The regulation of gap junctional permeability by phosphorylation was examined in a model system in which connexin 43 (Cx43) gap junction hemichannels were reconstituted in lipid vesicles. Cx43 was immunopurified from rat brain, and Cx43 channels were reconstituted into unilamellar phospholipid liposomes. The activities of the reconstituted channels were measured by monitoring liposome permeability. Liposomes containing the Cx43 protein were fractionated on the basis of permeability to sucrose using sedimentation in an iso-osmolar density gradient. The gradient allowed separation of the sucrose-permeable and -impermeable liposomes. Liposomes that were permeable to sucrose were also permeable to the communicating dye molecule lucifer yellow. Permeability, and therefore activity of the reconstituted Cx43 channels, were directly dependent on the state of Cx43 phosphorylation. The permeability of liposomes containing Cx43 channels was increased by treatment of liposomes with calf intestinal phosphatase. Moreover, liposomes formed with Cx43 that had been dephosphorylated by calf intestinal phosphatase treatment showed increased permeability to sucrose. The role of phosphorylation in the gating mechanism of Cx43 channels was supported further by the observation that phosphorylation of Cx43 by mitogen-activated protein kinase reversibly reduced the permeability of liposomes containing dephosphorylated Cx43. Our results show a direct correlation between gap junctional permeability and the phosphorylation state of Cx43.

Intercellular communication is necessary in multicellular organisms to maintain tissue homeostasis and is involved in diverse cellular activities including metabolic cooperation and the exchange of signaling information for harmonized control of cell growth and differentiation (1–4). Gap junction channels play an important role in intercellular communication by providing a direct pathway for the movement of molecular information among cells (4, 5). Gap junction channels mediate the communication between adjacent cells by the open-closed gating of an aqueous pore permeable to ions and small molecules. Thus, gap junction channels are regarded as cytoplasmic bridges that allow cell-to-cell passage of ions, nutrients, and second messenger regulatory molecules (6–9).

Intercellular communication through the gap junction is believed to be modulated during cellular processes such as cell cycle progression (10–12), embryogenesis, and development (13, 14). A correlation exists between decreases in gap junctional communication (GJC) and the cellular events that lead to cell cycle progression; for example, GJC is reduced during the G1 to S phase transition of the cell cycle (11, 12). In addition, factors that affect cell proliferation were found to inhibit GJC, including tumor promoters (15, 16), growth factors (10, 17), carcinogens (18, 19), and oncogene products (20–22). The temporal loss of GJC in proliferating cells was further supported by the observed growth retardation in cells transfected with overexpressing connexin genes (23–25).

It has been proposed that GJC is modulated by a mechanism that involves the posttranslational phosphorylation of gap junction proteins. Connexin 43 (Cx43) is a major gap junction protein found in animal heart and brain (26, 27). Studies have implicated phosphorylation of Cx43 on tyrosine and/or serine residues as a regulatory mechanism for channel gating. Two types of Cx43 phosphorylation events have been described. One type is the tyrosine phosphorylation of Cx43 by oncogene-induced tyrosine kinases (21, 22, 28). The other type involves the rapid phosphorylation of Cx43 on serine residues in cells stimulated to grow (10–12, 29, 30). However, the biochemical mechanism by which posttranslational modification is associated with the modulation of gap junction channels remains obscure.

It has been suggested that mitogen-activated protein (MAP) kinase plays an important role in the loss of GJC during mitotic division (31). Therefore, we sought to determine whether MAP kinase is directly involved in the gating mechanism of gap junction channels. Detailed studies of GJC have been severely constrained by the fact that gap junction channels are not exposed to the extracellular space, and access to the channels is only available via the cytoplasm or dialyzed cytoplasm in a whole-cell patch clamp configuration. Thus, it is difficult to correlate unequivocally the effects of agents that alter channel phosphorylation with channel permeability, as any of these agents might elicit secondary metabolic effects. One approach is to make gap junction channels accessible to study by splitting the isolated gap junctions and reconstituting them in an artificial lipid bilayer. Several studies report the induction of channel activities in a lipid bilayer by incorporating gap junction proteins into lipid vesicles (32–36).

