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Characterization of the Gap Junction Protein, Connexin45

J.G. Laing[1], E.M. Westphale[1], G.L. Engelmann[3], E.C. Beyer[1,2]

[1]Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri 63110

[2]Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

[3]Department of Medicine, Loyola University, Strich School of Medicine, Maywood, Illinois 60153

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Abstract. Connexin45 is a gap junction protein which forms channels with unique characteristics. RNA blots demonstrated that connexin45 is expressed in a number of cell lines including WB, SK Hep1, BHK, A7r5, CLEM, and BWEM cells. Connexin45 was further studied in BWEM cells using specific affinity-purified antibodies directed against a synthetic peptide representing amino acids 285–298 of its sequence. Immunofluorescence experiments demonstrated that the BWEM cells expressed both connexin43 and connexin45 and that these connexins colocalized. Connexin45 polypeptide, immunoprecipitated from BWEM cells metabolically labeled with [^{35}S]-methionine, consisted of a predominant 48 kD polypeptide. Connexin45 and connexin43 contained radioactive phosphate when immunoprecipitated from BWEM cells metabolically labeled with [^{32}P]-orthophosphoric acid. This phosphate label was removed from connexin45 by alkaline phosphatase digestion. Treatment of BWEM cells with the tumor promoting agent 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibited intercellular passage of microinjected Lucifer yellow. While TPA treatment induced phosphorylation of connexin43 in these cells, it reduced the expression of connexin45. Furthermore, the connexin45 expressed after TPA treatment was not phosphorylated. These results suggest that treatments which alter protein phosphorylation may regulate connexin43 and connexin45 in BWEM cells by different mechanisms.

Key Words: Connexin45 — Gap junction — Intercellular communication — Phosphorylation

Introduction

Gap junctions, which contain the channels allowing intercellular passage of ions and small molecules, are formed by members of a family of related proteins called connexins (Cx).[1] While over a dozen different connexins have been cloned (*reviewed in* Kumar & Gilula, 1992; Beyer, 1993), many of these sequences have been characterized only by analysis of RNA expression or by functional expression in exogenous systems. Only a few connexin proteins [e.g., Cx32, Cx43, and Cx26 have been analyzed biochemically after isolation from tissues or immunoprecipitation from cultured cells (*reviewed in* Musil & Goodenough, 1990; Revel et al., 1992)].

We recently identified an interesting member of this gene family, Cx45. Cx45 was originally found in chick embryo heart, where expression of its RNA is developmentally regulated (Beyer, 1990). Subsequent studies demonstrated that Cx45 is rather widely expressed in many tissues of the chick embryo, embryonic and adult mouse, and in adult canine ventricular myocytes (Beyer, 1990; Hennemann, Schwarz & Willecke; 1992; Kanter, Saffitz & Beyer, 1992). Expression of Cx45 by transfection of a communication-deficient cell line showed that it formed channels with unique characteristics: a small unitary conductance (~30 pS) and a pronounced voltage dependence (Veenstra et al., 1992).

In the present study, we showed that Cx45 is expressed in several mammalian cell lines of diverse origin. In some cases it is coexpressed with another com-

Correspondence to: Eric C. Beyer, Division of Pediatric Hematology/Oncology, Box 8116, Washington University School of Medicine, One Children's Place, St. Louis, Missouri 63110

[1] The abbreviations used are: Cx, connexin; pS, picoSiemens; nS, nanoSiemens; kb, kilobase pairs; TPA, 12-O-tetradecanoylphorbol-13-acetate.

mon connexin, Cx43. We prepared monospecific polyclonal anti-Cx45 antibodies, which we have used to localize Cx45 in a cell line derived by the transformation of fetal rat cardiac myocytes (BWEM). We have immunoprecipitated the Cx45 polypeptide and have shown that it is phosphorylated. Phosphorylation, as previously found in Cx43 (Musil & Goodenough, 1990), may be a modification common to many gap junction proteins. We have also demonstrated that the level of expression of connexin45 is altered by the addition of the tumor-promoting agent TPA.

Materials and Methods

Cell Lines and Reagents

Rat aorta smooth muscle A7r5 cells, Baby Hamster Kidney cells (BHK), SK Hep1 human hepatoma cells, and mouse neuroblastoma Neuro2A (N2A) cells were obtained from the American Type Culture Collection (Rockville, MD). Normal rat kidney fibroblasts (NRK) were a gift from Dr. Stuart Kornfeld (Washington University, St. Louis, MO). WB cells (a rat liver epithelial cell line) were the gift of Dr. James Trosko (Michigan State University, East Lansing, MI). UMR 106-01 rat osteoblasts were a gift of Dr. Roberto Civitelli (Washington University, St. Louis, MO). The preparation of BWEM and CLEM cells by the retroviral transformation of fetal rat cardiac myocytes has been described previously (Engelmann et al., 1993).

All radioactively labeled compounds were obtained from Amersham (Arlington Heights, IL). Buffers and other chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

RNA Blots

Total cellular RNA was prepared from cells or tissues according to Chomczynski and Sacchi (1987), separated on formaldehyde/agarose gels, and transferred to nylon membranes, as previously described (Beyer, Paul & Goodenough, 1987). Relative equivalence of loading of samples and RNA integrity was verified by ethidium bromide staining of gels or hybridization of blots with a probe for human fibroblast γ actin. Hybridization was performed using specific [^{32}P]-labeled DNA probes prepared using random hexanucleotide primers and the Klenow fragment of DNA polymerase I (Beyer, 1990). Specific probes for rat Cx43 and canine Cx45 were prepared as previously described (Beyer et al., 1987; Kanter et al., 1992).

