

# Properties and Regulation of Gap Junctional Hemichannels in the Plasma Membranes of Cultured Cells

Haiying Li,\* Tai-Feng Liu,\* Ahmed Lazrak,‡ Camillo Peracchia,‡ Gary S. Goldberg,§ Paul D. Lampe,\* and Ross G. Johnson\*

\*Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minnesota 55108; ‡Department of Physiology, University of Rochester, Rochester, New York 14642; and §Department of Biological Sciences, State University of New York at Buffalo, Buffalo, New York 14260

**Abstract.** During the assembly of gap junctions, a hemichannel in the plasma membrane of one cell is thought to align and dock with another in an apposed membrane to form a cell-to-cell channel. We report here on the existence and properties of nonjunctional, plasma membrane connexin43 (Cx43) hemichannels. The opening of the hemichannels was demonstrated by the cellular uptake of 5(6)-carboxyfluorescein from the culture medium when extracellular calcium levels were reduced. Dye uptake exhibited properties similar to those of gap junction channels. For example, using different dyes, the levels of uptake were correlated with molecular size: 5(6)-carboxyfluorescein (~32%), 7-hydroxycoumarin-3-carboxylic acid (~24%), fura-2 (~11%), and fluorescein-dextran (~0.4%). Octanol and heptanol also reduced dye uptake by ~50%. Detailed analysis of one clone of Novikoff cells transfected with a Cx43 antisense expression vector revealed a reduction in dye uptake levels according to uptake assays

and a corresponding decrease in intercellular dye transfer rates in microinjection experiments. In addition, a more limited decrease in membrane resistance upon reduction of extracellular calcium was detected in electrophysiological studies of antisense transfectants, in contrast to control cells. Studies of dye uptake in HeLa cells also demonstrated a large increase following transfection with Cx43. Together these observations indicate that Cx43 is responsible for the hemichannel function in these cultured cells. Similar dye uptake results were obtained with normal rat kidney (NRK) cells, which express Cx43. Dye uptake can be dramatically inhibited by 12-*O*-tetradecanoylphorbol-13-acetate-activated protein kinase C in these cell systems and by a temperature-sensitive tyrosine protein kinase, pp60<sup>v-src</sup> in LA25-NRK cells. We conclude that Cx43 hemichannels are found in the plasma membrane, where they are regulated by multiple signaling pathways, and likely represent an important stage in gap junction assembly.

**G**AP junctions are cell surface specializations that connect adjacent cells and provide cell-to-cell channels for the direct exchange of small molecules (Gilula et al., 1972; Bennett and Goodenough, 1978; Loewenstein, 1981). They are found between most animal cells and are thought to play important biological roles, which include transmitting electrical signals (Barr et al., 1965; De Mello, 1987), buffering concentrations of metabolites or regulatory molecules (Ledbetter and Lubin, 1979), affecting patterning processes during embryonic develop-

ment (Furshpan and Potter, 1968; Loewenstein, 1966; Loewenstein and Azarnia, 1988; Warner et al., 1984), and regulating cell growth (Atkinson et al., 1981; Azarnia and Loewenstein, 1984; Azarnia et al., 1988). Gap junction permeability can be modified by a variety of factors, including octanol, heptanol (Spray and Bennett, 1985; Burt and Spray, 1989), cAMP (adenosine 3':5'-cyclic monophosphate) (Bennett et al., 1991), 12-*O*-tetradecanoylphorbol-13-acetate (TPA)<sup>1</sup>-activated protein kinase C (PKC) (Reynhout et al., 1992; Lampe, 1994), tyrosine protein kinases (Atkinson et al., 1988), intracellular pH (Spray and Bennett, 1985), and low density lipoproteins (Meyer et al., 1991).

It has been demonstrated that gap junction protein subunits (connexins) are arrayed in hexamers to form hemi-

Address all correspondence to Dr. Ross G. Johnson, Department of Genetics and Cell Biology, University of Minnesota, 1445 Gortner Avenue, St. Paul, MN 55108. Tel.: (612) 624-3003. Fax: (612) 625-5754. E-mail: Gap-lab@maroon.tc.umn.edu

Tai-Feng Liu's present address is Institute of Life Science, Beijing University, Beijing, P.R. China.

Ahmed Lazrak's present address is Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831.

Paul D. Lampe's present address is Fred Hutchinson Cancer Research Center, Seattle, Washington 98104.

1. *Abbreviations used in this paper:* Cx, connexin; ESS, external salt solution; HCCA, 7-hydroxycoumarin-3-carboxylic acid; 5-nitro-BAPTA, 5-nitro-1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; NRK, normal rat kidney; PKC, protein kinase C; R<sub>m</sub>, membrane resistance; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SE, standard error.

channels (or connexons), and two apposed hemichannels interact to form an axial, aqueous channel connecting the cytoplasm of the participating cells (Makowski et al., 1977). Although much work has been done to identify different connexins and determine their tissue distribution, as well as certain physiological properties, relatively little is understood about how connexins are assembled into gap junctions. For example, one possibility is that hemichannels are inserted directly into gap junctions. Another theory, which currently has more support (Johnson et al., 1989; Musil and Goodenough, 1993), suggests that connexin proteins are assembled into hemichannels or connexons in an intracellular compartment before being inserted into the plasma membrane and distributed uniformly over the cell surface. Presumably, hemichannels are routinely prevented from opening until a hemichannel from one cell comes into contact and "docks" (or pairs) with another hemichannel from a neighboring cell. Although not yet fully characterized, the latter theory is supported by several findings related to a connexin pool in the nonjunctional, plasma membrane. It has been shown that newly synthesized connexin43 (Cx43) proteins form oligomers in the *trans*-Golgi network (Musil and Goodenough, 1993) and can then be constitutively transported to the plasma membrane in gap junction-competent cells (normal rat kidney [NRK]), as well as gap junction-deficient cells (S180 and L929 cells) (Musil et al., 1990). Cx43 localized to the nonjunctional, plasma membrane can be detected using cell surface biotinylation methods (Musil and Goodenough, 1991; Lampe, 1994).

Since hemichannels are formed in cytoplasmic membranes, and connexin proteins can be detected in the nonjunctional, plasma membrane, it is reasonable to assume that hemichannels occur in the plasma membrane. Although these hemichannels would presumably be closed to avoid direct passage of molecules between the cytoplasm and the external environment, specific conditions may allow them to open. For example, solitary horizontal cells from catfish retina display dopamine-sensitive channels, putative hemichannels, which have a much higher probability of being open when the extracellular calcium ( $\text{Ca}^{2+}$ ) concentration is reduced (DeVries and Schwarz, 1992). In addition, isolated horizontal cells from the skate retina display nonselective, voltage-gated ionic currents that can be blocked by acetate, halothane, cobalt, and octanol (Malchow et al., 1993). The above studies support the existence of nonjunctional, plasma membrane channels, which are likely gap junction hemichannels because of similarities in a number of channel properties (DeVries and Schwarz, 1992). However, these studies provide no information relative to the molecular nature of these channels. It has also been shown that *Xenopus* oocytes expressing exogenous rat Cx46 (Paul et al., 1991; Ebihara and Steiner, 1993; Trexler et al., 1996) or chick Cx56 (Ebihara et al., 1995) proteins develop novel, large, nonselective, and voltage-dependent currents that can be amplified when the extracellular  $\text{Ca}^{2+}$  concentration is reduced. It is concluded that (Cx46 and Cx56) functional hemichannels are found in the nonjunctional, plasma membrane of oocytes following the expression of exogenous connexins. Many of the properties of these hemichannels are also similar to gap junction channels (Ebihara and Steiner, 1993; Ebihara et al., 1995;

Trexler et al. 1996). A critical question is whether these hemichannels represent a normal feature of gap junction assembly or whether they result from an overexpression of connexins in the oocyte system. The fact that the endogenous Cx38 in *Xenopus* oocytes also appears to display hemichannel activity supports the notion that the presence of Cx46- and Cx56-hemichannels in this system is not an artifact of overexpression or faulty processing of foreign connexins (Ebihara, 1995).

Other nonjunctional, plasma membrane pathways without ion-selectivity and with a size limitation similar to gap junction channels have been described in mouse macrophages expressing Cx43 (Steinberg et al., 1987). These putative plasma membrane pathways, in contrast to the studies mentioned above, can be induced to open in response to extracellular ATP. Other nucleotides are ineffective, and the presence of high concentrations of extracellular divalent cations, such as  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ , inhibit the dye uptake through the pathways (Steinberg et al., 1987; Buisman et al., 1988; Beyer and Steinberg, 1991). Thus, the gating of these ATP-induced pathways is regulated differently than those mentioned above, and the precise nature of these pathways is not yet fully understood.

Here we demonstrate the existence of nonjunctional hemichannels in the plasma membranes of several well-characterized, gap junction-competent cell systems. The level of hemichannel activity correlates well with the extent of gap junction communication mediated by the endogenous Cx43 populations in both Novikoff cells and NRK cells. In addition, similar hemichannels are observed in HeLa cells transfected with Cx43. We postulate that, as an integral part of the connexin life cycle, hemichannels are found in the plasma membrane, where they represent an important stage in the gap junction assembly process. We also present methods for studying the function of hemichannels and their regulation during gap junction assembly in these cultured cells. Our data demonstrate that Cx43 is responsible for dye uptake from the medium through the hemichannels. We have also identified two regulatory pathways involving protein kinases, which influence the gating of the Cx43 hemichannels.

## Materials and Methods

### Reagents

Swim's 77 medium was purchased from Sigma Chemical Co. (St. Louis, MO). Newborn calf serum, penicillin/streptomycin, TEMED, and trypan blue were from GIBCO BRL (Grand Island, NY). 5(6)-carboxyfluorescein was from Eastman Kodak Laboratory Chemicals (Rochester, NY). Fura-2, neurobiotin (biotin ethylenediamine), fluorescein-conjugated avidin, fluorescein-dextran (molecular mass = 3,000 D), 7-hydroxycoumarin-3-carboxylic acid (HCCA), and 5-nitro-1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (5-nitro-BAPTA) were from Molecular Probes, Inc. (Eugene, OR). TPA was from LC Laboratories (Woburn, MA). Cell-Tak was from Collaborative Biomedical Products (Bedford, MA). All radioactive reagents were purchased from ICN Biomedicals (Costa Mesa, CA). The mix of SDS-PAGE protein standards was from Boehringer Mannheim Biochemicals (Indianapolis, IN). The remaining chemicals were obtained from Sigma Chemical Co. unless otherwise indicated.

