Homotypic Gap Junctional Communication Associated with Metastasis Suppression Increases with PKA Activity and Is Unaffected by PI3K Inhibition

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Introduction

The deadliest attribute of cancer cells is their ability to disseminate and colonize other tissues (1). The process of metastasis involves genetic changes that result in dysregulation of tumor cell interactions with other tumor cells and the host (2, 3). Breast cancer metastasis suppressor 1 (BRMS1) blocks the ability of breast (4–7), melanoma (8), ovarian (9), and non–small cell lung (10) cancer cells to metastasize but does not form the formation of orthotopic tumors following injection. BRMS1 is a component of multiple SIN3:HDAC complexes that alter the expression of numerous genes and proteins (11–13). Among the first identified phenotypic changes reported for BRMS1-expressing cells was the restoration of gap junctional intercellular communication (GJIC; ref. 14).

GJIC is defined as a process in which small molecules (e.g., inositol trisphosphate, ATP, Ca²⁺) can be transferred between adjoining cells through physical interaction of highly regulated channels located in the plasma membrane. These functional channels are composed of hexameric structures (connexons) made up of individual connexin proteins that interact with numerous other components within the plasma membrane (15). GJIC is associated with normal cellular homeostasis, but dysregulation is common in neoplastic progression and even further loss of communication is often associated with acquisition of a metastatic phenotype (16–19). However, exceptions to this trend exist, for example, Nicolson et al. showed that cells transfected with p21ras exhibited higher metastatic potential but did not always lose GJIC (20). Nevertheless, restoration of GJIC by expression of connexins in some models decreases proliferation and inhibits metastasis (21–25). Contradictory reports show an increase in migratory and metastatic potential when GJIC is increased [reviewed in (26, 27)], highlighting cell- and context-specific components of the process.

Because some connexins function in a plasma membrane–independent manner (28) and because posttranslational modifications and assembly of connexins into connexons can be regulated by altered signaling pathways (29, 30), we hypothesized that the selective alteration of phosphoinositide levels by BRMS1 (31) could be, at least partially, involved in the regulation of GJIC. Specifically, BRMS1 expression results in a dramatic greater than 95% reduction in PtdIns (4, 5)P₂ levels.

Abstract

Loss of gap junctional intercellular communication (GJIC) between cancer cells is a common characteristic of malignant transformation. This communication is mediated by connexin proteins that make up the functional units of gap junctions. Connexins are highly regulated at the protein level and phosphorylation events play a key role in their trafficking and degradation. The metastasis suppressor breast cancer metastasis suppressor 1 (BRMS1) upregulates GJIC and decreases phosphoinositide-3-kinase (PI3K) signaling. On the basis of these observations, we set out to determine whether there was a link between PI3K and GJIC in tumorigenic and metastatic cell lines. Treatment of cells with the well-known PI3K inhibitor LY294002, and its structural analogue LY303511, which does not inhibit PI3K, increased homotypic GJIC; however, we found the effect to be independent of PI3K/AKT inhibition. We show in multiple cancer cell lines of varying metastatic capability that GJIC can be restored without enforced expression of a connexin gene. In addition, while levels of connexin 43 remained unchanged, its relocalization from the cytosol to the plasma membrane was observed. Both LY294002 and LY303511 increased the activity of protein kinase A (PKA). Moreover, PKA blockade by the small molecule inhibitor H89 decreased the LY294002/LY303511-mediated increase in GJIC. Collectively, our findings show a connection between PKA activity and GJIC mediated by PI3K-independent mechanisms of LY294002 and LY303511. Manipulation of these signaling pathways could prove useful for antimitastatic therapy. Cancer Res 70(23): 10002–11. ©2010 AACR.

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(31), leading to a decrease in phosphorylation of AKT at Ser473. Because PtdIns (4, 5) P2 is a major substrate for the oncogenic phosphoinositide-3-kinase (PI3K) and is a major component of lipid rafts in which connexons form (32, 33), we tested whether inhibition of PI3K by the commonly used PI3K inhibitor LY294002 would itself mimic BRMS1 in BRMS1 null cancer cells by restoring or enhancing GJIC. Although LY294002 indeed enhanced GJIC in numerous cell lines of different origin, we report that the mechanism of action is not likely via its regulation of PI3K but rather PI3K-independent mechanisms related to protein kinase A (PKA).