Anti-Connexin Antibodies

A rabbit antiserum directed against a synthetic peptide representing amino acids 252–271 in Cx43 was produced previously and has been extensively characterized (Beyer et al., 1989). A mouse monoclonal antibody (IgG1) against Cx43 (amino acids 252–270) was purchased from Zymed (South San Francisco, CA). These antibodies have a similar reactivity in immunofluorescence microscopy with a variety of cell lines and tissues. As described previously, a rabbit polyclonal antiserum against Cx45 was raised to a synthetic peptide representing amino acids 285–298 of dog Cx45 conjugated to keyhole limpet hemocyanin via a cysteine residue added to the amino terminal end (Kanter et al., 1992). The anti-Cx45 antibodies were affinity purified by chromatography (Sulfolink coupling gel, Pierce Chemical, Rockford, IL) on agarose derivitized with this peptide, according to the manufacturer's instructions, and eluted with 0.1 M glycine pH 3. The anti-Cx43 and anti-Cx45 reagents have been shown to react with gap junction structures by electron microscopic immunocytochemistry (Beyer et al., 1989; Kanter et al., 1992). We have previously demonstrated the specificity of the anti-Cx43 and anti-Cx45 antibodies in recognizing the in vitro translated Cx43 and Cx45 polypeptides in an immunoprecipitation assay as well as their specificity in immunofluorescence microscopy (Kanter et al., 1993).

Immunofluorescence

Cells adherent to plastic microscope slides (Nunc, Naperville, IL) were fixed in 50% methanol/50% acetone for 2 min at room temperature and permeabilized in 1% Triton X-100/PBS. The permeabilized cells were then incubated in primary antibody (mouse monoclonal anti-Cx43 or affinity-purified rabbit polyclonal anti-Cx45, 1:500 dilution) and secondary antibody (rhodamine conjugated-goat anti-rabbit IgG, 1:1000 dilution and fluorescein conjugated-goat anti-mouse IgG, 1:1000 dilution) at room temperature with intervening washes. In double-labeling experiments, cells were incubated simultaneously with both anti-Cx45 and anti-Cx43 reagents (1:500 dilution of affinity-purified rabbit polyclonal anti-Cx45 and 1:500 dilution of monoclonal anti-Cx43), and then with secondary antibodies (rhodamine conjugated-goat anti-rabbit IgG, 1:1000 dilution and fluorescein conjugated-goat anti-mouse IgG, 1:1000 dilution). The cells were viewed on a Nikon epifluorescence microscope with the appropriate barrier filters. Secondary antibody reagents were obtained from Boehringer Mannheim (Indianapolis, IN).

Cell Cultures and Radioactive Labeling

N2A, WB, and SK Hep1 cells were grown in Minimal Essential Medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum (JRH Biosciences, Lenexa, KS), 1× nonessential amino acids (GIBCO), 2 mM L-glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO). BWEM and CLEM cells were grown in 50% Dulbecco's Modified Eagle Medium/50% F-12 medium buffered with HEPES (GIBCO) supplemented with 4% fetal calf serum (JRH Biosciences) and 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO). All other cells were grown in Dulbecco's Modified Eagle Medium (GIBCO) supplemented with 10% fetal calf serum and penicillin-streptomycin.

Cells were labeled for 2 hr in methionine-depleted 50% Dulbecco's Modified Eagle Medium/50% F-12 medium buffered with HEPES containing [^{35}S]-methionine (100 μCi/ml) at 37°C, or labeled for 2 hr in phosphate-depleted 50% Dulbecco's Modified Eagle Medium/50% F-12 medium buffered with HEPES in the presence of 200 μCi/ml [^{32}P]-orthophosphate. In the TPA-treatment experiments, the labeling was extended by 30 min in the presence of added TPA (initially dissolved in dimethyl sulfoxide, then added to a final concentration of 50 nM). The sham-treated cells were incubated for the same period of time after addition of a comparable amount of solvent without drug.

Immunoprecipitation

Labeled BWEM cells were harvested by scraping and then lysed by sonication (4 × 15 sec). The cell debris was concentrated by centrifugation (10 min, 14,000 × g), then boiled in RIPA buffer (PBS

containing 1% Triton X-100, 0.6% SDS, 100 U/ml aprotinin, 0.1% PMSF, and 1 mM sodium orthovanadate) for 5 min. Cellular debris was collected by centrifugation (14,000 × *g*, 15 min), and the supernatant was added to tubes containing 10 μl or normal rabbit serum and 20 μl rProtein A-IPA 300 (Repligen, Cambridge, MA) and centrifuged to preclear. This supernatant was added to tubes containing 10 μl anti-Cx43 antisera or 1 μg anti-Cx45 antibody and 20 μl rProtein A-IPA 300. These tubes were incubated with shaking at 4°C for 2 hr. The pellets were collected with a brief centrifugation, washed four times in RIPA buffer, and then analyzed by SDS-PAGE on a 12.5% gel and subjected to fluorography. Control immunoprecipitations were run in the presence of 100 μg/ml of the peptide corresponding to amino acids 285–298 of canine Cx45 or in preimmune antiserum.

Dephosphorylation of Cx45

Metabolically labeled Cx45 was immunoprecipitated and incubated in the presence of alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) as detailed in Musil, Beyer and Goodenough (1990*a*). Control reactions were incubated in the identical buffer without added enzyme.