### Cell Culture

The Novikoff hepatoma cell line (N1S1-67) was grown in complete Swim's

77 medium containing 10% newborn calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. NRK cells and LA25-NRK cells (infected with an avian sarcoma virus which is temperature-sensitive for the tyrosine kinase activity of pp60<sup>src</sup>, kindly provided by Dr. Michael Atkinson, University of Minnesota, St. Paul, MN) were grown in DMEM containing 2 mM L-glutamine and 10% iron-supplemental calf serum (Hyclone Labs, Logan, UT). The MDA MB231 cell line (kindly provided by Dr. David Kiang, University of Minnesota) was grown in MEM with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. The HeLa parental and Cx43-transfected HeLa cells (kindly provided by Dr. Klaus Willecke, University of Bonn, Bonn, Germany) were grown in DMEM with 10% fetal calf serum, and 0.5 mg/ml G418 was included in the Cx43-transfectant medium.

### Novikoff Cell Dissociation/Recovery and Dye Uptake Assay

Cells in logarithmic growth were centrifuged and resuspended to  $5 \times 10^5$  cells/ml in Swim's 77 medium (containing 2 mM L-glutamine) with 10 mM EDTA to facilitate dissociation. Cells were then 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 for 90 min at 37°C on a gyratory shaker at 200 rpm in Swim's 77 medium with 2 mM L-glutamine and 5% BSA, which eliminated remnants of previously existing junctions (Preus et al., 1981a,b). Cells were then washed through centrifugation/resuspension in Swim's 77 medium with no added serum or BSA. Cells were finally resuspended in Swim's 77 medium at normal or reduced external calcium concentrations for dye uptake assays. To reduce calcium, pelleted cells were routinely resuspended in Swim's 77 medium with 5 mM EGTA, although in some experiments, a series of EGTA concentrations was studied (see Results). All of these media contained 2 mg/ml of the membrane-impermeant dye, 5(6)-carboxyfluorescein (molecular mass 376 D), unless otherwise noted. After a 15-min incubation at 4°C and pH 8.0, cells were washed twice with Swim's 77 medium by centrifugation and resuspension. Cells were then viewed under a fluorescent microscope to monitor the uptake of 5(6)-carboxyfluorescein. Fields containing a total of approximately 250 cells were chosen randomly for each individual experiment and the percentage of cells that took up the dye was obtained by calculating the number of fluorescent cells divided by the total number of cells observed under phase contrast.

### Experimental Treatments for Novikoff Cells

**Varied Conditions.** Single Novikoff cells were tested for dye uptake under varied conditions to examine different parameters which could influence the assay. This included a range of cell concentrations ( $0.5 \times 10^6$ – $2 \times 10^6$  cells/ml), a range of dye uptake times (1–30 min), a series of different external pH values (6.5–8.5), and two temperatures (4 and 37°C). For only the temperature experiments, Lucifer yellow CH was substituted for 5(6)-carboxyfluorescein since it yielded lower backgrounds at 37°C.

**Different Alcohols.** Dissociated Novikoff cells were recovered at 37°C in the presence of 1 mM octanol, heptanol, or hexanol for 0, 15, 30, 60, or 90 min before dye uptake assays. Dissociated/recovered single Novikoff cells were also treated with 1 mM octanol or heptanol for 15 min and later washed with Swim's 77 medium before the dye uptake assays.

**Calcium Dependency.** Single Novikoff cells were tested for dye uptake in Swim's 77 medium prepared without Mg<sup>2+</sup> or Ca<sup>2+</sup> by treating with 5(6)-carboxyfluorescein in the presence or absence of 1.8 mM Ca<sup>2+</sup> and 0.83 mM Mg<sup>2+</sup>. Dye uptake was also tested in the presence of 1.8 mM Sr<sup>2+</sup> and 0.83 mM Mg<sup>2+</sup>, but in the absence of Ca<sup>2+</sup>. Single Novikoff cells were also tested for dye uptake in the presence of different concentrations of extracellular Ca<sup>2+</sup> obtained with different concentrations of EGTA or 5-nitro-BAPTA chelation. The final extracellular Ca<sup>2+</sup> concentrations following chelation by EGTA or 5-nitro-BAPTA were calculated with computer programs "Bound and Determined" or "Maxchelator," both of which calculate the concentrations of free Ca<sup>2+</sup> in a particular medium based on the interactions between various chemicals.

**Nucleotide Treatments.** Single Novikoff cells were tested for dye uptake in the presence or absence of 5 mM adenosine, AMP, ADP, ATP, GTP, or 5 mM ATP + 5 mM EGTA. The free extracellular Ca<sup>2+</sup> concentrations after the chelation by the above nucleotides were obtained with the "Bound and Determined" program.

**Different Sized Dyes.** Single Novikoff cells were tested in the presence or absence of 5 mM EGTA for dye uptake by 5(6)-carboxyfluorescein (5.3 mM; 1.2 mM when uptake was tested to compare with fura-2), neuro-

biotin (3.75 µM), fura-2 (1.2 mM), HCCA (1.2 mM), fluorescein-dextran, molecular mass = 3,000 D (3 mM), or trypan blue (0.83 mM).

**TPA Treatments.** Before dye uptake assays, dissociated Novikoff cells were recovered at 37°C in the presence of the indicated concentrations: 5, 10, 25, 50, 75, or 100 ng/ml of the 4β isoform of TPA or the 4α isoform of TPA at 100 ng/ml.

### Dye Uptake Assays For NRK, LA25-NRK, MDA MB231, and HeLa Cells

NRK, LA25-NRK, MDA MB231, or HeLa cells were plated onto 35-mm culture dishes and grown at 37°C overnight in a CO<sub>2</sub> incubator. Cells to be used for dye uptake assays were plated at relatively low densities so that by the next day, single cells would predominate. Overnight-grown LA25-NRK were shifted to a 35 or a 39°C incubator 1 h before being subjected to the dye uptake assays. The growth medium was removed and the cells were washed with DMEM (for NRK, LA25-NRK, and HeLa cells) or MEM (for MDA MB231 cells). Cells were then treated with or without 5 mM EGTA in dishes containing 2 mg/ml 5(6)-carboxyfluorescein at 4°C and pH 8.0 for 15 min. Cells were washed with the corresponding medium containing normal calcium (to close hemichannels) and viewed under a fluorescent microscope to determine the number of fluorescent cells as a measure of dye uptake.

### Antisense Transfection and Cloning

$12 \times 10^6$  Novikoff cells were pelleted and resuspended in 4.8 ml of standard medium and placed into 6-well culture plates at  $4 \times 10^6$  cells/1.6 ml per well. 4 µg of filter-sterilized plasmid DNA in 200 µl medium was mixed with 20 µl lipofectin/180 µl medium. The above mixture was added to Novikoff cells for 5 h and cells were then transferred to 10 ml of growth medium and grown in flasks overnight. The transfected plasmids were constructed with the entire Cx43 coding sequence in antisense orientation subcloned into a pRc/CMV vector. The construct conferred neomycin resistance, and expression of the insert was driven by the human cytomegalovirus promoter (Goldberg et al., 1994). Selection of stable transfectants was achieved by incubation with 600 µg/ml geneticin. Cells were then cloned using a series dilution method and maintained in medium containing geneticin.

### Western Immunoblot Analysis

SDS-PAGE was performed on 10% polyacrylamide gels following published methods (Laemmli, 1970). Novikoff cells were pelleted and washed once in cold PBS and solubilized in Laemmli sample buffer containing 2 mM EDTA, 50 mM NaF, 50 µM NaVO<sub>4</sub>, and protease inhibitors (1 µg/ml pepstatin A, 1 µg/ml leupeptin, 10 µg/ml aprotinin, and 2 mM PMSF). Protein transfer to derivatized paper was performed as previously described (Keeling et al., 1983; Lampe and Johnson, 1990), and Cx43 was detected using primary antibodies (HA12 rabbit polyclonal antibodies against the NH<sub>2</sub> terminus of Cx43, kindly provided by Dr. Barbara Yancey, Caltech, Pasadena, CA) and secondary <sup>125</sup>I-conjugated goat anti-rabbit antibodies followed by autoradiography (a 2% milk solution was used to block non-specific binding). The levels of Cx43 protein expression in different antisense clones were assessed by comparing the intensity of Cx43 bands on Western immunoblots after normalizing for protein loading.

### Microinjection of Dye and Measurement of Transfer

Microinjection was carried out as previously described (Biegon et al., 1987). 4% Lucifer yellow CH was microinjected into one cell of a Novikoff cell pair. Dye transfer rates were measured by calculating the fluorescence intensity difference between an injected cell and its recipient over time. Calculating the logarithm of this difference over time produces a linear relationship, the slope of which is proportional to the permeance of the cell-cell interface (Atkinson et al., 1988).

### Electrophysiological Analysis

For electrophysiological measurements, 10 ml of lightly trypsinized Novikoff cells suspended in growth medium were centrifuged and diluted in 10 ml of an external salt solution (ESS) of the following composition: 125 mM NaCl, 2.7 mM KCl, 1.9 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 15 mM TEA, 5.5 mM glucose, 10 mM Hepes, and adjusted to pH 7.2 at 22.5°C (Lazrak and Peracchia, 1993). A few drops of the cell suspension were added to the ex-

perimental chamber containing 0.5 ml of ESS. The cells were continuously superfused at 0.3 ml/min ( $T = 22 \pm 1.5^\circ\text{C}$ ). All the electrophysiological experiments were performed using a standard whole-cell patch clamp procedure (Hamill et al., 1981). The patch pipettes were made from capillary tubing (Kimax-51) with a vertical puller (700B; David Kopf Instr., Tujunga, CA). The pipettes displayed resistances which ranged from 3–6 M $\Omega$  when filled with an internal salt solution (ISS) of the following ionic composition: 6 mM NaCl, 120 mM KCl, 0.5 mM MgCl<sub>2</sub>, 15 mM CsCl, 3 mM ATP, 10 mM Hepes at pH 7.2. The pipette was connected to a patch clamp amplifier (model EPC-7, List Electronics, distributed by Medical Systems Corp., Greenvale, NY) and lowered onto the cells using a hydraulic micromanipulator (Narishige, distributed by Medical Systems Corp., Greenvale, NY). The steps involved in the gigaseal and whole-cell patch formation were followed on an oscilloscope screen (model V-355; Hitachi Denshi America, Woodbury, NY). Pulse generation and data acquisition were performed by means of an IBM-AT compatible computer equipped with P-clamp software (Axon Instruments, Inc., Foster City, CA) and an A/D-D/A interface (model Labmaster-TL1; Axon Instruments, Inc.).

