An inhibition of gap-junctional communication by cadherins

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SUMMARY

The action of Ca\(^{2+}\)-dependent cell-cell adhesion molecules (cadherins) on cell-to-cell channel-mediated intercellular communication was investigated in mouse L and rat Morris hepatoma cells. These cells fail to adhere to one another in aggregation assays and thus seem to lack cell adhesion molecules. Expression of exogenous cadherin induced strong cell-cell adhesion in both cell types, but had opposite effects on communication, causing inhibition in L cells and improvement in hepatoma cells. Both cells express the connexin43 cell-to-cell channel protein. By western blot we found no cadherin-specific changes in connexin43 protein in either cell type, but connexin43 gap junctional plaque staining, i.e. connexin43 localization to cell-cell junctions, was inhibited in L cells and facilitated in hepatoma cells. In addition we found that the inhibitory effect is largely abolished by blockers of glycosylation. Cadherin-cadherin interactions are known to trigger cell type-specific intracellular signal cascades resulting in diverse end effects, and gap junctional communication/plaque formation seems a further example of such cell type-specificity.

Key words: Gap junction, Cell-to-cell channel, Connexin43, Cadherin, Glycosylation, CAMP, Intercellular communication

INTRODUCTION

The cell-to-cell channels of gap junctions, the membrane channels that interconnect the cells of organs and tissues, are permeable to hydrophilic molecules of up to about 1 kDa, providing a means for direct intercellular communication (Loewenstein, 1981). Such channels form one by one when competent cells come into contact, each cell contributing a channel half (Loewenstein et al., 1978). The channel halves (connexons) are canalicular units made of six concentrically arrayed protein (connexin) subunits which bond to their opposite numbers across the intercellular gap by their extracellular protruding ends to form the cell-to-cell channels (Caspar et al., 1977; Unwin and Zampighi, 1980; Milks et al., 1988; Sosinsky et al., 1988). The channels are often found aggregated into junctional membrane structures called gap junctions (Revel and Karnovsky, 1967) that can be detected by electron microscopy (Robertson, 1963; Kreutziger, 1968; Goodenough and Revel, 1970) or by fluorescence microscopy after immunostaining with antibodies against connexins (Paul, 1986; Dermietzel et al., 1987; Beyer et al., 1989).

The initial focal contact between two adjoining cells’ membranes is probably stabilized by specific cell adhesion molecules, and that adhesive interaction somehow seems to pave the way for cell-to-cell channel formation. The first evidence to this effect came from work with dissociated sponge cells (haliclonia and microciona). When these cells were re-aggregated, they turned out to form cell-cell channels only in the presence of specific glycoprotein adhesion molecules (and Ca\(^{2+}\)) (Loewenstein, 1967). In vertebrates, a family of transmembrane glycoproteins called cell adhesion molecules (CAMs), which includes the (Ca\(^{2+}\)-dependent) cadherins (Takeichi, 1988, 1991), appears to have taken over this synergic role; there is a body of evidence attesting to a facilitatory effect of cell adhesion molecules on junctional communication and gap junction formation in a variety of vertebrate tissues (Kanno et al., 1984; Keane et al., 1988; Mege et al., 1988; Musil et al., 1990b; Jongen et al., 1991; Meyer et al., 1992).

Thus, it came as a surprise when we met with a cadherin-induced inhibition of junctional communication. The case in point is the mouse L cell, a transformed cell type with low adhesivity due to their lack of CAMs. These cells become strongly adhesive upon transfection with cadherin genes (Nose et al., 1988). But instead of the expected enhanced communication, there was just the opposite sequel: reduced communication was inferred from work with dissociated sponge cells (haliclonia and microciona). When these cells were re-aggregated, they turned out to form cell-cell channels only in the presence of specific glycoprotein adhesion molecules (and Ca\(^{2+}\)) (Loewenstein, 1967). In vertebrates, a family of transmembrane glycoproteins called cell adhesion molecules (CAMs), which includes the (Ca\(^{2+}\)-dependent) cadherins (Takeichi, 1988, 1991), appears to have taken over this synergic role; there is a body of evidence attesting to a facilitatory effect of cell adhesion molecules on junctional communication and gap junction formation in a variety of vertebrate tissues (Kanno et al., 1984; Keane et al., 1988; Mege et al., 1988; Musil et al., 1990b; Jongen et al., 1991; Meyer et al., 1992).

Thus, it came as a surprise when we met with a cadherin-induced inhibition of junctional communication. The case in point is the mouse L cell, a transformed cell type with low adhesivity due to their lack of CAMs. These cells become strongly adhesive upon transfection with cadherin genes (Nose et al., 1988). But instead of the expected enhanced communication, there was just the opposite sequel: reduced communication. We will show that this inhibitory cadherin effect is glycosylation-sensitive; it can be abolished by blockers of glycosylation.

