

Analyzing Phorbol Ester Effects on Gap Junctional Communication: A Dramatic Inhibition of Assembly

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Abstract. The effect of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on gap junction assembly between Novikoff hepatoma cells was examined. Cells were dissociated with EDTA to single cells and then reaggregated to form new junctions. When TPA (25 nM) was added to the cells at the onset of the 60-min reaggregation, dye transfer was detected at only 0.6% of the cell-cell interfaces compared to 72% for the untreated control and 74% for 4- α TPA, an inactive isomer of TPA. Freeze-fracture electron microscopy of reaggregated control cells showed interfaces containing an average of more than 600 aggregated intramembranous gap junction particles, while TPA-treated cells had no gap junctions. However, Lucifer yellow dye transfer between nondissociated cells via gap junctions

was unaffected by 60 min of TPA treatment. Therefore, TPA dramatically inhibited gap junction assembly but did not alter channel gating nor enhance disassembly of preexisting gap junction structures. Short term TPA treatment (<30 min) increased phosphorylation of the gap junction protein molecular weight of 43,000 (Cx43), but did not change the cellular level of Cx43. Cell surface biotinylation experiments suggested that TPA did not substantially reduce the plasma membrane concentration of Cx43. Therefore, the simple presence of Cx43 in the plasma membrane is not sufficient for gap junction assembly, and protein kinase C probably exerts an effect on assembly of gap junctions at the plasma membrane level.

GAP junctions allow molecules of molecular weight of <1,000 to pass between cells (26) via matched membrane channels composed of proteins from the "connexin" gene family (6). Cell-cell communication through these channels is thought to play an important role in the control of cell proliferation, embryonic development, cell differentiation, and the regulation of differentiated function in postmitotic cells (4, 22).

A number of studies have documented a correlation between neoplastic transformation and reduced gap junctional intercellular communication (e.g., references 2, 3, 10). Many laboratories have also confirmed the original reports (32, 57) that tumor promoters reduce gap junctional communication between cells grown in tissue culture. As a result of these and other studies, reduced cell-cell communication has been proposed as a critical step in multistage carcinogenesis (13, 51).

A common method used to study gap junctional communication is the intracellular microinjection of tracer dyes that can pass through gap junction channels. The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)¹ has been

shown to reduce dye spread in a wide variety of animal cell types (33). Since the maximal decrease in dye transfer usually requires 1–2 h to occur (e.g., 1, 9, 15, 44), the mechanism by which TPA addition decreases gap junction activity is not evident. However, treatment of cells with TPA for several hours has been shown to significantly reduce the number of gap junctions as assayed by freeze-fracture electron microscopy (53, 55). These results have been supported recently by immunofluorescence studies which have documented reductions in punctate labeling of connexin antibodies at the plasma membrane after 30 min–2 h of TPA treatment (1, 5, 9). A study on nucleotide transfer has also indicated that TPA may have some effects on gap junction assembly based on a reduction in the rate of appearance of gap junctional communication (40). In a few cases, no change or an increase (31) in junctional conductance has been observed with TPA treatment. Since the time course and extent of TPA effects on gap junctional communication seem to vary with cell type, it has been difficult to determine the mechanism(s) of TPA reduction in gap junctional communication.

TPA, a specific regulator of protein kinase C activity (38), could affect a wide variety of cellular processes related to gap junction communication. Protein kinase C has been reported to directly phosphorylate connexins with molecular weights of 32,000 and 43,000 (Cx43) (45, 46, 48, 49). Phosphorylation of gap junction proteins could regulate channel opening and closing (gating) or channel assembly or turnover. Gap junction assembly could be regulated when con-

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1. *Abbreviations used in this paper:* BFA, brefeldin A; CON, control; DiI, 1,1'-diiododecyl-3,3',3'-tetramethylindocarbocyanine; NT, not treated; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

nexins are trafficking to the plasma membrane or when putative hexamers of connexins (referred to as connexons or hemi-channels) are being assembled into gap junctions within the plasma membrane.

Gap junction assembly can be readily studied with Novikoff hepatoma cells, which can be grown in suspension culture and readily dissociated without proteases in EDTA-supplemented medium (16). A 90-min incubation period with agitation allows the dissociated cells to eliminate existing gap junction structures, so at the end of this "recovery" period more than 95% of the cells are single cells without any gap junction structures (41). When the cells are allowed to make contact and "reaggregate," new gap junctions form. Gap junction assembly can then be assayed in a quantitative manner with dye permeability and freeze-fracture electron microscopy. We have studied the influence of TPA on gap junction assembly using this system.

Materials and Methods

Cell Culture and Dissociation

Tissue culture reagents were purchased from GIBCO-BRL (Gaithersburg, MD). The Novikoff hepatoma cell line (NIS1-67) was maintained and dissociated as previously described (27). Briefly, cells in logarithmic growth were dissociated by centrifugation from standard growth medium (S210 medium supplemented with 10% newborn calf serum), resuspended in S210 with 10 mM EDTA at 6×10^5 cells/ml, and placed in a 37°C gyratory shaker incubator (200 rpm) for 15 min. The EDTA treatment was repeated, resulting in >95% single cells. The cells were then "recovered" by incubation with agitation in S210 with 5% BSA (A7030; Sigma Chemical Co, St. Louis, MO) for 90 min, which eliminated remnants of previously existing junctions. The cells remained as ~95% single cells during this recovery period. The cells were then allowed to reaggregate and make new gap junctions.

Freeze-Fracture and Electron Microscopy to Quantify Gap Junction Structures

Cells were reaggregated by centrifugation at 30 g for 5 min to yield loose pellets in 17 × 100 mm round bottom tubes, and then incubated for 60 min at 37°C in a 5% CO₂ atmosphere. Cell pellets were fixed in 2.5% glutaraldehyde in S210 medium for freeze-fracture and electron microscopy. Further processing was performed as previously reported (27). Methods of quantitation have been described (42). Briefly, an "interface" was defined as a fractured membrane area comprising at least 57 μm² (i.e., filling the screen at 10,000× on our microscope) and containing an indication of cell apposition. Interfaces were scored according to the presence or absence of one or more formation plaques or gap junctions. "Formation plaques" (42) were defined as specialized membrane areas with clusters or arrays of 9–11 nm intramembranous particles. Aggregated 9–11 nm particles are defined here as individual gap junction channels. Formation plaque area and particle number measurements were performed as reported in detail elsewhere (42).