### Statistical Analysis of the Data

To obtain the percentage of dye uptake, ~250 cells were chosen randomly for each treatment in a single experiment. Each experiment was repeated a minimum of three times. The total number of cells was counted under phase contrast conditions, while the number of fluorescent cells was counted with fluorescent optics. Data from similar treatments were combined and are presented as the mean  $\pm$  standard error (SE). A *t*-test was applied when the percentages of dye uptake from different treatments were compared, and mean values were considered significantly different when  $P < 0.05$ .

## Results

### Dye Uptake Assays

To examine the possible existence of hemichannels in Novikoff hepatoma cells, cells were treated with reduced Ca<sup>2+</sup>. Uptake of 5(6)-carboxyfluorescein from the medium was used as a measure of hemichannel opening. In a typical dye uptake assay, a substantial percentage (40–50%) of cells took up the dye when the extracellular calcium concentration was reduced by chelation with EGTA (Fig. 1, *B* and *D*), as compared to approximately 5% of the cells in the presence of normal extracellular Ca<sup>2+</sup> (Fig. 1, *A* and *C*).

A series of experiments was carried out to determine whether certain parameters might influence dye uptake levels. No difference in dye uptake is detected when treatments are done with cell densities ranging from  $0.5 \times 10^6$ – $2 \times 10^6$  cells/ml of reaction solution ( $n = 1522$  cells). Dye uptake levels are comparable when the pH of the medium ranges from 6.5–8.5 ( $n = 2664$  cells). The dye uptake process is rapid because a significant percentage of cells took up dye after only 1 min of reaction ( $44.5 \pm 0.32\%$ ,  $n = 721$  cells). Initial experiments were performed at 4°C to minimize the involvement of changes during the course of the labeling. Significant dye uptake occurred at 4°C in the presence of reduced Ca<sup>2+</sup> (Fig. 1), with little dye uptake in the presence of normal extracellular Ca<sup>2+</sup>. However, due to the extensive endocytosis of 5(6)-carboxyfluorescein at room temperature as well as 37°C, and due to additional diffuse labeling of cells, all of the cells tested for dye uptake at these elevated temperatures were at least faintly labeled with 5(6)-carboxyfluorescein at both control and reduced Ca<sup>2+</sup> concentrations (data not shown). Since the plasma membranes of many cells are less permeable to Lucifer yellow CH than to 5(6)-carboxyfluorescein, exper-

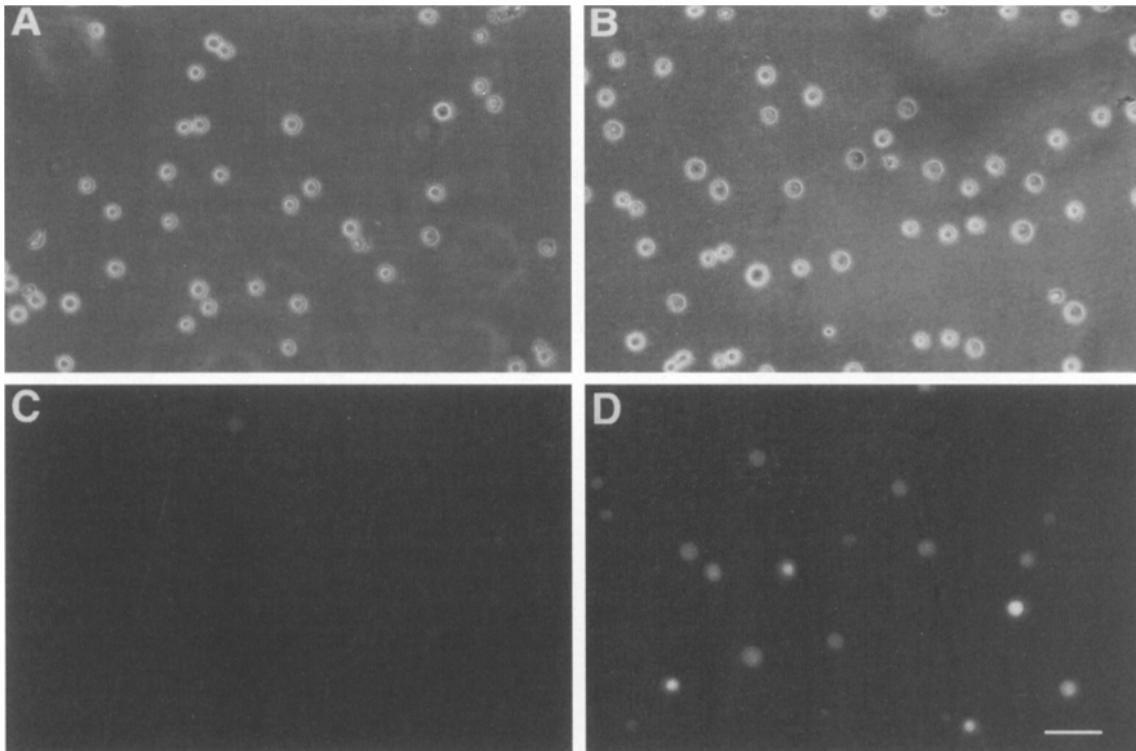
iments were carried out with Lucifer yellow CH to determine whether uptake levels with this dye in control cells were comparable at 4 and 37°C. Dye uptake assays performed with 1 mg/ml Lucifer yellow CH, where uptake could be clearly distinguished from endocytosis at 37°C, demonstrated similar uptake levels at 4 and 37°C ( $n = 2457$  cells). Thus, 5(6)-carboxyfluorescein was routinely used for all experiments at 4°C since it is much less expensive. However, Lucifer yellow CH was used and is recommended for studies at 37°C. To summarize, the dye uptake process is independent of cell labeling density within the range tested, medium pH, and incubation temperature and occurs over a time course of minutes. All the subsequent dye uptake assays reported here were done with 5 mM EGTA and 2 mg/ml 5(6)-carboxyfluorescein, at 4°C and pH 8.0, for 15 min unless otherwise specified.

### Induction of Dye Uptake by Reduced Extracellular Calcium

To determine whether dye uptake levels vary with different extracellular Ca<sup>2+</sup> concentrations, cells were incubated in media with different concentrations of free Ca<sup>2+</sup>, ranging from  $\sim 10^{-7}$ –1.8 mM. The dye uptake levels are inversely proportional to the varied extracellular Ca<sup>2+</sup> concentrations with the maximal levels obtained at approximately 10  $\mu\text{M}$  Ca<sup>2+</sup> or lower (Fig. 2 *A*). To confirm these data, 5-nitro-BAPTA, a Ca<sup>2+</sup> chelator that has a lower affinity for Ca<sup>2+</sup> than EGTA, and therefore buffers Ca<sup>2+</sup> more accurately at higher Ca<sup>2+</sup> concentrations, was also used to induce dye uptake (Fig. 2 *B*). Note that the results utilizing 5-nitro-BAPTA agree with those obtained using EGTA, e.g., the maximal dye uptake occurred at approximately 10  $\mu\text{M}$  extracellular Ca<sup>2+</sup>.

Since Mg<sup>2+</sup> is another important regulatory cation potentially acting upon the hemichannels, its effect on the dye uptake process was compared to that of reduced Ca<sup>2+</sup>. In Swim's 77 medium prepared without Mg<sup>2+</sup> or Ca<sup>2+</sup>, cells were tested for dye uptake by adding back 1.8 mM Ca<sup>2+</sup> and/or 0.83 mM Mg<sup>2+</sup>, which are the levels of these ions found in the control medium. In the presence of Ca<sup>2+</sup>, the lack of Mg<sup>2+</sup> does not induce dye uptake, while the lack of Ca<sup>2+</sup> in the presence of Mg<sup>2+</sup> results in significant dye uptake (Fig. 2 *C*). As a control, cells with both added Ca<sup>2+</sup> and Mg<sup>2+</sup> were examined and found to display levels of dye uptake equivalent to Swim's 77 medium with normal Ca<sup>2+</sup> and Mg<sup>2+</sup> concentrations, while cells without either Ca<sup>2+</sup> or Mg<sup>2+</sup> display dye uptake levels similar to those treated with EGTA (Fig. 2 *C*). These results suggest that the extracellular Mg<sup>2+</sup> has no effect on the induction of dye uptake. Sr<sup>2+</sup>, which is known to substitute effectively for Ca<sup>2+</sup>, was also tested and was found to prevent dye uptake in the absence of Ca<sup>2+</sup> (Fig. 2 *C*). The above results support the theory that the reduction of extracellular Ca<sup>2+</sup> is necessary and sufficient to induce dye uptake.

Although previous reports in the mouse macrophage system suggest that ATP<sup>4-</sup> binds to a purinergic receptor and induces putative pathways for dye uptake, 5 mM ATP chelates Ca<sup>2+</sup> in Swim's 77 medium down to 10  $\mu\text{M}$ , the free extracellular Ca<sup>2+</sup> concentration that leads to maximal dye uptake levels. GTP chelates Ca<sup>2+</sup> in a manner similar to ATP but would not induce dye uptake if a puri-



**Figure 1.** Dye uptake assay. Novikoff cells in logarithmic growth were dissociated/recovered into single, intact, and gap junction–competent cells. Uptake of 5(6)-carboxyfluorescein from the extracellular medium was tested in the presence or absence of 5 mM EGTA at 4°C and pH 8.0 for 15 min. The opening of putative plasma membrane hemichannels was monitored by calculating the percentage of fluorescent cells. This figure represents a typical dye uptake experiment showing cells under both phase contrast and fluorescent illumination. (A) Phase contrast of control cells, (B) phase contrast of EGTA-treated cells, (C) fluorescent view of control cells, and (D) fluorescent view of EGTA-treated cells. Approximately 40–50% of the Novikoff cells take up detectable levels of the dye in the presence of 5 mM EGTA, compared to approximately 5% of the cells in the absence of EGTA. Bar, 50  $\mu$ m.