Dye Transfer Studies

Functional assessment of intercellular coupling was performed by microinjection of a 1% solution of Lucifer yellow CH (Molecular Probes, Eugene, OR) using a Nikon picoinjector and visualized with a Nikon Diaphot inverted microscope equipped with epifluorescence and Hoffman modulation contrast optics. In the TPA-treatment experiments, the cells were incubated for 2 hr in the presence of 200 nM TPA prior to dye injection. Sham-treated cells were incubated with an equivalent amount of DMSO.

Immunoblots

BWEM cells were washed with PBS and harvested by scraping. The cells were concentrated by centrifugation (1,330 × *g*, 5 min) and ruptured by sonication (4 × 15 sec). The cellular residue was concentrated by centrifugation (14,000 × *g*, 10 min), and then resuspended in PBS. The protein concentration of these extracts was determined (Bradford, 1976), and 20 μg aliquots were separated by SDS-PAGE on 12.5% polyacrylamide gels (Laemmli, 1970) and blotted onto Immobilon P (Millipore, Bedford, MA) (Towbin, Staehelin & Gordon, 1979). Membranes were blocked in BLOTTO (5% nonfat dry milk in PBS) for 16 hr. The membrane was incubated for 2 hr in anti-Cx45 antibodies (5 μg/ml). The membranes were washed four times in PBS. The membranes were then incubated in alkaline phosphatase conjugated goat anti-rabbit IgG (Boehringer Mannheim), washed, and developed with Nitro Blue Tetrazolium and Bromo-Chloro Indolyl Phosphate (Promega, Madison, WI).

Results

To find tissue culture systems to examine Cx45 expression and synthesis, RNA was prepared from a number of established cell lines of diverse tissue origins and hybridized with a probe for canine Cx45 (Fig. 1). The Cx45 probe hybridized to a band of ~2.1 kb in RNA prepared from the liver-derived SKHep1 and WB, cardiac-derived BWEM and CLEM, smooth-muscle-derived A7r5, and fibroblastic BHK cells. RNA from the osteoblastic UMR cells and from primary cultures of neonatal rat ventricular myocytes also hybridized to the Cx45 probe (*data not shown*). A similar mobility 2.1 kb Cx45 has previously been demonstrated in a number of canine and murine tissues (Hennemann et al., 1992; Kanter et al., 1992). No hybridization was detected with RNA from the well-coupled NRK cells or the communication-deficient N2A cells. As expected from previous studies (Beyer et al., 1992), a rat Cx43 probe hybridized to a band of ~3.3 kb in many of these RNAs (WB, BWEM, CLEM, A7r5, BHK, and NRK). The relative levels of Cx43 and Cx45 showed little relation in the different samples. Rehybridization of the blots with an actin probe showed some differences in intensity, but confirmed the integrity of the RNA samples.

The Cx45 protein was further characterized in BWEM cells which clearly produced functional gap junctions, since microinjection of the low molecular weight dye Lucifer yellow CH into a single BWEM cell was consistently followed by rapid passage to many neighboring cells (Fig. 2). Analysis of cell pairs by double whole-cell patch-clamp techniques confirmed that these cells were well coupled electrically (junctional conductance >20 nS).[2] Coupling of these cells was inhibited by 2 mM heptanol.

Indirect immunofluorescence was performed on cultures of fixed and permeabilized BWEM cells with specific anti-Cx43 (Fig. 3*A*) or anti-Cx45 antibodies (Fig. 3*B*). The antibodies against both connexins produced punctate staining at cell-cell boundaries in a distribution consistent with that expected for gap junctions. A low level of cytoplasmic staining was seen with both antibodies. Nonimmune serum or antibodies to unrelated peptides showed no specific labeling of these cells (*data not shown*). Both rabbit polyclonal and mouse monoclonal anti-Cx43 antibodies gave similar staining patterns. In a double labeling experiment, BWEM cells stained with both anti-Cx43 monoclonal antibodies and affinity-purified anti-Cx45 antibodies exhibited identical staining patterns (Fig. 3*E,F*): punctate staining at the cell-cell boundary, as well as low level cytoplasmic staining. Control experiments showed that there was no crossover in the fluorescence detected with the two antibodies, and that the secondary antibodies did not cross-react with noncorresponding primary antibodies (Fig. 3*C,D*).

Immunoprecipitation of lysates of BWEM cells metabolically labeled with [^{35}S]-methionine confirmed the specificity of the anti-Cx45 antibodies. The anti-

[2] J.G. Laing, R.D. Veenstra, H.-Z. Wang, and E.C. Beyer, *unpublished observations.*