Assay of Dye Transfer

We assayed dye transfer between two populations of Novikoff cells using a procedure with elements that are similar to a previously published method (50). One was loaded with the membrane-permeant dye calcein-AM, which is intracellularly cleaved to membrane-impermeant calcein, and the other was labeled with the lipophilic dye, 1,1'-diiododecyl-3,3',3'-tetramethylindocarbocyanine (DiI). The cells were dissociated and recovered for 1.5 h as described above. In separate tubes, calcein-AM (Molecular Probes, Eugene, OR) at a dilution of 1,700× from a 0.6 mM stock solution in DMSO (0.35 μM final concentration) or DiI (Molecular Probes) at a dilution of 3,750× from a 0.85 mM stock solution in DMSO (0.2 μM final concentration) was added to the cells. The two populations of cells were separately pelleted, resuspended and washed three times with S210 medium. Calcein-containing cells (0.25×10^6) were then mixed with DiI-

labeled cells (0.75×10^6) in 2 ml of S210 medium containing 5% BSA in a 35 mm tissue culture dish. Cells were routinely allowed to settle out and "reaggregate" in a 5% CO₂ incubator at 37°C. TPA was added either at the beginning or during the reaggregation period. After reaggregation, the cells were removed from the incubator and viewed with a Zeiss IM35 inverted microscope. Calcein was viewed with a fluorescein filter set (BP 450–490, FT 510, LP 520). A 570 SP filter was added to the fluorescein emission set to eliminate low levels of orange DiI fluorescence observed when cells that were highly labeled with DiI were viewed. Rhodamine filters (BP 546, FT 580, LP 590) were used to view DiI labeling. Phase views containing a total of up to several dozen cells per field in groups of two to eight cells were selected in a "blind" manner without the microscopist knowing cell treatments. Phase and fluorescent views for calcein and DiI were recorded using either a 35 mm camera with TMax film at 400 ASA or a Dage silicon-intensified target video camera, Panasonic video recorder and monitor. The percentages of cells that transferred dye were determined prior to treatment identification by dividing the number of DiI labeled cells that contained calcein (i.e., transfers) by the number of cell interfaces between calcein loaded and DiI cells (i.e., total). The complete set of data was collected from eight separate experiments.

The effects of TPA on cell-cell aggregation were determined by totaling the number of aggregates of a given number of cells in a field (i.e., a four-cell cluster would be scored as one cluster under the "4" heading). A total of 287 clusters of untreated control and 239 clusters of TPA treated cells from two separate days and several dozen fields were scored and the frequencies determined.

Lucifer yellow was injected into nondissociated Novikoff cells as previously described (28). The mean permeance of cell-cell interfaces between Novikoff cell pairs was estimated according to published methods (8).

Connexin 43 (Cx43) Metabolic Labeling, Immunoprecipitation and Western Transfer

Dissociated Novikoff cells were labeled with [³²P]O₄ by incubation at 37°C for 3 h with agitation at 1×10^6 cells/ml in phosphate-free Dulbecco's modified Eagle medium (Sigma Chemical Co.) containing 3% BSA and 0.25 mCi [³²P]O₄/ml (New England Nuclear, Boston, MA). The cells were then allowed to "settle out" and reaggregate in the presence or absence of TPA for 1 h.

Novikoff cells were labeled with [³⁵S]methionine for Cx43 half-life studies. Cells (20×10^6) were starved in methionine-free minimum essential medium for 45 min. The cells were then pelleted and vortexed in 10 ml of fresh methionine-free minimum essential medium and 1 mCi [³⁵S]methionine (Amersham Corp., Arlington Heights, IL) was added. The cells were incubated for 2 h, pelleted, and chased with S210 medium containing 3% BSA for the times specified.

Cx43 was immunoprecipitated from Novikoff cells, run on SDS-PAGE, and subjected to autoradiography as previously described (44), except 2 μg of monoclonal antibody 3068 (Chemicon, Temecula, CA) specific for residues 252–270 of Cx43 and/or an antibody prepared to the amino terminal 20 residues (AT-2; generous gift of Barbara Yancey (Caltech, Pasadena, CA); described in reference 56) was used for each treatment. For [³⁵S]methionine turnover studies, the stained/detained gels were soaked in Fluoro-Hance (Research Products International, Mount Prospect, IL) prior to drying and autoradiography. Cx43 half-life determination was from densitometry of two autoradiographic exposures (3 and 6 d) from two different experiments.

Cells were biotinylated on the cell surface using a protocol modified from a previously published procedure (36). Approximately 2×10^7 cells per sample were dissociated and recovered as indicated above. The cells were then allowed to settle and reaggregate for 1 h at 37°C in the presence or absence of 50 nM TPA. All subsequent steps were performed in a 4°C room. The cells were then pelleted and washed twice with 50 ml of 4°C, PBS (Celox Corp., Minneapolis, MN). NHS-LC-biotin (0.5 mg/ml in PBS; Pierce Chemical Co, Rockford, IL) was added to the pelleted cells, and the mixture was vortexed and placed on a rocker platform for 30 min. The cells were pelleted and then vortexed in 10 ml S210 medium with 10 mM glycine. After rocking for 15 min, the cells were pelleted and sonicated in 1.0 ml of lysis buffer (0.025 M Tris-HCl, 0.1 M NaCl, 10 mM EDTA, 50 mM NaF, 500 μM Na₃VO₄, 0.5% Triton X-100, 1% SDS, 0.02% NaN₃, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 10 μg/ml aprotinin, 1 μg/ml leupeptin, pH 8.0). After sonication, 0.8 ml of 50 mM Tris-HCl, containing 50 mM NaF, 500 μM Na₃VO₄, and the protease inhibitors at the above concentrations, was added and mixture was pelleted in a microfuge at 12,000 g for 10 min. A portion (50 μl) was removed for the whole cell

samples, and the rest was added to 40 μ l of PBS washed, immobilized avidin beads (crosslinked, 6% beaded agarose, Pierce Chemical Co.). Free d-biotin (20 μ g; Sigma Chemical Co.) was preincubated for 20 min with the immobilized avidin beads that were to be used for the control sample. After rocking the samples for 30 min, the beads were pelleted at 2,000 g, and washed, once with lysis buffer without SDS, once with 50 mM Tris-HCl, 0.5% Tween-20, 2 M urea, 0.1 mM EDTA, pH 8.0, and twice with 50 mM Tris-HCl, 0.5% Tween-20, 0.1 mM EDTA, pH 8.0. The beads and whole cell samples were treated with sample buffer (the beads also had d-biotin added to 5 mM). Samples were boiled for 3 min, run on SDS-PAGE, and Western blotted to detect Cx43.

For Western immunoblotting, SDS-PAGE of Novikoff cells was performed on 10.0% polyacrylamide gels following published methods (23). For the TPA treatment time course experiments, Novikoff cells were dissociated as described above. Approximately 0.5×10^6 cells were centrifuged for 1 min at 300 g and allowed to reaggregate at 37°C for the time indicated in the presence of TPA. After treatment for 15, 30, or 60 min with TPA, cells were washed once in cold PBS and solubilized in Laemmli sample buffer containing 2 mM EDTA, 50 mM NaF, 50 μ M NaVO₄, and protease inhibitors (1 μ g/ml pepstatin A, 1 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 2 mM PMSF). Cells that were not treated (NT) with TPA were reaggregated for 5–10 min including the wash. Protein transfer was performed as previously described (20) and Cx43 was detected using antibody AT-2. Primary antibody was detected using ¹²⁵I-labeled goat anti-rabbit IgG (3 μ Ci/blot; ICN Biomedicals, Irvine, CA). Autoradiography was performed using Kodak XAR 5 film overnight with an intensifying screen.