MATERIALS AND METHODS

Materials

All culture media were from Gibco BRL (Gaithesberg, MD), fetal bovine serum from Hyclone (Logan, UT), tissue culture plasticware from Nunc (Roskilde, Denmark), Lucifer Yellow CH from Molecular Probes (Eugene, OR). Phosphodiesterase inhibitor Ro-20-1724 was a gift from Dr P. Sorter, Hoffman-LaRoche. Rhodamine- or fluorescein isothiocyanate-labeled goat anti-rabbit IgG and alkaline phosphatase were from Boehringer Mannheim (Indianapolis, IN), goat anti-rat IgG from Cappel (Durham, NC), forskolin from Calbiochem (La Jolla, CA) and all other reagents (Molecular Biology grade or highest purity) from Sigma (St Louis, MO). Monoclonal antibody NCD-2 (Hatta and Takeichi, 1986) and N-cadherin expression vector pMiwcN (Nose et al., 1988) were gifts from Professor M. Takeichi (Kyoto Uni-
versity, Japan). Plasmid pMC1neo was from Stratagene (La Jolla, CA).

Cell culture
Mouse L cells and L cells transfected with E-cadherin (clone ELB1) or P-cadherin (clone PLB2) were obtained from Prof. M. Takeichi (Kyoto University). They were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s (DMEM) and Ham’s F12 media supplemented with 10% fetal bovine serum. The medium for the stocks of cadherin expression clones contained 200 μg/ml geneticin (G418). Cells from the MHD-1 clone (Wang et al., 1995) of rat Morris hepatoma 5123 cells (Borek et al., 1969) were grown at 37°C in basal minimal essential medium supplemented with 5% fetal bovine serum. Cells were incubated in an atmosphere of 5% CO2/95% air. Stock cultures were maintained in 10 ml medium in 10 cm dishes and passed weekly at 103 cells/dish, with a medium change at 3 or 4 day intervals. For experiments, cells were plated at 2×103/35 mm dish and used 2 days later when newly confluent.

Transfection of cells
Mouse L cells and MHD1 cells were cotransfected with the N-cadherin expression construct pMiwC and plasmid pMC1neo by electroporation. Stably transfected clones were obtained and screened by an aggregation assay and by immunostaining for N-cadherin. Several N-cadherin expression clones were obtained for both types of cells and used for the studies described in this work. All clones gave similar results for each type of cells. We show the results of two clones (N1 and N2) for L and one clone, D1-N1, for MHD1 cells.

Aggregation assay
Cadherin-mediated cell aggregation was assayed as described (Nose et al., 1988). Briefly, newly-confluent cells were treated with 0.25% trypsin in the absence of Ca2+ to obtain a single-cell suspension. Cells were then spun and resuspended in 1 ml culture medium in 35 mm Falcon Petri dishes and incubated for 5 hours at 37°C with constant shaking after which time they were inspected for aggregation under the microscope.

Treatments
All test reagents were applied with a medium change; forskolin, Ro-20-1724, and tunicamycin were added from 1,000× stock solutions in dimethylsulfoxide (DMSO), with final DMSO not exceeding 0.4%, a concentration which did not affect any of the parameters we measured. Forskolin was always used in combination with 50 μM phosphodiesterase inhibitor Ro-20-1724. For controls, cultures received fresh medium containing 0.4% DMSO, the highest DMSO concentration used in any experiment.

Communication assay
Individual cells were pressure-injected with the fluorescent dye Lucifer Yellow CH (5%) and viewed on a Nikon Diaphot fluorescence microscope; 15-20 randomly selected, distantly spaced cells were injected in each dish. The number of neighbor cells fluorescent 5 minutes after injection was used as an index of communication. Data are presented as means ± s.e.m. (for >20 injections, 2-4 dishes) or ± s.d. (for <20 injections, 1 dish).