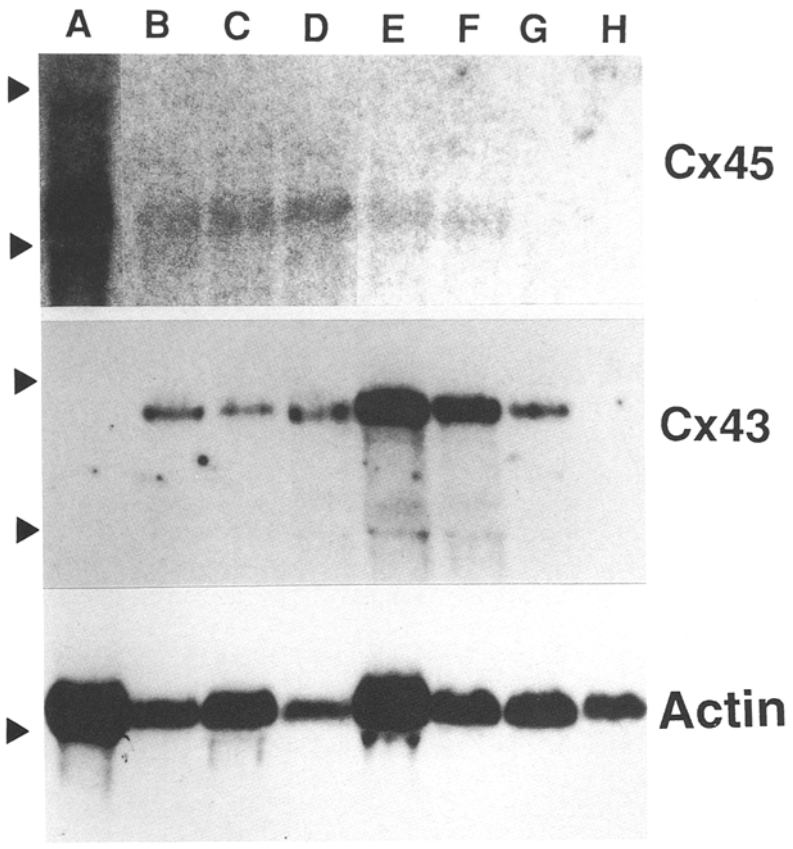

Fig. 1. Expression of Cx45 and Cx43 mRNAs by cultured cells. Total cellular RNA was prepared from SK Hep1 (*A*), BWEM (*B*), CLEM (*C*), WB (*D*), A7r5 (E), BHK (*F*), NRK (*G*), and N2A (*H*) cells. Ten micrograms of each RNA was resolved on a 1% agarose-formaldehyde gel, transferred to nylon membranes, and hybridized with specific probes for canine Cx45 (*A*), rat Cx43 (*B*), or human fibroblast γ actin (*C*). Arrowheads indicate the migration of 18 and 28 S rRNA. Exposure times were five days for Cx45, 18 hr for Cx43, and 2 hr for actin.

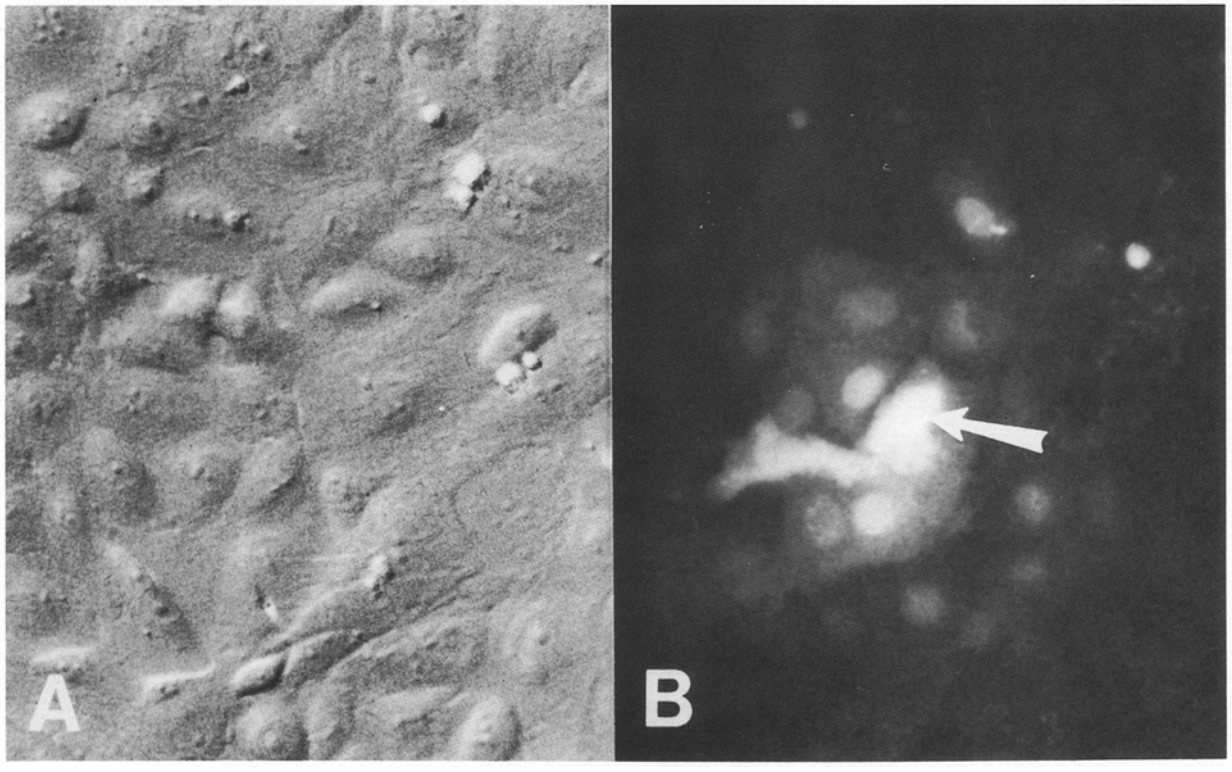

Fig. 2. Dye transfer demonstration of functional intercellular communication among cultured BWEM cells. Lucifer Yellow CH microinjected into a single cell (arrow) rapidly spread to many adjacent cells. The cells were visualized by fluorescence (*A*) and Hoffman modulation contrast (*B*) microscopy.

