C-erbB2/neu Transfection Induces Gap Junctional Communication Incompetence in Glial Cells

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Astrocytes form functional networks that participate in active signaling in which external stimuli are generated and amplified in many of the same ways as in neurons. Gap junctions between astrocytes offer the structural avenue by which the electrical and metabolic signals are propagated from one cell to another. Little is known about the trafficking, assembly, and degradation mechanisms of the major astrocytic gap junction protein connexin43. We have studied a glial cell line transfected with the C-erbB2/neu oncogene (neu

phorylation isoforms is disturbed. Confocal laser imaging indicates that the major deficit in the neu

cells is attributable to a lack in plaque assembly of connexin43. Because the neu

cells also lack N-CAM proteins and because work from others has indicated a close relationship between communication competence and constitutive CAM expression, our data suggest that expression of C-erbB2/neu oncogene alters cell–cell association via CAM proteins, which thereby affects gap junction plaque assembly and appropriate phosphorylation of connexin43.

Key words: gap junctions; astrocytes; oncogene; connexin43; phosphorylation; N-CAM

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Gap junctions provide intercellular pathways for electrical and metabolic coupling between adjoining cells and are found in almost all tissues except for a few cell types, such as mature skeletal muscle cells and circulating blood cells. In the CNS, gap junctions have been found between neurons and various other cell types (for recent review, see Dermietzel and Spray, 1993), and it is generally well accepted that they constitute the structural link by which electrically and/or metabolically coupled compartments of the CNS are created. Gap junctions are composed of transmembrane channels that directly couple the cytoplasm of adjoining cells. The channels are composed of hemichannels (connexons) in one plasma membrane joined in mirror symmetry with a connexon in the adjacent cell (Caspar et al., 1977; Makowski et al., 1977). Each hemichannel is an oligomer of six protein subunits (connexins). Recent cloning works have elucidated that connexins form a protein family, the members of which show a highly diversified distribution among different cell types (Bennett et al., 1991; Beyer, 1993; Dermietzel and Spray, 1993). In the CNS, a cell-specific and developmentally regulated expression of three connexin isoforms has been demonstrated (Dermietzel et al., 1989). Via the molecular characterization of the connexin protein family and the subsequent generation of specific probes, i.e., isotopic antibodies, cDNAs etc., it has become feasible to better address the issue of occurrence and the potential importance of electronic transmission in mammalian brain and spinal cord. Astrocytes present one cell type in which these issues can now be addressed, because a detailed characterization of the major astrocytic connexin type and its functional properties recently has been achieved (Dermietzel et al., 1991; Giaume et al., 1991a).

Astrocytes form an extensively coupled syncytium, which is assumed to play a crucial role in the homeostatic balance of the interstitial cerebral fluid. A variety of regulatory mechanisms have been assigned to astrocytes in which the presence of intercellular coupling is essential. A prominent feature is the uptake and buffering of K+ surrounding active neurons. Gap junctions may provide a direct pathway from the site of potassium disposal to the perivascular compartment or, alternatively, may increase the volume of the astrocytic buffer sink by connecting multiple cytoplasmic units (Kuffler et al., 1966; Orkand et al., 1966; Gardner-Melvin, 1983; Newman, 1986; Walz, 1989). Another feature of considerable functional significance is the ability of astrocytes to create spontaneous Ca2+ waves after topical glutamate application or mechanical stimulation (Cornell-Bell et al., 1990; Finkbeiner, 1992). Such transjunctional oscillatory Ca2+ signaling provides the possibility of a dynamic population response in an electrically coupled astrocytic network. The demonstration that astrocytes possess neurotransmitter receptors in culture and in slice preparations has been taken as a strong indication that astrocytes participate in active signaling in which external stimuli are generated and amplified as in neurons (Barres et al., 1990; Parpura et al., 1994). In all likelihood, astrocytic gap junctions offer the structural avenue by which the electrical and metabolic signals are mediated (Dermietzel and Spray, 1993).
Very little is known about the molecular mechanisms that regulate the coupling efficiency between astrocytes. In a recent series of experiments on sarcoma cell lines, Musil et al. (1990) showed that cell adhesion is an important prerequisite for con

