J. Membrane Biol. 155, 275–287 (1997)

The Journal of
Membrane
Biology

Loss of Gap Junction Plaques and Inhibition of Intercellular Communication in Ilimaquinone-treated BICR-M1R$_k$ and NRK Cells

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Received: 16 January 1996/Revised: 20 September 1996

Abstract. To examine the mechanism(s) and pathways of gap junction formation and removal a novel and reversible inhibitor of protein secretion, ilimaquinone (IQ), was employed. IQ has been reported to cause the vesiculation of Golgi membranes, block protein transport at the *cis*-Golgi and depolymerize cytoplasmic microtubules. Connexin43 (Cx43) immunolabeling and dye microinjection experiments revealed that gap junction plaques were lost and intercellular communication was inhibited following IQ treatment for 1 hr in BICR-M1R$_k$ rat mammary tumor cells and for 2 hr in normal rat kidney (NRK) cells. Gap junction plaques and intercellular communication recovered within 2 hr when IQ was removed. IQ, however, did not affect the distribution of zonula occludens-1, a protein associated with tight junctions. Western blot analysis revealed that the IQ-induced loss of gap junction plaques was accompanied by a limited reduction in the highly phosphorylated form of Cx43, previously shown to be correlated with gap junction plaques. The presence of IQ inhibited the formation of new gap junction plaques in BICR-M1R$_k$ cells under conditions where preexisting gap junctions were downregulated by brefeldin A treatment. Treatment of BICR-M1R$_k$ and NRK cells with other microtubule depolymerization agents did not inhibit plaque formation or promote rapid gap junction removal. These findings suggest that IQ disrupts intercellular communication by inhibiting the events that are involved in plaque formation and/or retention at the cell surface independent of its effects on microtubules. Our results also suggest that additional factors other than phosphorylation are necessary for Cx43 assembly into gap junction plaques.

Key words: Ilimaquinone — Gap junction plaque — Connexin43 — Intercellular communication

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Introduction

Gap junctions are membrane channels between closely apposed cells that allow for intercellular exchange of secondary messengers and other small molecules (Flagg-Newton, Simpson & Loewenstein, 1979; Loewenstein, 1981). Gap junction proteins (connexins) have been shown to oligomerize into hemichannels (connexons) after they exit the endoplasmic reticulum *en route* to the plasma membrane (Musil & Goodenough, 1993). These hemichannels assemble further at the cell surface and become densely clustered to form gap junction plaques (Caspar et al., 1977). Unlike the long half-life (>20 hr) of many integral membrane proteins (Chu & Doyle, 1985; Hare & Taylor, 1991), gap junction plaques are structures that turnover rapidly with in vivo half-lives of 5 hr in hepatocytes (Fallon & Goodenough, 1981) and ~1.5 hr in cultured rat mammary BICR-M1R$_k$ tumor cells (Laird, Castillo & Kasprzak, 1995). In addition to being regulated through formation and removal of gap junctions, intercellular exchange can also be regulated through gating of gap junction channels which undergo reversible conformational changes that temporarily close the channel in response to changes in the intracellular or extracellular environment (Loewenstein, 1978; Makowski et al., 1984; Spray et al., 1984). Regulated intercellular communication is essential for proper embryonic development (Guthrie & Gilula, 1989; Réaume et al., 1995) and for controlled cell growth and differentiation (Loewenstein, 1979; Mehta, Bertram & Loewenstein, 1986).

The gap junction protein, connexin43 (Cx43), has been extensively studied over the past ten years and is the best understood member of the connexin family. It is known to have several states of phosphorylation (Crow et al., 1990; Musil et al., 1990*a,b;* Laird, Puranam & Revel, 1991; Laird et al., 1995; Wang & Rose, 1995). The two most characterized of these phosphorylated species are commonly referred to as P_1 and P_2 (Musil et al.,

1990*b*) although other phosphorylated species of Cx43 have been identified (Lau et al., 1992; Laird et al., 1995). Studies by Musil and colleagues have correlated the phosphorylation of Cx43 with gap junction plaque formation and communication competence (Musil et al., 1990*b;* Musil & Goodenough, 1991). Phosphorylation of Cx43 to the P_1 and P_2 species was shown to occur at the cell surface and the P_2 species of Cx43 was linked to plaque formation (Musil & Goodenough, 1991). However, in WB F-344 cells, Cx43 was present in nonfunctional gap junction plaques in the apparent absence of the P_2 species of Cx43 (Oh et al., 1993). Conversely, Wang, Mehta & Rose (1995) showed that gap junction plaques did not form in tunicamycin-treated mouse hepatoma cells transfected with Cx43 even though Cx43 was extensively phosphorylated.

The intracellular trafficking, maturation and assembly of Cx43 into functional gap junctions has been elucidated further by the use of brefeldin A (BFA) (Musil & Goodenough, 1993; Laird et al., 1995). In a recent study, we used brefeldin A to reversibly block protein secretion in BICR-M1R_k tumor cells and uncouple the events that lead to gap junction formation from processes that result in their removal from the cell surface (Laird et al., 1995). Gap junctions and functional intercellular communication were inhibited in BICR-M1R_k cells that were treated with BFA for 6 hr, a finding consistent with the prevention of new gap junction formation and the rapid turnover kinetics of Cx43 (Laird et al., 1995). Recently, a novel inhibitor of protein secretion, ilimaquinone (IQ), has been shown to possess some properties similar to BFA. IQ is a major metabolite of several dictyoceratids sponges and was first obtained from Hippospongia metachromia (Luibrand et al., 1979). IQ has been shown to rapidly and reversibly disassemble Golgi membranes, including the *trans* Golgi network, into small vesicles and to inhibit protein transport between successive Golgi cisternae (Takizawa et al., 1993; Veit et al., 1993; Acharya et al., 1995*a,b*). However, unlike BFA, IQ does not cause ER/Golgi fusion and retrograde trafficking of Golgi proteins (Takizawa et al., 1993). In addition, IQ induces the depolymerization of cytoplasmic microtubules independently of its effects on the Golgi membranes (Veit et al., 1993).

In the present study, we examined the effect of IQ on gap junction plaques in a fibroblastoid (BICR-M1R_k) cell line from a rat mammary tumor and in NRK cells which both express moderate to high levels of Cx43. We showed that IQ inhibits gap junction formation and regulates the level of gap junction plaques found on the cell surface independent of its effects on microtubules. Furthermore, the IQ-induced inhibition of intercellular communication was correlated with a limited reduction in the most highly phosphorylated form of Cx43 suggesting that additional factors other than phosphorylation may govern gap junction plaque formation and communication competence.

Materials and Methods

Cell Culture

The rat BICR-M1R_k cell line, derived from a rat mammary tumor (characterized by Rajewsky & Gruneisen, 1972) and normal rat kidney cells (NRK cell line obtained from the American Type Culture Collection, Rockville, Maryland; 1571-CRL) were maintained as previously described (Laird et al., 1995). Subconfluent to confluent cultures (70–100%) were treated in some studies with 10 μM IQ (provided by Dr. V. Malhotra) for 1–1.5 hr (BICR-M1R_k cells) or 1.5–2 hr (NRK cells), 2–5 μg/ml BFA (CedarLane Laboratories, Hornby, ON) for 6 hr, 100 μg/ml nocodazole for 1.5 hr (Boehringer Mannheim, PQ, Canada), or 1 μM colcemid for 1.5 hr (Boehringer Mannheim, PQ, Canada). In recovery studies, cells were washed and allowed to recover in the presence of BFA, IQ, colcemid or nocodazole for 1–2 hr. In other recovery studies, BICR-M1R_k cells were treated with 2 μg/ml BFA for 6 hr, washed and allowed to recover for 1–2 hr in the presence or absence of 10 μM IQ. All experiments involving IQ, nocodazole and colcemid were carried out in complete culture medium supplemented with 25 mM Hepes, pH 7.4 (Veit et al., 1993).