Densitometry of autoradiographs was performed with NIH Image 1.44 using a COHU CCD video camera and a frame grabber board (Data Translation, Inc., San Diego, CA) connected to a Macintosh microcomputer. The public domain NIH Image program was written by Wayne Rasband at the U.S. National Institutes of Health (Gaithersburg, MD) and is available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS (part number PB93-504868; Springfield, VA).

RNA Isolation from Novikoff cells

Novikoff cells were grown and dissociated as described above. Cells were allowed to reaggregate for zero, one or two hours with or without 50 nM TPA. Cells were harvested by centrifugation at 1,000 g for 5 min. Cells were lysed and homogenized in a dounce with the addition of 7.5 ml cold 5 M guanidine-HCl, 0.2 M sodium acetate, 0.5% sodium sarkosyl, 1 mM EDTA, and 0.5 M 2-mercaptoethanol. The suspension was repeatedly extracted with phenol/chloroform/isoamyl alcohol (25:24:1), followed by a single chloroform extraction, and RNA was selectively precipitated by adding one-half volume 95% ethanol overnight at 20°C. After centrifugation at 12,000 g, pelleted RNA was resuspended in water treated with diethyl pyrocarbonate. Northern blots were obtained by electrophoresis of total RNA, 30 μ g per lane, in formaldehyde gels (0.8–1.0% agarose, 6.6% formaldehyde, 20 mM MOPS, pH 7.0, 2.5 mM sodium acetate, 1 mM EDTA). Gels were blotted to Zeta-Probe nylon membranes (Bio-Rad Laboratories, Beverly, MA) by capillary action using 20 \times SSPE. Blots were hybridized in 0.25 M NaCl, 7% SDS, 1 mM EDTA, 0.25 M sodium phosphate, pH 7.0, 150 mg/ml herring sperm DNA, 50% formamide at 48°C. Filters were washed at 55°C with 0.2 \times SSPE, 0.1% SDS.

Probes for rodent connexins Cx26, Cx31.1, Cx32, Cx33, Cx37, Cx40, Cx46 (kindly provided by D. Paul, Harvard University, Boston, MA) and Cx43 cDNA clone G2 (the entire rat heart Cx43 coding sequence, kindly provided by E. Beyer, Washington University, St. Louis, MO; described in 7) were radiolabeled (12) and used as hybridization probes for Northern analysis.

Results

Dye Transfer between Nondissociated Novikoff Cells

Rapid dye transfer between neighboring cells has been observed when the dye Lucifer yellow was microinjected into clusters of Novikoff hepatoma cells (8, 27–29). We wanted to determine if TPA treatment would affect dye transfer between nondissociated cells. Approximately 80–90% of nondissociated Novikoff cells will transfer dye to adjacent cells. Treatment of these cells for 1 h with 50 nM TPA did not change the percentage that transferred dye. We also obtained a measure of the rate of gap junctional communication be-

tween cells by evaluating “permeance” values. The dye permeance of a cell–cell interface (fully defined in reference 8) depends on the total cross-sectional area and permeability of the gap junction. Comparison of the mean dye permeance of nondissociated Novikoff cells that were TPA (50 nM) treated for 1 h ($2.2 \times 10^{-11} \pm 0.9 \times 10^{-11}$ cm³/s, $n = 17$) with untreated cells ($1.5 \times 10^{-11} \pm 0.9 \times 10^{-11}$ cm³/s, $n = 20$) showed no significant difference in the rate of dye transfer. Since cells treated for one hour continued to readily pass Lucifer yellow, TPA had little effect on the regulation or gating of gap junctions between Novikoff cells which had formed dye-permeable gap junction channels prior to application of TPA.

Inhibition of Gap Junction Assembly by TPA: Dye Transfer

We examined the effect of TPA on gap junction assembly between Novikoff hepatoma cells which had been dissociated with EDTA. The dissociated cells were divided into two groups and labeled either with calcein-AM or DiI. Non-fluorescent calcein-AM is cleaved by intracellular esterases to fluorescent, membrane impermeant calcein (mol wt = 622) which is small enough to pass through gap junction channels. DiI labels membranes and is used here simply as a marker of potential recipient cells. The two populations of cells were washed, mixed and allowed to reaggregate for 1 h in a culture dish.

Fig. 1 shows phase (*A* and *a*) and calcein (*B* and *b*) and DiI (*C* and *c*) fluorescence views of untreated control cells (*A*, *B*, and *C*) and cells TPA-treated during the reaggregation period (*a*, *b*, and *c*). Donor cells emitted calcein but not DiI fluorescence. Recipient cells were identified in the microscope by their yellow-green calcein fluorescence with the fluorescein filters and red DiI fluorescence with rhodamine filters (shown in Fig. 1 in black and white). When evaluated for calcein, both donor and recipient cells were quite uniformly fluorescent over the entire cell, but donors were brighter than recipients. Cells were labeled with DiI in a distinctly more punctate manner consistent with internal and plasma membrane localization of the label. In the untreated control cells shown in Fig. 1 (*top*), dye transfer was demonstrated from calcein-labeled donor cells to DiI labeled cells in 22 out of 32 calcein–DiI cell interfaces. One example of an interface and transfer between a donor and recipient cell is indicated by an arrow. Interfaces were only evaluated at the light microscope level and may not represent true cell–cell contact. In TPA-treated cells, no transfer was observed (Fig. 1, *bottom*).

Data from over 1,300 cell–cell interfaces are summarized in Table I. When TPA (25 nM) was added to the cells at the onset of the 60 min reaggregation, dye transfer was detected at only 0.6% of the cell interfaces compared to 72% for the untreated control and 74% for 4- α TPA, an inactive isomer. At 50 nM TPA no transfers were detected. Thus, TPA completely blocked dye transfer between cells when assembly of new gap junction structures was required.

TPA had no obvious influence on the ability of the cells to aggregate (Table II). The size distributions of cell aggregates were nearly identical in the two treatments. The cells appeared healthy by phase contrast microscopy even after much higher concentrations of TPA and prolonged treatments.