nexion43 (Cx43) insertion into the plasma membrane. In addition, they provided evidence that post-translational phosphorylation of Cx43 is an important event in gap junction formation and/or activation. Communication incompetence can also be obtained by transfection of cell lines with various viral oncongenes (Azarnia and Loewenstein, 1987; Bignami et al., 1988; Dotto et al., 1989; Martin et al., 1991). For the src oncogene product p60-src, phosphorylation of Cx43 at tyrosine 265 has been shown to be responsible for the communication deficiency (Swenson et al., 1990). From these studies, it seems reasonable to expect that similar mechanisms may be influential on interglial coupling. We therefore transfected a communication-competent glial cell line with the rat C-erbB2/neu oncogene (neu1), which encodes a 185 kDa transmembrane protein with intrinsic tyrosine kinase activity, and which exhibits extensive structural homology with the epidermal growth factor receptor (Coussens et al., 1985; Bargmann et al., 1986a,b). We found that transfection of the glial cell line resulted in a significant reduction of cell–cell communication. In contrast to the effect reported for the v-src gene product, we did not detect phosphorylation at the tyrosine sites; rather, we found a remarkable reduction of the higher molecular weight isoforms of Cx43 that are believed to arise from phosphorylation of serine/threonine residues. In addition, the C-erbB2/neu oncogene transfection also resulted in a loss of expression of the cell adhesion molecule N-CAM, which might largely explain the reduced gap junctional communication.

MATERIALS AND METHODS

Cultures of purified astrocytes and rat glial cells. Cultures of purified astrocytes were obtained as described (Dermietzel et al., 1991) and cultured for 12 d. A wild-type Sprague–Dawley rat glial cell line (gift from Dr. A. Koestner, Michigan State University) has been previously reported to be well coupled under subconfluent conditions (Sutter et al., 1987). Growth media. Cultures of purified astrocytes were grown in a medium containing 45% minimal essential medium (MEM), 45% Ham’s F12, 10% fetal calf serum, penicillin (50 3 M), streptomycin (50 mg/ml), and 2 mM glutamate, buffered with 25 mM bicarbonate. The rat glial cell line was grown in MEM supplemented with 1 mM sodium pyruvate, 0.05% non-essential amino acids (Biochrom, Berlin, Germany), 0.1 mg/ml gentamycin, and 10% fetal calf serum, buffered with 25 mM bicarbonate. Medium for culturing the C-erbB2/neu transformed rat glial cell line contained 0.05 mg/ml G418 as an additive. All cultures were grown at 37°C in 5% CO2, 95% air atmosphere in humidified water-jacketed incubators.

Transfection and subculture. The C-erbB2/neu transforming oncogene, with a substitution mutating valine to glutamic acid at residue 664, was derived originally from DNA of ethylnitrosourea-induced rat glioblastomas. The neu proto-oncogene (tr+, with val 664) and its neu transforming oncogene (tr−, with glu 664) were inserted into the SalI cloning site of a modified version of pDOL retrovirus vector, containing the selectable G418 neomycin-resistance marker. As a control, cells were infected by a virus with no neomycin-resistance marker. For transfection of subconfluent rat glial cells, undiluted viral supernatant (containing 1 3 106 cells/ml) was added separately with 8 mg/ml polybrene for 2 hr at 34°C. Resistant cells were selected in medium containing 0.5 mg/ml G418 2 d after viral infection and allowed to continue to grow for 7 d. For this study, the clone (GN-7) that stably transfected with the transforming oncogene (designated neu+) was compared with parental wild-type cells (designated neu−) transfected with the virus containing the neomycin-resistant marker but not the neu insert (here designated neo). In addition, clones overexpressing the neu proto-oncogene (tr−) were screened for phenotypical appearance and dye transfer.

Immunofluorescence. Processing for immunofluorescence of Cx43, N-CAM, and glial fibrillary acidic protein (GFAP) was performed as described (Dermietzel et al., 1984). Briefly, cells were fixed in absolute ethanol at −20°C. After washing twice with PBS (10 mM phosphate buffer, 0.9% NaCl, 140 mM NaCl), cells were incubated with 10% horse serum and 1% bovine serum albumin (BSA) to block nonspecific labeling. The primary antibody was applied for 60 min at room temperature, removed by three washes with PBS supplemented with 1% BSA, and followed by incubation with the secondary fluorescein isothiocyanate (FITC)-labeled antibody (Sigma, Munich, Germany).