Immunostaining
For immunostaining, cells were seeded on coverslips; when newly confluent, they were fixed at −20°C for 15-20 minutes in 50:50 acetone:methanol, washed in phosphate buffered saline (PBS, pH 7.2), incubated in blocking solution (PBS containing 5% bovine serum albumin) at 4°C for 1 hour, and incubated overnight at 4°C in blocking solution containing affinity-purified polyclonal (rabbit) antibody against amino acids 252-271 of rat heart Cx43 or monoclonal antibody (NCD-2) for N-cadherin. After an additional 1 hour at room temperature, cells were washed 3× with PBS, incubated with rhodamine-labeled goat anti-rabbit or anti-mouse IgG (1:400 dilution in blocking solution) for 1 hour and washed 3× with PBS. Cells were mounted in 50% glycerin/50% PBS and viewed on a Nikon Diaphot fluorescence microscope with a 100× oil immersion objective. Images were photographed or captured on an optical disk (Panasonic Model TQ-2026F) with a SIT66 (DAGE MTI) videocamera, and reproduced on a videoprinter (Hitachi). For confocal fluorescence microscopy (Zeiss laser scanning microscope LSM410), cells were fixed in 1% paraformaldehyde and permeabilized with acetic acid/methanol (1:1:1).

RESULTS
Cadherin expression inhibits communication in mouse L cells
Mouse L cells lack cell-cell adhesion molecules (Nose et al., 1988). They fail to adhere to one another in aggregation assays and remain as single cells. This deficiency was corrected by transfection with N-cadherin cDNA when the cells that expressed N-cadherin became adhesive to one another and formed large clusters in aggregation assays (Fig. 1). Similar corrections have been shown with E- and P-cadherin by Nose et al., 1988.

Tests for gap junctional communication, cell-to-cell transfer of the microinjected fluorescent tracer Lucifer Yellow, revealed

<table>
<thead>
<tr>
<th>Table 1. Communication in cadherin-lacking and cadherin-expressing L cells</th>
<th>Communication*</th>
<th>Control†</th>
<th>Forskolin‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>Cadherin type expressed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>None</td>
<td>1.0±0.3 (31)</td>
<td>13.8±6.0 (52)</td>
</tr>
<tr>
<td>N1</td>
<td>N-cadherin</td>
<td>0.3±0.1 (47)</td>
<td>8.5±0.6 (56)</td>
</tr>
<tr>
<td>ELB1</td>
<td>E-cadherin</td>
<td>0.0±0.0 (29)</td>
<td>2.4±0.3 (79)</td>
</tr>
<tr>
<td>PLB2</td>
<td>P-cadherin</td>
<td>0.0±0.0 (37)</td>
<td>5.2±0.4 (86)</td>
</tr>
</tbody>
</table>

*Communication was determined by cell-to-cell transfer of microinjected Lucifer Yellow and is expressed here as the mean number of fluorescent neighbors of the injected cell ± s.e.m., scored 5 minutes after injection. Each mean is derived from the number of injections indicated in parentheses, from 2 separate experiments.
†Cells were treated for 8 hours with 4 μl/ml DMSO.
‡Cells were treated for 8 hours with 20 μM forskolin and 50 μM Ro-20-1724. These reagents were dissolved in DMSO at 1,000-fold the final concentration.
that L cells have a low level of communication. On average, only 1 neighbor cell was fluorescent 5 minutes after injection (Fig. 2a,b and Table 1). To our surprise, communication was less in the N-cadherin-expressing cells: few injections resulted in tracer transfer to any neighbor, the average being 0.3 (Fig. 2c,d and Table 1). In contrast to findings with cadherin expression in other cell types where communication was improved (Keane et al., 1988; Mege et al., 1988; Jongen et al., 1991; Musil et al., 1990b), N-cadherin in L cells apparently depresses communication.

To investigate whether this inhibition of communication was specific for N-cadherin, we turned to L cell clones expressing E- or P-cadherin. These clones (ELβ1 and PLβ2, respectively) also exhibited strong cell-cell adhesion (data not shown, but see Nose et al., 1988) and their communication, too, was depressed by the cadherins. In fact, neither in E- nor in P-cadherin expressors could any tracer transfer be detected at all (Table 1).

The communication inhibition associated with cadherin expression was seen even more clearly when the level of communication in the cells was increased by elevation of intracellular cAMP which has been shown to upregulate communication in a wide variety of cells (Flagg-Newton et al., 1981; Loewenstein, 1985; Stagg and Fletcher, 1990). We used forskolin here, a stimulator of adenylylcyclase, in conjunction with the phosphodiesterase inhibitor Ro-20-1724, to elevate cAMP. This treatment has been shown to greatly...

Fig. 1. Cell-cell adhesion and N-cadherin immunostaining in L cells (L) and an N-cadherin-transfected L cell clone (N1). Aggregation: in aggregation assays L cells remain single (a) whereas N1 cells adhere to one another, forming tight cell clusters (c). N-cadherin immunostaining: L cells lack N-cadherin-specific staining (b) whereas N1 cells exhibit abundant N-cadherin staining, particularly at cell-cell contacts (d, arrows).