The abbreviations used are: GJC, gap junctional communication; Cx43, connexin 43; PE, phosphatidylethanolamine; octylglucoside; N-octyl-β-D-glucopyranoside; CIP, calf intestinal phosphatase; CNBr, cyanogen bromide; CHAPS, 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate; DMS, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; Cx32, connexin 32; Cx26, connexin 26; MAP, mitogen-activated protein.
In the present study, we reconstituted Cx43 gap junction channels in liposomes and measured the permeability with the use of iso-osmolar sucrose/urea density gradient sedimentation (34). Our results indicate that MAP kinase phosphorylation of Cx43 directly modulates channel gating.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Lissamine rhodamine B-labeled phosphatidylethanolamine (PE) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Bovine phosphatidylcholine, egg phosphatidylserine, N-octyl-β-D-glucopyranoside (octylglucoside), leupeptin, phenylmethylsulfonyl fluoride, and cyagen bromide (CNBr)-activated Sepharose 4B beads were obtained from Sigma. CHAPS and dimethyl sulfoxide (DMS) were purchased from Pierce. MAP kinase (Erk2) was purchased from New England Biolabs (London, United Kingdom). γ-32P]ATP (5000 Ci/mmol) was from Amersham Pharmacia Biotech.

**Preparation of anti-Cx43 Antibody**—The sequence of synthetic peptide SSRASSPRPPDDE1 correspended to amino acid residues 368 to 382 of the C-terminal region of Cx43 (26). The peptide was conjugated to keyhole limpet hemocyanin, and antisera were then prepared by the method of Beyer et al. (38).

**Immunofluorescence** of Cx43—Cx43 was immunoaffinity-purified by the method of Beyer et al. (36). Brain membranes were homogenized in 5 m M NaHCO3 (pH 8.3), 5 mM triethanolamine, 80 mM urea, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 0.75% CHAPS. After centrifugation at 100,000 g for 1 h, the supernatant was loaded onto an immunoaffinity column in which anti-Cx43 antibody was cross-linked to CNBr-activated Sepharose 4B. After successive washes with buffer A and buffer A plus 0.5 M NaCl, the column was equilibrated with buffer A in which CHAPS was replaced with 80 m M octylglucoside. Bound Cx43 was eluted from the column by brief exposure to 50 m M sodium phosphate (pH 7.4), 50 m M NaCl, 5 mM EDTA, 50 m M NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 0.75% CHAPS. After centrifugation at 100,000 g for 1 h, the supernatant was loaded onto an immunoaffinity column in which anti-Cx43 antibody was cross-linked to CNBr-activated Sepharose 4B. After successive washes with buffer A and buffer A plus 0.5 M NaCl, the column was equilibrated with buffer A in which CHAPS was replaced with 80 m M octylglucoside. Bound Cx43 was eluted from the column by brief exposure to 50 m M sodium phosphate (pH 7.4), 50 m M NaCl, 5 mM EDTA, 50 m M NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 0.75% CHAPS.

**Gel Electrophoresis and Immunoblot Analysis**—Immunoblot analysis with anti-Cx43 antibody. The permeability of Cx43 channels reconstituted in liposomes was examined by the technique of transport-specific density shift as described (34). The liposomes were layered onto linear, 0 to 400 mM, iso-osmolar sucrose density gradients. The distribution of the lipid vesicles was determined by measuring the fluorescence of rhodamine labeled PE in each fraction with a spectrofluorometer (LS-3B, Perkin-Elmer) at 590 nm excited at 560 nm. To measure the channel activity of Cx43 in liposomes, the loss of lucifer yellow fluorescent communication dye from the liposomes was monitored for the fluorescence of lissamine rhodamine B-labeled PE in the liposome membrane. The amount of lucifer yellow retained in the liposomes was quantified by measuring its fluorescence intensity at 530 nm excited at 425 nm.