**Materials and Methods**

**Cell lines**

MDA-MB-231, MDA-MB-435, MDA-MB-436, MDA-MB-468, T-47D, and C8161.9 were grown in Dulbecco’s modified Eagle’s medium mixed 1:1 (v:v) with Ham’s F-12 medium (DMEM/F12, #11330; Invitrogen, Carlsbad, CA) supplemented with 2 mmol/L of l-glutamine, 0.2 mmol/L of nonessential amino acids with 5% fetal bovine serum (FBS). MDA-MB-231, MDA-MB-435, MDA-MB-436, MDA-MB-468, and T-47D are human breast carcinoma–derived cell lines, whereas C8161.9 is a clone derived from the C8161 human melanoma. The origin of MDA-MB-435 has been questioned (34); however, recent literature strongly confirms its use as a breast carcinoma cell line (35–37). S2VP10 pancreatic cancer cell line (38) was maintained in RPMI-1640 medium (#11875; Invitrogen) supplemented with 5% FBS. All cell lines were tested and found to be free of Mycoplasma spp. contamination, using a PCR-based kit (#ab27478; AbCam) was used as an isotype control. Images were obtained on a Nikon Eclipse TE2000-U microscope using Q Imaging QICAM software for image analysis.

**Chemicals**

All chemicals were prepared as stock solutions and stored at –20°C in aliquots; working solutions were diluted fresh at the time of experiment. Calcein-AM (#C1430) and CM-DiI (#C1409) were obtained from Invitrogen and dissolved in DMSO at 10-mmol/L stock. Vehicle controls (DMSO, purified water) had no effect on assays performed in DPBS and permeabilized with ice-cold methanol for 30 minutes at –20°C. Cover slips were blocked with 5% bovine serum albumin (BSA) in DPBS and primary antibodies overnight at 4°C. Anti-connexin 43 (C43; #3512; Cell Signaling) was used. Rabbit polyclonal IgG secondary antibodies were diluted at 1:2,500 in 5% nonfat dry milk in TBST. For Cx43 immunoblot analysis, lysates were sonicated on ice; all membranes were blocked for 1 hour in 5% nonfat dry milk in TBST. For Cx43 immunoblot analysis, membranes were washed 4 times in TBST. Secondary antibodies were diluted at 1:2,500 in 5% nonfat dry milk in TBST. Secondary horseradish peroxidase–conjugated antibodies were purchased from GE Healthcare: anti-rabbit (#NA934V) and anti-mouse (#NA931V). Membranes were developed by chemiluminescence (#32106; Thermo Scientific). ImageJ (National Institutes of Health) processing and analysis

**Immunofluorescence**

Cells were grown on glass cover slips with indicated treatments, followed by performing the GJIC assay described earlier. Cells were fixed with 3.7% neutral buffered formalin in DPBS and permeabilized with ice-cold methanol for 30 minutes at –20°C. Cells were rinsed with PBS and incubated with 5% normal goat serum in PBS for 1 hour at room temperature. Cells were rinsed with PBS and permeabilized with ice-cold methanol for 30 minutes. Cells were blocked for 1 hour with 5% BSA in PBS, followed by the addition of secondary antibody (488; Alexa Fluor 488–conjugated antibody #ab27478; Sigma) at 1:160 in 2% BSA in DPBS for 1 hour at room temperature. Cover slips were then washed 3 times with DPBS and mounted using VECTASHIELD with DAPI (#H-1200; Vector Labs). For phospho-CREB Ser133 analysis, Alexa Fluor 488–conjugated antibody (#ab9187; Cell Signaling) was used. Rabbit polyclonal IgG secondary antibody (AbCam) was used as an isotype control. Images were obtained on a Nikon Eclipse TE2000-U microscope using Q Imaging QICAM software for image analysis.

**Immunoblot analysis**

Cells were lysed on ice in 25 mmol/L of Tris-HCl, 1% Triton X-100, 5% glycerol, 0.5 mmol/L of EDTA, 50 mmol/L of β-glycerophosphate supplemented with 1.0 mmol/L of NaVa, 0.5 mmol/L of phenylmethylsulfonyl fluoride and 1 × HALT Protease Inhibitor Cocktail (#78430; Thermo Scientific). For connexin protein analysis, lysates were sonicated on ice; all other samples were boiled at 95°C for 5 minutes. Proteins were resolved using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis before transfer to polyvinylidene fluoride membranes and blocked for 1 hour in 5% nonfat dry milk in Tris buffered saline with Tween-20 (TBST). For Cx43 immunoblots, proteins were transferred onto nitrocellulose membranes. Primary antibodies were diluted at 1:1,000 in 5% BSA in TBST.