Fig. 2. Communication in L cells is depressed by N-cadherin expression. Intercellular transfer of Lucifer Yellow injected into cadherin lacking (L) or N-cadherin expressing (N1) cells after 8 hours control (top row), forskolin/Ro-20-1724 (middle row) or forskolin/Ro-20-1724/tunicamycin treatment (bottom row). (a,c,e,g,i,k) Brightfield images of the corresponding communication test cell areas depicted in fluorescence images b,d,f,h,j,l, respectively (injected cells marked with asterisks; photos taken 5 minutes after injection). Control: DMSO, 4 μl/ml; forskolin: 20 μM; Ro-20-1724: 50 μM; tunicamycin: 10 μg/ml; all reagents were administered (from 1:000x stock solutions in DMSO) with a medium change. Concentrations and protocol apply to all further experiments unless stated otherwise.
increase communication in cultured cells (Mehta et al., 1986) by stimulating transcription of cx43 and increasing Cx43 protein (Mehta et al., 1992), and causing clustering of Cx43 cell-to-cell channels into gap junction plaques (Wang and Rose, 1995a). As seen in Fig. 3, communication in L cells increased greatly and in the E- and P-cadherin expressing cells (ELβ1 and PLβ2) it became measurable after about 3 hours and continued to increase over the course of 13 hours. The final communication level was, however, much higher in the cadherin-lacking than in any of the cadherin-expressing cells (Table 1).

**Inhibition of communication in the cadherin expressors is Ca²⁺ dependent**

Since cadherin-cadherin interaction and protection of cadherin from proteolysis require extracellular Ca²⁺ (Atsumi and Takeichi, 1980), one might expect the inhibition of communication in the cadherin expressors to be Ca²⁺-dependent, too, if it is related to cadherin expression rather than clonal variability. We therefore investigated what effect lowering extracellular Ca²⁺ had on communication in the cadherin-expressing clones compared to their parental, cadherin-lacking cells. For this study, we used cells with elevated communication, namely after 8-12 hours forskolin/Ro-20-1724 treatment.

Table 2 shows that when we lowered extracellular Ca²⁺ by addition of 5 mM EGTA to the medium, communication was not affected in the cadherin-lacking cells but it increased about twofold in the cadherin-expressing clones (ELβ1 and PLβ2), and after return to Ca²⁺ containing medium, it slowly diminished again to the initial levels. (A delay in communication inhibition after re-addition of Ca²⁺ can be expected because of cadherin proteolysis at low extracellular Ca²⁺.) This result of Ca²⁺-sensitivity of communication in the cadherin-expressing but not in the cadherin-lacking cells supports our interpretation that the depression of communication in cadherin expressors is mediated by cadherin and is not due to clonal variability of the cells.

**Cadherin expression inhibits localization of Cx43 to cell junctions**

We have shown previously (Wang and Rose, 1995b) that L cells express the cell-to-cell channel protein connexin43 (Cx43). Since cadherin expression affected communication, we were curious whether it also had an effect on cellular localization of this protein. By fluorescent immunostaining of the cells with anti-Cx43 antibody, we found that cadherin expression reduced the frequency of Cx43 gap junction plaques detectable by fluoromicroscopy. Fig. 4 illustrates this for N-cadherin expressors (N1). In control conditions, plaques, brightly fluorescent dots at cell junctions, were not detected in either L or N1 cells (Fig. 4a and b), but after forskolin treatment, plaques appeared at cell junctions; however, there were fewer in N1 than in L cells (Fig. 4c and d).

**Cadherin expression does not alter Cx43 expression level or Cx43 mobility on SDS-PAGE**

One possible mechanism by which communication might be depressed is by a downregulation of the cell-to-cell channel protein; alternatively, the protein itself might be modified in a manner that interferes with the formation or opening of the channels. For example, Cx43 has been shown to undergo multiple phosphorylations, resulting in Cx43 forms that are

![Fig. 3. Time course of communication increase with forskolin/Ro-20-1724 treatment. Plotted is the number of fluorescent cell neighbors 5 minutes after injection versus hours of treatment, measured in L cells (L), the E-cadherin expressing clone ELβ1 (E) and the P-cadherin expressing clone PLβ2 (P). Data points are means of 15-22 injections each; all measurements are from one experiment of parallel cultures.](image)