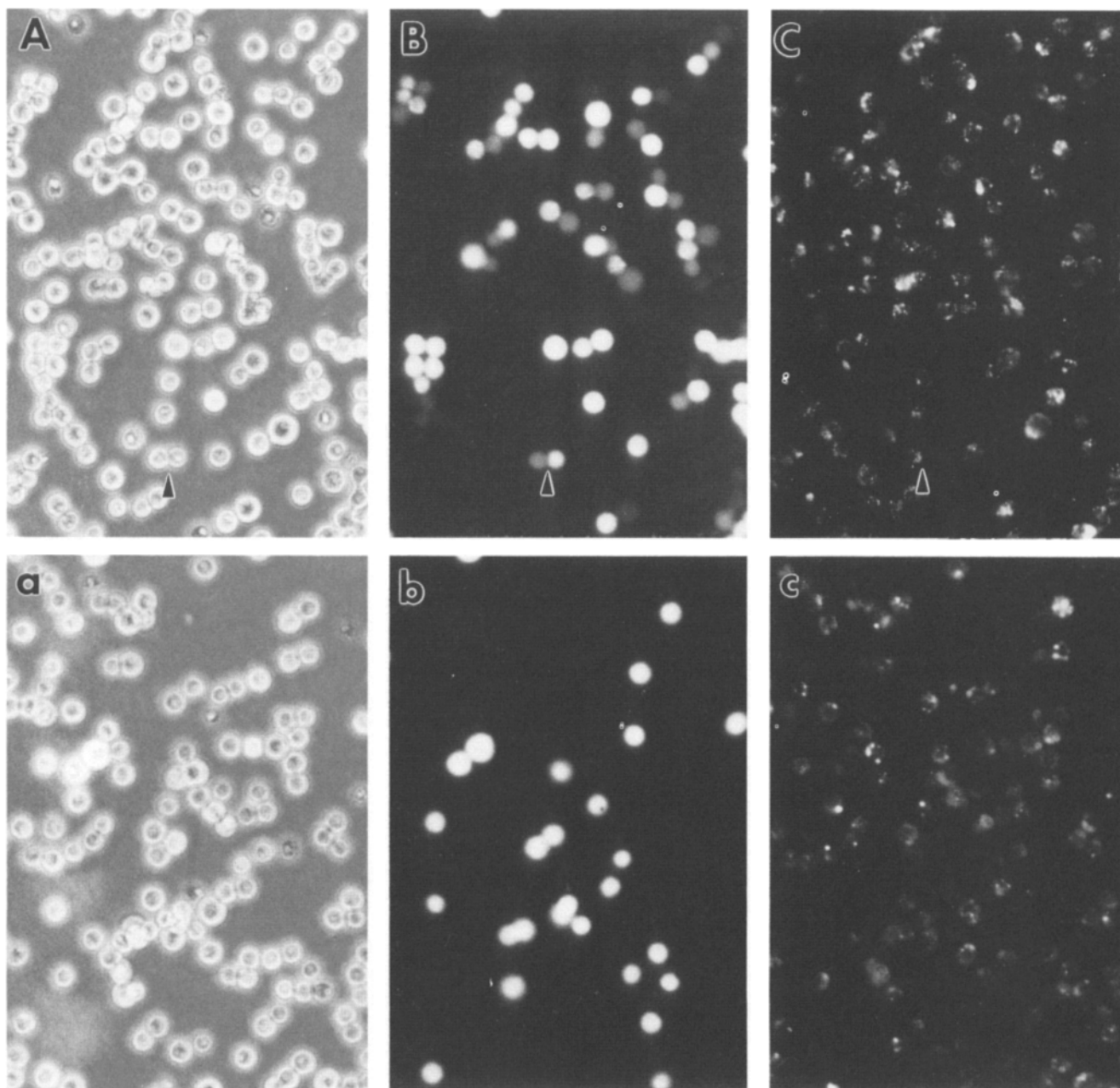


Figure 1. Transfer of dye from calcein-loaded cells to DiI-labeled cells. Phase (*A* and *a*) and calcein (*B* and *b*) and DiI (*C* and *c*) fluorescence views of untreated control (*A*, *B*, and *C*) and 50 nM TPA-treated (*a*, *b*, and *c*) Novikoff cells. TPA was only present during the 60 min reaggregation period (see Materials and Methods for cell dissociation and labeling methods). The arrow points out one cell pair showing calcein dye transfer from the cell on the right to a DiI-labeled recipient cell on the left. The phase pictures were taken with both visible and UV light sources on.

Table I. The Effect of TPA on Calcein Transfer to DiI-labeled Cells

Treatment	Number of cell interfaces*	Percent of interfaces with transfer
Untreated control	683	72
4- α TPA (25 nM)	247	74
TPA (25 nM)	308	0.6
TPA (50 nM)	176	0

* The complete set of data was compiled from eight separate experiments on different days.

Inhibition of Gap Junction Assembly by TPA: Freeze-Fracture Electron Microscopy

Since freeze-fracture revealed that prior to reaggregation essentially none of the EDTA-dissociated cells were linked by gap junctions (41), gap junctions observed after reaggregation were products of nascent assembly. Formation plaques are areas of membrane specialization with clusters or arrays of putative gap junction precursors (16, 17). A formation plaque from control cells reaggregated for 60 min containing four small gap junction aggregates is shown in Fig. 2. Freeze-fracture electron microscopy of cells reaggregated

Table II. Effects of TPA on Cell-Cell Aggregation: Frequency of Clusters Containing Different Numbers of Cells

Treatment	Number of cells in a cluster						
	1	2	3	4	5	6	>6
Control	32%	24%	15%	7%	6%	4%	12%
TPA*	35%	23%	14%	9%	5%	2%	11%

* 50 nM TPA was added at the beginning of the 60 min reaggregation period.

for 60 min in the presence of 50 nM TPA showed that the interfaces between Novikoff cells contained no formation plaques and no aggregated 9–11 nm intramembranous particles (i.e., gap junction channels; 17). Control cell interfaces and those treated with 4- α TPA had values for aggregated gap junction channels per interface and formation plaque areas that were indistinguishable (Table III).

The time course of TPA inhibition is shown in Fig. 3. Cells were reaggregated for the time specified before fixation. Untreated control cells show a steady increase in aggregated gap junction channels with increasing time of reaggregation (\square , solid line) consistent with earlier work (16, 42). To our surprise, TPA treated cells had some gap junction channels present at 15 min before decreasing to near zero levels at 30 and 60 min (\circ , dashed line). Specifically, untreated control and TPA-treated cells that were allowed to reaggregate for 15 min before fixation had gap junctions at 63 and 49% of the interfaces and formation plaque areas of 0.32 and 0.21 μm^2 , respectively. The gap junctions that were present at 15 min of TPA treatment appeared to be newly formed, since they were smaller than junctions between nondissociated cells and had formation plaques that were morphologically consistent with nascent gap junctions (16). Since junctions were not detected at the end of a 60-min reaggregation, TPA might

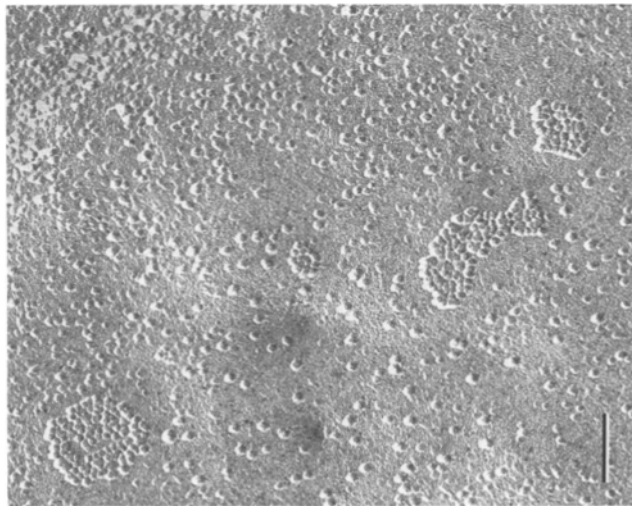


Figure 2. Freeze-fracture electron microscopy of a Novikoff cell membrane from a preparation of cells which had been reaggregated for 60 min. Four small gap junction aggregates of 9–11 nm intramembranous particles can be observed. Bar equals 100 nm. When the cells were treated with TPA during the 1 h reaggregation, no gap junction aggregates or formation plaques were observed (not shown).