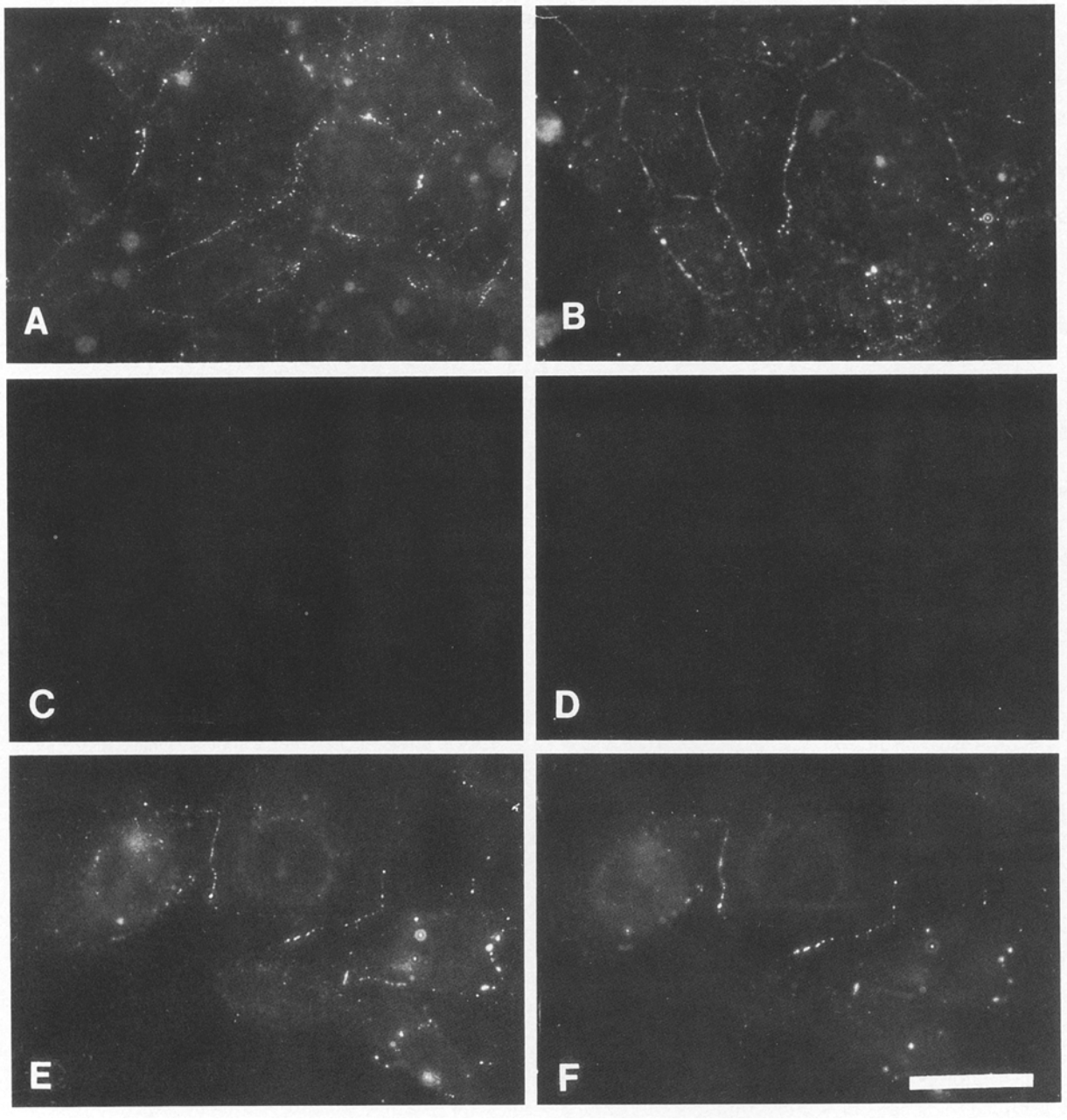

Fig. 3. Immunofluorescence analysis of connexins in BWEM cells. Cultured BWEM cells were fixed and permeabilized with Triton X-100, then stained with mouse anti-Cx43 (*A,C,E*) or rabbit anti-Cx45 (*B,D,F*) antibodies followed by rhodamine-conjugated goat anti-rabbit IgG (*B,C,F*) or fluorescein-conjugated goat anti-mouse IgG (*A,D,E*). Cx43 and Cx45 reactivity both localized to bright spots between cells in a virtually identical distribution (*A,B*) confirmed in panels (*E,F*) which show the same cells double labeled with antibodies directed against both connexins. Panels (*C,D*) show cells reacted with primary antibodies followed by the noncorresponding secondary antibodies and demonstrate the lack of cross-reactivity of these reagents. Bar, 25 μm.

Cx45 antibodies precipitated a predominant 48 kD polypeptide and a minor 46 kD polypeptide; (Fig. 4, lane *B*). The 46 kD polypeptide was present in differing amounts in different experiments and may correspond to a proteolytic degradation product of the 48 kD polypeptide, since Cx45 translated in vitro from the cloned sequence had a mobility indistinguishable from the 48 kD band (*data not shown*) Preimmune serum or anti-Cx45 antibodies which were preincubated with the immunizing peptide (100 μg/ml) did not precipitate any labeled proteins from the BWEM cell lysates (Fig. 4, lanes *A,C*).