Immunofluorescent Labeling, Microinjection and Confocal Microscopy

Control and drug-treated BICR-M1R_k and/or NRK cells grown on coverslips were fixed, blocked of nonspecific binding sites and immunolabeled as described by Laird and Revel (1990). In single labeling or double labeling experiments cells were labeled with 1–5 μg/ml affinity purified anti-Cx43 antibody (CT-360) raised against the amino acid segment, 360–382 of Cx43 as described by Laird and Revel (1990) followed by a 500-fold dilution of goat anti-rabbit antibody conjugated to rhodamine or fluorescein. In double labeling experiments, cells were labeled with 100-fold diluted monoclonal anti-Cx43 antibody (Ingram & Bell, Montreal, PQ) followed by goat anti-mouse secondary antibody conjugated to fluorescein. The same cells were then labeled with either rabbit anti-MG-160 serum (resident protein of *medial* Golgi cisterna) (Gonatas et al., 1989; Croul et al., 1990) or with rat monoclonal anti-ZO-1 (tight junction associated protein) (Developmental Studies Hybridoma Bank, Iowa City, IA and Baltimore, MD) followed by goat anti-rabbit antibody or goat anti-rat IgG antibody, respectively, conjugated to rhodamine.

Untreated, IQ-treated for 1–1.5 hr (BICR-M1R_k), 1.5–2 hr (NRK) or cells that were allowed to recover from IQ treatment for 1 hr (BICR-M1R_k) or 2 hr (NRK) were pressure microinjected with 5% Lucifer yellow in 10 mM Hepes (pH 7.4) to assay for gap junction coupling (n = 15–25 injections/treatment). Microinjected dye was allowed to spread for 15 min before fixing the cells for 5 min in Phosphate Buffered Saline (PBS) containing 3.7% paraformaldehyde. All fluorescent images were visualized and photographed using a Zeiss Axiophot fluorescent microscope and Kodak Tmax 400 film at 800 ASA. Double immunolabeled cells were analyzed on a Zeiss LSM 410 inverted confocal microscope as described by Laird et al. (1995). Images were saved on a Bernoulli multidisk and printed on a Kodak XLS 8300 high resolution (300 DPI) color printer.

Metabolic Labeling, Cell Lysates and Membrane Preparations

In metabolic labeling studies, BICR-M1R_k cells were first grown on 60 mm tissue culture dishes. Cells were labeled with ^{35}S-trans-label for

100 min as described in Laird et al. (1995) in the presence of IQ. After washing the cells in culture medium, the cells were chased for 3 hr in complete medium supplemented with 2 mM methionine and 5 μg/ml brefeldin A. The cells were lysed in RIPA buffer and protein concentrations were determined using the BCA assay and albumin as a standard. Cx43 was immunoprecipitated, resolved by SDS-PAGE and detected by fluorography as previously described (Laird et al., 1995).

In other studies, BICR-M1R_k and NRK cells were grown in 75 cm^2 flasks. Control and drug-treated cells were rinsed three times and scraped in 10 ml of cold PBS. The cells were pelleted in a clinical centrifuge and sonicated in 300–600 μl of modified Laemmli sample buffer as described by Laird et al. (1995).

Cell membrane fractions were prepared as described by Pollak, Polychronakos & Richard (1990) with the following modifications. Equal cells treated with or without IQ for 1–1.5 hr (BICR-M1R_k) or 1.5–2 hr (NRK), were rinsed two times in cold PBS and lysed in hypotonic buffer (0.01 M Tris, pH 7.4, 1 mM EDTA, 2 mM PMSF, 1 μg/ml leupeptin, 500 μM sodium orthovanadate) on ice. Cells were homogenized with 10 strokes of a Potter-Elvehjem teflon homogenizer and centrifuged at 1,000 × *g* for 10 min (pellet = nuclei). Supernatants were then centrifuged at 15,000 × *g* for 15 min to reduce lysosomal contamination. Finally, supernatants were centrifuged at 50,000 × *g* for 35 min and the pellet was resuspended in Laemmli sample buffer.

Western Blotting

Total cell lysates from an equal number of cells and prestained standards were resolved on a 10% SDS-polyacrylamide gel, transferred onto nitrocellulose paper and immunostained for Cx43 as described by Laird et al. (1995). Western blots of membranes from control and IQ-treated cells were loaded equally and compared to total cell lysates taken from control cells (*see* Fig. 10). All Western blots were performed in triplicate with the exception of the NRK membrane blot (Fig. 10*B*) which was performed twice with similar results. The blots were exposed to Amersham Hyperfilm-MP with an intensifying screen and/or quantified by exposing to a Molecular Dynamics phosphorimager. Cx43 species in Western blots exposed to the phosphorimager (Figs. 9 and 10) were quantified by determining volume counts and subtracting background counts from equal volumes of the blot where no Cx43 signal was observed. The signals obtained from the blots were quantifiable as the phosphorimager is linear over five orders of magnitude.

Results

Loss of Intercellular Communication and Gap Junction Plaques in IQ-treated Cells

IQ is known to cause Golgi membranes to breakdown in a number of culture cells, including BHK, CHO, HeLa, MDCK and NRK cells (Takizawa et al., 1993). The effect of IQ on gap junction plaques, Golgi membranes and intercellular communication was examined in rat BICR-M1R_k tumor cells. Cells were double immunofluorescent-labeled for Cx43 and MG-160 (resident of *medial* Golgi cisternae) or microinjected with Lucifer yellow. In control cells, a punctate Cx43 immunostaining pattern was observed by confocal microscopy between neighboring cells (Fig. 1*A*) and MG-160 was organized in a paranuclear location (Fig. 1*B*). Following treatment with IQ for 1 hr, gap junction plaques were not observed (Fig. 1*C*), the Golgi became vesicular and dispersed throughout the cytoplasm (Fig. 1*D*). When IQ-treated cells were allowed to recover in the absence of IQ for 1 hr, punctate Cx43 immunostaining was again observed at locations of cell-cell apposition (Fig. 1*E*) and MG-160 had a paranuclear distribution (Fig. 1*F*). Lucifer yellow spread extensively from the microinjected cell (Fig. 2*A* and *B;* asterisk) to neighbor cells in untreated cell cultures. However, a less fibroblastoid morphology was exhibited in IQ-treated BICR-M1R_k cells and Lucifer yellow remained within the boundaries of the microinjected cell (Fig. 2*C* and *D*, asterisk). Dye spread extensively from an IQ-treated cell that was allowed to recover for 1 hr in the absence of IQ (Fig. 2*E* and *F;* asterisk).

Analysis of gap junction plaques and intercellular communication in NRK cells after IQ treatment revealed similar effects to those seen in BICR-M1R_k cells. Punctate Cx43 immunostaining was seen at sites of cell-cell apposition (Fig. 3*A*) and dye spread extensively from the microinjected cell (asterisk) to neighboring cells (Fig. 3*B* and *C*). Cells that were treated with IQ for 2 hr did not have a distinct cell surface punctate staining pattern (Fig. 3*D*) and dye failed to spread from the microinjected cell (asterisk) (Fig. 3*E* and *F*). Distinct gap junction plaques (Fig. 3*G*) and dye transfer from the microinjected cell (asterisk) (Fig. 3*H* and *I*) were observed 2 hr after the removal of IQ. In all cases, the morphology of the drug-treated and microinjected cells was unchanged as indicated by the corresponding phase images (Fig. 3*C, F, I*). In both BICR-M1R_k and NRK cells that were treated with IQ, Lucifer yellow remained within the microinjected cell or transferred to first order cells.