Table III. Freeze-Fracture Analysis of the Effects of TPA on Gap Junction Assembly

Treatment*	Number of cell interfaces	Gap junction channels/interface [‡] (x \pm SEM)	Formation plaque area μm^2 /interface (x \pm SEM)
Control	56	670 \pm 44	0.27 \pm 0.04
4- α TPA	31	606 \pm 57	0.25 \pm 0.03
TPA	42	0	0
BFA	48	154 \pm 24	0.35 \pm 0.06
Monensin	53	160 \pm 19	0.32 \pm 0.05

* 50 nM TPA or 4- α TPA was added at the beginning of the 60-min reaggregation period. BFA (36 μM) and monensin (5 μM) were present during the recovery (2 h) and reaggregation (1 h) periods.

[‡] Average number of intramembranous particles within gap junctions on a per interface basis.

be having an effect on gap junction disassembly and/or turnover.

Effects of TPA on Gap Junction Assembly/Disassembly

TPA had a dramatic effect on net gap junction assembly. To determine the extent to which TPA was inhibiting assembly and/or increasing disassembly, we added TPA at different times during the reaggregation period and assayed dye transfer at the end of the 60-min reaggregation. If cells were reaggregated for 15 min and then had TPA added for the remaining 45 min, 6% of the interfaces showed dye transfer after the 60 min reaggregation was complete (Table IV). After 30 min of untreated reaggregation followed by 30 min with TPA present, 39% of the cells transferred dye. Freeze-fracture data also demonstrated that when gap junctions were allowed to form for 15 or 30 min prior to TPA addition, some gap junction structures were detected after completion of the 60-

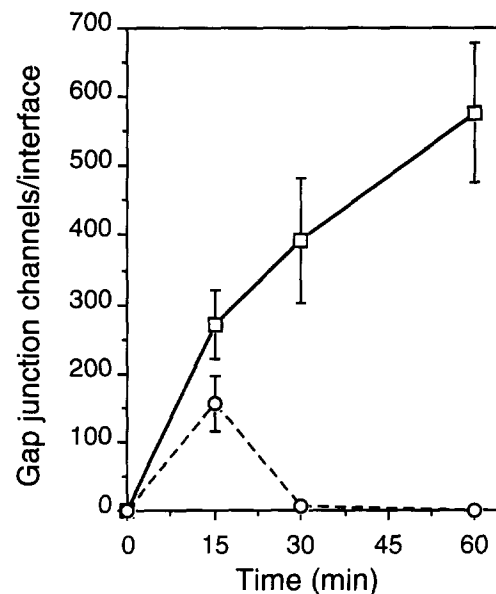


Figure 3. Time course of TPA inhibition of the assembly of gap junction channels at a cell-cell interface. Quantitative freeze-fracture electron microscopy was used to count the number of aggregated gap junction channels per interface in the absence (\square , solid line) and presence (\circ , dashed line) of 50 nM TPA during the reaggregation time indicated. Cells were fixed immediately at the times indicated. Error bars indicate the standard error of the mean.

Table IV. Effects of TPA Addition at Different Times during the Reaggregation Period

Time of 50 nM TPA addition during 60-min reaggregation	Dye transfer:		Electron microscopy: gap junction channels/interface (x \pm SEM)
	n	Percent transfer	
0	308	0	0
15	198	6.1	177 \pm 33
30	259	39	261 \pm 52
none	683	72	577 \pm 100

min reaggregation (Table IV). Therefore, addition of TPA after reaggregation has proceeded for at least 15 min did not cause complete loss of gap junction structure and function. TPA must exert its most extensive effects on assembly with much less if any effect on disassembly.

The Effects of TPA on Cx43 Phosphorylation, Protein, and mRNA Levels

Novikoff cells contain Cx43 as detected by Western, Northern, and polymerase chain reaction methods, but no mRNA has been detected for connexins 26, 31.1, 32, 33, 37, 40, or 46 (17, 27, 28, and data not shown). Several investigators have shown that one band representing Cx43 in an immunoblot is not phosphorylated and one to four bands that run at higher apparent molecular weight correspond to phosphorylated forms of Cx43. These phosphorylated species can be converted to the nonphosphorylated form upon alkaline phosphatase treatment (e.g., 5, 9, 19, 24, 34). Alkaline phosphatase treatment of Novikoff cell membrane preparations has also been previously shown to convert the higher apparent molecular weight bands corresponding to Cx43 to a single species (27).

Since phosphorylation of Cx43 has been implicated in the assembly of gap junctions (25, 36), we wanted to determine

if TPA had an effect on the phosphorylation of Cx43 in Novikoff cells. Fig. 4 A shows immunoprecipitations of Cx43 from cells metabolically labeled with [32 P]O $_4$. The first lane corresponds to an untreated control labeling and immunoprecipitation performed with the Cx43 antibody omitted (Fig. 4 A, No Ab); no band corresponding to Cx43 is observed. Both the control and 4- α TPA lane show a band at 44 kD and less distinct but significant phosphorylated species between 44 and 52 kD. Addition of TPA (50 nM) during the reaggregation period led to a reduction of label at 44 kD with a corresponding increase in the abundance of higher apparent molecular weight forms detected by densitometry. Despite these shifts, densitometric measurements of the label from 44–52 kD showed essentially the same level of overall labeling with 1 h of TPA addition as with the controls (Untreated control = 8,120; 4- α TPA = 7,520; TPA = 7,980; arbitrary units). Cx43 protein levels in the presence of TPA are considered below.

Phosphorylation was also evaluated via Western immunoblot analysis of Novikoff cell preparations NT or treated for 15, 30, or 60 min with TPA from the onset of reaggregation (Fig. 4 B). Immunoblots of Novikoff cells allowed much easier evaluation of the time course of Cx43 phosphorylation and appeared to yield more distinct bands corresponding to phosphorylated Cx43 than the 32 P immunoprecipitates. Other investigators have also observed less distinct Cx43 band patterns with 32 P immunoprecipitation compared to [35 S]methionine metabolic labeling or immunoblots (e.g., 24, 35), possibly due to the high energy of the 32 P radioisotope (35). The major Cx43 band in the untreated sample, 41 kD, did not co-migrate with a phosphorylated species while two higher molecular weight bands (44 and 48 kD) co-migrated with bands present in phosphorylated Cx43 (Fig. 4 A). In the presence of TPA, the 41-kD species appeared to be almost completely converted to the 44- and 48-kD species. Densitometric analysis (Fig. 4 C) of the different bands