**Table 2. Removal of Ca²⁺ increases communication in cadherin-expressing but not in cadherin-lacking cells**

<table>
<thead>
<tr>
<th>Cell</th>
<th>Cadherin type expressed</th>
<th>Forskolin/Ca²⁺†</th>
<th>Forskolin/EGTA‡</th>
<th>Ca²⁺§ (3 hours)</th>
<th>Ca²⁺¶ (15 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>None</td>
<td>17.2±0.7 (11)</td>
<td>17.2±3.9 (15)</td>
<td>14.8±3.0 (13)</td>
<td>14.8±4.7 (15)</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>16.3±4.3 (14)</td>
</tr>
<tr>
<td>ELβ1</td>
<td>E-cadherin</td>
<td>4.1±2.8 (9)</td>
<td>10.4±5.0 (12)</td>
<td>8.4±3.4 (11)</td>
<td>3.9±3.0 (12)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.1±2.2 (10)</td>
</tr>
<tr>
<td>PLβ2</td>
<td>P-cadherin</td>
<td>6.5±3.3 (10)</td>
<td>11.9±4.3 (11)</td>
<td>10.7±4.7 (7)</td>
<td>4.2±2.9 (12)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.2±3.3 (9)</td>
</tr>
</tbody>
</table>

*Mean number of fluorescent cell neighbors ± s.d., 5 minutes after injection of Lucifer Yellow; in parentheses, the number of injection trials.
†Cells were treated with 20 μM forskolin and 50 μM Ro-20-1724 in normal, Ca²⁺-containing medium for 15 hours before testing communication.
‡5 mM EGTA was added to the forskolin/Ro-20-1724 medium of cells after 10 hours with forskolin/Ro-20-1724, and communication was tested 5 hours later (total forskolin/Ro-20-1724 treatment time, 15 hours).
§After EGTA treatment, normal Ca²⁺-containing medium was applied for 3 hours; for comparison, the time-corresponding (18 hours) data from cultures that underwent forskolin/Ro-20-1724 but not EGTA treatment are listed in the second row in this column.
¶Communication was tested 15 hours after return to Ca²⁺ containing medium.
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distinguishable from one another and from non-phosphorylated Cx43 by their different mobilities on SDS-PAGE (Musil et al., 1990a; Musil and Goodenough, 1991). In one communication-deficient, cadherin-lacking cell line, mouse sarcoma S180, gap junctional plaques and Cx43 phosphorylation were largely absent but appeared, together with communication, when the cells expressed E-cadherin (Musil et al., 1990b), suggesting a link between Cx43 phosphorylation and presence of channels in gap junctional plaques. On the other hand, channel closure, too, has been associated with Cx43 phosphorylation, namely on tyrosine (Crow et al., 1990; Filson et al., 1990; Swenson et al., 1990) or serine residues (Brissette et al., 1991; Berthoud et al., 1992). We thus investigated whether cadherin expression in L cells resulted in any differences in Cx43 protein level and/or mobility by comparing Cx43 in lysates of cadherin-expressing and cadherin-lacking cells in western blots.

Cx43 of L cells in control conditions appeared as a major band corresponding to a molecular mass of about 42-43 kDa, and a much weaker band at about 48 kDa. In addition, a very faint band of less than 42-43 kDa was detected in some blots. The same pattern was seen in all cadherin expressing clones, as illustrated in Fig. 5A for E- and P-cadherin-expressing clones ELβ1 and PLβ2, and in Fig. 5C for the N-cadherin expressing clone N1: neither intensity nor position of the Cx43 bands was different from that of L cells. This result is at variance with that reported by Musil et al. (1990b) who had found that Cx43 of slower mobility (phosphorylated Cx43) appeared in S180 cells after transfection with E-cadherin.

Also after forskolin treatment, the Cx43 pattern was the same in all cells: total Cx43 increased and bands of slower mobility 43-45 kDa appeared in both cadherin-lacking and cadherin-expressing L cells alike. The forms with slower mobility represent phosphorylated Cx43 as shown by 32P labelling (data not shown here, but see Wang and Rose, 1995b).

Fig. 4. Inhibition of Cx43 localization to cell-cell contacts by cadherin-expression in L cells. Cx43-immunostaining of cells after 8 hours control (a,b) or forskolin/Ro-20-1724 (c,d) treatment. No punctate staining at cell junctions typical of Cx43 gap junction plaques was detected in controls; such punctate staining appeared after forskolin treatment (arrows); it was consistently less in the cadherin-expressing (N1) than in the cadherin-lacking (L) cells.