Rapid Recovery of Gap Junction Plaques Following the Removal of IQ

To determine the time course of gap junction plaque reassembly upon the removal of IQ, IQ-treated BICR-M1R_k cells were allowed to recover in drug-free medium for up to 60 min. Control (Fig. 4*A*) and drug-treated (Fig. 4*B*) cells were immunolabeled for Cx43 using a sensitive polyclonal antibody to Cx43 and visualized by epifluorescent microscopy. Similar to results obtained using an anti-Cx43 monoclonal antibody (Fig. 1*C*), gap junction plaques were not observed after IQ treatment (Fig. 4*B*), however, a reticular intracellular network of light immunofluorescent labeling was seen throughout these cells. Gap junction plaques that were beginning to form were observed as early as 10 min (Fig. 4*C*, arrows) after the removal of IQ. Plaques were easily recognizable after 20 min (Fig. 4*D*) 30 min (Fig. 4*E*) and 60 min (Fig. 4*F*) of recovery.

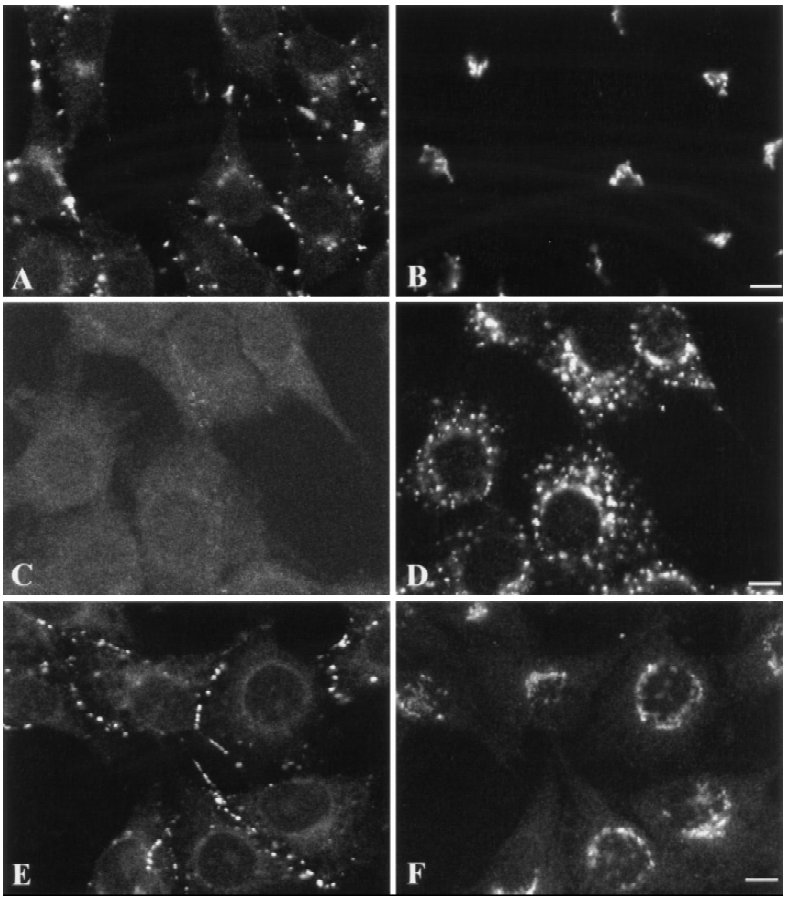

Fig. 1. IQ-induced reversible loss of gap junction plaques in BICR-M1R_k cells. Rat BICR-M1R_k cells were untreated, treated with IQ for 1 hr or recovered from IQ-treatment for 1 hr. Cells were permeabilized and double immunolabeled for Cx43 (mouse anti-Cx43 monoclonal antibody) and MG-160. Immunolabeled cells were imaged using a confocal microscope. In control cells, Cx43 was seen in abundance between neighboring cells (*A*) and MG-160 was organized in a paranuclear location (*B*). Following treatment with IQ, Cx43 punctate labeling was lost (*C*) and MG-160 was found in dispersed vesicles (*D*). When IQ-treated cells were allowed to recover in the absence of IQ, punctate Cx43 labeling (*E*) and a paranuclear MG-160 staining pattern were observed (*F*). Bar = 10 μm.

Recovery of Gap Junction Plaques Is Not Dependent on Cx43 Trafficking to the Cell Surface from BFA-sensitive Compartments

IQ inhibits protein transport at the *cis*-Golgi and, upon removal of the drug, protein transport is restored (Takizawa et al., 1993). To determine whether the rapid recovery of gap junction plaques upon the removal of IQ was dependent upon Cx43 trafficking to the cell surface from the endoplasmic reticulum or Golgi compartments, BICR-M1R_k cells were allowed to recovery from IQ in the presence or absence of BFA (Fig. 5). BFA has previously been shown to block protein transport within a fused ER/Golgi compartment (Misumi et al., 1986; Chege & Pfeffer, 1990; Lippincott-Schwartz et al., 1989, 1991; Klausner et al., 1992) and also to prevent Cx43 trafficking to the cell surface and its maturation to highly phosphorylated species (Laird et al., 1995). The punctate cell surface Cx43 immunostaining pattern seen in control cells (Fig. 5*A*), was not evident in IQ-treated cells (Fig. 5*B*). However, when IQ-treated cells were allowed to recover in the absence of BFA (Fig. 5*D*) or presence of BFA (Fig. 5*E*) numerous gap junction plaques were observed. In NRK cells, distinct punctate Cx43 immunostaining at sites of cell-cell apposition (Fig. 6*A*) was significantly reduced following IQ treatment (Fig. 6*B*). Upon the removal of IQ, gap junction plaques formed both in the absence (Fig. 6*D*) or presence of BFA (Fig. 6*E*). These observation would suggest that the Cx43 involved in the reformation of gap junction plaques is derived from a pool of Cx43 found distal to the *trans* Golgi, possibly residing within the *trans* Golgi network (TGN) or plasma membrane.

To provide evidence that BFA was an effective blocker of protein trafficking after IQ treatment, BICR-M1R_k cells were labeled with ^{35}S-trans label in the presence of IQ and chased in IQ-free medium supplemented with BFA (Fig. 7). A doublet of Cx43 at ~42/43 kD was immunoprecipitated from cells labeled in the presence of IQ (Fig. 7, lane a). This same Cx43 doublet was observed in cells that were labeled with ^{35}S-trans label in the presence of IQ and chased in the presence of BFA for 3 hr. The presence of BFA prevented the maturation of Cx43 to the well-characterized higher molecular weight phosphorylated species (Musil et al., 1990*b;* Musil & Goodenough, 1991; Laird et al., 1995) that are thought to occur after the protein exits that Golgi apparatus.