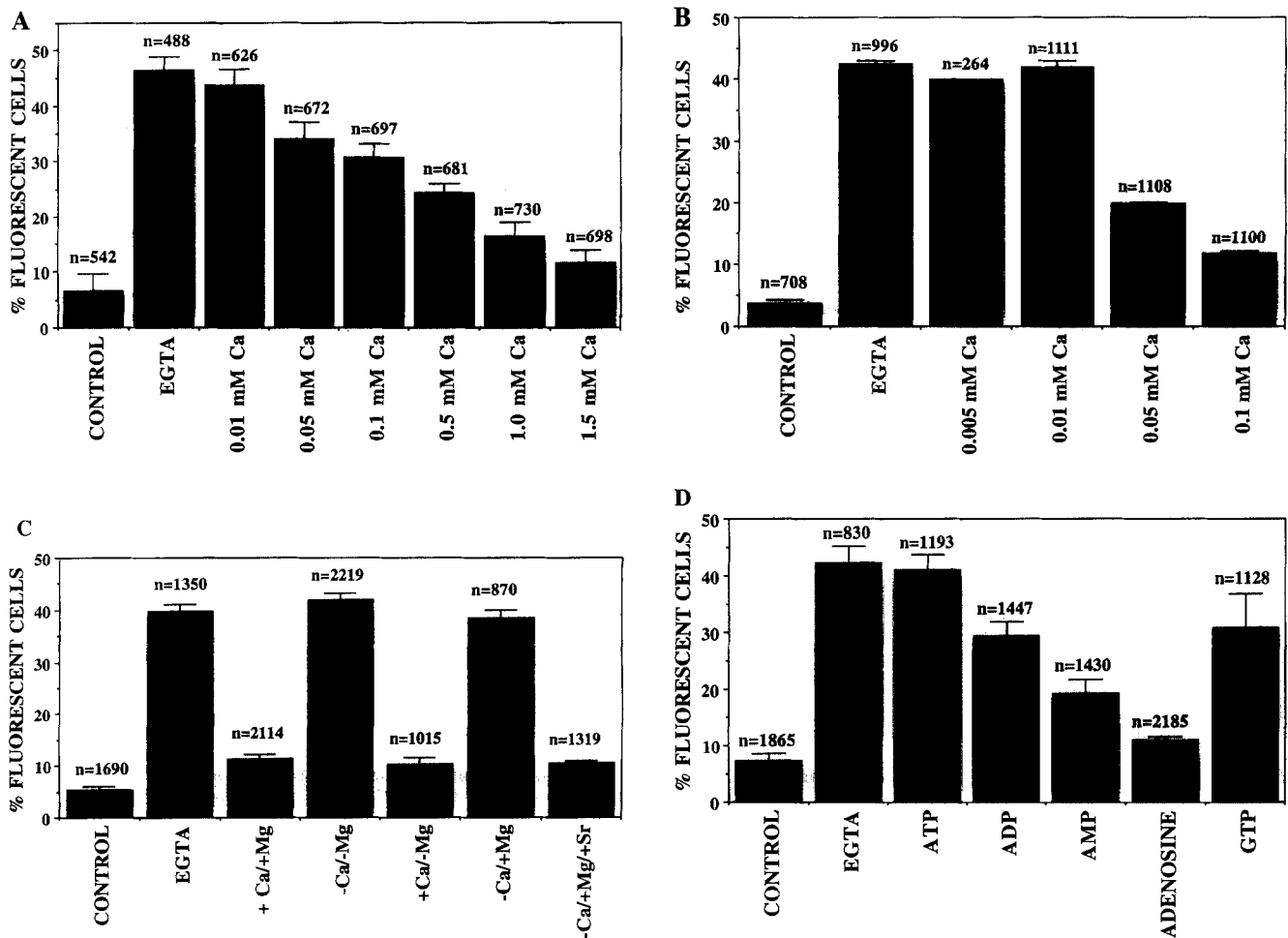
nergic receptor specific for ATP was involved. To explore the effects of various nucleotides on dye uptake in the Novikoff system, cells were tested for dye uptake in the presence or absence of 5 mM adenosine, AMP, ADP, ATP, and GTP. The data show that cells treated with ATP or GTP display similar dye uptake levels, while ADP, AMP, and adenosine induce respectively lower levels of dye uptake, likely due to their lower efficiencies in terms of chelating  $\text{Ca}^{2+}$  (Fig. 2 D). These results suggest that, in the Novikoff system, nucleotides induce dye uptake by chelating  $\text{Ca}^{2+}$ , independent of the involvement of a purinergic receptor. That dye uptake occurs with incubation times as short as 1 min and at temperatures as low as 4°C also argues against an involvement of the purinergic receptor pathway in hemichannel regulation in Novikoff cells.

#### **Maintenance of Membrane Integrity during the Dye Uptake Process**

The following observations strongly argue against the possibility that Novikoff cells take up dye because of membrane damage caused by reduction of extracellular  $\text{Ca}^{2+}$ . First, the growth properties of Novikoff cells subjected to the dye-loading procedure with EGTA were compared to those of the control cells. No differences were found (e.g., 1.0 doubling in 12 h versus 1.05 doubling in 12 h), which minimized the possibility of significant, long-term damage

caused by EGTA chelation. Secondly, EGTA-treated cell membranes are impermeant to trypan blue (data not shown), a dye used to test for cell viability and membrane damage. Thirdly, cells loaded with Lucifer yellow CH in the presence of reduced extracellular  $\text{Ca}^{2+}$  did not show dye leakage after being washed with normal  $\text{Ca}^{2+}$ -containing medium. This analysis with a SIT-camera and computer-based system for monitoring dye intensity (Biegon et al., 1987) demonstrated the same level of membrane integrity typically seen in control cells (data not shown). Finally, experiments were also done to ask whether cells treated with EGTA twice have higher dye uptake levels due to membrane damage compared to the cells treated with EGTA only once. The results demonstrate no difference between these two groups of cells (data not shown), which again argues against EGTA-damaged cell membranes during the dye uptake process.

However, the strongest support for a functional attribute underlying the dye uptake process, in contrast to membrane damage, was obtained from experiments in which dye uptake was blocked by a PKC activator, TPA at 100 ng/ml (this refers to the active isoform of TPA, TPA-4 $\beta$ ). TPA affects gap junctions dramatically in a variety of cell systems (Reynhout et al., 1992; Lampe, 1994). When EGTA is used to reduce extracellular  $\text{Ca}^{2+}$ , incubation of cells with TPA almost completely inhibits dye uptake, while the inactive isoform (TPA-4 $\alpha$ ) has no effect on dye



**Figure 2.** Effects of different cations and nucleotides on the dye uptake levels. (A) Individual Novikoff cells were tested for dye uptake under different extracellular calcium concentrations using a range of EGTA levels at 4°C and pH 8.0 for 15 min. Note that dye uptake levels varied corresponding to the extracellular Ca<sup>2+</sup> concentrations, obtained with varying EGTA levels. In this and all subsequent figures, “EGTA” refers to 5 mM EGTA in the medium. (B) Individual Novikoff cells were tested for dye uptake under a range of 5-nitro-BAPTA concentrations, which resulted in different extracellular Ca<sup>2+</sup> concentrations. (C) Individual Novikoff cells were tested for dye uptake in specially prepared media with or without 1.8 mM Ca<sup>2+</sup>, 0.83 mM Mg<sup>2+</sup>, or 1.8 mM Sr<sup>2+</sup> at 4°C and pH 8.0 for 15 min. Note that dye uptake was induced by the reduction of the extracellular Ca<sup>2+</sup>, but not Mg<sup>2+</sup>. Sr<sup>2+</sup> effectively blocked dye uptake in the absence of extracellular Ca<sup>2+</sup>. (D) Individual Novikoff cells were tested for dye uptake induced by 5 mM adenosine, AMP, ADP, ATP, or GTP at 4°C and pH 8.0 for 15 min. Note that the dye uptake levels induced by these nucleotides corresponded to their Ca<sup>2+</sup>-chelating abilities.

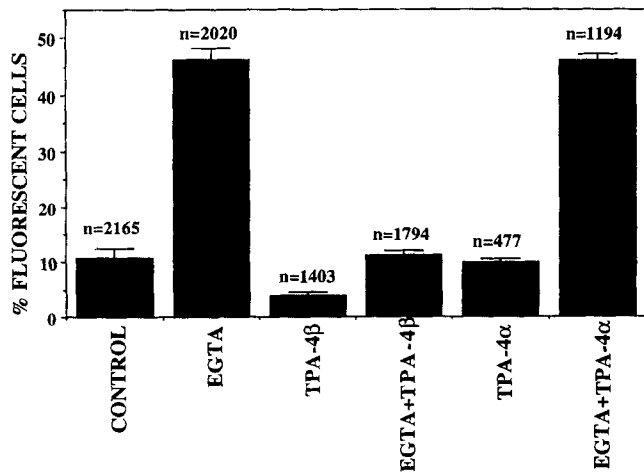
uptake (Fig. 3). The inhibition of dye uptake by TPA-4β is dosage-dependent over a range of concentrations from 5–100 ng/ml, with 100 ng/ml being most effective (data not shown). If cell membranes were permeabilized by EGTA treatment, it would be highly unlikely that TPA-4β could reverse this process while TPA-4α could not. Instead, it is likely that TPA-4β acts through regulation of a specific dye uptake pathway, e.g., a connexin hemichannel.

### Characteristics of the Putative Hemichannels

Octanol and heptanol have been shown to inhibit gap junction communication in a variety of systems. In previous experiments with the Novikoff cells, transfer of microinjected Lucifer yellow CH between cells was thoroughly blocked after a 15-min incubation in 1 mM octanol (Lazrak and Peracchia, 1993). When cells were tested for dye uptake with EGTA in the presence of octanol or heptanol,

the dye uptake levels were reduced by 50% (Fig. 4). Since the incomplete inhibition was somewhat unexpected, several different experiments were performed. Incubating cells in 1 or 2 mM octanol for 15, 30, 60, and 90 min at 37°C before EGTA treatment also demonstrated a similar reduction in dye uptake levels, indicating that the partial inhibition was not due to an insufficient treatment period or octanol concentration (data not shown). This effect was seen in reactions done at both 4 and 37°C. Dye uptake levels were also examined in cells pretreated with octanol or heptanol but then washed to remove the alcohol before EGTA and dye treatment. No inhibition was observed, suggesting that the effects of octanol or heptanol are fully reversible (Fig. 4). In contrast, cells treated with 1, 2, and 3 mM hexanol, an alcohol not affecting gap junctions, showed no inhibition of dye uptake (Fig. 4).