**Cell Culture**—COS-1 cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

**Electrophysiological Measurements**—The whole cell patch clamp technique was used to record membrane currents (Axopatch 200B; AXON Instruments, Foster City, CA) in voltage clamp mode, and currents were sampled at 10-kHz and low-pass filtered at 5-kHz. Cells were maintained at 37 °C in a humidified atmosphere of 95% (v/v) air and 5% (v/v) CO2.

**Data Analysis**—Data were recorded and stored using pCLAMP acquisition and analysis software (Axon Instruments, Foster City, CA). The data were analyzed using an EPX/11 program (Atoll Software, San Diego, CA). If not stated otherwise, the standard error of the mean is shown. Differences were considered statistically significant if P < 0.05.
kinase. The reaction mixture was incubated for 3 h at 30 °C in the presence or absence of [γ-32P]ATP (0.1 mCi/ml). Protein samples phosphorylated in the presence of [γ-32P]ATP were resolved by 8% SDS-PAGE followed by autoradiography. The permeability of liposomes formed with the MAP kinase-treated Cx43 was compared with that of untreated control Cx43 by sedimentation analysis in an iso-osmolar density gradient.

RESULTS

Purification of Cx43—Cx43, immunoaffinity-purified from rat brain using anti-Cx43 antibody, was resolved on an SDS-polyacrylamide (8%) gel, and a protein band with a molecular size of ~41 kDa was detected by silver staining. Immunoblot analysis identified the band as Cx43 (Fig. 1). The anti-Cx43 antibody used in this study was directed against a nonconserved cytoplasmic epitope and may not cross-react with other types of connexins (27, 38). Other connexin family proteins expressed in rat brain (27) were also probed using antibodies against connexin 32 (Cx32) and connexin 26 (Cx26), but no corresponding protein bands were detected (data not shown).

Oligomeric State of Immunoaffinity-purified Cx43—The assembly of immunoaffinity-purified Cx43 was examined by velocity sedimentation in a sucrose density gradient, and the composition of the purified Cx43 complex was deciphered by chemical cross-linking with DMS. In previous studies, connexins were isolated from insoluble lipid membranes under denaturing conditions with the use of ionic detergents. It is possible that these isolation conditions alter the secondary structure of connexin proteins, influencing the ability of connexin subunits to assemble into gap junctional connexons, which are composed of six connexin subunits. Therefore we sought to determine whether Cx43 solubilized with a nonionic detergent (octylglucoside) under nondenaturing conditions maintained its oligomeric state after purification. To accomplish this, we subjected immunoaffinity-purified Cx43 to sucrose density gradient sedimentation. The gradient was fractionated, and the amount of Cx43 in each fraction was quantified by enzyme-linked immunosorbent assay using the same antibody (lane 2).

The quaternary structure of immunoaffinity-purified Cx43 was examined by chemical cross-linking procedures using DMS as a coupling agent. Cx43 was incubated either in the presence or absence of 10 mM DMS for 8 h at 4 °C, and the treated proteins were resolved on a 4 to 15% SDS-polyacrylamide gradient gel followed by immunoblot analysis using anti-Cx43 antibody. DMS cross-linked the ~41 kDa Cx43 monomers (lane 1) to form complexes that migrated at ~210 kDa (lane 2).

Permeability of Cx43 Channels Formed in Unilamellar Liposomes—We next incorporated immunoaffinity-purified Cx43 protein into unilamellar phospholipid liposomes. The size of the liposomes is strongly influenced by the lipid:detergent ratio and by the chain length of the detergent molecules. In Rhee et al. (36), phospholipid unilamellar vesicles were constructed under the same experimental conditions as ours. The size distribution of the vesicles in that study was examined by filtra-

![Image of SDS-PAGE and Immunoblot](http://www.jbc.org/)

**Fig. 1. SDS-PAGE of Cx43 and the corresponding immunoblot.** Solubilized membrane proteins from rat brain (lane 1) and Cx43 immunopurified with an antibody specific to the C-terminal region of Cx43 (amino acids 368 to 382) (lane 3) were subjected to electrophoresis on an SDS-polyacrylamide (8%) gel and visualized by silver staining. Immunoblot analysis was carried out using the same antibody (lane 2).
tion over a size exclusion high performance liquid chromatography column, and the average vesicle diameter was estimated to be 45 nm. Assuming a bilayer thickness of 4.0 nm and an average area of 0.7 nm²/phospholipid molecule (43), the protein:lipid ratio used in this study should give rise to contain an average of one gap junction channel per vesicle (36). The Poisson distribution predicts that 67% of the vesicles will contain at least one connexon.