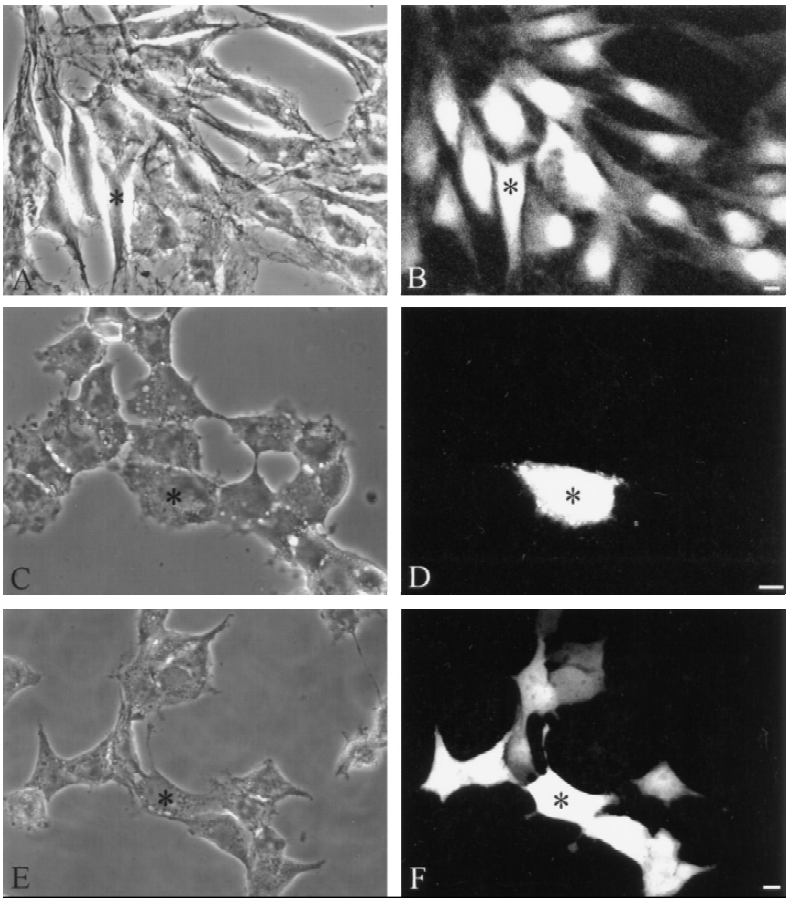

Fig. 2. IQ-induced reversible loss of dye transfer in BICR-M1R$_k$ cells. Untreated (*A* and *B*), IQ-treated (*C* and *D*) or IQ-treated and recovered (*E* and *F*) rat BICR-M1R$_k$ cells were microinjected with Lucifer yellow (asterisks). Transmitted light (*A, C, E*) or fluorescent (*B, D, F*) images were collected from a confocal microscope. Note that IQ reversibly inhibited dye transfer. Bar = 10 μm

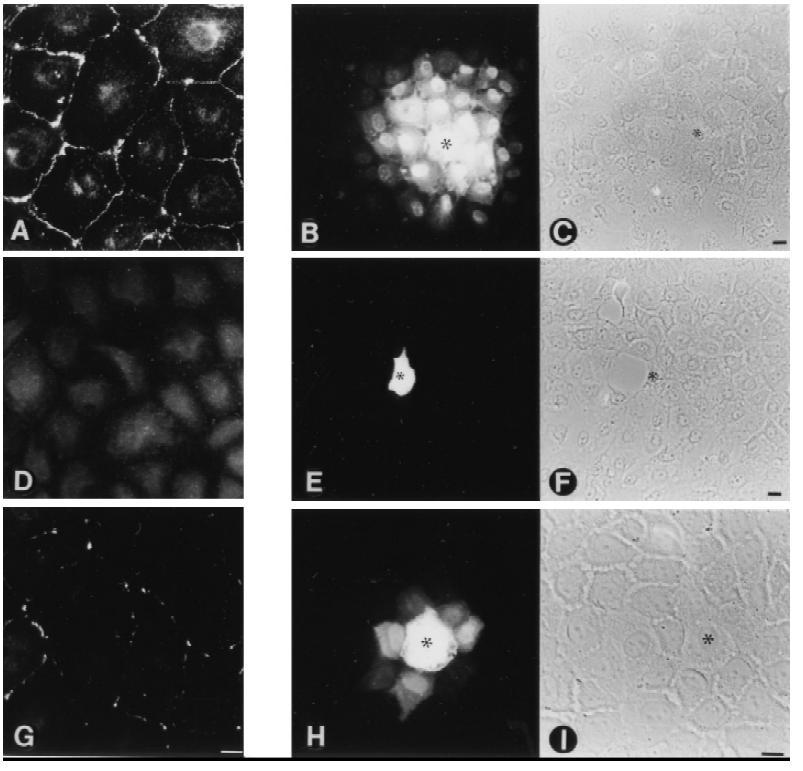

Fig. 3. IQ-induced reversible loss of intercellular communication and gap junction plaques in NRK cells. In untreated NRK cells, a punctate Cx43 immunostaining was seen between neighboring cells (*A*) and Lucifer yellow spread extensively from the microinjected cell (asterisk) to neighboring cells (*B* and *C*). Following treatment with IQ for 2 hr, gap junction plaques (*D*), and dye transfer from microinjected cell (asterisk) were not observed (*E* and *F*). When IQ-treated cells were allowed to recover in the absence of IQ for 2 hr, punctate immunofluorescent labeling was observed (*G*) and dye transferred from the microinjected cell (asterisk) (*H* and *I*). The morphology of the drug-treated and microinjected cells was unchanged as indicated by the corresponding phase images (*C, F, I*). Bar = 10 μm

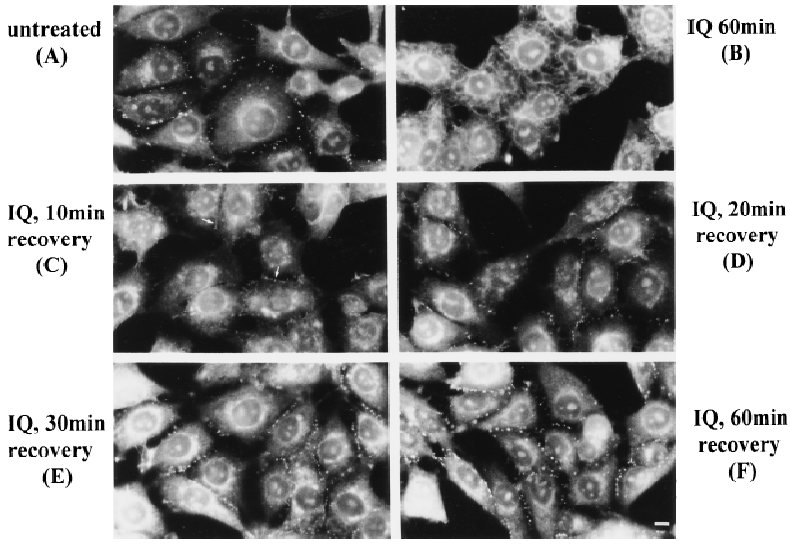

Fig. 4. Gap junction plaques reassemble rapidly upon removal of IQ. BICR-M1R_k cells were untreated (*A*) or treated with IQ for 1 hr (*B*), then rinsed and allowed to recover in drug-free medium for periods of 10 min (*C*), 20 min (*D*), 30 min (*E*), and 60 min (*F*). The cells were permeabilized and immunostained with an anti-Cx43 polyclonal antibody (CT-360) (Laird & Revel, 1990). The loss of Cx43 punctate immunostaining at the cell surface in IQ-treated cells was accompanied by a reticular network of light immunofluorescent labeling. Throughout the time course of recovery from IQ treatment, a gradual increase in gap junction plaques (arrows) was observed. Bar = 10 μm