Northern blots and in situ hybridization. Isolation and Northern blots of total RNA were performed according to standard procedures (Sambrook et al., 1989) with the following modifications: total RNA of subconfluent cultures was extracted with 4 M guanidinium thiocyanate, 25 mM sodium acetate, pH 6.0, 1.0% mercaptoethanol followed by centrifugation at 100,000 g (rotor: Sorvall TH-641) in 5.7 M sodium acetate buffer, pH 7.5, 140 mM NaCl), cells were incubated in PBS for 2 hr in 0.1% SDS at 62°C. Blots were exposed to X-OMAT AR 5 film (Eastman Kodak, Rochester, NY) at −70°C. Subconfluent monolayers of neu+ and neu− cells were grown on glass coverslips overnight and used for in situ hybridization as described (Dermietzel et al., 1992). Briefly, cells were rinsed in PBS and fixed four times repeatedly in 4% freshly prepared paraformaldehyde in PBS for 20 min at room temperature, followed by fixation in a solution of 25% acetic acid and 75% methanol at −20°C. Cells were washed through PBS and blocked three times for 10 min in 100 mM glycine in PBS. Monolayers were rinsed with 100 mM triethanolamine, pH 8.0, and incubated in 100 mM triethanolamine, pH 8.0, with acetic anhydride for 10 min. Then they were rinsed in water, washed in 2× SSC for 5 min and immersed in 100% ethanol followed by ethanol air drying. Prehybridization was performed at 42°C for 4−5 hr in pHmix (2× SSC, 5× Denhardts, Salmon Sperm DNA 1:20, 50% formamide), followed by an additional wash in ethanol and air drying. The 1.3 kb fragment of Cx43 (see above) was labeled with digoxigenin (DIG) according to the manufacturer’s recommendations (Boehringer Mannheim, Mannheim, Germany) and hybridized at 42°C for 15 hr in Hmix (2× SSC, 5× Denhardts, Salmon Sperm DNA 1:20, 10% dextran sulfate, 50% formamide). Successively, cells were washed in 2×, 1×, and 0.1× SSC at 40°C and in 0.1× SSC and PBS at room temperature. In a final step, cells were blocked in 1% DIG-blocking reagent for 30 min, washed with PBS, and incubated with anti-DIG antibody labeled with alkaline phosphatase.

Subfractionation and Western blotting. Neu+, neo, and neu− cells were scraped with a rubber policeman, pelleted, and washed with ice-cold PBS. Homogenization was performed at 4°C in 10 mM potassium phosphate, pH 7.2, with 2 mM phenylmethylsulfonyl fluoride (PMSF) with a glass dounce homogenizer. The homogenates were centrifuged at 10,000 g (Sigma 3K20, rotor 12154) for 2 min at 4°C. The sediment was resuspended in 10 mM potassium phosphate, pH 7.2, containing a crude fraction enriched with cell nuclei and unbroken cells. Supernatants were diluted in a 10-fold volume of 10 mM potassium phosphate, pH 7.2, 2 mM PMSF, and centrifuged at 100,000 g (Sigma 3K20, rotor 12154) at 4°C for 10 min to yield plasma membrane and mitochondrial fraction (crude membrane fraction). Microsomal fractions were obtained after centrifugation of the 10,000 g supernatant at 100,000 g (Sorvall OTD 65, rotor TH641) for 1 hr at 4°C. The supernatants containing the cytosol were precipitated with 20% trichloroacetic acid. The concentration of protein in cell subfractions was determined using the enhanced alkaline copper protein assay (Lowry et al., 1951) with BSA as a standard.