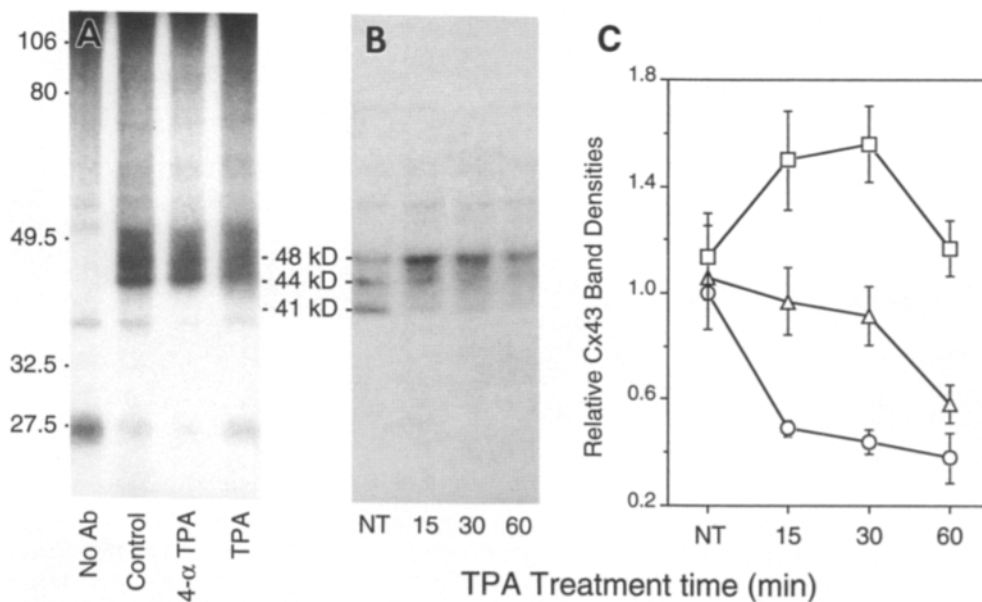


Figure 4. Immunoprecipitation and immunoblots of Cx43. An autoradiograph representing 32 P-labeled Cx43 immunoprecipitated from Novikoff cells is shown in A. Cells were metabolically labeled with [32 P]O $_4$ and were either not treated (No Ab and Control) or treated with 50 nM TPA or 50 nM of the inactive analogue 4- α TPA (as labeled) for the 1-h reaggregation period. In the No Ab lane, no Cx43 monoclonal antibody was used in the immunoprecipitation incubation. The effects of TPA on Cx43 were also investigated by western immunoblotting as shown in B. Cells were either NT or were treated with 50 nM TPA for a reaggregation period of 15, 30, or 60 min as

labeled. In C the mean Western immunoblot band density values from four separate experiments were normalized to their respective not treated, 41-kD band density values determined from densitometric scans of the 41- (○), 44- (Δ), and 48-kD (□) bands and were plotted with standard errors indicated.

from four separate experiments confirmed the loss of the nonphosphorylated species and gain of the phosphorylated 48-kD form. This result is in agreement with those of others who have documented similar changes in Cx43 phosphorylation within 5 min of TPA addition (5, 39). Summation of the densitometric values for the three species showed no significant change in Cx43 for up to 30 min of TPA treatment in four separate experiments. After 60 min of TPA treatment, the Cx43 protein levels dropped to an average of 72% of the not treated, control level. Thus, up to 30 min of treatment with 50 nM TPA did not affect Novikoff Cx43 cellular protein levels even though gap junction assembly (Fig. 3) was dramatically reduced at that time. These results may also explain why no significant increase in ^{32}P incorporation into Cx43 was detected after 60 min of TPA treatment (Fig. 4A), since a 28% loss in Cx43 would be expected. Treatment of cells with 4- α TPA for 30 min did not cause a shift in apparent molecular weight, and cells that were not allowed to reaggregate but were treated with TPA for 1 h showed a shift in the same manner as those that were reaggreated (results not shown).

Northern analysis was performed to determine if the loss of Cx43 found at 60 min of TPA treatment was due to changes in Cx43 mRNA. TPA treatment (50 nM) of Novikoff cells during reaggregation for 1 or 2 h did not significantly affect Cx43 mRNA levels (Fig. 5). Densitometric values calculated from the autoradiograph for no reaggregation, 1 h control, 1 h TPA, 2 h control, and 2 h TPA were 1,826, 2,131, 2,043, 2,310, and 2,210 (arbitrary units), respectively. Several other investigators also have observed no change in Cx43 mRNA with TPA treatment in a variety of cell types (1, 5, 9, 39).

Since there was essentially no change in Cx43 mRNA, the loss of Cx43 at 60 min of TPA treatment observed in the immunoblot may have been due to increased turnover of Cx43. Turnover was investigated in pulse-chase [^{35}S]methionine labeling experiments followed by immunoprecipitation of Cx43 (Fig. 6). In these experiments, an excess of anti-Cx43 polyclonal antibody was used for immunoprecipitation and some compression of the bands (44 and 48 kD) that corresponded to phosphorylated Cx43 was observed due to large amounts of IgG heavy chain. TPA treatment at the beginning of the chase period eliminated the nonphosphor-

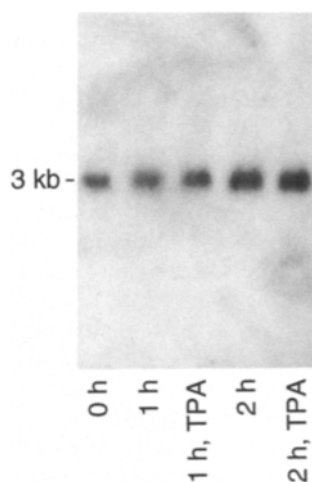


Figure 5. TPA effect on Cx43 mRNA. Cells were allowed to reaggregate for 0, 1, or 2 h with or without 50 nM TPA. Northern blots with 30 μg total RNA/lane were hybridized to a radiolabeled Cx43 clone G2 probe (see Materials and Methods). A single band, determined to be 3 kb from the position of 18S and 28S rRNA, was detected in all samples.