The anti-Cx43 and anti-Cx45 antibodies were used to immunoprecipitate Cx43 and Cx45 from extracts of cells metabolically labeled with [^{35}S]-methionine; the precipitated material was then analyzed in parallel by

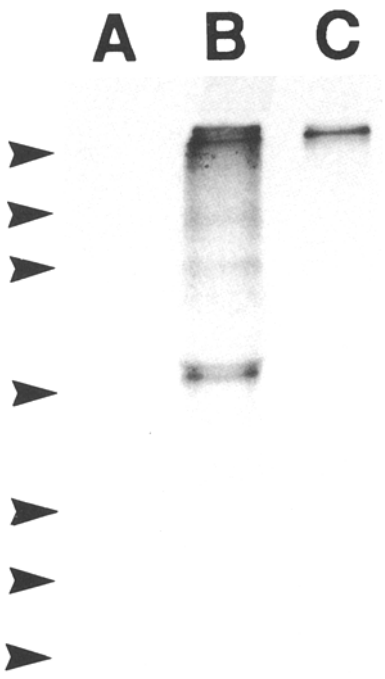

Fig. 4. Demonstration of anti-Cx45 antibody specificity by immunoprecipitation of Cx45. BWEM cells were metabolically labeled with [^{35}S]-methionine, and lysates were immunoprecipitated with preimmune serum (*A*), anti-Cx45 antibodies (*B*), or anti-Cx45 antibodies which had been preincubated with 100 μg/ml of the immunizing peptide. The immunoprecipitated materials were analyzed on a 12.5% polyacrylamide gel and subjected to fluorography. Protein molecular weight standards indicated by arrowheads are (kD): 200, 97.4, 69, 46, 30, 21.5, and 14.3.

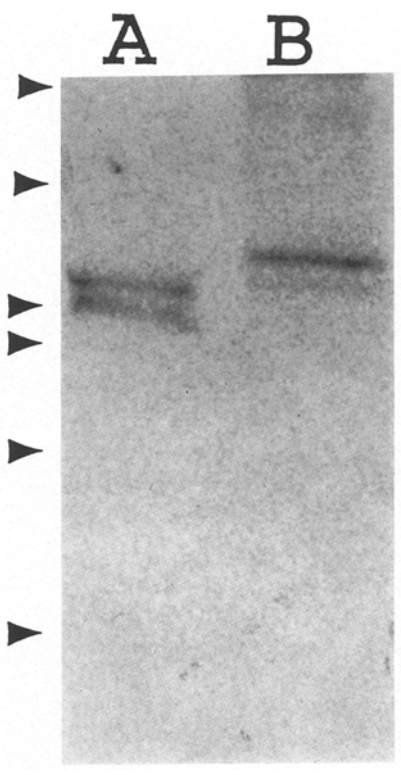

Fig. 5. Immunoprecipitation of connexins from BWEM cell extracts. BWEM cells were metabolically labeled with [^{35}S]-methionine, and lysate was divided in half and then immunoprecipitated with anti-Cx43 (*A*) or anti-Cx45 antibodies (*B*). Protein molecular weight standards indicated by arrowheads are (kD): 67, 55, 42.7, 40, 31, and 21.5.

SDS-PAGE. The Cx43 immunoprecipitate contained 42 and 44 kD polypeptides (Fig. 4, lane *A*) which likely correspond to the unmodified (NP) and phosphorylated (P_1) forms of Cx43 previously characterized in a number of laboratories (Crow et al., 1990; Laird, Puranam & Revel, 1991; Musil & Goodenough, 1991). The Cx45 immunoprecipitate again contained 48 and 46 kD bands (Fig. 5, lane *B*); polypeptides of the same size were immunoprecipitated from several of the other cell lines that express Cx45 mRNA (*data not shown*). With longer labeling times, an additional form of Cx43 of 46 kD (P_2) was also observed, while no additional Cx45 bands were seen (*data not shown*).

To determine if Cx45 like Cx43 was modified by phosphorylation, we metabolically labeled BWEM cells with [^{32}P]-orthophosphoric acid or [^{35}S]-methionine prior to immunoprecipitation. Half of the immunoprecipitated material was digested with alkaline phosphatase; the other half was subjected to a sham digestion by incubation in phosphatase buffer with no added enzyme. The immunoprecipitated material was analyzed by SDS-PAGE and autoradiography (Fig. 6). The [^{35}S]-methionine labeled anti-Cx45 immunoprecipitate again contained a major 48 kD polypeptide. Anti-Cx45 precipitation of material labeled with [^{32}P]-phosphate yielded a single 48 kD band. Alkaline phosphatase did not alter the mobility of the 48 kD [^{35}S]-methionine-labeled polypeptide; however, [^{32}P]-label was not detectable after phosphatase treatment confirming removal of phosphate from the Cx45 protein. These results indicate that Cx45 is subject to phosphorylation, but unlike Cx43, this modification does not alter the mobility of Cx45 on SDS-PAGE.

Treatment with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) affects intercellular communication and connexin phosphorylation in a number of different cell lines (Yada, Rose & Loewenstein, 1985; Brissette et al., 1991; Berthoud et al., 1992). Lucifer yellow dye passage in BWEM cells was dramatically reduced by the addition of TPA, as shown in Fig. 7. In 20 injections of sham-treated BWEM cells, all injections showed intercellular dye passage to a median of 15 neighbors after 2 min. After treatment with 200