Fig. 5. Western blot analysis of Cx43 in cadherin-lacking and cadherin-expressing cells. (A) Comparison of Cx43 in lysates from cadherin-lacking L cells (L); E-cadherin expressing L cell clone ELβ1 (E); and P-cadherin expressing L cell clone PLβ2 (P). Cells had been treated for 10 hours with 4 μM forskolin and 50 μM Ro-20-1724 (F), 10 μg/ml tunicamycin (T) or forskolin/Ro-20-1724/tunicamycin (F/T). 50 μg total protein/lane. (B) The Coomassie blue stained portion of the gel from which the western blot in A was derived. (C) Comparison of Cx43 in lysates from L cells (L) and N-cadherin expressing L cell clone N1 (N1). 40 μg total protein/lane. (D) Comparison of Cx43 in lysates from cadherin-lacking Morris hepatoma cell clone MHD1 (D1) and N-cadherin expressing Morris hepatoma cell clone MHD1-N1 (D1-N1). 50 μg total protein/lane. Cell treatments in C and D as in A.

An increase in total Cx43 level is expected from the reported stimulation of Cx43 transcription rate by elevation of cAMP (Mehta et al., 1992), and the increase in phosphorylated (slower-moving) Cx43 forms has also been seen in other cells (Wang et al., 1995; Wang and Rose, 1995a). The lack of a difference in Cx43 protein between cadherin-lacking and cadherin-expressing cells, both in control condition and after
forskolin treatment, suggests that cadherins do not inhibit communication here by downregulation of Cx43 expression. Whether or not Cx43 phosphorylation is involved in the cadherin effect cannot be resolved by these experiments; however, there is no indication of a change in abundance of phosphorylated Cx43 (forms of slower mobility).

**N-cadherin expression improves communication in hepatoma cells**

Since our results of an inhibition of communication by cadherins were quite different from those reported in the literature, we decided to examine the effect of cadherin expression in another transformed cell line. We therefore transfected a clone (MHD1) of rat Morris hepatoma H5123 cells (Wang and Rose, 1995a) with N-cadherin cDNA and compared communication in N-cadherin expressing and N-cadherin lacking MHD1 cells. We chose these cells because of their similarity to L cells in several respects: (a) they fail to aggregate in aggregation assays (Fig. 6A) and thus seem to lack cell adhesion molecules; (b) they express Cx43 at a level similar to L cells (data not shown); and (c) they also respond to forskolin with an increase in communication (Wang and Rose, 1995a,b). We obtained several hepatoma clones which showed cell-cell adhesion and stably expressed N-cadherin (Fig. 6B).

In hepatoma cells it turned out that N-cadherin-expression increased communication; in control conditions some injections now resulted in tracer transfer, and after forskolin treatment communication in N-cadherin transfectants (MHD1-N1) was enhanced about double that of the cadherin-lacking cells (Table 3). Also gap junctional plaque staining (absent in control conditions in both MHD1 and MHD1-N1, Fig. 6C,D) was more abundant in the forskolin-treated N-cadherin expressing MHD1-N1 than the MHD1 cells (Fig. 6E,F). This result is clearly in contrast to L cells where both communication and Cx43 plaques were reduced by cadherin expression. Since N-cadherin expression in the hepatoma cells induced communication and increased Cx43 junctional plaque formation, we investigated whether there might be a relationship between the localization at cell-cell contacts of N-cadherin and Cx43. We double-immunostained forskolin-treated cells for Cx43 and N-cadherin and examined the cells by confocal fluorescence microscopy. We found that Cx43 junctional plaque staining was highly co-localized with N-cadherin staining (Fig. 6G,H). As in the L cells, cadherin expression did not seem to have a significant effect on Cx43 protein in the hepatoma cells either, as seen in the western blot of Fig. 5D.

**Inhibition of glycosylation blocks the cadherin-mediated inhibition of communication**

We had shown previously that communication and the formation of gap junction plaques can be improved in many mammalian cell types by cell treatment with inhibitors of gly-

**Table 3. N-Cadherin expression increases communication in Morris hepatoma (MHD1) cells**

<table>
<thead>
<tr>
<th>Cell</th>
<th>Cadherin expressed</th>
<th>Communication*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control†</td>
</tr>
<tr>
<td>MHD1</td>
<td>None</td>
<td>0.0±0.0 (34)</td>
</tr>
<tr>
<td>MHD1-N1</td>
<td>N-cadherin</td>
<td>0.1±0.1 (54)</td>
</tr>
</tbody>
</table>

*Mean number of fluorescent cell neighbors ± s.e.m. 5 minutes after injection; in parentheses, the number of injection trials.
†8 hour treatment with 4 μM forskolin.
‡8 hour treatment with 10 μg/ml tunicamycin.
§8 hour treatment with 20 μM forskolin and 50 μM Ro-20-1724.