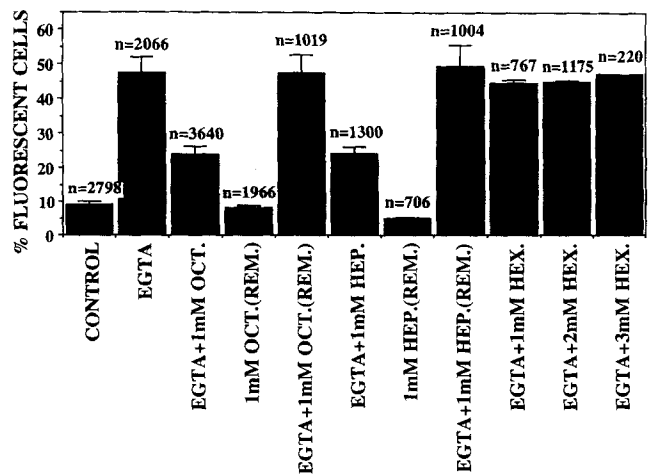
Since gap junction communication is inhibited dramatically following the reduction of intracellular pH with



**Figure 3.** Effects of TPA-4 $\alpha$  and TPA-4 $\beta$  on the dye uptake levels. Cells were dissociated and then recovered in the presence of TPA-4 $\alpha$  or TPA-4 $\beta$  for 1 h at 37°C. Dye uptake was tested at 4°C and pH 8.0 for 15 min in the presence or absence of TPA-4 $\alpha$  or TPA-4 $\beta$ , alone or together with 5 mM EGTA. Note that TPA-4 $\beta$ , but not TPA-4 $\alpha$ , inhibited dye uptake dramatically.

100% CO<sub>2</sub> perfusion (Spray and Bennett, 1985), its effect on dye uptake was also examined. Dye uptake levels obtained after CO<sub>2</sub> perfusion were significantly reduced (data not shown).

The molecules that can pass through gap junctions vary widely but display a size limitation of about 1000 D (Schwarzmann et al., 1981). To explore further the properties of the hemichannels, permeability was assayed with dyes of different molecular weights. Since the uptake levels as well as the efficiency of detecting a particular dye can be influenced by a variety of factors, e.g., concentration, size, extinction coefficient, etc., it is important to consider these factors when comparing the levels of uptake with different dyes. At the same molar concentration (1.2 mM), 5(6)-carboxyfluorescein (molecular mass = 376 D) displayed higher uptake ( $32.5 \pm 1.2\%$ ,  $n = 1269$  cells) than fura-2 (molecular mass = 832 D,  $10.8 \pm 0.6\%$ ,  $n = 1198$  cells). Since the extinction coefficient of 5(6)-carboxyfluorescein is higher ( $70 \times 10^{-3} \text{ M}^{-1}\text{cm}^{-1}$ ) than that of fura-2 ( $27 \times 10^{-3} \text{ M}^{-1}\text{cm}^{-1}$ ), HCCA, which has a more comparable extinction coefficient ( $32 \times 10^{-3} \text{ M}^{-1}\text{cm}^{-1}$ ) to that of fura-2, was tested for dye uptake. At the same molar concentration mentioned above, HCCA (molecular mass = 206 D) displayed higher uptake levels ( $24.3 \pm 1.2\%$ ,  $n = 2625$  cells) than fura-2. Since HCCA and fura-2 are both negatively charged, the above results suggest that uptake levels correlate with the relative molecular sizes of the dyes. Barely detectable levels ( $0.42 \pm 0.14\%$ ,  $n = 1782$  cells) of fluorescein-dextran (molecular mass = 3,000 D) were obtained with the Novikoff cells, likely due to a very low level of damaged cells, suggesting that this dextran is beyond the size limit of the hemichannels. All of the above dyes are membrane impermeant in the presence of normal Ca<sup>2+</sup> levels. The fact that smaller dyes pass more readily through the putative hemichannels than the larger ones and that the upper limit of the dyes appeared to be over 832 D (fura-2) and less than 3,000 D (fluorescein-dextran) indicates that the relative size of a putative hemichannel is



**Figure 4.** Effects of octanol, heptanol, and hexanol on the dye uptake levels. Individual Novikoff cells were tested for dye uptake at 4°C and pH 8.0 for 15 min in the presence or absence of the reagents indicated. The results were compared to dye uptake levels induced with 5 mM EGTA after removal (by washing) of octanol or heptanol (OCT., octanol; HEP., heptanol; HEX., hexanol; REM., removal). Note that octanol and heptanol reduced dye uptake levels reversibly by 50%, while hexanol demonstrated no effects on dye uptake.

similar to that of a gap junction channel. Studies with neurobiotin and trypan blue further supported these conclusions (data not shown).

#### **Effects of Cx43 Antisense Transfection on the Putative Hemichannels**

All of the above data support the theory that gap junction hemichannels are responsible for dye uptake. Since Cx43 is the only detectable connexin in the Novikoff system (Lampe et al., submitted), transfecting a Cx43 antisense expression vector into this system should result in a comparable reduction in gap junction permeability and hemichannel activity. Several stable antisense transfectants were obtained through cloning. Immunoblot analyses performed with Cx43 antibodies revealed the conventional series of three Cx43 bands (data not shown), migrating with apparent molecular weights of  $\sim 43$  kD (Musil and Goodenough, 1991; Berthoud et al., 1992; Lampe, 1994). One clone, AA20, expressing the lowest level of Cx43, was selected for additional studies.

Gap junction permeability was compared between clone AA20 cells and control, nontransfected cells, as described in Materials and Methods. The distribution of dye permeance values in nontransfected cells was oppositely skewed and significantly different from that for Cx43 antisense-transfected cells (Table I). It is important to note that 99% of the control Novikoff cell pairs transfer dye, as opposed to less than 50% of the antisense-transfected cells ( $n = 84$ ). Electrophysiological data on clone AA20 further support the reduction in communication with antisense transfection. Cx43 antisense-transfected cell pairs are coupled by gap junctions with a mean initial conductance of  $9.24 \pm 4.73$  nS ( $n = 22$ ), while in nontransfected cell pairs, the junctional conductance is  $53.55 \pm 17.62$  nS ( $n = 84$ ).

**Table I. Comparison of Dye Transfer Between Nontransfected Cell Pairs and Cx43 Antisense-Transfected Cell Pairs**

Slope of transfer $\times 10^{-3}$ *	Nontransfected cells	Antisense-transfected cells
	(Number of pairs)	(Number of Pairs)
0	1	46
< -1.0 to -1.9	11	23
-2.0 to -3.9	16	7
-4.0 to -5.9	12	3
-6.0 to -7.9	7	2
-8.0 to -9.9	7	1
-10.0 to > -11.0	30	2
	Total 84	Total 84

\*The larger negative numbers correspond to more rapid dye transfer.

(Lazrak and Paracchia, 1993). These results illustrate that transfection with Cx43 antisense, as expected, leads to decreased gap junction communication in Novikoff cells.

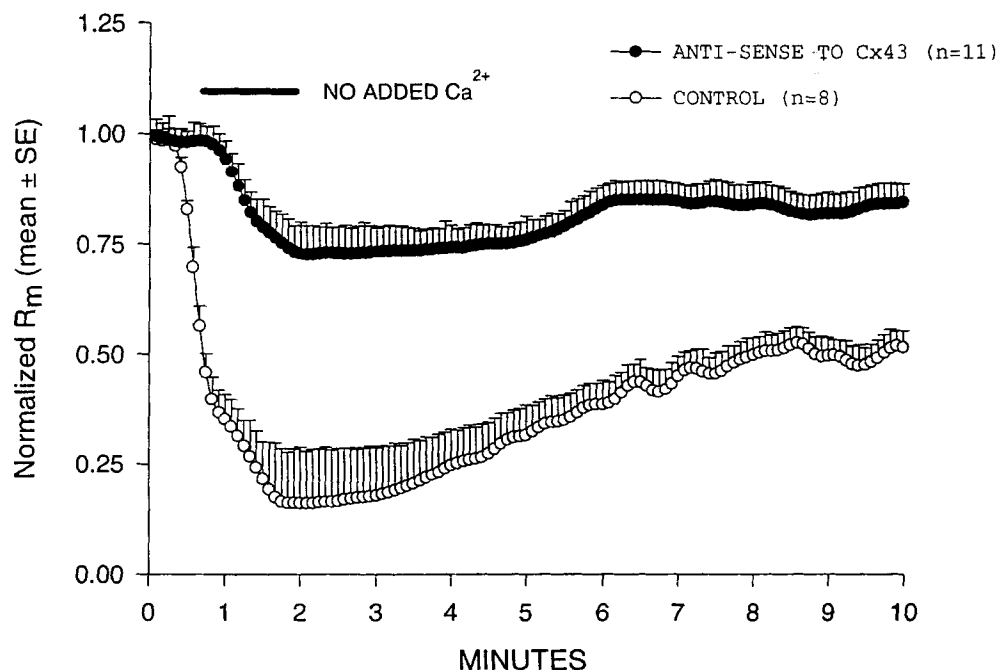
It was reasoned that with the effect of antisense on junctional communication, this treatment was also likely to alter the function of hemichannels. To study these possible effects, dye uptake levels were compared between nontransfected cells and several antisense-transfected clones, including clone AA20. Dye uptake levels were reduced to the greatest extent in the AA20 clone (from a control value of  $44.45 \pm 0.85\%$  to  $14.28 \pm 0.67\%$ ), which also showed the most reduction in Cx43 protein levels (data not shown). These data, in combination with the antisense data on junctional communication, strongly support the concept that Cx43 proteins are part of the dye uptake pathway.

To further examine the effects of Cx43-antisense transfection on hemichannels, whole cell currents of single cells were studied using the patch clamp technique. Membrane resistance was measured for antisense-transfected (clone

AA20 cells) and nontransfected cells before, during, and after perfusion of ESS with no calcium for 1.5 min. Exposure of single cells in a whole cell configuration to solutions with no added calcium induces a decrease in membrane resistance ( $R_m$ ) because of the opening of a variety of membrane channels in both control and antisense-transfected cells. The membrane resistance of nontransfected cells is lower than that of antisense-transfected cells by approximately one order of magnitude, the input resistances of cells similar in size being  $\sim 800$  and  $\sim 4000$  M $\Omega$ , respectively. In nontransfected cells,  $R_m$  decreases to less than 10% of the initial values, whereas in antisense-transfected cells,  $R_m$  decreases only to  $\sim 70\%$  of the initial values (Fig. 5). The difference between nontransfected and antisense-transfected cells is consistent with the observations on dye uptake above, namely that there are fewer channels open in the antisense-transfected cells in the absence of extracellular  $Ca^{2+}$ .