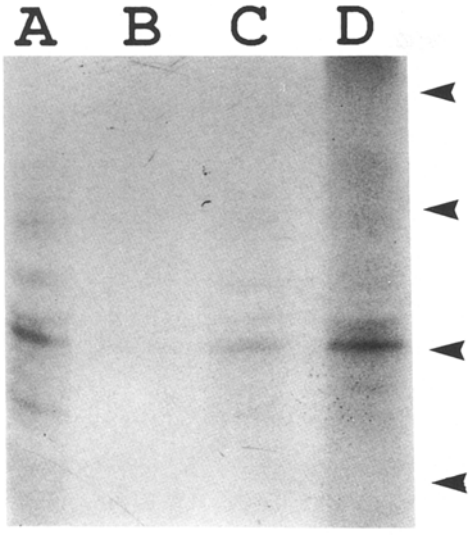

Fig. 6. Phosphorylation of Cx45 in BWEM cells. BWEM cells were metabolically labeled with [^{35}S]-methionine (*A,C*) or [^{32}P]-orthophosphoric acid (*B,D*) prior to immunoprecipitation with affinity-purified anti-Cx45 antibodies. Precipitated materials were divided: half was treated with alkaline phosphatase (*A,B*); half was reacted with buffer, but no added phosphatase (*C,D*). The immunoprecipitated material was resolved by SDS-PAGE on a 12.5% polyacrylamide gel and analyzed by autoradiography. Protein molecular weight standards indicated by arrowheads are (kD): 97.4, 69, 46, and 30.

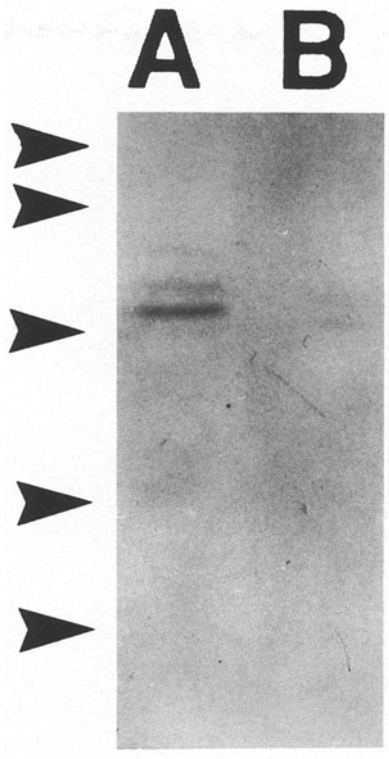

Fig. 8. Effects of TPA on Cx45 expression. Immunoblots reacted with anti Cx45 antibodies were prepared from whole cell lysates of control BWEM cells (*A*), or BWEM cells treated for 30 min with 50 nM TPA (*B*). Protein molecular weight standards indicated by arrowheads are (kD): 97, 69, 46, and 30.

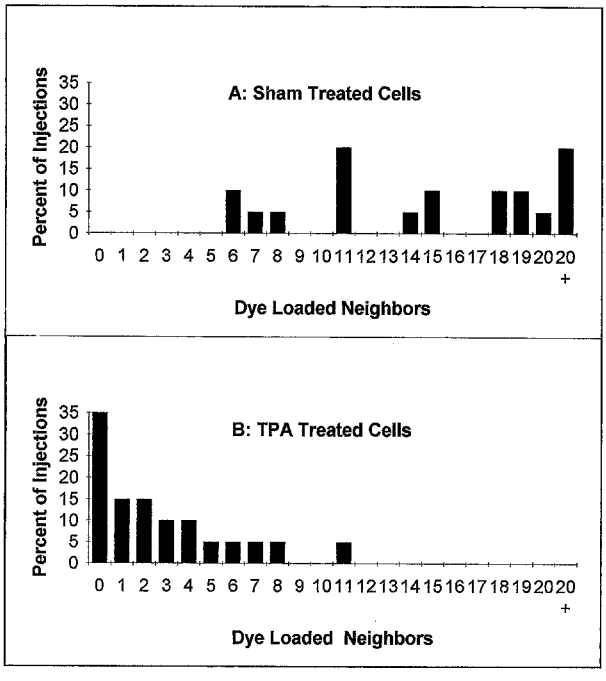

Fig. 7. Dye transfer in BWEM cells treated with solvent alone (*A*) or treated with 200 nM TPA for 2 hr (*B*). The cells that contained dye were scored 2 min after dye injection.

nM TPA for 2 hr, 35% of injected cells showed no dye transfer; the mean number of cells receiving dye was 2.9 and the median was 1 (n = 20).

Lysates of TPA-treated BWEM cells were analyzed by immunoblotting. Sham-treated BWEM cell extracts contained 46 and 48 kD Cx45 polypeptides similar to those in the immunoprecipitates (Fig. 8, lane *A*). BWEM cells treated with 50 nM TPA contained much less Cx45 (Fig. 8, lane *B*). The consequences of the TPA treatments were further analyzed by immunoprecipitation of Cx43 and Cx45 from BWEM cells labeled with [^{35}S]-methionine or [^{32}P]-orthophosphoric acid in the presence or absence of 50 nM TPA. Cx43 precipitated from TPA-treated cultures had a reduced electrophoretic mobility, compared to that from control cultures (Fig. 9, lanes *A* and *B*); this change likely represents a TPA-induced phosphorylation of Cx43, as observed by others (Yada et al., 1985; Brissette et al., 1991; Berthoud et al., 1992). In contrast, the mobility of [^{35}S]-methionine-labeled Cx45 from TPA-treated cultures was not altered; however, the amounts of [^{35}S]-methionine-labeled Cx45 appeared significantly reduced (Fig. 9, lanes *C,E*). In addition, the precipitated Cx45 polypeptide did not incorporate [^{32}P]-label, suggesting