The following primary antibodies were purchased from Cell Signaling Technology: C43 (#3512), phospho-AKT Ser473 (#9271), pan-AKT (#4685), α-tubulin (#2125), cAMP response element binding (CREB; #9104), phospho-CREB Ser133 (#9198). Secondary antibodies were diluted at 1:2,500 in 5% nonfat dry milk in TBST. Secondary horseradish peroxidase–conjugated antibodies were purchased from GE Healthcare: anti-rabbit (#NA934V) and anti-mouse (#NA931V). Membranes were developed by chemiluminescence (#32106; Thermo Scientific). ImageJ (National Institutes of Health) processing and analysis.
was used for quantification of Cx43 blots and results represented as fold change compared with nontreated (NT) cells.

**Statistical analysis**

Data for GJIC are presented as mean ± SEM and represented as fold change. Student’s *t* test was used for statistical analysis between groups.

**Results**

**Treatment of cells with LY294002 increases calcein dye transfer**

Donor cells were labeled with calcein-AM and CM-DiI before plating with nonlabeled acceptor cells. We observed that treatment with 10 μmol/L of LY294002 exhibited significantly higher rates of dye transfer from donor to acceptor cells (Fig. 1A). We extended these results to a total of 5 breast cancer cell lines (MDA-MB-231, MDA-MB-435, MDA-MB-436, MDA-MB-468, and T-47D), a metastatic melanoma cell line (C8161.9), and a metastatic pancreatic cell line (S2VP10) (Fig. 1B). All of the cell lines exhibited varying levels of dye spread in NT conditions, but all cells displayed a significant and consistent increase in GJIC when incubated in LY294002. Cell lines that exhibited the lowest baseline rates of dye spread (MDA-MB-231 and S2VP10) experienced the greatest fold change. Calcein could be seen spreading between cells within an hour of addition of donor cells and increased throughout the duration of the experiment with readily noticeable differences at 6 hours.

**Treatment with wortmannin and direct inhibition of AKT do not increase GJIC compared with LY294002**

To determine whether inhibition of AKT phosphorylation by LY294002 was mediating the effects on GJIC, MDA-MB-231, MDA-MB-435, and C8161.9, cells were treated with increasing concentrations of wortmannin (50, 100, and 500 nmol/L), another PI3K inhibitor. Phosphorylation of AKT at Ser473 was potently inhibited (Fig. 2A). However, in comparison with LY294002, no increase in GJIC was noted compared with NT cells in MDA-MB-231, MDA-MB-435, and C8161.9 (Fig. 2B) whereas slight decreases were observed, signifying that inhibition of phosphorylation of AKT was not responsible for the LY294002-mediated increase in GJIC. To further confirm that signaling factors downstream of AKT do not increase GJIC in these cell lines, we used a direct inhibitor of AKT, AKTinbVII, which binds to the pleckstrin homology domain of AKT. Treatment of MDA-MB-231 and MDA-MB-435 with AKTinbVII reduced phosphorylation of AKT in MDA-MB-231 cells and completely abolished phosphorylation in MDA-MB-435 (Fig. 3A). However, despite these decreases, no significant increase was observed in GJIC (Fig. 3B). Similar to treatment with wortmannin, the cells exhibited slight decreases in GJIC upon direct AKT inhibition. These results demonstrate that directly decreasing AKT activity does not cause an increase in GJIC.

**LY303511 increases GJIC similar to LY294002**

Because PI3K/AKT signaling was ruled out as the mechanism for the LY294002-mediated increase in GJIC, we examined whether the stated effect was related to its chemical structure. LY303511 is structurally identical to LY294002, except for a substitution of −O for −NH in the morpholine ring (Supplementary Fig. S2), and does not potently inhibit PI3K. Treatment of cells with LY303511 caused an increase in calcein spread similar to levels of LY294002 (Fig. 4A). The ability of LY303511 to increase GJIC did not occur concomitant with inhibition of phosphorylation of AKT as measured by immunoblotting (Fig. 4B).

**Cx43 is relocalized after treatment with LY294002 and LY303511**

Because connexins are the functional units of connexon channels, we examined whether LY294002 and LY303511...
altered the regulation of Cx43, a connexin frequently dysregulated in breast and other cancers (39–42). Immunoblot analysis of Cx43 levels in C8161.9 and MDA-MB-231 showed a consistent decrease in Cx43 levels after treatment with LY294002; however, this effect was not observed with LY303511 (Fig. 5A and B). This result may be due to other nonspecific effects of LY294002 that are not shared by LY303511 or the simultaneous inhibition of PI3K. However, significant changes in trafficking of Cx43 from the cytosol to the plasma membrane were observed following treatment with the LY compounds (Fig. 5C). These results show that both LY294002 and LY303511 are capable of causing a dramatic subcellular relocalization of Cx43 and that this effect is likely responsible for the increases in GJIC.