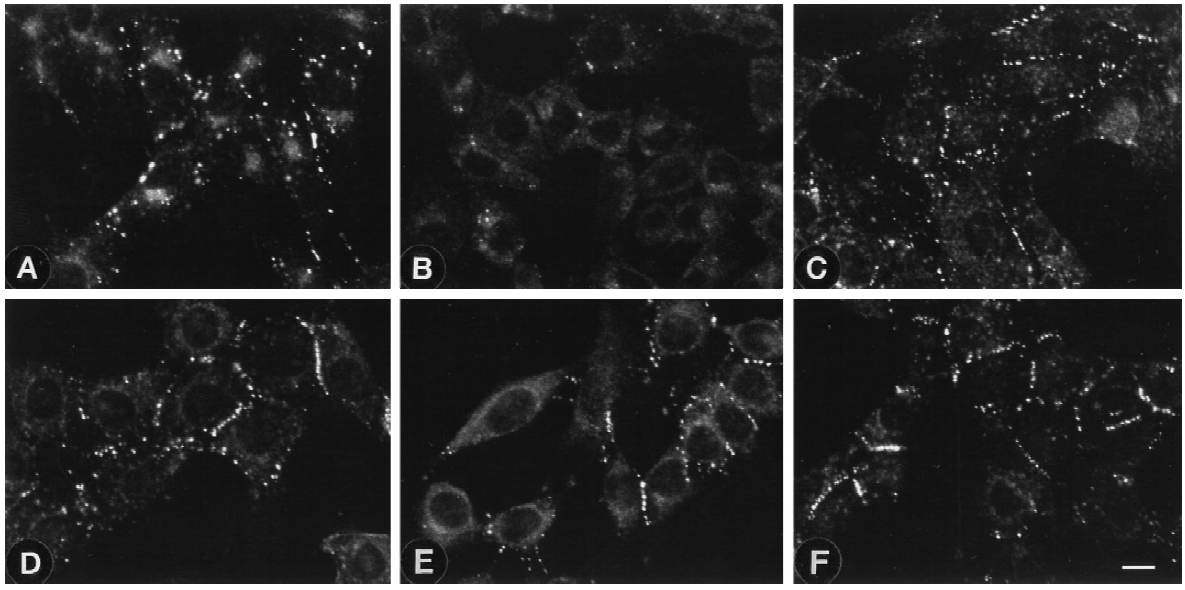

Fig. 5. IQ-induced loss of gap junction plaques and its effect on plaque formation in BICR-M1R_k cells are not due to the depolymerization of microtubules. Cells were untreated (*A*), treated with IQ for 1 hr (*B*) or nocodazole for 1.5 hr (*C*). IQ-treated cells were washed and allowed to recover in drug-free medium (*D*), in medium supplemented with BFA (*E*) or in medium supplemented with nocodazole (*F*) for 1 hr. Cells were permeabilized and immunolabeled for Cx43 (CT-360 antibody). Cx43 punctate immunolabeling was observed at sites of cell-cell apposition following nocodazole treatment (*C*). Moreover, cell surface punctate Cx43 immunolabeling was observed in cells that were recovering from IQ in the presence of BFA and nocodazole. All confocal images were collected using identical contrast and brightness settings. Bar = 10 μm

IQ-induced Loss of Gap Junction Plaques and Its Inhibitory Effect on Plaque Formation Are Not Due to the Depolymerization of Microtubules

IQ acts as a microtubule depolymerizing agent (Veit et al., 1993). To determine if the loss of preexisting gap junction plaques and the inhibition of gap junction plaque formation were likely the result of IQ's affect on microtubules, BICR-M1R_k cells were either treated with nocodazole (Fig. 5*C*) or allowed to recover from IQ treatment in the presence of nocodazole (Fig. 5*F*). Similar to control cells (Fig. 5*A*), a punctate Cx43 immunostaining pattern was observed at sites of cell-cell apposition in cells treated with nocodazole (Fig. 5*C*) and in cells that were recovering from IQ in the presence of nocodazole (Fig. 5*F*). As in BICR-M1R_k cells, punctate Cx43 immunostaining was localized at sites of cell-cell apposition in NRK cells pretreated with nocodazole (Fig. 6*C*), as well as in cells recovering from IQ in

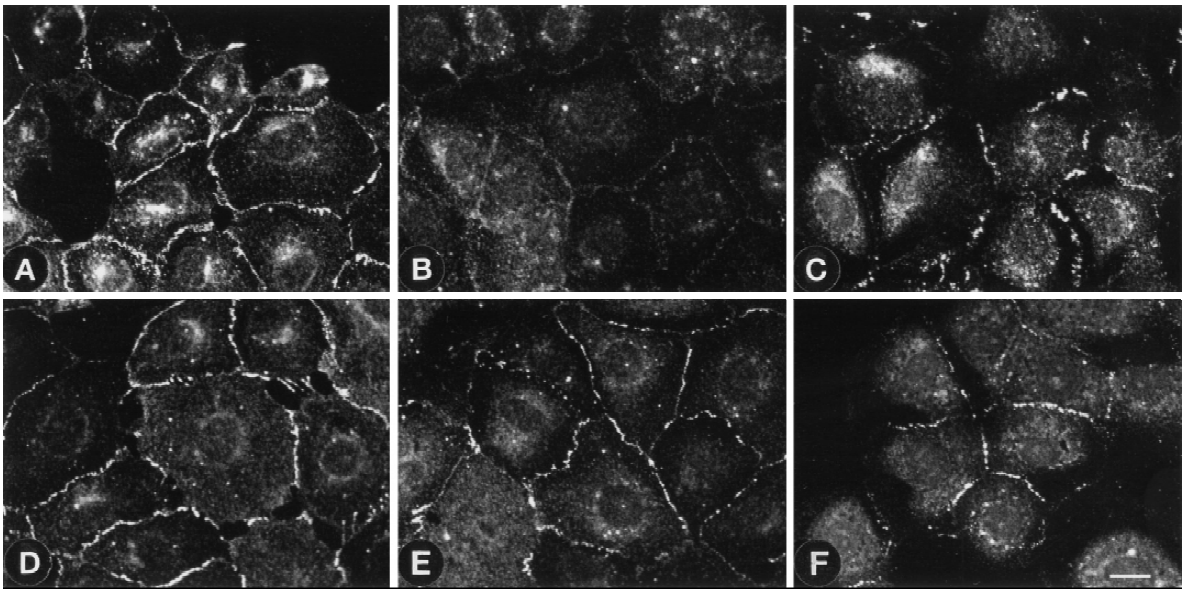

Fig. 6. Loss and recovery of gap junction plaques in IQ-treated NRK cells is not dependent on microtubule assembly or on protein trafficking to the cell surface. Cells were untreated (*A*), treated with IQ for 2 hr (*B*) or nocodazole for 1.5 hr (*C*). IQ-treated cells were washed and allowed to recover in drug-free medium (*D*), in medium supplemented with BFA (*E*) or in medium supplemented with nocodazole (*F*) for 2 hr. Cells were permeabilized and immunolabeled for Cx43 (CT-360 antibody). Cell surface Cx43 punctate immunolabeling was observed following nocodazole treatment (*C*). In addition, punctate immunostaining for Cx43 was seen in cells that were recovering from IQ in the presence of BFA and nocodazole. All confocal images were collected using identical contrast and brightness settings. Bar = 10 μm

the presence of nocodazole (Fig. 6*F*). Similar results as described above were observed when the microtubule depolymerization agent colcemid was used (*results not shown*). These results suggest that IQ disrupts gap junction plaques independently of its effects on microtubules.

Effect of IQ on the Tight Junction Associated Protein, ZO-1

To examine whether IQ causes a redistribution of other junction proteins, we examined IQ's effect on the distribution of zonula occludens-1, ZO-1. ZO-1 is a tight junction associated protein which is localized on the cytoplasmic surface just beneath the membrane of tight junctions (Stevenson et al., 1986; Anderson et al., 1988; Furuse et al., 1993). Untreated NRK cells (Fig. 8*A* and *B*), cells treated with IQ (Fig. 8*C* and *D*) or cells recovered from IQ (Fig. 8*E* and *F*) were double-labeled for Cx43 and ZO-1. The punctate Cx43 labeling at sites of cell-cell contact was lost in IQ-treated cells but was restored upon recovery from IQ. However, the beltlike staining pattern of ZO-1 around the periphery of the cell was not altered by IQ treatment. These results indicate that IQ does not affect the spatial distribution of ZO-1 in NRK cells by inducing its dissociation from the plasma membrane.