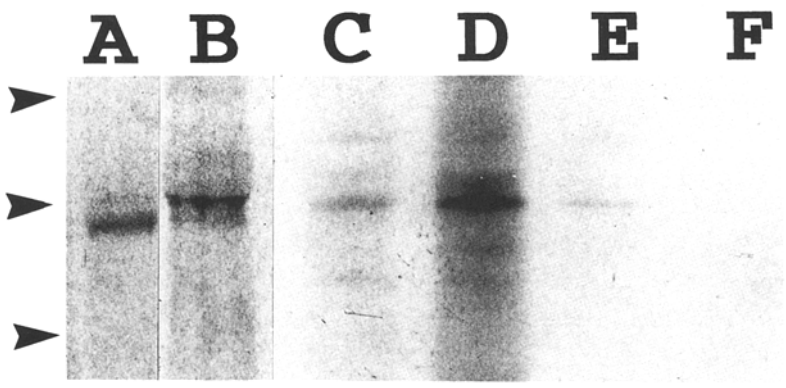

Fig. 9. Effects of TPA on Cx43 and Cx45 polypeptides in BWEM cells. Cx43 was immunoprecipitated from [^{35}S]-methionine labeled sham-treated cells (lane *A*) or TPA-treated cells (lane *B*). Cx45 was precipitated from sham-treated cells labeled with [^{35}S]-methionine (lane *C*) or [^{32}P]-orthophosphoric acid (lane *D*) and from TPA-treated cells labeled with [^{35}S]-methionine (lane *E*) or [^{32}P]-orthophosphoric acid (lane *F*). Protein molecular weight standards indicated by arrowheads are (kD): 69, 46, and 30.

that this treatment inhibited phosphorylation of newly synthesized Cx45 (or stimulated its dephosphorylation) (Fig. 9, lane *F*).

Discussion

We have shown by RNA blotting that Cx45 is widely expressed in cell lines originating from many different tissue sources. Some of the cells showing the highest levels of Cx45 expression originated from organs with low overall levels of Cx45 (Hennemann et al., 1992); however, some of these cell lines (e.g., SKHep1) have retained few differentiated features of their original tissues. We have utilized BWEM cells because of their advantages as a model system for investigating intercellular communication. Our previous transfection studies demonstrated that chick Cx45 forms gap junctional channels with a unitary conductance of ~30 pS and a strong voltage dependence (Veenstra et al., 1992). In mammalian Cx45, 83% of amino acids are identical to those in the chick (Hennemann et al., 1992; Kanter et al., 1992); therefore, it is likely to have similar physiological characteristics. A small gap junctional channel has been characterized in a number of cells including SKHep1 cells (Moreno, Eghbali & Spray, 1991), WB cells (Spray et al., 1991), and cardiac myocytes (Rook, Jongsma & van Gienneken; 1988; Wang et al., 1992). It is reasonable to speculate that many of these may be formed by Cx45. Indeed, preliminary immunofluorescence studies demonstrate the reactivity of anti-Cx45 antibodies with these cells.[3]

Our RNA blotting and immunofluorescence studies have demonstrated that Cx45 has a unique pattern of expression, but that in many cases it is present in the same cell as the widely expressed Cx43. In the BWEM cells, Cx43 and Cx45 colocalized as detected by immunofluorescence. A number of studies have previously demonstrated the localization of multiple connexins to the same gap junctional plaques within a single cell (Traub et al., 1989; Spray et al., 1991; Kanter et al., 1993). However, the physiological significance or consequences of this colocalization remain to be elucidated.

Our data demonstrate that Cx45 is a phosphoprotein. This is shown by the metabolic [^{32}P] labeling of the Cx45 polypeptide, as well as by the removal of this label with alkaline phosphatase. We have not yet determined which amino acids are phosphorylated. Cx43 has previously been shown to be phosphorylated, primarily on serine residues (Crow et al., 1990; Filson et al., 1990; Musil et al., 1990*b*). Stimulation of protein kinases or alterations in connexin phosphorylation appear to have a number of effects, including involvement in gating of junctional channels (Loewenstein & Rose, 1992; Sáez et al., 1993*a*) and in assembly of connexins into gap junctional plaques (Musil & Goodenough, 1991). Several connexins (including Cx45, Cx43, Cx40, Cx37, Cx46, and Cx56) share a serine-rich region near the carboxyl terminus containing predicted phosphorylation sites for protein kinase C and other kinases (Beyer et al., 1987, 1990, 1992; Kennelly & Krebs, 1991; Paul et al., 1991; Haefliger et al., 1992; Rup et al., 1993). Indeed, synthetic peptides corresponding to carboxyl terminal Cx43 sequences can act as a substrate for protein kinase C in vitro (Sáez et al., 1993*b*). Our data concerning Cx45 demonstrate phosphorylation of a second member of this group.

Our data indicate that intercellular coupling between BWEM cells is diminished by treatment with the tumor promoter TPA, as previously observed in a number of transformed cell lines (Yada et al., 1985; Chanson et al., 1988; Brissette et al., 1991; Berthoud et al., 1992). In several of these systems, TPA stimulates phosphorylation of Cx43 (Berthoud et al., 1992). We have demonstrated that TPA treatment of BWEM cells induces a similar hyperphosphorylation in Cx43. In contrast, TPA treatment reduces Cx45 expression, and either eliminates the phosphorylation of this protein or stimulates its dephosphorylation. These observations

[3] J.G. Laing and E.C. Beyer, *unpublished observations.*

demonstrate that an agent which modulates protein phosphorylation leads to cellular uncoupling accompanied by different biochemical effects on two gap junction proteins.

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