**PKA activity is upregulated in response to LY294002 and LY303511**

PKA activity leads to phosphorylation of specific residues of Cx43, promoting its transport from the Golgi apparatus to the plasma membrane and increasing GJIC (43–47). Because LY294002 and LY303511 do not dramatically alter protein levels of Cx43 but rather change Cx43 subcellular localization, we set out to determine whether an increase in PKA activity was responsible for the enhancement in GJIC. PKA phosphorylates the transcription factor CREB at Ser133, which was used as an indicator of PKA activity. Using an Alexa Fluor 488–conjugated antibody that binds CREB when phosphorylated at Ser133, a significant increase in fluorescence within the nucleus following a 2-hour treatment with LY294002 or LY303511 was observed (Fig. 6A), indicative of PKA activation. To determine how quickly PKA is activated, nuclear extracts were isolated following treatment with LY294002 at varying time points. Increased phosphorylation of CREB was evident as early as 15 minutes posttreatment (Fig. 6B). In support of PKA playing a role in increased GJIC in these cells, MDA-MB-231 cells were treated with increasing concentrations of 8-BR-cAMP, a cell-permeable analogue of cAMP that upregulates PKA activity. 8-BR-cAMP increased GJIC in a dose-dependent manner in these cells (Fig. 6C). To determine whether PKA activity was responsible for the LY294002-mediated enhancement in GJIC, cells were cotreated with LY294002 (10 μmol/L) and the PKA inhibitor H89 (10 μmol/L). Addition of H89 caused a significant reduction in GJIC induced by LY294002 (Fig. 6D and E), indicating that upregulation of PKA by LY294002 is at least, in part, responsible for the increase in GJIC.

**Discussion**

GJIC is a complex process capable of inhibiting or promoting the migratory and invasive qualities of cancer cells. Importantly, GJIC is defined in a context-dependent manner, because tumor cells can form homotypic (tumor cell–tumor cell) or heterotypic (tumor cell–host cell) interactions using multiple permutations of greater than 20 mammalian connexins (48). Further complexity exists because connexin proteins are now known to mediate multiple cellular effects independent of their involvement...
in gap junctions. Because a number of studies show that restoration of gap junctional proteins and/or GJIC can reverse tumorigenicity or metastatic potential (18, 19, 21, 23–25), understanding how GJIC is dysregulated in cancer cells and delineating such (dys)regulatory signaling pathways will allow insight into defining the role(s) of GJIC in tumor progression and metastasis.

Because the metastasis suppressor BRMS1 inhibits metastasis in numerous models, and has previously been reported to inhibit AKT phosphorylation at Ser473 while increasing GJIC, we initially set out to determine whether a link between these effects existed. We utilized 2 PI3K inhibitors, LY294002 and wortmannin, to recapitulate the effect of BRMS1 on AKT phosphorylation in cells that do not express detectable endogenous BRMS1, followed by measuring GJIC.

Treatment of cells with the relatively more “selective” PI3K inhibitor LY294002 (49) significantly increased GJIC in multiple cancer cell lines. No clear trends were observed in metastatic potential and baseline GJIC between the cell lines used. For example, MDA-MB-231 and C8161.9 are both highly metastatic in murine models; however, MDA-MB-231 exhibited low baseline GJIC whereas C8161.9 displayed moderate levels (Fig. 1A), which was higher in comparison with some of the nonmetastatic cell lines (i.e., MDA-MB-468). Regardless of baseline communication rates, all cell lines experienced a significant increase in GJIC when treated with LY294002. Unexpectedly, treatment of the same cell lines with wortmannin did not increase GJIC, raising doubts regarding the connection between PI3K and GJIC. This was further substantiated when cells treated with a direct AKT inhibitor failed to induce GJIC as well. Interestingly, inhibition of AKT with these treatments caused decreases in GJIC, signifying that AKT appears to be partially involved in the regulation of GJIC; however, the magnitude of these changes did not match the level of GJIC increase observed with LY294002. Reasoning that the effect of LY294002 could be due to signaling through the inositol trisphosphate arm, cells were treated with phospholipase C inhibitors without increasing GJIC (data not shown). Taken together, these data required exploration of potential PI3K-independent effects of LY294002. To facilitate dissection of the possible nonspecific effects, a chemically related molecule, LY303511 (50), was used. We found that LY303511 enhanced GJIC similarly to LY294002.

We examined a number of published dose-dependent, nonspecific effects reported for these compounds including inhibition of casein kinase 2 [Ref. 51; Supplementary Fig. S3] and increased intracellular calcium levels [Ref. 52; Supplementary Fig. S4A and B]; however, none of the effects tested corresponded with GJIC enhancement.