IQ-induced Reduction in the 46 kD Species of Cx43

Gap junction plaque formation and intercellular communication competence have been associated with Cx43

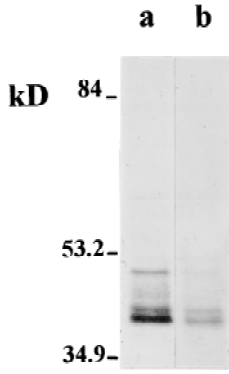

Fig. 7. Inhibition of extensive phosphorylation of Cx43 in the presence of IQ and brefeldin A. BICR-M1R$_k$ cells were metabolically labeled with ^{35}S-trans in the presence of IQ (lane *a*) and subsequently chased for 3 hr in the presence of brefeldin A (lane *b*). Cx43 was immunoprecipitated from cell lysates, resolved by SDS-PAGE and detected by fluorography. A doublet of Cx43 was identified at ~42/43 kD in both IQ-treated cells and cells that were allowed to recover from IQ in the presence of brefeldin A

phosphorylation to the more mature forms (Musil & Goodenough, 1991). We therefore wanted to examine whether the IQ-induced loss of intercellular communication and gap junction plaques resulted in a loss of Cx43 phosphorylation in BICR-M1R$_k$ and NRK cells. Untreated, IQ-treated or recovering BICR-M1R$_k$ and NRK cells were lysed and subjected to SDS-polyacrylamide gel electrophoresis and probed for Cx43. Control lanes

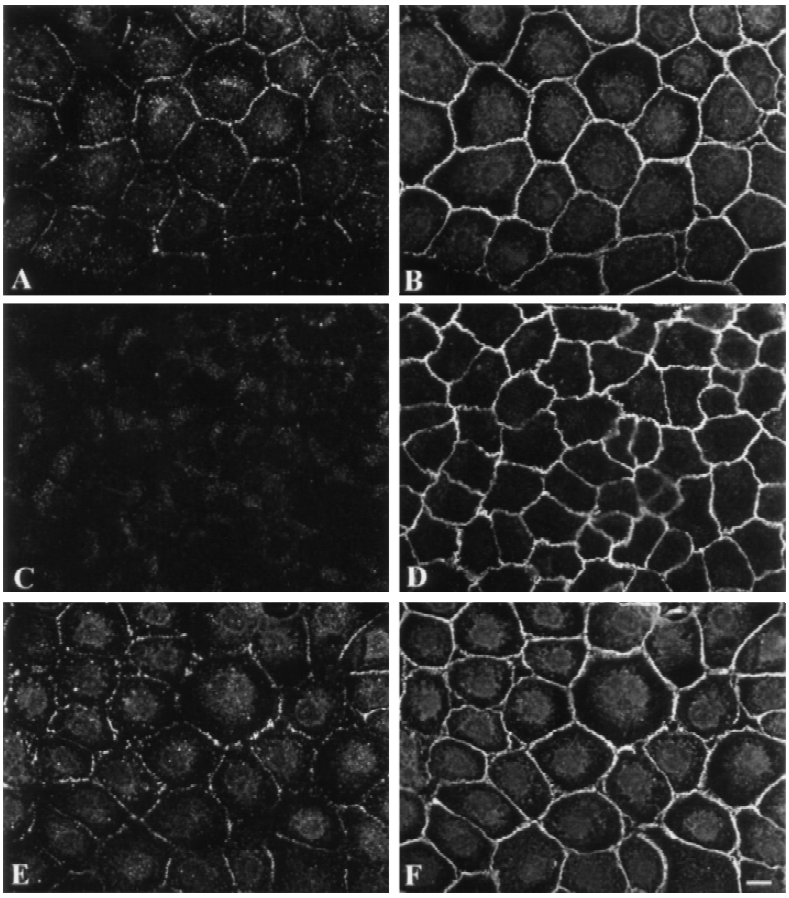

Fig. 8. IQ does not affect the spatial distribution of ZO-1, a tight junction associated protein. NRK cells were untreated (*A* and *B*) IQ-treated for 2 hr (*C* and *D*) or recovered from IQ (*E* and *F*) for 2 hr, permeabilized and double immunofluorescently labeled for Cx43 and ZO-1. Immunolabeled cells were imaged using a confocal microscope. The punctate Cx43 labeling at sites of cell-cell apposition (*A*) was lost upon IQ treatment (*C*) and restored when cells were allowed to recover from IQ (*E*). The beltlike pattern of ZO-1 immunostaining at the cell periphery (*B, D, F*) was not altered by IQ treatment. Bar = 10 μm

(Fig. 9*A* and *B*, lane *a*) revealed a series of bands between 42 and 46 kD characteristic of unphosphorylated and phosphorylated Cx43 (Musil et al., 1990; Laird et al., 1995). In BICR-M1R_k cells, phosphorimager analysis revealed that the Cx43 doublet at 46 kD was reduced by 26% after IQ treatment while the remaining phosphorylated forms of the protein were relatively unchanged (Fig. 9*A*, lane *b*). However, when NRK cells were treated with IQ there was a 38% increase in Cx43 at 42/43 kD and a 27% increase in the band at 44 kD with an ~40% decrease in the Cx43 doublet at 46 kD (Fig. 9*B*, lane *b*). The increase in Cx43 at 42/43 kD was anticipated since IQ also acts as an inhibitor of protein transport at the *cis*-Golgi, allowing for protein accumulation in this early compartment. In recovering cells (Fig. 9*A* and *B*, lane *c*), there was an increase in all forms of Cx43 including the doublet at 46 kD.

Transport of Cx43 to the cell surface has been demonstrated to precede Cx43 phosphorylation to the mature P_1 and P_2 forms of the protein (Musil & Goodenough, 1991). To further examine the effect of IQ on Cx43 species, membrane fractions were prepared from control and IQ-treated BICR-M1R_k and NRK cells. Western blot analysis revealed that, as in the total cell lysates (Fig. 10*A* and *B*, lane *a*), all forms of the Cx43 protein were represented in the control membrane fractions (Fig. 10*A* and *B*, lane *b*). In IQ-treated NRK cells (Fig. 10*A* and *B*, lane *c*) a nearly identical pattern of Cx43 was seen with only a 20% reduction in the Cx43 doublet at 46 kD.