The permeability of reconstituted Cx43 channels was examined by sedimentation velocity analysis of liposomes in an iso-osmolar sucrose density gradient (34). Liposomes that were formed in the absence of Cx43 or with denatured Cx43 were sucrose-impermeable; thus they migrated only a short distance into the gradient and banded near the top of the gradient (Fig. 3A, tubes 1 and 3). In contrast, liposomes formed with intact Cx43 were fractionated into two populations within the gradient, a sucrose-permeable fraction that migrated near the bottom of the gradient and a sucrose-impermeable fraction that migrated near the top (Fig. 3A, tube 2). It has been suggested that sucrose-permeable liposomes that contain open Cx43 channels continuously equilibrate their internal solution with the surrounding external solution and thus migrate to a more dense position in the gradient. Liposomes that are impermeable to sucrose, either because they lack Cx43 channels or contain a closed form of the channels, are buoyed by the internal urea buffer, which is lighter than the external sucrose buffer. Impermeable liposomes are therefore retained at the top of the gradient (36).

We next monitored the transfer of lucifer yellow, a well known communicating dye molecule, through reconstituted Cx43 channels. Liposomes were formed with or without Cx43 in the presence of 3 mM lucifer yellow, and the loss of the entrapped lucifer yellow from the liposomes was monitored. After velocity sedimentation, the liposomes in the iso-osmolar density gradient were eluted from the bottom of the tube. The amount of lucifer yellow retained in liposomes was quantified by measuring its fluorescence intensity at 530 nm excited at 428 nm (panel 2).
4. The data indicate that the fraction of sucrose-permeable liposomes increases with increasing concentrations of Cx43 incorporated.

**Increased Channel Permeability by Dephosphorylation of Cx43**

The effect of Cx43 phosphorylation on gap junctional permeability was presented (Fig. 5). Cx43 preparations from rat brain are known to contain both the phosphorylated and nonphosphorylated forms of the protein (27). Our immunoadfinity-purified Cx43 was treated with CIP either before or after incorporation into liposomes. Treatment of Cx43 with CIP converted the 43-kDa phosphorylated form (Fig. 5, lanes 1 and 3) into the dephosphorylated 41-kDa form (Fig. 5, lanes 2 and 4). The fraction of permeable liposomes containing Cx43 was increased by treating the liposome with CIP (Fig. 5, tube 2). The distribution analysis of liposomes after sedimentation revealed that 15% of the total liposome population was sucrose-permeable. However, 27% of the total liposome population became sucrose-permeable if the liposomes were treated with CIP (5 units for 3 h) before sedimentation. The hypothesis that dephosphorylation of Cx43 increases gap junctional permeability is further supported by our subsequent experiment, which showed an increase in the permeability of liposomes formed with dephosphorylated Cx43 (Fig. 5, tube 4). The fraction of permeable liposomes formed with dephosphorylated Cx43 was about 2-fold greater than that observed with control liposomes incorporated with intact Cx43 (Fig. 5, tubes 3 and 4).
Reduced Channel Permeability by MAP Kinase Phosphorylation of Cx43—In an effort to further characterize the role of phosphorylation in the gating of gap junction channels, we used MAP kinase to phosphorylate solubilized Cx43 (Fig. 5, lane 5b). The fraction of permeable liposomes containing CIP-treated Cx43 was reduced by 79% if the dephosphorylated Cx43 became phosphorylated by MAP kinase (Fig. 5, tubes 5a and 5b). MAP kinase-dependent gating mechanism of Cx43 channels was further supported by the observation that MAP kinase-induced inhibition of channel permeability was reversed by treatment of Cx43 with CIP (Fig. 5, tube 5c). These data suggest that phosphorylation of Cx43 by MAP kinase may be directly involved in the channel gating process. Because protein kinase C has also been implicated in the regulation of GJC, we tested whether protein kinase C could phosphorylate the immunoaffinity-purified Cx43. Only a trace amount of phosphorylated Cx43 was detected by autoradiography, whereas histone H1, a known substrate of protein kinase C, was readily phosphorylated under the same experimental conditions (data not shown).