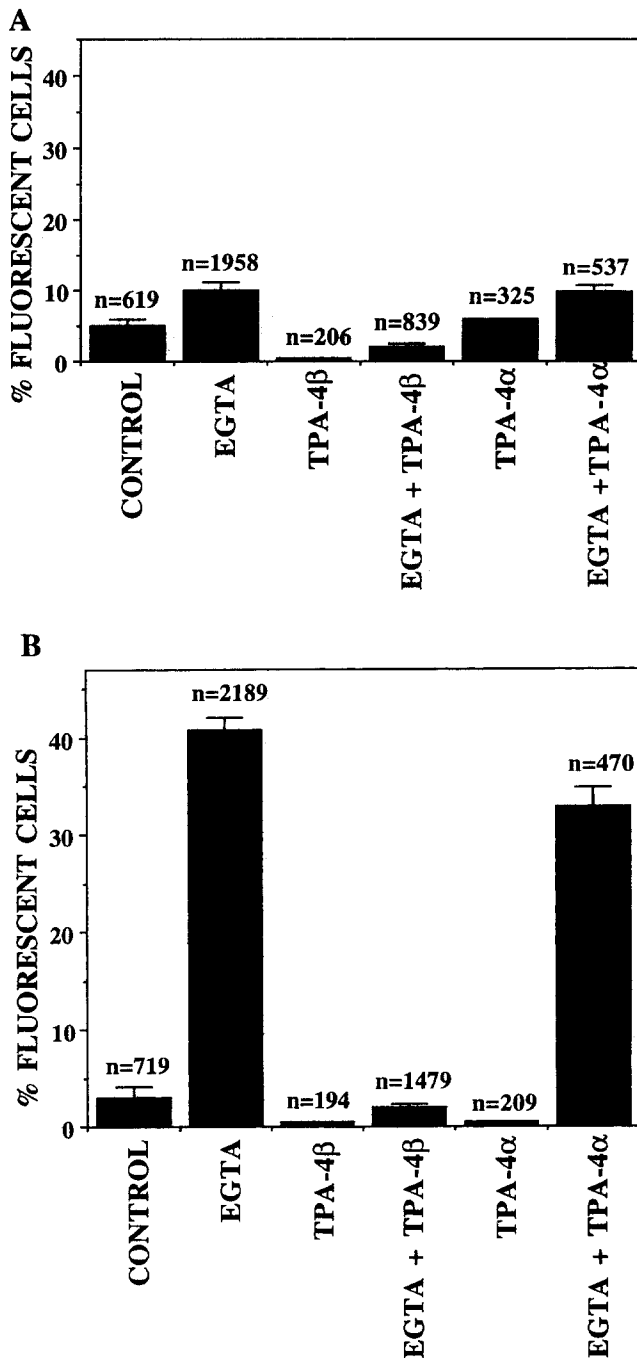
### Hemichannel Activity in Cx43-transfected HeLa Cells

Although a strong correlation was established between the levels of Cx43 expression and hemichannel activity in the Novikoff cells, further support was provided by testing the hemichannel activity in Cx43-transfected human HeLa cells. HeLa cells have no transcripts detectable by northern analysis for Cx26, 30.3, 31, 31.1, 32, 37, 40, 43, or 45 (Elfgang et al., 1995). In the presence of reduced extracellular  $Ca^{2+}$ , HeLa parental cells display limited dye uptake due to a small amount of unidentified connexin(s) (Elfgang et al., 1995) (Fig. 6 A), while Cx43-transfected HeLa cells show significant uptake levels (Fig. 6 B). Dye uptake was inhibited by TPA-4 $\beta$  in both HeLa parental and Cx43-transfected cells, while TPA-4 $\alpha$  displayed no effects (Fig. 6, A and B). These data provide the strongest evidence for the role of Cx43 in the observed hemichannel activity, as assayed with dye uptake.



**Figure 5.** Comparison of membrane resistance changes in nontransfected and Cx43 antisense-transfected cells upon reduction of extracellular  $Ca^{2+}$ . Membrane resistance before, during, and after perfusion with ESS buffer with no  $Ca^{2+}$  for 1.5 min (perfusion period indicated by the *no added  $Ca^{2+}$*  bar), as measured with whole-cell patch clamp methods. The decreases in membrane resistance in the nontransfected cells were approximately five times greater than in the Cx43 antisense-transfected cells (line AA20).



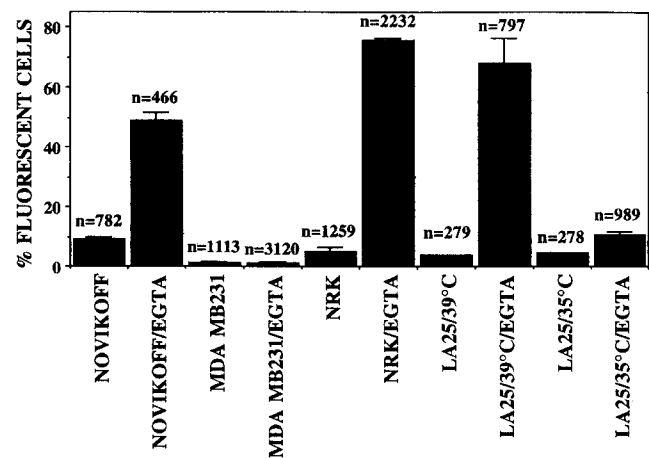


**Figure 6.** Comparison of dye uptake levels and the effects of TPA-4 $\alpha$  and TPA-4 $\beta$  on HeLa parental cells and Cx43-transfected HeLa cells. Dye uptake levels were obtained at 4°C and pH 8.0 for 15 min. To assess TPA effects, cells were incubated with 100 ng/ml of TPA-4 $\alpha$  or TPA-4 $\beta$  at 37°C for 1 h before dye uptake assays. (A) HeLa parental cells. (B) Cx43-transfected HeLa cells. Note that Cx43-transfected HeLa cells display significant levels of dye uptake, while the HeLa parental cells only have limited uptake. Dye uptake was inhibited by TPA-4 $\beta$ , but not TPA-4 $\alpha$  in both HeLa parental and Cx43-transfected cells.

### Dye Uptake Assays in Other Cell Lines and Potential Regulatory Pathways for Hemichannels

To study the hemichannel activities in other cell lines, NRK, MDA MB231, and LA25-NRK cells were also tested for dye uptake. NRK cells, which contain cellular levels of Cx43 severalfold higher than those in the Novikoff cells (P.D. Lampe, personal communication), display higher levels of dye uptake following EGTA (Fig. 7). On the other hand, MDA MB231 cells, a human mammary tumor cell line which does not have detectable levels of Cx31.1, 32, 40, and 43 by Northern blots (D. Kiang, personal communication), display only background levels of dye uptake, even in the presence of EGTA (Fig. 7). The correlation between Cx43 protein expression levels and dye uptake further substantiates the idea that Cx43 is responsible for the plasma membrane hemichannels.

LA25-NRK cells are NRK cells infected with a temperature-sensitive mutant of avian sarcoma virus. The pp60<sup>v-src</sup> tyrosine kinase is active when the cells are grown at the permissive temperature (35°C). The permeability of gap junctions in these cells is dramatically reduced upon activation of pp60<sup>v-src</sup> at the permissive temperature, while at the nonpermissive temperature (39°C), gap junction communication is well developed (Atkinson et al., 1986). To determine whether hemichannel activities are also affected by activation of pp60<sup>v-src</sup> tyrosine kinase, dye uptake was evaluated at permissive and nonpermissive temperatures. At the nonpermissive temperature, when the pp60<sup>v-src</sup> tyrosine kinase is inactive, dye uptake levels obtained with EGTA treatment are similar to those in uninfected NRK cells. At the permissive temperature, dye uptake levels obtained with EGTA treatment are dramatically reduced compared to uninfected NRK cells (Fig. 7). These results parallel the TPA data and introduce another potential regulatory mechanism for controlling the permeability of the hemichannels.



**Figure 7.** Comparison of dye uptake levels between cells with different levels of Cx43 proteins. Dye uptake levels were obtained at 4°C and pH 8.0 for 15 min in Novikoff, NRK, MDA MB231, and LA25-NRK cells in the presence or absence of 5 mM EGTA. Note that the dye uptake levels corresponded to the levels of Cx43 in these cells: NRK > Novikoff > MDA MB 231. Activation of pp60<sup>v-src</sup> tyrosine kinase at the permissive temperature, 35°C, caused a decrease in dye uptake levels in LA25-NRK cells.

## Discussion

We have studied the existence and properties of the non-junctional, plasma membrane hemichannels in the Novikoff, NRK, and HeLa systems. Dye uptake from the medium upon reduction of extracellular  $\text{Ca}^{2+}$  is used to assay for the opening of the hemichannels. Membrane damage is not likely the cause for dye uptake, since dye uptake can be regulated by both TPA and  $\text{pp60}^{\text{v-src}}$ . We have shown a strong correlation between the nonjunctional, plasma membrane hemichannels and Cx43 in the Novikoff culture system. First of all, dye uptake through the hemichannels displays similar characteristics to gap junctional channels, including the size limit of molecules that can pass through the channels. Secondly, experiments with Cx43 antisense-transfected Novikoff cells support the notion that the hemichannels are composed of Cx43, for several reasons: (a) The effectiveness of Cx43 antisense transfection was demonstrated with a reduction in dye transfer via gap junction channels. (b) The levels of dye uptake through hemichannels were also reduced in the transfected cells. (c) The decrease in membrane resistance upon reduction of extracellular  $\text{Ca}^{2+}$  was more limited in Cx43 antisense-transfected cells compared to the nontransfected ones, which implies that there is less hemichannel activity in the Cx43 antisense-transfected cells. Thirdly, higher dye uptake levels are obtained with cells expressing more Cx43 (e.g., NRK cells), while dye uptake is essentially undetectable in cells that lack Cx43, as well as four other connexins (MDA MB231 cells). Finally, and most critically, when parental HeLa cell lines (which display little dye uptake) are transfected with Cx43, the resulting dye uptake and its regulation by TPA parallel that seen in Novikoff cells. Thus, we conclude that Cx43 hemichannels in the plasma membrane can be assayed by the dye uptake methods described in this study.

