THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 19, Issue of May 7, pp. 20058–20066, 2004
Printed in U.S.A.

Regulation of Purified and Reconstituted Connexin 43 Hemichannels by Protein Kinase C-mediated Phosphorylation of Serine 368*

Received for publication, October 9, 2003, and in revised form, February 11, 2004
Published, JBC Papers in Press, February 17, 2004, DOI 10.1074/jbc.M311137200

Xiaoyong Bao‡, Luis Reuss, and Guillermo A. Altenberg§

From the Membrane Protein Laboratory, Sealy Center for Structural Biology, and Department of Physiology and Biophysics, The University of Texas Medical Branch Galveston, Texas 77555-0437

Indirect evidence suggests that the permeability of connexin 43 (Cx43) gap-junctional channels (connexons) to small organic molecules (M_r < 1,000) is decreased by protein kinase C (PKC)-mediated phosphorylation of Ser-368. However, it is currently unknown whether this effect is produced directly by phosphorylation of this residue or whether cytoplasmic regulatory factors are required for the decrease in Cx43 gap-junctional channel permeability. Here we studied the effects of PKC-mediated phosphorylation on purified recombinant wild-type Cx43 and a PKC-unresponsive mutant (S368A). Our studies show that (*a*) PKC phosphorylates Ser-368, (*b*) the phosphorylation by PKC of purified and reconstituted connexons abolishes sucrose and Lucifer Yellow permeability, (*c*) the regulation of Cx43 by PKC is the direct result of phosphorylation of Ser-368 and does not involve intermediary regulatory factors, and (*d*) phosphorylation of Ser-368 produces a conformational change in purified Cx43 as demonstrated by changes in intrinsic Trp fluorescence and proteolytic digestion pattern. We conclude that phosphorylation of Ser-368 by PKC induces a conformational change of Cx43 that results in a decrease in connexon permeability.

Gap-junctional channels are aqueous channels responsible for cell-to-cell communication that allow for the exchange of solutes of $M_r \leq 1{,}000$. Gap-junctional channels are formed by the docking of two connexons or gap-junctional hemichannels (GJHs)[1], one from each neighboring cell; each connexon is formed by six connexin molecules that contain four transmembrane domains each (for a review, see Ref. 1). Cx43 is expressed in several cell types in organs such as brain, myocardium, and kidney as well as in vascular endothelial cells (2–6). Its functional significance in cell-to-cell communication has been clearly established (1). Cx43 GJHs have also been shown on the plasma membrane of a number of cell types (7–13), and there is evidence supporting a role of their activation in cell injury because of uncompensated water and solute fluxes that overwhelm the normal membrane transport mechanisms, thus altering cell composition. ATP depletion may result in a decrease in the phosphorylation state of GJHs, which activates these channels, leading to cell damage (7–9, 12, 13).

It has been established that phosphorylation of specific Ser or Tyr residues in the C-terminal domain of Cx43 reduces gap-junctional intercellular communication (for reviews, see Refs. 14 and 15) and that PKC phosphorylates Cx43 at Ser-368 and Ser-372 of the C-terminal domain (16, 17). Recent evidence suggests that decreased phosphorylation of Cx43 at Ser-368 of the C-terminal domain increases gap-junctional channel dye permeability in cells exposed to PKC inhibitors (16, 17). However, it is currently unknown whether phosphorylation of Ser-368 by PKC regulates Cx43 directly or whether this effect requires intermediary regulatory factor(s). In this context, it is believed that the decrease in Cx43 permeability produced by lowering intracellular pH, which also depends on the C-terminal domain (18–20), requires a cytosolic component (21). Since studies on the regulation of Cx43 by PKC in cells are difficult to interpret because of the complexity of the PKC signaling pathways and the possible presence of cytosolic regulatory factors, we decided to assess the regulation of purified Cx43. Our results using purified recombinant Cx43 GJHs indicate that phosphorylation of Ser-368 produces a conformational change that reduces GJH permeability and that this effect does not require cytosolic factors.