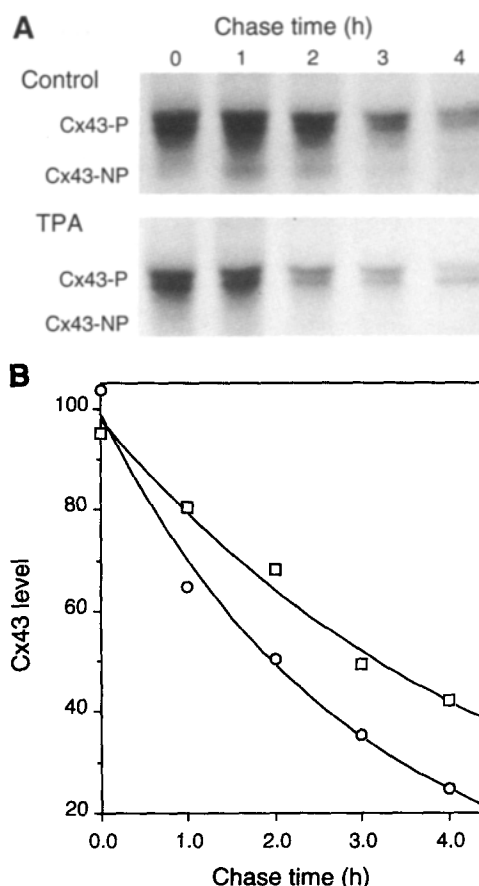


Figure 6. TPA effect on Cx43 half-life. Cx43 was immunoprecipitated from [^{35}S]methionine-labeled cells that had been chased for the time indicated. In A, an example of an autoradiograph representing Cx43 immunoprecipitated from control and 50 nM TPA-treated cells is shown. The bands that represent phosphorylated Cx43 are labeled Cx43-P and the nonphosphorylated band is labeled Cx43-NP. Note that in the TPA treated cells no NP form was found. In part B, plots of the mean relative densitometric values for total Cx43 (P + NP) in the absence (\square) and presence (\circ) of 50 nM TPA versus chase time are shown.

ylated form of Cx43 before the cells could be aliquoted, pelleted, and lysed for immunoprecipitation (~ 5 min). This result was consistent with the time course of conversion of the 41-kD band to 48 kD found in the immunoblot experiments. Mean normalized densitometric values of the total Cx43 through the 4 h chase were fit to a single exponential decay. Control pulse-chase analysis yielded a 3.1-h half-life for Cx43, while TPA treatment reduced the half-life to 2 h. TPA reduced the half-life of Cx43 in each of three separate experiments relative to the untreated controls.

Cx43 Protein Trafficking and TPA Inhibition of Gap Junction Assembly

TPA could affect the trafficking of connexins to the plasma membrane or connexin-connexin assembly within the plasma membrane. We addressed these possibilities using a cell surface biotinylation technique to determine if TPA affects the level of Cx43 in the plasma membrane. A TPA-dependent reduction in plasma membrane Cx43 could indicate that TPA

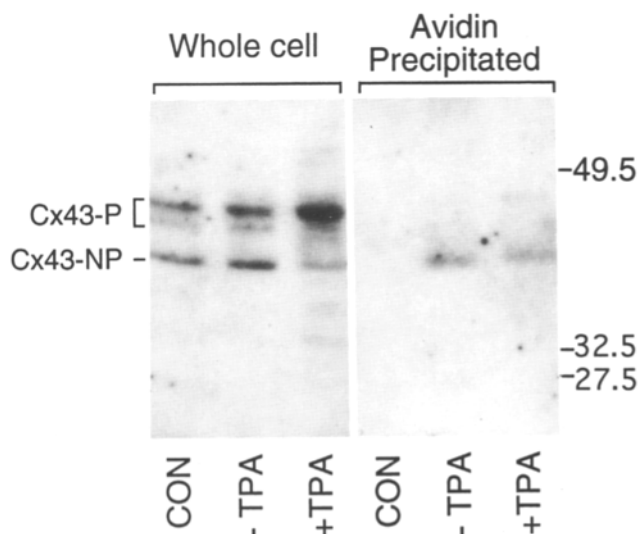


Figure 7. TPA effect on the levels of plasma membrane Cx43. After dissociation, recovery, and reaggregation, intact cells were reacted with a membrane-impermeant, biotin-linked molecule. TPA was either present (+TPA) or absent (-TPA, CON) during the reaggregation. After solubilization, whole cell portions (first three lanes) were removed and the rest was precipitated with avidin-agarose (latter three lanes). The avidin-agarose beads for the control sample (CON) were preincubated with free biotin prior to addition of the solubilized cell mixture and showed no precipitated Cx43.

acts by inhibiting Cx43 trafficking to the cell surface. Novikoff cells were dissociated, recovered and incubated for 60 min with or without TPA. The cells were then cell surface biotinylated using NHS-LC-biotin, a membrane impermeant, amine reactive molecule with biotin attached via a spacer arm, in a modification (see Materials and Methods) of a previously published technique (36) which also studied plasma membrane Cx43. After solubilization and removal of an aliquot for determination of whole cellular levels of Cx43, biotinylated Cx43 was avidin precipitated and Cx43 was quantitated in an immunoblot using Cx43 antibodies (Fig. 7). The whole cell portion of the immunoblot shows that TPA does not cause large changes in the total Cx43, but does increase the abundance of the phosphorylated forms as previously shown in Fig. 4. The avidin precipitated portion shows the phosphorylated and nonphosphorylated forms of Cx43 that were present in the plasma membrane and able to bind NHS-LC-biotin. When no TPA was present during the reaggregation of the cells (-TPA), a clearly discernable band was observed that comigrated with the nonphosphorylated form of Cx43. This preference for biotinylation of the nonphosphorylated form has been previously observed for normal rat kidney cells and likely indicated a preference for Cx43 that was in the bulk plasma membrane and not within a gap junction (36). When TPA was present (+TPA) both a nonphosphorylated and phosphorylated band were readily apparent. Densitometry from five separate biotinylation experiments showed that the sum of the amount of label in both forms for TPA treatment was 0.82 ± 0.14 of the value for untreated cells.

In the sample represented in the control lane (CON), the immobilized avidin beads were preincubated with free biotin to block the avidin binding sites; no Cx43 was discerned. An

additional control where the NHS-LC-biotin was omitted in the cell surface reaction also showed a complete lack of Cx43 (data not shown). A nonspecific, 66-kD band was observed in all samples that were precipitated with avidin beads or when a sample buffer extract of avidin beads alone (no cellular component) was probed (not shown). This band was not observed in the whole cell extracts.

Comparison of the level of Cx43 in the whole cell and avidin precipitated material indicated that 0.4–0.9% ($n = 5$) of the Cx43 molecules were biotinylated; a value which is only slightly less than the 1% efficiency obtained for biotinylation of Cx43 in NRK cells (36). These low efficiencies probably do not suggest that only 1% of the Cx43 is in the plasma membrane, but likely indicate that biotinylation is a relatively inefficient process at 4°C (36) especially since only two to three lysines are present on putative extracellular portions of Cx43.

The role of protein trafficking in TPA-dependent inhibition of gap junction assembly was also investigated using two well-known reagents that affect protein trafficking, brefeldin A (BFA) and monensin. BFA specifically inhibits protein transport between the endoplasmic reticulum and Golgi network (21). Monensin, a Na⁺ ionophore, impairs Golgi function and protein trafficking past the *trans*-Golgi network (30). Both reagents have been shown to inhibit Cx43 protein trafficking to the plasma membrane (37, 43). Dissociated cells were incubated with agitation in the presence of BFA or monensin for 2 h and then reaggregated in the presence of BFA or monensin for 1 h. While the average number of gap junction channels per interface was reduced to less than one-third of control levels in both of these treatments (Table III), some assembly still occurred. This incomplete inhibition of gap junction assembly, probably represents connexin proteins that are in the cytoplasm and have passed the sites of BFA or monensin blockage and/or that are already present in the plasma membrane. Other studies support the concept of gap junction assembly from connexin precursors present in the plasma membrane (11, 36). This plasma membrane "pool" of connexins, given time and cell–cell contact, assembled small yet functional gap junctions even in the presence of BFA or monensin. However, when TPA was present no gap junctions were observed, consistent with activity at the plasma membrane, and not protein trafficking.