The electrophysiological data are consistent with the evidence from dye uptake assays, both of which demonstrate an opening of membrane channels when the external  $\text{Ca}^{2+}$  concentration is reduced. Since the decrease in membrane resistance upon exposure to ESS with no  $\text{Ca}^{2+}$  is dramatically reduced in Cx43 antisense-transfected cells, it is reasonable to believe that the opening of Cx43 hemichannels is responsible for the decrease in membrane resistance. The channels responsible for the decrease in membrane resistance with  $\text{Ca}^{2+}$ -deficient external solutions are found to be voltage independent, as square-shaped current traces are recorded both in normal and in Cx43 antisense-transfected cells, although much reduced in amplitude in the latter, when the cells are exposed to families of square voltage pulses ranging from  $-80$  to  $+80$  mV (data not shown). This is consistent with an involvement of Cx43 hemichannels, because gap junction channels in cells expressing Cx43 are known to be insensitive to membrane potential and to have low transjunctional voltage sensitivity (Wang et al., 1992). The partial recovery of membrane resistance following exposure to  $\text{Ca}^{2+}$ -deficient solutions can result from incomplete closure of connexin hemichannels as well as other factors. For example, in  $\text{Ca}^{2+}$ -deficient solutions, the cells are likely to load external  $\text{Na}^+$  via open hemichannels, and with return to normal external  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$  influx may occur via  $\text{Na}^+/\text{Ca}^{2+}$  exchange. The

resulting increase in internal  $\text{Ca}^{2+}$  concentrations may then reduce membrane resistance by opening  $\text{K}^+$  and/or  $\text{Cl}^-$  channels and may affect other membrane channels indirectly by perturbing the cytoskeleton.

A feature of the dye uptake assays carried out on the Novikoff cells is that only  $\sim 45\%$  of the cells took up the dye. This raises the question of what the differences are between the dye-positive and negative cell populations. The simplest interpretation, which we favor, is that the number of hemichannels in the nonjunctional, plasma membrane of some cells may have been too low to detect dye uptake. Consistent with this is the possibility that the dye uptake assay, although convenient for analyzing hemichannel properties, may not be the most sensitive way of measuring hemichannel opening. Hemichannel opening is currently being tested by measuring dye leakage, in contrast to dye uptake, from Lucifer yellow CH-injected single Novikoff cells in the presence of reduced extracellular  $\text{Ca}^{2+}$ . It appears that low levels of hemichannel activity can be detected in a quantitative leakage experiment, but not via dye uptake. Novikoff cells may contain varied amounts of Cx43 protein or hemichannels on the cell surface at different cell cycle stages. Several studies have shown that gap junctions are regulated during cell proliferation, with a general negative correlation between gap junction communication and proliferation (Weinstein and Pauli, 1987). An alternative interpretation is that hemichannel numbers are relatively consistent, but that these channels possess different phosphorylation states during the cell cycle. Both TPA-activated PKC and  $\text{pp60}^{\text{v-src}}$ -associated tyrosine kinase inhibit dye uptake through hemichannels, which suggests that hemichannels may display different levels of dye uptake when phosphorylated to different degrees. Possibly related to this, single channel conductance values for Cx43-based gap junctions are thought to be modified by phosphorylation (Moreno et al., 1994).

Although octanol and heptanol block gap junctional communication almost completely in Novikoff cells and many other cells (Spray and Bennett, 1985; Burt and Spray, 1989; Lazrak and Peracchia, 1993), there are reports where octanol failed to block gap junction permeability completely. For example, Cx56 channels are not affected by octanol (Rup et al., 1993). It has been proposed that octanol and heptanol interrupt the specific lipid environment in the plasma membrane where gap junction channels reside, thereby inhibiting communication (Spray and Bennett, 1985). The fact that the actual mechanism of octanol and heptanol action is unclear makes it difficult to explain the observation that these reagents only inhibit dye uptake by 50%. One possible explanation is that the nonjunctional plasma membrane hemichannels reside in a different lipid environment in the plasma membrane than the mature gap junction channels (Johnson et al., 1989). Alternatively, the protein conformation of the hemichannels may differ from that of mature gap junction channels.

Reduction of extracellular  $\text{Ca}^{2+}$  seems to be the key factor for hemichannel opening. The fact that dye uptake through hemichannels, upon reduction of extracellular  $\text{Ca}^{2+}$ , is a fast process and occurs at low temperature ( $4^\circ\text{C}$ ) leads to a hypothesis that a physical interaction is involved in the dye uptake process, instead of metabolic mechanisms. The acidic side chains of Asp and Glu ( $\text{pK}_a < 5$ )

have a net negative charge at near neutral pH, which could provide candidates for electrostatic  $\text{Ca}^{2+}$  binding. In fact, there are conserved Asp and Glu residues on both extracellular loops of all connexins studied to date, as first observed for a smaller subset of connexins (Peracchia et al., 1994). These Asp and Glu residues may be the sites on the nonjunctional, plasma membrane hemichannels that respond to the changes in extracellular  $\text{Ca}^{2+}$  and therefore influence the gating of the hemichannels. However, extracellular  $\text{Mg}^{2+}$  levels do not modify the hemichannels, and one would expect  $\text{Mg}^{2+}$  to be as effective as  $\text{Ca}^{2+}$  because the negatively charged residues in Cx43 do not appear to be part of known  $\text{Ca}^{2+}$ -specific binding domains. Alternatively, since an increase for a period of minutes in intracellular  $\text{Ca}^{2+}$  to high nanomolar concentrations has been shown to close Novikoff gap junction channels (Lazrak and Peracchia, 1993), external  $\text{Ca}^{2+}$  diffusing through open hemichannels could close them momentarily by activating their intracellular gating mechanism. In this case, the hemichannels would be expected to flicker on and off as  $\text{Ca}^{2+}$  continuously binds and unbinds, their open and closed times depending on opening and closing kinetics as well as on intracellular  $\text{Ca}^{2+}$  buffering efficiency. Consistent with this idea is the observation that even with a normal external  $\text{Ca}^{2+}$  concentration (1.8 mM) the membrane conductance of nontransfected, control Novikoff cells is substantially greater than that of antisense-transfected cells, which indicates that a significant number of hemichannels are open at any given time even at normal external  $\text{Ca}^{2+}$  concentrations.

Phosphorylation has been proposed as a key mechanism for regulating gap junction communication (Musil et al., 1990). In the Novikoff system, the primary effect of TPA is a dramatic inhibition of gap junction assembly, in contrast to a change in channel gating or an enhanced disassembly of preexisting gap junction structures (Lampe, 1994). Thus, phosphorylation of Cx43 by TPA-activated PKC is thought to be critical for the regulation of gap junction assembly, and the kinetics of phosphorylation support this idea. Dye uptake results suggest that PKC, upon activation by TPA, also regulates the gating of the nonjunctional, plasma membrane hemichannels. If phosphorylation of Cx43 by PKC is the cause of TPA effects on hemichannel gating, the site of this phosphorylation may or may not coincide with a site related to assembly. In fact, it will be interesting to determine whether the gating of hemichannels is in any way related to hemichannel docking during assembly. In the studies with LA25-NRK cells, a pp60<sup>v-src</sup>-induced inhibition of dye uptake by hemichannels was observed, reminiscent of the effects of this kinase on gap junction communication. It is possible that tyrosine phosphorylation of Cx43 is involved in both cases (Atkinson and Sheridan, 1988; Swenson et al., 1990). Again, the question is whether the pp60<sup>v-src</sup>-associated tyrosine kinase acts on the same site or sites in both hemichannels and gap junction channels.

The dye uptake methods for the nonjunctional, plasma membrane hemichannels have provided important functional evidence for this connexin pool. A critical question now relates to the nature of this hemichannel population. One attractive possibility is that the plasma membrane hemichannels are involved in the gap junction assembly

process. The present findings reinforce, from a physiological perspective, the possibility that hemichannels are dispersed over the cell surface and subsequently "dock" (i.e., a hemichannel in one cell membrane pairs with another hemichannel in the neighboring cell membrane) before they are incorporated into a developing gap junction and form functional cell-to-cell channels. Evidence supporting this interpretation comes from the observation that gap junction formation is inhibited by Cx43 antibodies (Meyer et al., 1992), where hemichannel docking is thought to be interrupted by the antibodies to external Cx43 domains. The studies described here provide powerful approaches for studying hemichannels at this critical stage of gap junction assembly. They permit quantification of hemichannel levels in the plasma membrane and will allow important questions regarding the regulation of gap junction assembly to be addressed. For example, can a "pulse" of hemichannels delivered to the plasma membrane be subsequently "chased" into gap junctions? It will also be important to determine whether the nonjunctional, plasma membrane hemichannel is a universal phenomenon in all gap junction-containing systems. Other questions include the following: Are all plasma membrane hemichannels capable of participating in the gap junction assembly process? Does the regulation of hemichannel opening influence other aspects of gap junction assembly, e.g., hemichannel docking? Finally, it is also possible that hemichannels fulfill functions distinct from those involving these channels as gap junction precursors.

We wish to thank Dr. Michael Atkinson for providing us with NRK and LA25-NRK cells, Dr. David Kiang for providing us with MDA MB231 cells, and Drs. Claudia Elfgang and Klaus Willecke for providing HeLa parental cells and transfectants. We are grateful to Drs. Michael Atkinson, Erica TenBroek, Alison Krufka, and Alicia Paulson for critically reading the manuscript.

This work was supported by National Institutes of Health Grants GM 46277 and GM 20113.

Received for publication 4 August 1995 and in revised form 10 June 1996.