![Fig. 6. N-cadherin (green) and Cx43 (red) immunostaining in the Morris hepatoma cell clone MHD1 and the N-cadherin-expressing clone MHD1-N1. N-cadherin immunostaining in MHD1 (A) and MHD1-N1 (B) cells. (MHD1 cells lack N-cadherin-specific immunostaining; non-specific cytoplasmic staining shows up here because we exposed the film longer than in b so as to visualize the cells in the field). (C-F) Cx43 immunostaining of MHD1 (C,E) and MHD1-N1 (D,F) in control condition (C,D) and after 10 hours forskolin/Ro-20-1724 treatment (E,F). No punctate staining typical of Cx43 gap junction plaques was detected at cell junctions in controls; punctate staining appeared after forskolin/Ro-20-1724 treatment in both, cadherin-lacking MHD1 and cadherin-expressing MHD1-N1, but it was much more abundant in D1-N1 cells. (G,H) Colocalization of Cx43 gap junctional plaques with N-cadherin plaques. Confocal microscopy images of MHD1-N1 cells double immunostained for Cx43 and N-cadherin. Pictured is one cell and its junction (J) with a neighbor cell. The cell’s nucleus (N) is surrounded by Cx43 and N-cadherin, presumably in the endoplasmic reticulum. Single arrows point to examples of co-localization of the immunostains at the junction, double arrows to where there is no co-localization.**
The major findings of this study are: (i) that cadherin expression inhibits communication and gap junction plaque formation in one cell type (L cells) and enhances communication and plaque formation in another (hepatoma cells); and (ii)

that the inhibitory effect is largely abolished in the absence of glycosylation.

There are several ways by which opposite, cell type-dependent effects of cadherins on cell communication could come about. For instance, intracellular signaling cascades resulting from cadherin-cadherin interaction (Doherty and Walsh, 1994), such as opening of Ca$^{2+}$ channels, changes in intracellular [Ca$^{2+}$] or pH, and activation of particular protein kinases can be cell-type specific (von Bohlen und Halbach et al., 1992). Since activity of some kinases depresses communication (protein kinase C, src) and EGF receptor kinase (Murray and Fitzgerald, 1979; Yotti et al., 1979; Atkinson et al., 1981; Azarnia and Loewenstein, 1984; Maldonado et al., 1988; Kanemitsu and Lau, 1993) whereas activation of others, e.g. protein kinase A, improves communication (Flagg-Newton et al., 1981; Wiener and Loewenstein, 1983; DeMello, 1984) in a variety of cells, cadherin-cadherin interaction may bring about inhibition or enhancement of communication, depending on which kinases are activated in the particular cell’s signaling cascade. Changes in Cx43 phosphorylation or Cx43 protein level have been associated with activity of all of the above kinases (Crow et al., 1990; Filson et al., 1990; Swenson et al., 1990; Brisette et al., 1991; Oh et al., 1991; Berthoud et al., 1992; Mehta et al., 1992). However, we observed no cadherin-induced decrease in Cx43 protein level in L cells and hence find no evidence for inhibition of communication as a result of a downregulation of Cx43 in these cells. L cells do not express Cx26 or Cx32 (our unpublished observations) but the possibility remains that they express another member of the connexin family, whose expression is somehow regulated by cadherins. Although we saw no cadherin-specific alteration in Cx43 mobility on SDS-PAGE indicative of a change in relative amounts of phosphorylated Cx43, lack of such evidence does not preclude a change in phosphorylation. For instance, if a new site were phosphorylated roughly stoichiometrically to dephosphorylation of another site, one might not detect a difference on western blots. This leaves open the possibility that a particular kinase or set of kinases, activated by cadherin interactions in one cell type but not another, modifies Cx43 in a manner that determines whether there are fewer or more open channels. The finding that the cell type-specific differences in cadherin action on communication persist in the presence of forskolin suggests that the CAMP-signaling path is not the mediator of these cadherin effects.

Cadherins are tightly associated with cytoskeletal elements and may regulate the cytoskeletal organization in their immediate vicinity (Ozawa and Kemler, 1992; Hirano et al., 1992), cause recruitment of specific membrane proteins or protein kinases to cell-cell contacts (McNeill et al., 1990), and are involved in the formation of other intercellular structures such as the adherens and occludens type of junctions (Itoh et al., 1993; for a recent review see Gumbiner, 1996). Any of these cadherin-initiated events may be cell-type specific; some may interfere with cell-to-cell channel formation or cause channels to close, and others may foster channel formation or opening. Of particular interest here is that expression of adhesion molecules has been found to induce the assembly of tight junctions in L cells (Itoh et al., 1993), but not in mouse sarcoma (S180) cells (Howarth et al., 1994). Whether there is any causal relation between this finding and the opposite effect that the inhibitory effect is largely abolished in the absence of glycosylation.