IQ Inhibits the Assembly of Cx43 into Gap Junction Plaques without Affecting its Maturation

To examine the effect of IQ on newly forming gap junction plaques we used an inhibitor of protein trafficking, BFA (Misumi et al., 1986) to uncouple events leading to gap junction assembly from those events related to gap junction removal (Laird et al., 1995). BICR-M1R_k cells were treated with BFA for 6 hr, recovered in the presence or absence of IQ for 1 hr and Cx43 was detected by immunofluorescence (Fig. 11) or by Western blotting (Fig. 12). Gap junction plaques were cleared from the cell surface after BFA treatment for 6 hr (Fig. 11*A*). Plaques were restored following a 1 hr recovery from BFA in the absence of IQ (Fig. 11*B;* arrows). However, cells recovering from BFA in the presence of IQ for 1 hr (Fig. 11*C*) did not restore plaques. Cells recovered from BFA in the presence of IQ for 1 hr fol-

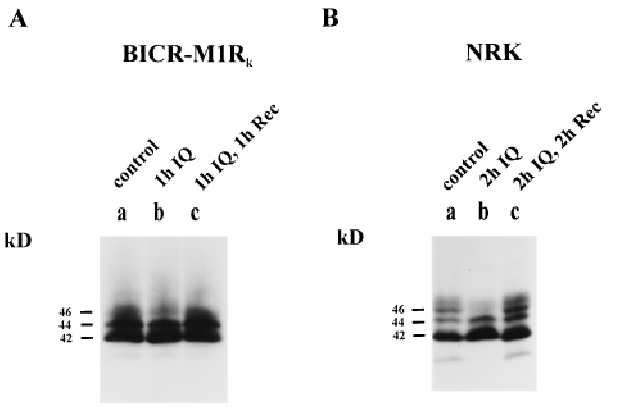

Fig. 9. Western blot analysis of total Cx43 in IQ treated BICR-M1R$_k$ and NRK cells. Untreated (*A* and *B,* lane *a*), IQ-treated (1 hr—BICR-M1R$_k$; 2 hr—NRK cells) (*A* and *B,* lane *b*), or cells recovered from IQ (1 hr—BICR-M1R$_k$; 2 hr—NRK) (*A* and *B,* lance *c*) were lysed and subjected to SDS-PAGE and Western blotting for Cx43. A series of bands between 42 and 46 kD was seen in untreated cells (lane *a*). In both BICR-M1R$_k$ and NRK cells there was a reduction in the Cx43 doublet at 46 kD after IQ treatment. In recovering cells there was an increase in all forms of Cx43 including the doublet at 46 kD.

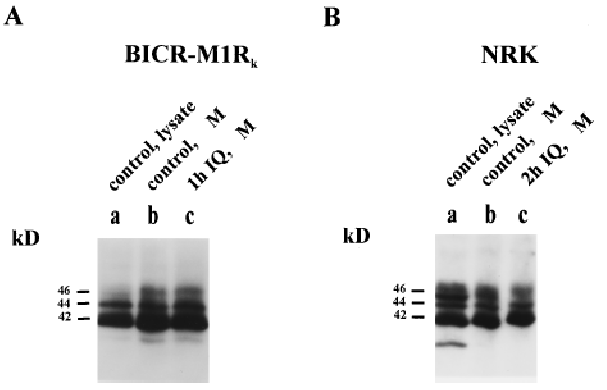

Fig. 10. Cx43 was found in membrane fractions following IQ treatment. Western blots of membrane (M) fractions from untreated and IQ-treated BICR-M1R$_k$ and NRK cells were immunolabeled for Cx43. As compared to cell lysates (*A* and *B,* lane a) and membrane fractions (*A* and *B,* lane *b*) from untreated cells, all forms of Cx43 were represented in the membrane fractions of IQ-treated cells (*A* and *B,* lane *c*) with only a slight reduction in the Cx43 doublet at 46 kD in NRK cells.

lowed by an additional 1 hr in the absence of IQ assembled gap junction plaques (Fig. 11*D;* arrows). Western blot analysis of BFA-treated cells revealed the 42/43 kD doublet of Cx43 while the more mature phosphorylated species of Cx43 were lost (Fig. 12, lane *a*) as previously described (Laird et al., 1995). The maturation of Cx43 to the 44 and 46 kD phosphorylated species of Cx43 was observed following a 1 hr recovery from BFA in the absence (Fig. 12, lane *b*) or presence of IQ (Fig. 12, lane *c*). All species of Cx43 were clearly seen in cells that were allowed to recover in IQ for 1 hr followed by an additional hour in the absence of any inhibitors of protein trafficking (Fig. 12, lane *d*).

Discussion

Regulated intercellular communication has been proposed to be essential in embryonic development (Larsen & Wert, 1988; Réaume et al., 1995), differentiation (Ginzberg & Gilula, 1979), proliferation (Mehta et al., 1986; Dermietzel et al., 1987; Mehta et al., 1991; Naus et al., 1992) and cell cycle control (Yee & Revel, 1978; Su et al., 1990; Xie, Huang & Hu, 1991; Lee et al., 1992). At some stage during these processes there is a temporal uncoupling of cells from their neighbors followed by reestablishment of intercellular communication with the same or new neighbors. One of the principle mechanisms of downregulating intercellular coupling is the removal of preexisting channels and gap junction plaques from the cell surface. In the present study, we used a novel microtubule depolymerizing and protein trafficking inhibitor, ilimaquinone (IQ), to examine the mechanisms and factors involved in gap junction plaque formation and retention in rat mammary tumor BICR-M1R$_k$ and NRK cells.

Previous studies have examined the effect of IQ on the structural organization of Golgi membranes, protein transport and cytoplasmic microtubules (Takizawa et al., 1993; Veit et al., 1993; Acharya et al., 1995*a,b*). IQ causes a complete breakdown of Golgi membranes into small vesicles and in the process inhibits protein trafficking (Takizawa et al., 1993). A similar vesicularization of the Golgi membranes and inhibition of protein transport occurs in cells undergoing mitosis (Featherstone, Griffiths & Warren, 1985; Lucocq & Warren, 1987). In addition, IQ causes the depolymerization of cytoplasmic microtubules resulting in the dispersal of small Golgi vesicles throughout the cytoplasm (Veit et al., 1993). These processes are reversed and recovery follows quickly after the removal of IQ.

In our studies, IQ induced the loss of gap junction plaques and inhibited intercellular communication within 2 hr in both BICR-M1R$_k$ and NRK cell cultures. In a previous study on BICR-M1R$_k$ cells, the loss of gap junction plaques and inhibition of intercellular communication occurred after a 6 hr BFA treatment (Laird et al., 1995). Considering that the half-life of Cx43 in these cells is ~1.5 hr, we proposed that removal of Cx43 and gap junction plaques resulted from mechanisms endogenous to BICR-M1R$_k$ cells. However, in the present study, since gap junction plaques are lost in <2 hr we suggested that IQ is inducing their dispersal and/or removal from the cell surface. Alternatively, it is possible that IQ may only be blocking gap junction formation while preexisting gap junction plaques are internalized under normal cellular control. However, for gap junc-

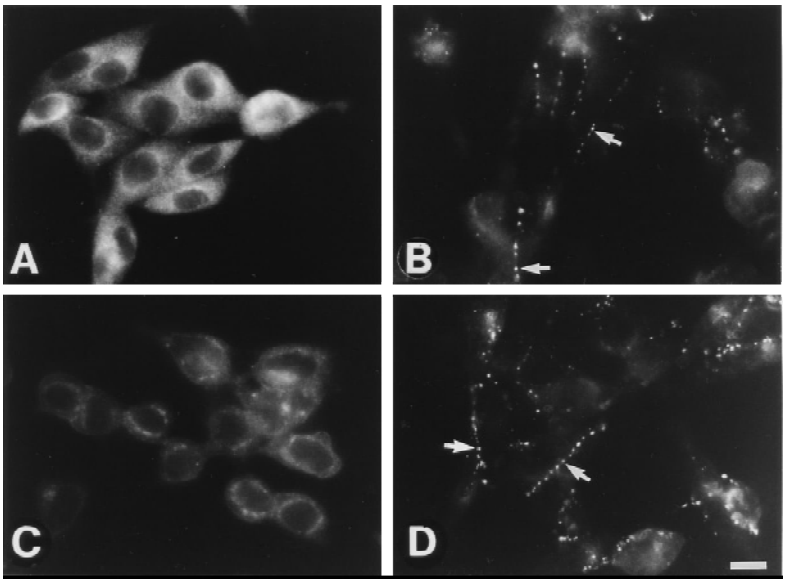

Fig. 11. Immunolocalization of Cx43 in IQ treated BICR-M1R_k cells. Cells treated with BFA for 6 hr (*A*), were allowed to recover in the absence (*B*) or presence (*C*) of IQ for 1 hr or BFA-treated cells were recovered for 1 hr in IQ followed by an additional 1 hr in the absence of IQ (*D*). All cells were permeabilized and immunostained for Cx43. No Cx43 punctate immunostaining was observed after BFA treatment. Cx43 punctate immunostaining was restored following a 1 hr recovery from BFA in the absence of IQ but was not restored in the presence of IQ until the cells were allowed to recover for an additional 1 hr. Arrows denote areas of gap junction plaques. Bar = 10 μm

tions plaques to be lost from the cell surface in <2 hr it would require that the kinetics of gap junction removal be substantially faster than the half-life of Cx43 in BICR-MIR$_k$ cells (1.5 hr; Laird et al., 1995) and in NRK cells (2-2.5 hr; Musil et al., 1990*b*).