DISCUSSION

Gap junctions are membrane channels that link end-to-end across the space between juxtaposed cells. In this study, we present evidence that the phosphorylation of connexin is directly involved in the gating mechanism of gap junction channels. We showed that the activity of Cx43 channels reconstituted in liposomes was dramatically increased by dephosphorylation of Cx43.

The activity of the reconstituted Cx43 channels was monitored by measuring the permeability of the liposomes to sucrose in an iso-osmolar sucrose density gradient. Several other studies have reported the functional reconstitution of connexons consisting of Cx26 (35) and Cx32 (36).

Information regarding the gating mechanism of gap junction channels can be obtained from the assessment of the permeability of hemichannels to small molecules. Structural, biochemical, and physiological data show the hemichannels have properties that are similar to those of intact gap junctions. For example, the activities of hemichannels are, like those of intact gap junction channels, voltage-dependent. Trexler et al. (44) demonstrate the voltage-dependent gating properties of Cx46 hemichannels in excised patches from Xenopus oocytes. Hemichannels have also been shown to exist in the plasma membrane (45, 46). Several studies have proposed that the electrical coupling between horizontal cells of the fish retina occurs through hemichannels. Recently, Li et al. (47) presented data in support of the existence of nonjunctional plasma Cx43 hemichannels in Novicoff hepatoma cells. In this cell line, they measured properties of fluorescent dye transfer through nonjunctural hemichannels that were similar to those observed with gap junction channels. The hemichannels are believed to be typically in the closed state to prevent the loss of cytoplasmic solutes and the entry of extracellular ions. But some of the hemichannels formed in Xenopus oocyte membranes open by membrane depolarization (48) and induce cation fluxes (44). Open hemichannels were also shown to form in a system in which Cx32 channels were reconstituted in lipid vesicles that lacked the cytoplasmic or membrane factors necessary for maintaining the channels in a closed state (32, 34, 36). From the result of our study, we propose that Cx43 forms open hemichannels in lipid vesicles. The results herein showed that liposomes containing open Cx43 channels were permeable to sucrose and communicating dye (Fig. 3). Indeed we observed an increase in the fraction of permeable liposomes as more connexin protein was incorporated (Fig. 4).

Although the gating mechanism of gap junction channels remains obscure, the rapid and reversible changes in the GJC by protein kinase activators have led to the hypothesis that gap junction channels undergo a conformational change by phosphorylation to gate the channels. Cx43 has been identified as a substrate of MAP kinase (30), protein kinase C (49), and p34cdc2 kinase (29), and it has been shown that GJC is rapidly reduced by the increased phosphorylation of Cx43 on serine residues (11, 12, 29). MAP kinase constitutes one of the most important protein kinase families for the progression of the cell cycle. Activated MAP kinase phosphorylates a variety of targets including other protein kinases and transcription factors (50). Recently it was shown that Cx43 is a substrate of MAP kinase and that the phosphorylation of Cx43 is mitosis-specific in many types of cells (11, 12, 29, 30). Comparison of the amino acid sequences of MAP kinase substrates has identified PKP_3(S/T)P as a consensus phosphorylation site (51–53). Sequence analysis of Cx43 revealed MAP kinase consensus phosphorylation sites at serine 255, 279, and 282. In fact Warn-Cramer et al. (37) confirm that MAP kinase phosphorylates Cx43 at Ser-255, -279, and -282. Data presented in this study suggest that phosphorylation of Cx43 by MAP kinase is directly involved in the gating mechanism of Cx43 channels.

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