Proteins of cell subfractions and total homogenate were resolved by electrophoresis on 15% polyacrylamide gels (Laemmli, 1970) and electroblotted with a wet blotting system (Hoefer, San Francisco). Blotted nitrocellulose was blocked in 5% dry milk and probed with site-specific antibodies against Cx43 or N-CAM (see monoclonal and polyclonal antibodies). A gold-conjugated anti-rabbit IgGs served as secondary antibody. A band-conjugated anti-rabbit secondary antibody after an additional incubation for 1 hr and was visualized by silver staining. Immunoprecipitation and half-time measurement. Neu+ and neu− cells were seeded at 2.5 3 105 cells/ml in 20 mm culture dishes. Three days after replating, the medium was replaced by a deficient medium containing 90% of MEM Eagle’s Medium (Sigma) supplemented with 1 mM sodium pyruvate, 0.05% nonessential amino acids, 0.1 mg/ml gentamycin,
buffered with 25 mM bicarbonate and 10% of normally used medium as described above. Cells were labeled with 3.7–4.4 MBq/ml of L-[35S]methionine or with 5.5–11.8 MBq/ml [32P]orthophosphate for 3 hr. Cells were rinsed three times with growth medium and chased for various time intervals (1, 2, 3, 5, and 8 hr, respectively) until further processing. Cells were treated and lysed according to the method described elsewhere (Musil et al., 1990). The lysate was then precleared for 30 min at 4°C with Protein A bound to Sepharose CL-4B. The immunoprecipitation was performed with 1.8–3.5 μg of affinity-purified anti-Cx43 antibody (pAb2, see Monoclonal and polyclonal antibodies) at 4°C for 4 hr. The antigen–antibody complexes were collected by the addition of 10 μl of Protein A Sepharose CL-4B for 40 min and processed according to the method described (Musil et al., 1990). The immunoprecipitates were analyzed on SDS-PAGE using a 10% acrylamide/0.27% bisacrylamide system (Laemmli, 1970). Phosphoamino acid analysis was performed as described (Sáez et al., 1990). 32P-labeled protein bands were excised from dried polyacrylamide gels using the autoradiography as a guide. Gel slices were rehydrated and subjected to trypsin digestion. The digests were hydrolyzed with 6 M HCl for 45–60 min at 110°C in a N2 environment. Phosphoamino acids were separated by two steps: one-dimensional electrophoresis at pH 1.9 in 8.7% acetic acid/2.5% formic acid, followed by electrophoresis in the same direction at pH 3.5 in 10% acetic acid/1% pyridine. Phosphoamino acids were detected by autoradiography and identified by the specific mobilities of the P-Ser, P-Thr, and P-Tyr standards after developing with 0.1% ninhydrine.

Lucifer yellow injection and Ca2+ imaging. For dye-coupling assays, one cell was injected with Lucifer yellow (4% in 150 mM LiCl) iontophotically, and cells were viewed on a Zeiss Axiovert microscope (Thornwood, NY) equipped with fluorescence illumination and FITC filters.

For Ca2+ imaging, neu−, neo, and neu+ cells were seeded onto glass coverslips at 1 × 104 cells/ml. After 3 d, cells were washed three times with immersion (IM) buffer containing (in mM) 10 HEPES, 131 NaCl, 5 KCl, 4 CaCl2, and 25 glucose and incubated in 16 μM Fura-2 AM for 30 min at 37°C in a humidified incubator. Loaded cells were washed three times with IM buffer.
times with IM-buffer before use. For testing gap junctional intercellular coupling, cells were microinjected with 1 mM Ca\(^{2+}\) iontophoretically. Analysis of calcium spread was performed with a Ca\(^{2+}\) imaging system (Technology Transfer, Martinsried, Germany). Excitation wavelengths (340 and 380 nm) were alternately selected by a monochromator, and the shutter driver (model D122, Uni Blitz) was illuminated for 400 and 250 msec, respectively. Ca\(^{2+}\) images were captured using a silicon-intensified target camera at 450 nm emission and were analyzed by Fucal 2.0 software (Technology Transfer). Calibration of dual wavelength fluorescence data using values obtained in free solutions was performed as described (Thomas and Dellaville, 1991).

Monoclonal and polyclonal antibodies. Several distinct anti-Cx43 antibodies were used in this study. Two rabbit antisera generated against Cx43 peptide sites were used: the antibodies are directed to position 346–360 (pAb1; see Dermietzel et al., 1989) and position 359–381 (pAb2). Affinity purification of pAb2 was obtained using the oligopeptides as ligands. No immunoreactivity to the carrier proteins (ovalbumin) was detectable by Western blotting after purification. Specific reaction of pAb2 for Cx43 was proven by immunofluorescence and Western blotting. A polyclonal rabbit antisera specific for N-CAM was used for immunofluorescence and Western blotting (gift from Dr. M. Schachner, Zurich, Switzerland). Cell cultures were tested for GFAP immunoreactivity with an anti-GFAP rabbit antibody (Sigma) to check for astrocytic features of cultured neu\(^+\) and neu\(^-\) cells, respectively. Successful transfection with the C-erbB2 oncoprotein was detected with a monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY).

RESULTS

C-erbB2/neu transfection results in a communication deficiency in glial wild-type cells

The morphology of the neu\(^+\) cells was indistinguishable from that of cells transfected with vector carrying the neomycin resistance marker (neo4) or with the nontransforming version of the C-erbB2/neu oncoprotein (tr\(^-\)). Their typical appearance was in the form of flat cells with filopodia resembling the configuration of primary cultured astrocytes (Fig. 1A–C). Successful transfection with the transforming oncogene (tr\(^+\)) resulted in a remarkable change in phenotypic appearance of the neu\(^+\) cells, assuming round shapes and growing in foci (Fig. 1D). Staining with a monoclonal antiphosphotyrosine antibody revealed significant increase of phosphotyrosine proteins in neu\(^+\) cells compared with the neu\(^-\) and neo cells. Cells transfected with the nontransforming version of the oncoprotein showed variable staining levels, indicating inconsistent overexpression of the proto-oncoprotein (data not shown).