#### References

- Atkinson, M.M., and J.D. Sheridan. 1988. Altered junctional permeability between cells transformed by v-ras, v-mos or v-src. *Am. J. Physiol.* 255:C674-C683.
- Atkinson, M.M., A.S. Menko, R.G. Johnson, J.R. Sheppard, and J.D. Sheridan. 1981. Rapid and reversible reduction of junctional permeability in cells infected with a temperature-sensitive mutant of avian sarcoma virus. *J. Cell Biol.* 91:573-578.
- Atkinson, M.M., S.K. Anderson, and J.D. Sheridan. 1986. Modification of gap junctions in cells transformed by a temperature-sensitive mutant of Rous sarcoma virus. *J. Membr. Biol.* 91:53-64.
- Azarnia, R., and W.R. Loewenstein. 1984. Intercellular communication and control of cell growth. X. Alteration of junctional permeability by the src gene. *J. Membr. Biol.* 82:191-205.
- Azarnia, R., S. Reddy, T.E. Kmiecik, D. Shalloway, and W.R. Loewenstein. 1988. The cellular src gene regulates junctional cell-to-cell communication. *Science (Wash. DC)*. 239:398-400.
- Barr, L., M.M. Dewey, and W. Berger. 1965. Propagation of action potentials and the structure of the nexus in cardiac muscle. *J. Gen. Physiol.* 48:797-823.
- Bennett, M.V., and D.A. Goodenough. 1978. Gap junctions, electrotonic coupling, and intercellular communication. [Review]. *Neurosci. Res. Program Bull.* 16:1-486.
- Bennett, M.V.L., L.C. Barrio, T.A. Bargiello, D.C. Spray, E. Hertzberg, and J.C. Saez. 1991. Gap junctions: New tools. New answers. New questions. *Neuron*. 6:305-320.
- Berthoud, V.M., M.L. Ledbetter, E.L. Hertzberg, and J.C. Saez. 1992. Connexin43 in MDCK cells: regulation by a tumor-promoting phorbol ester and  $\text{Ca}^{2+}$ . *Eur. J. Cell Biol.* 57:40-50.
- Beyer, E.C., and T.H. Steinberg. 1991. Evidence that the gap junction protein connexin-43 is the ATP-induced pore of mouse macrophages. *J. Biol. Chem.*

- 266:7971-7974.
- Biegion, R.P., M.M. Atkinson, T.-F. Liu, E.Y. Kam, and J.D. Sheridan. 1987. Permeance of Novikoff hepatoma gap junctions: quantitative video analysis of dye transfer. *J. Membr. Biol.* 96:225-233.
- Buisman, H.P., T.H. Steinberg, J. Fischberg, S.C. Silverstein, S.A. Volgelzang, C. Ince, D.L. Ypey, and P.C.J. Leigh. 1988. Extracellular ATP induces a large non-selective conductance in macrophage plasma membranes. *Proc. Natl. Acad. Sci. USA.* 85:7988-7992.
- Burt, J.M., and D.C. Spray. 1989. Volatile anesthetics block intercellular communication between neonatal rat myocardial cells. *Circ. Res.* 65:829-837.
- De Mello, W.C. 1987. Cell-to-cell coupling assayed by means of electrical measurements. *Experientia (Basel).* 43:1075-1079.
- DeVries, S.H., and E.A. Schwartz. 1992. Hemi-gap-junction channels in solitary horizontal cells of the catfish retina. *J. Physiol. (Lond.).* 445:201-230.
- Ebihara, L. 1995. Connexin38, a *Xenopus* embryonic gap junctional protein, forms functional hemi-gap-junctional channels. *Mol. Biol. Cell.* 6:297a.
- Ebihara, L., and E. Steiner. 1993. Properties of a nonjunctional current expressed from a rat connexin46 cDNA in *Xenopus* oocytes. *J. Gen. Physiol.* 102:59-74.
- Ebihara, L., V.M. Berthoud, and E.C. Beyer. 1995. Distinct behavior of connexin56 and connexin46 gap junctional channels can be predicted from the behavior of their hemi-gap junctional channels. *Biophys. J.* 68:1-8.
- Elfgang, C.R.E., H. Lichtenberg-Frate, A. Butterweck, O. Traub, R.A. Klein, E.F. Hulser, and K. Willecke. 1995. Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. *J. Cell Biol.* 129:805-817.
- Furshpan, E.J., and D.D. Potter. 1968. Low-resistance junctions between cells in embryos and tissue culture. *Curr. Top. Dev. Biol.* 3:95-127.
- Gilula, N.B., O.R. Reeves, and A. Steinbach. 1972. Metabolic coupling, ionic coupling, and cell contacts. *Nature (Lond.).* 235:262-265.
- Goldberg, G.S., K.D. Martyn, and A.F. Lau. 1994. A connexin 43 antisense vector reduces the ability of normal cells to inhibit the foci formation of transformed cells. *Mol. Carcinog.* 11:106-114.
- Hamill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Archiv. Eur. J. Physiol.* 391:85-100.
- Johnson, R.G., R.A. Meyer, and P.D. Lampe. 1989. Gap junction formation: a "self-assembly" model involving membrane domains of lipid and protein. In *Cell Interactions and Gap Junctions*. N. Sperelakis and W. Cole, editors. CRC Press, Boca Raton, FL. 159-179.
- Keeling, P., K. Johnson, D. Sas, K. Klukas, P. Donahue, and R.G. Johnson. 1983. Arrangement of MP26 in lens junctional membranes: Analysis with proteases and antibodies. *J. Membr. Biol.* 74:217-228.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680-685.
- Lampe, P.D. 1994. Analyzing phorbol ester effects on gap junction communication: A dramatic inhibition of assembly. *J. Cell Biol.* 127:1895-1905.
- Lampe, P.D., and R.G. Johnson. 1990. Amino acid sequence of in vivo phosphorylation sites in the main intrinsic protein (MIP) of lens membranes. *Eur. J. Biochem.* 194:541-547.
- Lazrak, A., and C. Peracchia. 1993. Gap junction gating sensitivity to physiological internal calcium regardless of pH in Novikoff hepatoma cells. *Biophys. J.* 65:2002-2012.
- Ledbetter, M.L., and M. Lubin. 1979. Transfer of potassium. A new measure of cell-cell coupling. *J. Cell Biol.* 80:150-165.
- Loewenstein, W.R. 1966. Permeability of membrane junctions. *Ann. NY Acad. Sci.* 137:441-472.
- Loewenstein, W.R. 1981. Junctional intercellular communication: The cell-to-cell membrane channel. *Physiol. Rev.* 61:829-913.
- Loewenstein, W.R., and R. Azarnia. 1988. Regulation of intercellular communication and growth by the cellular src gene. *Ann. NY Acad. Sci.* 551:337-346.
- Makowski, L., D.L.D. Caspar, W.C. Phillips, and D.A. Goodenough. 1977. Gap junction structures. II. Analysis of the x-ray diffraction data. *J. Cell Biol.* 74:629-645.
- Malchow, R.P., H. Qian, and H. Ripps. 1993. Evidence for hemi-gap junctional channels in isolated horizontal cells of the skate retina. *J. Neurosci. Res.* 35:237-245.
- Meyer, R.A., P.D. Lampe, B. Malewicz, W. Baumann, and R.G. Johnson. 1991. Enhanced gap junction formation with LDL and apolipoprotein B. *Exp. Cell Res.* 196:72-81.
- Meyer, R.A., D.W. Laird, J.-P. Revel, and R.G. Johnson. 1992. Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. *J. Cell Biol.* 119:179-189.
- Moreno, A.P., J.C. Saez, G.I. Fishman, and D.C. Spray. 1994. Human connexin43 gap junction channels: regulation of unitary conductances by phosphorylation. *Circ. Res.* 74:1050-1057.
- Musil, L.S., and D.A. Goodenough. 1991. Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *J. Cell Biol.* 115:1357-1374.
- Musil, L.S., and D.A. Goodenough. 1993. Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. *Cell.* 74:1065-1077.
- Musil, L.S., B.A. Cunningham, G.M. Edelman, and D.A. Goodenough. 1990. Differential phosphorylation of the gap junction protein connexin-43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol.* 111:2077-2088.
- Paul, D.L., L. Ebihara, L.J. Takemoto, K.I. Swenson, and D.A. Goodenough. 1991. Connexin 46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of *Xenopus* oocytes. *J. Cell Biol.* 115:1077-1089.
- Peracchia, C., A. Lazrak, and L. Peracchia. 1994. Molecular models of channel interaction and gating in gap junctions. In *Handbook of Membrane Channels: Molecular and Cellular Physiology*. C. Peracchia, editor. Academic Press, San Diego, CA. 361-377.
- Preus, D., R.G. Johnson, and J. Sheridan. 1981a. Gap junctions between Novikoff hepatoma cells following dissociation and recovery in the absence of cell contact. *J. Ultrastruct. Res.* 77:248-262.
- Preus, D., R. Johnson, J. Sheridan, and R. Meyer. 1981b. Analysis of gap junctions and formation plaques between reaggregating Novikoff hepatoma cells. *J. Ultrastruct. Res.* 77:263-276.
- Reynhout, J.K., P.D. Lampe, and R.G. Johnson. 1992. An activator of protein kinase C inhibits gap junction communication between cultured bovine lens cells. *Exp. Cell Res.* 198:337-342.
- Rup, D.M., R.D. Veenstra, H. Wang, P.R. Brink, and E.C. Beyer. 1993. Chick Connexin-56, a novel lens gap junction protein. *J. Biochem.* 268:706-712.
- Schwarzmann, G., H. Weigandt, B. Rose, A. Zimmerman, D. Ben-Haim, and W.R. Loewenstein. 1981. Diameter of the cell-to-cell junctional membrane channels as probed with neutral molecules. *Science (Wash. DC).* 213:551-553.
- Spray, D.C., and M.V.L. Bennett. 1985. Physiology and pharmacology of gap junctions. *Ann. Rev. Physiol.* 47:281-303.
- Steinberg, T.H., A.S. Newman, J.A. Swanson, and S.C. Silverstein. 1987. ATP<sup>4-</sup> permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. *J. Biochem.* 262:8884-8888.
- Swenson, K.I., H. Pivnicka-Worms, H. McNamee, and D.L. Paul. 1990. Tyrosine phosphorylation of the gap junction protein connexin43 is required for pp60<sup>src</sup>-induced inhibition of communication. *Cell Regul.* 1:989-1002.
- Trexler, E.B., M.V.L. Bennett, T.A. Bargiello, and V.K. Verselis. 1996. Voltage gating and permeation in a gap junction hemichannel. *Proc. Natl. Acad. Sci. USA.* 93:5836-5841.
- Wang, H.Z., J. Li, L.F. Lemanski, and R.D. Veenstra. 1992. Gating of mammalian cardiac gap junction channels by transjunctional voltage. *Biophys. J.* 63:139-151.
- Warner, A.E., S.C. Guthrie, and N.B. Gilula. 1984. Antibodies to gap-junctional protein selectively disrupt junctional communication in the early amphibian embryo. *Nature (Lond.).* 311:127-131.
- Weinstein, R.S., and B.U. Pauli. 1987. Cell junctions and the biological behavior of cancer. *Ciba Found. Symp.* 125:240-260.