EXPERIMENTAL PROCEDURES

Recombinant Baculoviruses—Three constructs coding for Cx43 were created for these studies: (*a*) wild-type rat Cx43 (Cx43), (*b*) Cx43 fused to the enhanced green fluorescent protein (EGFP) at the C terminus (Cx43-EGFP), and (*c*) Cx43 in which Ser-368 was substituted with Ala (Cx43-S368A). Cx43 was amplified by PCR using the rat Cx43 DNA as template (a gift from Dr. Scott John, see Ref. 9) with XhoI and EcoRI restriction sites added at the 5′ and 3′ ends. The PCR products digested with XhoI and EcoRI were ligated to the plasmid pBlueBac 4.5/V5-His (Invitrogen) cut with the same enzymes. EGFP was amplified by PCR from pEGFP-C1 (Clontech) with EcoRI and HindIII flanking sites for in-frame cloning at the 3′ end of the Cx43 DNA. The PCR product digested with EcoRI and HindIII was ligated to Cx43 into pBlueBac 4.5/V5-His cut with the same enzymes to create the Cx43-EGFP fusion protein. The pBlueBac 4.5/V5-His vector was used as a source of the His_6 tag added to the C-terminal end of all constructs for Ni^{2+} affinity purification. The DNAs coding for Cx43 and Cx43-EGFP, plus the His tag at the C terminus, were excised with BamHI and SalI and ligated into the baculovirus transfer vector pFastBac (Invitrogen). The S368A mutant was obtained by site-directed mutagenesis (QuikChange multisite site-directed mutagenesis kit, Stratagene, La Jolla, CA) using the following oligonucleotide: 5′-CGACCTTCCAGCAGAGCCGCCTCCCGCGCCAGCAGCAGGCCTCGG-3′. Recombinant baculoviruses were generated following the instructions of the Bac-to-Bac system (Invitrogen). The viruses were produced in Sf9 cells grown at 27 °C in Grace's medium supplemented with 10% fetal calf serum and 0.05 mg/ml gen-

* This work was supported in part by American Heart Association Grant-in-aid 0050353N. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ A Jeane B. Kempner Postdoctoral Fellow.

§ To whom correspondence and requests for reprints should be addressed: Dept. of Physiology and Biophysics, The University of Texas Medical Branch, Galveston, TX 77555-0437. Tel.: 409-772-3026; Fax: 409-772-1301; E-mail: galtenbe@utmb.edu.

[1] The abbreviations used are: GJH, gap-junctional hemichannel; Cx43, connexin 43; EGFP, enhanced green fluorescent protein; Cx43-EGFP, Cx43 with the EGFP fused to its C-terminal end; Cx43-S368A, Cx43 with Ser-368 substituted with Ala; OG, *n*-octyl-β-D-glucopyranoside; PMSF, phenylmethylsulfonyl fluoride; PKC, protein kinase C.

tamycin. Cell density was ~10^6 cells/ml. A scheme of the Cx43s engineered is shown in Fig. 1*A*. DNA sequencing of the constructs was carried out at the Protein Chemistry Core Laboratory of The University of Texas Medical Branch.

Western Blot Analysis—Western blots were performed as described previously (22) using a rabbit anti-rat Cx43 polyclonal antibody against the Cx43 C terminus (Zymed Laboratories Inc.) and a horseradish peroxidase-labeled goat anti-rabbit antibody.

Purification of Wild-Type Cx43 and Cx43-S368A—Protein expression was carried out in High-Five insect cells in suspension, grown in 300-ml baffled flasks containing 100 ml of Excell 401 medium supplemented with 200 units/ml penicillin, 200 μg/ml streptomycin, and 2 mM glutamine. Cells (10^6 cells/ml) grown at room temperature were shaken at 200 rpm and infected at a multiplicity of infection of 10. Generally cells were harvested 72 h postinfection by centrifugation at 700 × *g* for 10 min at 4 °C. Cells were washed twice with phosphate-buffered saline solution, and the final pellet was frozen in liquid nitrogen. The frozen pellets were used for purification immediately or stored at −80 °C. For purification, cells were thawed in a buffer containing 1 mM bicarbonate and 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysed with a Dounce homogenizer. The membranes were alkali-extracted as described previously (23, 24). Briefly, after addition of NaOH to a 20 mM final concentration, the lysate was sonicated with a probe sonicator, incubated on ice for 30 min, and then centrifuged at 35,000 × *g* for 30 min at 4 °C. In some experiments, purification was performed from native membranes without alkali extraction. The membranes were solubilized with 2.5% *n*-dodecyl-β-D-maltoside or 2.3% *n*-octyl-β-D-glucopyranoside (OG) in 2 M NaCl, 10 mM EDTA, 10 mM dithiothreitol, 10 mM PMSF, and 10 mM glycine/NaOH, pH 10, at a protein concentration <2 mg/ml. The suspension was sonicated and incubated for 2 h at 4 °C with gentle rotation. Unsolubilized material was separated by ultracentrifugation at 100,000 × *g* for 40 min at 4 °C. Solubilized Cx43s were diluted with 15 volumes of 10 mM β-mercaptoethanol, 1 mM PMSF, 100 mM HEPES, and 0.2% *n*-dodecyl-β-D-maltoside or 2.3% OG, pH 8.0. Diluted samples were loaded at a speed of 0.5 ml/min, at 4 °C, on a nickel-nitrilotriacetic acid column (Qiagen) pre-equilibrated with the dilution buffer. The column was washed with 10 volumes of buffer (10 mM KCl, 0.1 mM EDTA, and 10 mM HEPES/KOH, pH 7.4, containing 0.01% *n*-dodecyl-β-D-maltoside or 2.3% OG and 20 mM imidazole). Imidazole, 250 mM in washing buffer, was added for elution. For the isoosmolar sucrose gradient experiments (see below), 459 mM urea was present in the elution buffer.