We then determined coupling efficiency in nontransformed and transformed cell lines by intracellular injections of Lucifer yellow into subconfluent and confluent cells. Whereas extensive dye spread occurred in the neu\(^-\) cells [the neo cells and the tr\(^-\) cells reaching third-order cells within minutes (Fig. 2A,B,C)], the neu\(^+\) cells showed significantly reduced dye transfer even after excessive duration times (>15 min; Fig. 2C, Table 1).

In addition to the dye-transfer experiments, we performed ratiometric imaging of Ca\(^{2+}\) after intracellular calcium injection. This technique offers the advantage of exploiting a physiological indicator for the detection of effective cell coupling (Sáez et al., 1989). Intracellular Ca\(^{2+}\) levels were measured in Fura-2-loaded neu\(^-\), neo, and neu\(^+\) cells beginning 45 sec after calcium injection. The basal levels of free calcium were 40 nm in all three cell lines. Microinjection of a 10 μM Ca\(^{2+}\) into a single cell led to rapid elevation of Ca\(^{2+}\) in second- and third-order cells within seconds in neu\(^+\) cells (Fig. 3A–H). Calcium levels revealed an increase in neighboring neu\(^-\) (Fig. 3a) and neo cells (Fig. 3b) to 500–700 nm and partially recovered within <60 sec to basal levels.

Neu\(^+\) cells behaved entirely differently. First, transfer was entirely random, leaving some first-order cells completely excluded from the Ca\(^{2+}\) increase (Fig. 3A’–H’). Second, there was a considerable time lag in Ca\(^{2+}\) transfer compared with the neu\(^-\) and neo cells (Fig. 3c). The dye injection and the calcium-imaging studies document a significant coupling deficiency of the neu\(^+\) cells. One reasonable explanation for this deficiency in coupling could be the loss of connexin expression after the transfection. We therefore examined these cells for the presence of Cx43 mRNA and protein, which constitutes the major connexin expressed in astrocytes (Dermietzel et al., 1991; Giaume et al., 1991a). Because no differences in the phenotypic appearance between neu\(^-\) and neo cells and cells transfected with the proto-oncopogene were evident, we continued further work with neu\(^-\) and neu\(^+\) cells exclusively.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No. of coupled cells(^a)</th>
<th>No. of uncoupled cells</th>
</tr>
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<tbody>
<tr>
<td>neu(^-)</td>
<td>33 ± 5</td>
<td>7 ± 4</td>
</tr>
<tr>
<td>neo (clone 1)</td>
<td>25 ± 3</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>neu(^+) (clone 7)</td>
<td>2 ± 2</td>
<td>23</td>
</tr>
<tr>
<td>tr(^-)</td>
<td>27 ± 6</td>
<td>6 ± 3</td>
</tr>
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\(^a\)Accumulated numbers of coupled first and higher-order cells from three different experiments.
The major effect of C-erbB2/neu transfection is reduction of the phosphorylated isoforms of Cx43. mRNAs from neu− and neu+ cells were subjected to Northern blot analyses (Fig. 4, lanes 1–3). Both cell lines revealed high levels of Cx43 mRNA, with the neu+ cells having a significantly higher relative level, about two times more when identical amounts of mRNA (20 μg as determined from ethidium bromide staining) were compared by densitometry. We also checked for the expression of various other connexin mRNAs (Cx26, Cx32, Cx37, and Cx40), which have previously been described to be expressed in brain tissues (Dermietzel et al., 1989; Willecke et al., 1991; Hennemann et al., 1992). No mRNAs for these connexins were found either in the wild-type or in the C-erbB2/neu transfected cells (not shown).
Insitu hybridization using a 1.3 kb cDNA of Cx43 (see Beyer et al., 1987) showed a dispersed cytoplasmic distribution of the transcript in both cell types (Fig. 4A, B). Control experiments were performed by omitting the cDNA in the hybridization mixture and by prehybridization of the sections with an unlabeled antisense RNA, which was transcribed from the linearized cDNA. Both controls were negative with respect to Cx43 mRNA labeling (not shown).