Table 4. Inhibition of glycosylation increases communication in cadherin-expressing cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cells</th>
<th>Cadherin type expressed</th>
<th>Forskolin†</th>
<th>Forskolin + tunicamycin‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>L</td>
<td>None</td>
<td>13.8±0.9 (52)</td>
<td>15.5±1.4 (23)</td>
</tr>
<tr>
<td></td>
<td>ELβ1</td>
<td>E-cadherin</td>
<td>2.4±0.3 (79)</td>
<td>10.4±0.7 (56)</td>
</tr>
<tr>
<td></td>
<td>PLβ2</td>
<td>P-cadherin</td>
<td>5.2±0.4 (86)</td>
<td>12.7±0.7 (57)</td>
</tr>
<tr>
<td></td>
<td>N1</td>
<td>N-cadherin</td>
<td>8.5±0.6 (56)</td>
<td>14.2±1.1 (64)</td>
</tr>
<tr>
<td>B</td>
<td>L</td>
<td>None</td>
<td>9.4±3.2 (20)</td>
<td>10.8±3.3 (9)</td>
</tr>
<tr>
<td></td>
<td>ELβ1</td>
<td>E-cadherin</td>
<td>3.0±2.1 (16)</td>
<td>8.6±2.9 (14)</td>
</tr>
<tr>
<td></td>
<td>PLβ2</td>
<td>P-cadherin</td>
<td>3.0±2.1 (22)</td>
<td>6.4±1.7 (15)</td>
</tr>
<tr>
<td></td>
<td>N1</td>
<td>N-cadherin</td>
<td>3.0±1.6 (13)</td>
<td>8.1±5.9 (11)</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>N-cadherin</td>
<td>5.4±2.8 (10)</td>
<td>9.3±2.7 (15)</td>
</tr>
</tbody>
</table>

*Mean number of fluorescent cell neighbors (±s.e.m. in A and ±s.d. in B) 5 minutes after injection of Lucifer Yellow (A) or FITC-glutamic acid (B); in parentheses, the number of injection trials.

†Cells were treated for 8 hours with 20 µg/ml forskolin and 50 µM Ro-20-1724.

‡Cells were treated for 8 hours with 10 µg/ml glycosylation inhibitor tunicamycin plus forskolin and Ro-20-1724.

In experiment A, Lucifer Yellow (mol. mass 430) was used as a communication probe, and in B, FITC-glutamic acid (mol. mass 536). The larger probe does not permeate the channels as easily as Lucifer Yellow, resulting in fewer fluorescent neighbors at 5 minutes after injection, permitting a more accurate count when cells have a high degree of communication.
on gap junctional communication in these two cell types remains to be explored.

The result of co-localization of Cx43 gap junction plaques with N-cadherin in the hepatoma cells junctions is interesting. It points to an intimate relationship here between sites of cadherin-cadherin interaction and connexin channel formation or aggregation, perhaps even a causal one: the cadherin interaction sites might become nucleation sites for cell-to-cell channel formation simply by providing stable and close membrane apposition; or the particular intracellular signal cascade triggered by cadherin-cadherin interaction at cell-cell contacts in hepatoma cells might also trigger recruitment of cell-to-cell channels or hemichannels to those contact sites.

What role glycosylation plays in the communication-inhibitory action of cadherins is not clear. Although cadherins normally are glycosylated, neither their expression on the cell surface nor their cell-cell adhesive function is affected by the absence of glycosylation (Shirayoshi et al., 1986). Perhaps the extent of cadherin glycosylation depends on cell type involved: if the carbohydrate moieties are particularly extensive and reach far beyond the cadherin protein core, the freedom of lateral movement, i.e. movement within the plane of the membrane, of other membrane proteins including connexons, might be curtailed and the chance for connexon encounters, alignment and interlocking be reduced. However, our finding that Cx43 gap junction plaques colocalize (at the confocal light microscope level) with N-cadherin, at least in the hepatoma cells where communication is enhanced by N-cadherin (see also Hertig et al., 1996, for N-cadherin/Cx43 colocalization in cardiomyocytes), would indicate that the carbohydrate moieties do not prevent the connexons, once aligned, from getting into reach of one another across the intercellular gap. Other explanations for the sensitivity of the communication-inhibitory effect to glycosylation might be that certain glycosylated receptors interacting with medium components set the intracellular baseline for triggering the signal transduction events that follow cadherin-cadherin interaction, or that intracellular glycoproteins play a role.

In summary, the effect of cadherins on cell-to-cell communication and Cx43 gap junctional plaque formation depends on cell type. It can be facilitatory in one cell and inhibitory in another, and the inhibition is sensitive to glycosylation.

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REFERENCES


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