamate-induced depolarization could activate voltage-dependent Ca^{2+} channels also present in type 2 astrocytes as is suggested by electrophysiological evidence (Barres et al., 1988) and by the recent demonstration of intracellular Ca^{2+} oscillation using the fluorescent dye fluo-3 after glutamate perfusion of astrocytic cultures (Cornell-Bell et al., 1990). Because gap junctions have been found to be nonselective for ions (Harris et al., 1981; Verselis et al., 1986) and permeable to second-messenger molecules (Sáez et al., 1989a), one possible role of astrocytic gap junctions could be the coordination of local neural-glial interactions, either by shunting ionic gradients (see above) or by serving as a route for second-messenger exchanges (Sáez et al., 1989a). Hence, the proposed existence of dynamic neural-glial signaling processes within parts of the nervous system may depend on astrocytic gap junction coupling.

Gating properties of glial connexins

We have reported that connexins are coexpressed in a variety of tissues (Nicholson et al., 1987; Dermietzel et al., 1989; Traub et al., 1989; Spray et al., 1991b). In astrocytes, however, we found only one type of connexin with our set of anti-connexin antibodies and corresponding cDNA probes. These data are consistent with the observed unitary channel conductance of 50–60 pS. In this context, it is noteworthy that oligodendrocytes, unlike astrocytes, express connexin 32, which was also found in a number of neurons in rat brain tissue (Dermietzel et al., 1989).

The fact that the two classes of macroglia express different types of connexins may be of considerable functional significance. According to the different connexin composition we have observed for astrocytes and oligodendrocytes, we suggest that the gap junctions observed between these glial types (Massa and Mugnaini, 1982, 1985) represent the first report of naturally occurring heterologous coupling. This pairing of different types of hemichannels suggests the possibility of differential regulation by factors acting on each side of the channel. Although no experimental evidence of heterologous glial coupling *in vivo* exists, such an asymmetrical glial coupling could ultimately result in rectification, because the connexin 32 channel expressed in mammalian cells has been demonstrated to be voltage dependent (Spray et al., 1991a), while the connexin 43 channel is much less so (Burt and Spray, 1988b). Rectifying junctions created by pairing of connexins to form heteromolecular channels have been demonstrated recently by injecting RNA coding for connexin 43 into *Xenopus* oocytes and pairing these with water-injected oocytes that express endogenous voltage-sensitive connexins (Swenson et al., 1989; Werner et al., 1989).

Such a heterologous construction at sites of glial contact would allow unidirectional closure of junctional channels whenever significant transjunctional voltage gradients exist between astrocytes and oligodendrocytes. In addition, it would permit a bidirectional exchange of small metabolites, second messengers, and ions when the transjunctional voltage gradient is low. Asymmetrical voltage dependence of heterologous glial coupling would thus result in an effective sharpening of astrocytic current spread

Figure 8. Pharmacological modification of electrical coupling between astrocyte cell pairs. *A*, Uncoupling of astrocyte cell pairs during exposure to 1.5 mM heptanol. During exposure to heptanol (line above recordings), the amplitude of the upward current deflections decreased to unmeasurably low levels and then recovered during rinsing. Traces are as in Figure 7, inset. *B*, Uncoupling of an astrocyte cell pair during exposure to external solution equilibrated with 100% CO_2 . 1, Under control conditions, g_j in this cell pair was low (about 2 nS). 2, Two minutes after continuous exposure to saline solution equilibrated with 100% CO_2 , g_j was less than 100 pS. 3, Two minutes after return to normal saline, g_j recovered substantially. Traces are as in Figure 7, inset.

generated by neuronal impulse activity on the astrocytic side and a blockage of shunting to the oligodendrocytic side.

In vitro studies of mixed glial cultures may thus serve as an advantageous tool to definitely prove whether heterologous functional coupling exists between these species of macroglia.

Conclusions

The findings presented here demonstrate that the astrocytic gap junction protein (and its mRNA) are indistinguishable from connexin 43, the gap junction protein of heart. Immunocytochemical studies demonstrate that the same protein is expressed between astrocytes in the brain *in vivo* (Dermietzel et al., 1989; Yamamoto et al., 1990) as in pure astrocytic cultures. Our electrophysiological studies indicate that the properties of gap junction channels between astrocytes are indistinguishable from those of heart: Conductance in both systems is reduced by heptanol, CO_2 , and halothane, and conductances of the unitary events are predominantly about 50–60 pS. Thus, connexin type as distinct from cellular environment may be a primary determinant of

Figure 9. Single-channel recordings from pairs of cultured astrocytes. *A* and *B*, Halothane exposure (2 mM) uncouples astrocytes and reveals single gap junction currents between an astrocyte pair. Current recordings from both cells are shown, with voltage of the upper cell of the pair at 0 mV and voltage of the lower cell at -50 mV. The calibration bar thus represents 2.5 pA, corresponding to 50 pS; records shown are chart recordings obtained with a Gould Brush recorder with frequency response limited to about 80 Hz. At the start of the recording shown in *A1*, g_j is fluctuating between 50 and 100 pS. After the addition of 1 mM halothane to the perfusate (between the arrows in *A* and *B*), g_j declines to a very low level (≤ 5 pS). Upon rinsing with normal saline, g_j recovers with steplike conductance changes of about 50–60 pS. *C*, Frequency distribution of unitary conductances calculated from three experiments in which amplitudes of more than 100 transitions were measured and normalized to the total number of events of each size category. Error bars represent SD of the frequencies among the experiments.

junctional phenotype, as is also indicated when connexin properties are compared in the same cell type stably transfected with connexin 32 or connexin 43 (Eghbali et al., 1990; Fishman et al., 1990).

Despite the apparent identity of gap junction proteins in heart and in astrocytes, the cellular types in which they are expressed would be expected to give rise to differential regulatory properties. For example, second messengers would be expected to be regulated by separate sets of membrane receptors in these cell types. At the organ level, heterologous coupling with connexin 32 gap junction hemichannels would be expected to produce rectifying junctions in brain.

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