We next examined expression of Cx43 protein because the presence of the mRNA does not necessarily indicate translation into its gene product. Western blot analyses of neu2 cell homogenates using two site-specific antibodies directed to position 346–360 (pAb1) and position 359–381 (pAb2) of Cx43 revealed a triplet of bands at positions 40, 43, and 45 kDa, which, according to Musil et al. (1990), represent the NP, P1, and P2 forms (Fig. 5, lane 3). Here we refer to this nomenclature, although in high-resolution Western blots a fourth band around 41 kDa was detectable (see Fig. 6), which according to Laird et al. (1991) represents a phosphorylated state of the nascent 40 kDa form that is associated with the Golgi apparatus. In the neu1 cells, the upper two bands (i.e., at position 43 and 45 kDa) were missing (Fig. 5, lane 4). These data indicated a loss of the less mobile isoforms of Cx43 in the neu1 cells. The isoforms have been repeatedly shown to represent different states of phosphorylation of the Cx43 protein (Musil et al., 1990; Laird et al., 1991; Lau et al., 1991; Berthoud et al., 1992).

To obtain information regarding the subcellular distribution and differences in processing of the Cx43 isoforms, we performed subfractionation of both cell lines. In the neu2 cells, all Cx43 forms were equally abundant in the membrane-containing subfractions corresponding to different cellular components (Fig. 6A), whereas in the neu1 cells, a relative enrichment of the NP Cx43 species was present in the microsomal fraction (Fig. 6B, lane c) compared with the cell homogenate and crude membrane fraction (Fig. 6B, lanes a, b). This finding is suggestive of an enrichment of the NP form in the microsomal compartment of neu1 cells. However, the total amount of the Cx43 proteins in the neu1 cells appeared to be reduced compared with the neu2 cells (~50% when the three isoforms in the subfractions are scanned.
by densitometry). One reason for a reduction, besides inhibitory effects on translational and/or post-translational regulatory mechanisms, could be an enhanced degradation of the Cx43 under the influence of the oncogene. We therefore checked for the half-time of the Cx43 protein in neu− and neu+ cells. Pulse-chase experiments on both cell lines indicated a half-time of 2–4 hr of the Cx43 protein, revealing no differences in the overall turnover of the Cx43 protein (Fig. 7A,B). Thus, an influence of the oncogene on the survival rate of the protein seems highly unlikely. A more trivial explanation of the apparent reduction of Cx43 in the neu+ cells is that the relative ratio of Cx43 to the total amount of protein loaded on the gels for Western blotting (20 μg per lane) decreased because the neu+ cells overexpress the oncogene product. We therefore checked the amount of protein per cell, which gave an approximate concentration of 0.45 ± 0.3 ng (n = 2) for neu− and 0.46 ± 0.2 ng (n = 2) for neu+, indicating no significant difference in overall protein concentration. This finding renders the notion of a relative reduction of Cx43 concentration attributable to a simple shift in protein ratios unlikely.

Differences in Cx43 phosphorylation were confirmed by metabolic labeling of wild-type cells and neu-transformed cells with [32P]orthophosphate for 4 hr followed by immunoprecipitation with anti-Cx43 (pAb2) antibody (Fig. 8A). Approximately 50% less 32P-labeled Cx43 was immunoprecipitated from neu+ compared with neu−. We also examined the phosphoamino acid content of 32P-labeled Cx43 in the neu− and neu+ cells. Immunoprecipitates of [32P]phosphate-labeled Cx43 were separated using SDS-PAGE. Gel pieces corresponding to the Cx43 bands

Figure 7. Half-life measurements of Cx43 protein in neu− and neu+ cells. A, Autoradiograms of immunoprecipitated Cx43 after pulse chase. Cell lines were labeled with [35S]methionine for 7 hr and cultivated for various durations in medium with unlabeled methionine (lanes 1–6 correspond to 0, 1, 2, 3, 5, and 8 hr). Arrows indicate the three phosphorylated isoforms of Cx43. After separation of the precipitates on SDS gels, the gels were processed for fluorography and exposed for 3 weeks to X-ray films. Autoradiograms were digitized and standardized to maximal intensity. B, Diagram depicting the lifetime of Cx43 in neu− and neu+ cells as taken from the autoradiograms. The indicated values represent the densitometrically measured mean values of three independent experiments. The half-time of the 41 kDA isoform of Cx43 is between 1 and 3 hr for both cell lines.
Transformation with C-erbB2/neu leads to N-CAM deficiency in glial cells