IQ did not appear to alter the integrity of the NRK cell sufficiently to cause ZO-1 to redistribute from the plasma membrane. The tight junction associated protein, ZO-1, is linked to actin based filaments (Madara, 1987) which are not affected by IQ as its effect on the cytoskeletal network is limited to microtubules (Veit et al., 1993). Wang and Rose (1995) demonstrated in MHD1-43A cells that the cAMP-induced clustering of channels composed of Cx43 into plaques is abolished by the disruption of microfilaments suggesting that actin based filaments play a direct role in gap junction formation. To date there is no evidence that gap junction plaques interact with microtubules (Kerjaschki, 1978; Kidder, Rains & McKeon, 1987; Wang & Rose, 1995). Moreover, when microtubules were depolymerized with nocodazole or colcemid, no changes in gap junction plaque distribution at the cell surface of BICR-M1R_k and NRK cells occurred. Consequently, our results suggest that the effect of IQ on gap junction plaques is independent of its effects on microtubules.

The reassembly of Cx43 into communication-competent gap junctions and gap junction plaques following the removal of IQ may be derived from three possible Cx43 pools; (i) accumulated Cx43 in a compartment within the endoplasmic reticulum or Golgi apparatus as IQ blocks protein transport between successive Golgi compartments (Takizawa et al., 1993); (ii) molecules of Cx43 that were assembled into gap junction plaques prior to IQ treatment; or (iii) Cx43 within the plasma membrane that was not previously assembled into gap junction plaques.

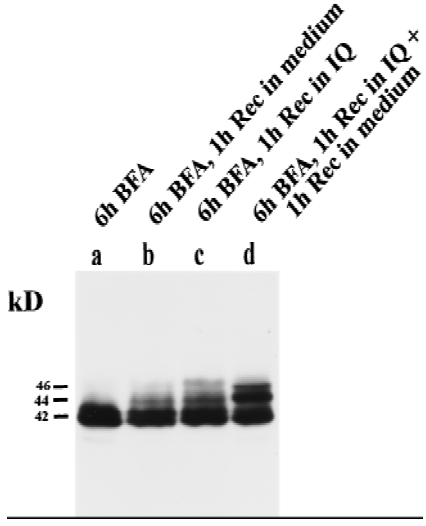

Fig. 12. Maturation of Cx43 was not affected in IQ treated BICR-M1R_k cells. Cells treated with BFA for 6 hr (lane *a*), were allowed to recover in the absence (lane *b*) or presence (lane *c*) of IQ for 1 hr or BFA-treated cells were recovered for 1 hr in IQ followed by an additional 1 hr in the absence of IQ (lane *d*). Western blots were probed with anti-Cx43 antibody (CT-360). In the presence of BFA a 42-43 kD doublet was seen while the remaining phosphorylated forms of Cx43 were lost. Upon recovery from BFA in the absence or presence of IQ, the 44 kD and 46 kD bands reappeared. The additional 1 hr recovery period from IQ showed a similar Cx43 banding profile.

To examine whether gap junctions were being reassembled by using endoplasmic reticulum or Golgi stores of Cx43, we allowed IQ-treated cells to recover in the presence of BFA which is known to block protein trafficking to the plasma membrane in Golgi compartments excluding the TGN (Chege & Pfeffer, 1990). The reassembly of gap junction plaques under these conditions suggests that Cx43 was being recruited for plaque as-

sembly from either the TGN or the plasma membrane but not from the endoplasmic reticulum, intermediate compartment, *cis* Golgi network, *cis, medial* or *trans* Golgi. Evidence that BFA was indeed capable of inhibiting Cx43 trafficking to the cell surface even after IQ treatment was provided by a pulse-chase experiment where BFA inhibited extensive phosphorylation of Cx43 that is thought to emanate at the cell surface.

To determine if a pool of Cx43 remained available within IQ-treated cells to reassemble gap junction plaques, we examined the levels and phosphorylated state of Cx43 in both total cell lysates and membrane fractions. The life cycle of Cx43 in BICR-M1R_k cells involves a series of phosphorylation events during which Cx43 proceeds from an unphosphorylated form to an early ER/Golgi phosphorylated species to the more extensively phosphorylated species at 44 and 46 kD (Laird et al., 1995) designated as P_1 and P_2 in NRK cells (Musil et al., 1990*b*). Based on cell surface biotinylation techniques, Musil and Goodenough (1991) determined that Cx43 is inserted into the plasma membrane of communication-competent NRK cells prior to conversion into either the P_1 or P_2 forms and that the P_2 form is associated with plaques. Our results show that upon IQ treatment of both NRK and BICR-M1R_k cells for the time periods that reflect inhibition of intercellular communication and loss of gap junction plaques, all phosphorylated forms of Cx43 continue to be represented with a moderate reduction in the most highly phosphorylated form in BICR-M1R_k and NRK cells. Consequently, our results reveal that extensive phosphorylation of Cx43 does not necessarily reflect the presence of gap junction plaques or cause gap junction plaque formation. Since phosphorylation of Cx43 is complex and involves many serine residues, it can not be assumed that the Cx43 forms seen in Western blots of IQ-treated cells are identical to the Cx43 species found in unperturbed cells even though they may appear to have the same apparent molecular weight.

Finally, we used BFA to downregulate preexisting gap junctions in BICR-M1R_k cells in an assay to determine if IQ would impair the assembly of new gap junctions upon the removal of BFA. Our studies showed that IQ inhibited gap junction plaque formation but did not obstruct the maturation of Cx43 to the 44 and 46 kD species further suggesting that extensive phosphorylation of Cx43 is not causal of gap junction plaque formation or retention. This finding is consistent with that described by Wang et al. (1995) where tunicamycin treatment of MHD1-43A cells resulted in extensive phosphorylation of Cx43 in the absence of channel clustering.

In summary, we suggest that BICR-M1R_k and NRK cells have two pools of Cx43 at the cell surface; (i) a fully assembled gap junction plaque pool, and (ii) a nonplaque pool of connexons (hemichannels). We propose that these two pools are differentially regulated independent of microtubules. IQ inhibits the assembly and promotes the loss of gap junction plaques while a nonplaque pool of Cx43 is available to reassemble gap junction plaques upon the removal of IQ. Such a mechanism of regulation may facilitate transient gap junction coupling in cellular processes such as mitosis and development.

The authors would like to thank Dr. V. Malhotra for his generous gift of IQ. We would also like to thank Dr. N. Gonatas for providing us with anti-MG-160 antibody. The hybridoma R26.4C (anti-ZO-1) developed by D. Goodenough was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, and the Department of Biology, University of Iowa, Iowa City, IA 52242, under contract N01-HD-2-3144 from NICHD. This work was supported by the Medical Research Council of Canada to D.W.L. (Grant MT-12241) and a FCAR fellowship to P.A.F.

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