Although there is a correlation between connexin levels and GJIC in many cancer cells, functional connexons are assembled by, among other posttranslational modifications, phosphorylation through protein kinases (53, 54). Because we observed relatively slight changes in Cx43 protein levels after treatment with LY294002 and LY303511, but highly substantial changes in Cx43 localization to the plasma membrane, we hypothesized that Cx43 was being modified posttranslationally and that these effects were likely to be inducing the effect on GJIC. Among the most well-defined phosphorylating molecules resulting in connexin trafficking to the plasma membrane is PKA (43–47). Although Davies et al. previously reported that LY294002 did not directly affect PKA activity in cell-free kinase assays (51), it is important to emphasize that they could not preclude indirect effects of LY294002 on PKA activation (as would occur in whole cells). We consistently found a robust increase in CREB phosphorylation at Ser133 by both immunoblot and immunofluorescence analyses, confirming that PKA was indeed activated by both LY294002 and LY303511. These results agree with our data involving relocation of Cx43 to the plasma membrane and a corresponding increase in GJIC that could be expected upon activation of PKA.

Further strengthening our hypothesis that PKA activation by LY294002/LY303511 enhanced homotypic GJIC are observations that cells treated with 8-BR-cAMP (an agonist of PKA)
or H89 (a PKA inhibitor) increased or decreased GJIC, respectively. Because Davies et al. showed that LY294002 did not directly affect PKA activity (51), we set out to determine whether LY294002/LY303511 induced activation of adenylate cyclase that would lead to increased cAMP levels and PKA activation. Pretreatment of cells with the adenylate cyclase inhibitors 2',5'-dideoxyadenosine and SQ 22,536 (Fig. 6F and G) did not reduce the ability of LY294002 to induce GJIC in contrast to direct PKA inhibition with H89. These data suggest that LY294002/LY303511 are acting downstream of adenylate cyclase, most likely through other indirect cellular interactions that have yet to be determined.

Because H89 significantly reduced LY294002/LY303511-mediated increase in GJIC, it seems that activation of PKA is, at least in part, responsible for the changes in GJIC. Collectively, our results highlight the fact that cancer cells may reduce GJIC not by causing a downregulation of connexin expression but rather by altering the regulatory pathways related to connexin function and/or localization. This can readily be appreciated because we induced an increase in GJIC

Figure 4. Both LY303511 and LY294002 increase gap junctional intercellular communication. LY303511 treatment (10 μmol/L) increases calcein transfer in MDA-MB-231, MDA-MB-435, and C8161.9 cells (A) despite not potently inhibiting PI3K (B). NT, nontreated. *, P < 0.05; **, P < 0.01.
in 7 cancer cell lines without exogenous expression of a connexin gene. In addition, with literature building on membrane-independent roles of connexin proteins, it is possible that cancer cells may not just cause a relocalization of connexins away from the plasma membrane but utilize these proteins for other membrane-independent tasks related to cancer cell function. Although this report is limited to observations with Cx43, these results warrant further investigation of other connexins and highlight an important principle to consider for future studies in this area.

Although not a central tenet for the studies recorded here, our data highlight the caution necessary when interpreting results using any pharmaceutical reagent (in this case LY294002), no matter how selective that agent is expected to be. More important, the findings also have important therapeutic implications for adjuvant cancer therapies. Because GJIC restoration was possible by exogenous drug treatment, it may be possible to accomplish the same in vivo. Cell-permeable compounds such as LY294002 and LY303511 that can induce GJIC in cancer cells may be further developed for treatments aimed at increasing the penetrance of chemotherapeutic agents throughout a tumor via an increase in gap junction activity. Doing so would be much easier to accomplish with a drug than by attempting to reexpress or overexpress connexins in tumor cells. However, whether PKA acts as a true convergence point for dysregulation in cancer cells remains to be determined.

Figure 5. Cx43 localizes to the plasma membrane after LY294002 or LY303511 treatment. A, representative immunoblots from 3 independent experiments of Cx43 in C8161.9 and MDA-MB-231 6 hours posttreatment with 10 μmol/L of LY294002 and LY303511. B, quantification of Cx43 immunoblot analyses (n = 3). **, P < 0.01. C, exposure of C8161.9 or MDA-MB-231 cells to LY294002 (10 μmol/L) or LY303511 (10 μmol/L) for 6 hours resulted in substantially more plasma membrane localization of Cx43 than nontreated (NT) cells. Arrows highlight Cx43 plaques between cells.
Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interest.

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