Neu + cells showed a remarkable reduction in cell adhesion and cell spreading (see above). In recent reports, it has been shown that CAM expression is crucial for establishment of functional gap junctions in different cell lines (Musil et al., 1990; Meyer et al., 1992). Lack of L-CAM has also been correlated with communication deficiency and inability of Cx43 to be phosphorylated to the less mobile protein isoforms (Musil et al., 1990). We therefore checked the neu − and neu + cells for the presence of N-CAM with a polyclonal antiserum that detects N-CAM on glial cells (Faisnner et al., 1984). Immunofluorescence (Fig. 10A,B) as well as Western blots (Fig. 10C) indicated that neu − cells are devoid of N-CAM expression. Comparison of primary cultured astrocytes and neu − cells with the neu + transfectants clearly showed a lack of the two dominant isoforms of the N-CAM, which display relative masses of 120 and 150 kDa in the neu − cell line (Fig. 10C, lane 3).  

DISCUSSION  

Gap junction communication appears to be essential for the syncytial behavior of astrocytes, because these channels provide the structural link that couples individual cells and thereby allows coordination of simultaneously occurring functions (for recent review, see Dermietzel and Spray, 1993). The coordinating properties of gap junctions have been suggested to include such basic cell biological functions as differentiation, cell growth, and synchronization of various cellular activities, i.e., contraction of smooth muscle and cardiac myocytes (Spray et al., 1994), secretion of epithelial and endocrine cells (Yamamoto and Kataoka, 1988; Meda et al., 1993), and synchronization of neuronal activity (Conners et al., 1983; Kessler et al., 1985; Linás, 1985). The high degree of gap junction presence and functional coupling between astrocytes (Dermietzel et al., 1991) allows the direct exchange of information essential for the coordinated behavior of the astrocytic syncytium.  

The recent documentation of Ca2+ waves by topical glutamate application or mechanical stimulation (Cornell-Bell et al., 1990) and its sensitivity to the gap junction inhibitor heptanol (Finkbeiner, 1992) is a clear demonstration of the gap junctional coupling efficiency between astrocytes. The coordinated properties of the coupled astrocytic syncytium is likely to be subject to functional modifications requiring dynamic capabilities. Modulation of astrocytic coupling has been demonstrated at the level of dye coupling, where norepinephrine and endothelins led to rapid decoupling in cultured astrocytes (Giaume et al., 1991b; Giaume et al., 1992). In situ modulation of Cx43 in response to exogenous stimulation has also recently been reported. For instance, intracerebral kainic acid alters accessibility to Cx43 antisera (Vukelic et al., 1991; Hossain et al., 1994), and ligation of the facial nerve leads to a rapid upregulation of Cx43 expression in the corresponding nuclei in the brainstem (Rohllmann et al., 1993). The latter event seems to occur as an early response (<1.5 hr; Rohllmann et al., 1994), indicating rapid regulation of Cx43 expression in astrocytes.
Coupling efficiency can be modulated at different levels, includes transcriptional and translational regulation sites as well as post-translational events such as phosphorylation of the protein, trafficking, membrane insertion, and assembly into functional gap junction plaques. It has been suggested that Cx43 phosphorylation might be a crucial step in the assembly of functionally competent gap junction plaques (Musil and Goodenough, 1991). Our findings in C-erbB2/neu transfected glial cells allow extrapolation of these data originally obtained from mouse sarcoma cell lines to astrocytes. Communication-deficient neu1 glial cells consistently showed a reduction of the higher phosphorylated Cx43 species compared with the neu2 cells. Unlike the effect of the pp60v-src oncogene product (Swenson et al., 1990), the coupling deficiency induced by C-erbB2/neu transfection is not related to tyrosine phosphorylation of Cx43. A further difference from transformation by pp60v-src, which leads to an elevation of Cx43 in Rat 1 fibroblasts (Goldberg and Lau, 1993), is the apparent reduction of total Cx43 in our glial cell line after C-erbB2/neu transfection. As already indicated, a simple explanation of this effect attributable to a shift of protein concentration by oncogene expression seems unlikely. We therefore consider that, in addition to the inhibition of phosphorylation (see below), the C-erbB2/neu oncogene may influence on the post-translational biosynthetic pathway of Cx43.