Discussion

Our results with short term (<1 h) TPA treatment are consistent with those of several others who have shown little or no change in Cx43 mRNA or cellular Cx43 protein levels (1, 5, 9, 39). With no obvious effect of TPA on transcription or translation of Cx43, two likely alternative points of gap junction regulation include gap junction assembly and/or channel open time (channel gating). Although several investigators have concentrated on the latter point, differentiating between these two possibilities has proven to be very difficult to date. We approached this problem by using Novikoff cells which were either microinjected with dye to measure preexisting gap junctions or dissociated with EDTA to single cells and reaggregated to measure nascent gap junction assembly.

When the dye Lucifer yellow was microinjected into one cell of a pair of Novikoff cells which had not been subjected to EDTA-dissociation, the rate of dye passage through gap

junctions from the injected cell to the recipient cell did not significantly change if TPA was present for 1 h prior to microinjection. That is, these nondissociated cells, which have moderate levels of gap junctional communication prior to TPA addition, were unaffected by TPA. Therefore, TPA must affect Novikoff gap junctional communication by a mechanism other than channel gating.

TPA completely inhibited the assembly of functional gap junctions as measured in a dye transfer method with cells that had been dissociated and reaggregated. Freeze-fracture electron microscopy confirmed that no gap junctions or formation plaques were present after 30–60 min of TPA treatment, while only slight changes were detected in the levels of total cellular Cx43 or levels of cell surface Cx43 that could be biotinylated. The observation that similar levels of plasma membrane Cx43 can be biotinylated with and without TPA treatment can be interpreted in at least two ways. The level of Cx43 in the plasma membrane may not be significantly affected by TPA and Cx43 trafficking or turnover may not be important in the inhibition of gap junction assembly. Alternatively, TPA could reduce the Cx43 plasma membrane concentration below a threshold level necessary to form gap junctions by inhibiting Cx43 trafficking or increasing Cx43 turnover, with the true difference between the control and TPA treated level of plasma membrane Cx43 being masked by preferential biotinylation of nonjunctional Cx43 over Cx43 present within gap junction structures. Several pieces of data support the idea that Cx43 trafficking and turnover are not critical in TPA inhibition of gap junction assembly. Indeed, enough Cx43 is present after 15 min of TPA treatment to form gap junctions (Fig. 3), while evidence indicated there was no increase in protein turnover up to 30 min of TPA treatment (Figs. 4 and 6). Experiments with BFA and monensin indicated that a sizeable plasma membrane pool exists and inhibition of trafficking via these reagents can not eliminate gap junction assembly. Finally, Lucifer yellow readily transferred between TPA-treated, nondissociated cells, so a mechanism of increased gap junction disassembly or Cx43 turnover caused by TPA is unlikely.

To further explore the possibility that TPA affects the disassembly of a subset of gap junction structures, TPA was added at different times during the reaggregation process. Cells that had been reaggregated for 15 or 30 min and then had TPA added for the remainder of the 60-min reaggregation still transferred dye and had gap junctions present (Table IV). That is, 45 min in the presence of TPA is insufficient to dispose of gap junctions if they are first allowed to form for 15 min in the absence of TPA. Furthermore, the numbers of gap junction channels/interface after this 15–45 protocol were similar to those observed after 15 min of reaggregation (Fig. 3). Therefore, TPA had an immediate effect of inhibiting any additional gap junction assembly, while gap junctions formed prior to TPA treatment were stable in the presence of TPA. Normal or TPA-enhanced disassembly of gap junctions which had not been fully stabilized could contribute to the disappearance of gap junctions observed after 15 min of reaggregation in the presence of TPA (Fig. 3). The results in this study indicate that at least one stage or event that occurs after the transport of Cx43 to the plasma membrane is sensitive to TPA. These results are not necessarily in conflict with immunofluorescence studies which have documented reductions in punctate, plasma membrane im-

munolabeling of Cx43 after 30 min–2 h of TPA treatment (1, 5, 9), because dispersal of connexin molecules from a gap junction plaque to the bulk plasma membrane would decrease the extent of Cx43 punctate labeling. In any case, disassembly of stable gap junctions can not be considered a major factor in the TPA-dependent inhibition of net gap junction assembly.

Whether TPA-dependent phosphorylation of Cx43 directly affects gap junction assembly is unclear. A direct correlation is difficult to prove and is far beyond the scope of this report. A putative phosphorylation event on Cx43 has been associated with the presence of Cx43 in the gap junction versus the non-junctional plasma membrane (36). Possibly TPA-dependent phosphorylation of Cx43 negates or prevents this phosphorylation event.

Since cell–cell adhesion and adhesion molecules appear to play an important role in gap junction assembly (18, 27, 35), the possibility that protein kinase C indirectly regulates gap junction assembly via changes in cell–cell adhesion should also be considered. Cell adhesion was addressed to a limited degree in this study by counting the number of cells in Novikoff cell aggregates; TPA had no effect in this simple adhesion assay. Several reports indicate that TPA actually increases cell adhesion via molecules such as E-cadherin and members of the integrin family (14, 47, 52, 54). In addition, assembly studies with Novikoff cells reaggregated at nanomolar levels of extracellular calcium concentration where no adherens junctions should be present indicate that gap junction assembly still occurs but is reduced by approximately 60% (Lampe and Johnson, personal communication). Therefore, TPA might have to be inhibiting multiple adhesion systems in order to eliminate gap junction assembly via general effects on adhesion. This study, however, was not meant to define the role of adhesion in gap junction assembly. The goal was to define how TPA can have dramatic effects on cell communication by determining TPA-dependent changes in connexin levels, connexin cellular distribution, and gap junction assembly/disassembly rates.

In conclusion, our results with TPA treatment of Novikoff cells are consistent with a number of other reports describing decreases in cell communication under different conditions, little or no changes in Cx43 mRNA or Cx43 protein levels, and rapid phosphorylation of Cx43 to higher apparent molecular mass forms. Further, we have shown that TPA acts by inhibiting gap junction assembly. We developed quantitative assays for gap junction assembly that show that TPA completely blocked cell–cell communication on a time scale of minutes rather than hours, but only when cell communication was dependent on nascent gap junction assembly. If gap junction structures were present prior to TPA application, TPA had no effect on channel permeance for treatment times of up to 1 h. TPA caused less than a 20% reduction in the apparent level of Cx43 in the plasma membrane. Therefore, TPA must be affecting the ability of gap junctions to assemble within the plasma membrane even though Cx43 is present. Given that a rapid response of Novikoff cells and many cell types to TPA is Cx43 phosphorylation, a regulatory mechanism involving protein kinase C-mediated Cx43 phosphorylation is quite plausible. Alternatively, other events including cell–cell adhesion, may play a key role.

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