From the confocal imaging of the neu1 cells, it is apparent that the major effect of the transfection on Cx43 distribution is in plaque assembly rather than the insertion of Cx43 into the plasma membrane. Because of the marked depletion in more highly phosphorylated isoforms of Cx43 in these cells, we conclude that the final step in junctional assembly in astrocytes depends on accurate phosphorylation of the junctional protein. The residual transfer of Ca2+ that we found in the neu1 cells can be explained simply as a random distribution of small clusters of channels, which may provide sufficient junctional conductance to permit Ca2+ passage.

Assembly of gap junction plaques apparently requires close cell-to-cell apposition. In a number of experiments, it has been shown that cell adhesion molecules are involved in establishment of cell-to-cell communication. Lack of L-CAM expression correlates with communication deficiency in sarcoma cell lines (Musil et al., 1990), and ordinarily communication-competent cells could be converted to communication-defective cells by exposure to anti-A-CAM antibodies (Meyer et al., 1992). Re-establishment of communication by transfection with a specific cDNA encoding the cell–cell adhesion molecule L-CAM corrected both the Cx43 phosphorylation deficiency and restored junctional communication (Mage et al., 1988; Musil et al., 1990; Jongen et al., 1991). The data suggest a functional link between gap junction formation and strong cell–cell association via CAM proteins. The inability of the neu1 cells to establish appropriate gap junctions also seems correlated with the proper expression of N-CAM, because neu transfection resulted in a loss of expression of this cell adhesion

Figure 9. Immunocytochemical detection of Cx43 in neu− and neu+ cells. a, b, Immunolabeling showed a pattern of discrete punctate staining at adjacent cell membranes in neu− cells (a) and diffuse intracytoplasmic labeling in neu− cells (b). A–D′. Confocal scanning laser microscopy of anti-Cx43-labeled cells. Plaque-like plasmalemmal staining and some juxtanuclear staining is evident in neu− cells (A–D), whereas neu+ cells (A′–D′) reveal diffuse membrane and juxtanuclear staining without any plaque-like formations. Scale bar, 20 μm.
Figure 10. Immunofluorescence (A, B) and immunohistochemical detection (C) of N-CAM. A depicts well defined immunolabeling of the plasma membrane in neu cells. B, Neu cells are devoid of anti-N-CAM immunoreactivity above background levels of intracellular autofluorescence. Scale bar, 20 μm. C, Western blots of homogenates of cultured astrocytes (lane 1), neu (lane 2), and neu (lane 3) cells using an anti-N-CAM antibody. Bands at the two major isoforms of N-CAM at positions 120 and 150 kDa for primary astrocytes (lane 1) and wild-type cells (neu; lane 2, arrows); no bands are found at corresponding positions in the neu cells (lane 3). Scale bar, 20 μm.

molecule. Thus, a scenario seems feasible by which activation of protein kinase activities are modulated directly by adhesion molecules, or indirectly, through signal transduction proteins. It is important to point out that the correlation that we and others have observed between Cx43 phosphorylation and gap junction assembly is not the only effect of phosphorylation on gap junction function. Biophysical properties of the Cx43 channel depend on its phosphorylation state, with an increase in unitary conductance and more rapid kinetics of closure by transjunctional voltage after dephosphorylation (Moreno et al., 1992, 1994). A possible link between changes in phosphorylation and physiological status of the cell has recently been shown by Lau et al. (1992), who provided evidence that epidermal growth factor (EGF) can transiently disrupt gap junctional communication. This effect correlates with an increase in Cx43 phosphorylation mainly in serine residues and not in tyrosine residues as might have been expected, because EGF receptor has an intrinsic tyrosine kinase activity (Yarden and Ullrich, 1988). In this process, phosphorylation of Cx43 serine residues is induced, perhaps attributable to microtubule-associated protein (MAP) kinase activity (Lau et al., 1992; Kanemitsu and Lau, 1993) or other serine/threonine kinases. The phosphorylation site of MAP kinase is independent from that of TPA-induced protein kinase C serine phosphorylation, which also disrupts junctional communication in at least some cell types (Brissette et al., 1991) presumably by inducing differential phosphorylation of serine residues of Cx43 (Berthoud et al., 1993). The regulation of gap junctional intercellular communication seems to involve multisite phosphorylation orchestrated by a complex interplay of intracellular protein kinases, thereby working on distinct levels of Cx43 biosynthesis. From further studies of this interplay, including the assembly mechanism of gap junction plaques, we expect to obtain a more complete understanding of the plasticity of the astrocytic network under normal conditions and in situations of pathological stress.

REFERENCES