Analysis of the Oligomeric State of Purified Cx43—The oligomerization of solubilized Cx43 was determined by gel filtration on Sephacryl S300HR (16/60 prepacked column, Amersham Biosciences) using an ÄKTA FPLC system (Amersham Biosciences). For these experiments, Cx43-EGFP in 2.3% OG, 150 mM NaCl, 0.1 mM EDTA, and 10 mM HEPES/NaOH, pH 7.4, was run at a flow rate of 0.5 ml/min (0.5-ml total volume, ~0.5 mg/ml Cx43-EGFP, with or without standard molecular weight markers added) on the column equilibrated with the same buffer. Molecular weights were calculated from the linear relationship between K_{av} and the log of the molecular weight. The standards were run under the same conditions as the Cx43-EGFP samples and had the following molecular masses: 669 kDa (thyroglobulin), 440 kDa (ferritin), 232 kDa (catalase), 158 kDa (aldolase), and 67 kDa (albumin). Blue dextran 2000 (~2,000 kDa) was used to determine the void volume.

Phosphorylation and Dephosphorylation of Cx43—Purified proteins were dephosphorylated as described by Kim *et al.* (26). Approximately 30 μg of purified Cx43 or Cx43-S368A in 10 mM KCl, 0.1 mM EDTA, 2.3% OG, and 10 mM potassium phosphate, pH 7.4, were mixed with 0.1 volumes of agarose-conjugated calf intestinal phosphatase (50 units/ml, Sigma), and the mixture was incubated for 3 h at 25 °C with gentle agitation. Reactions were stopped by the removal of the enzyme-conjugated agarose beads by centrifugation.

Dephosphorylated Cx43 and Cx43-S368A were phosphorylated by PKC as described by Sáez *et al.* (27) for Cx32 with minor modifications. Briefly 30 μg of dephosphorylated Cx43 were phosphorylated by rat brain PKC (1–2 μg, Calbiochem) in the presence of 200 μM ATP, 5 mM Ca^{2+}, 5 mM Mg^{2+}, 50 μg/ml phosphatidylserine (Avanti, Alabaster, AL), and 3 μg/ml 1,2-dioleoyl-*sn*-glycerol (Sigma). The reaction volume was generally 100 μl, and the reaction proceeded for 2 h at room temperature. Phosphorylation was confirmed by autoradiography using 10 nCi of [γ-^{32}P]ATP (1,000 cpm/pmol, American Radiolabeled Chemicals, St. Louis, MO) in the reaction described above. For these experiments, either Cx43 or Cx43-S368A was precipitated in 10% trichloroacetic acid and then subjected to SDS-PAGE (16% Tris, glycine gels). Films were exposed at −80 °C before development. For quantitative assessments of the phosphorylation level, Cx43 at a concentration of 0.1 mg/ml was phosphorylated in the presence of 10 nCi of [γ-^{32}P]ATP. The phosphorylated protein was then labeled with 100 μM fluorescein maleimide (Molecular Probes, Eugene, OR) for 15 min at 25 °C. The reaction was terminated by addition of a 5-fold molar excess of dithiothreitol, and then the free radioactive label and the free dithiothreitol-reacted fluorescein maleimide were removed from 50 μl of the reaction by filtration through a G-25 column (Amersham Biosciences). Measurements of radioactivity and fluorescence were performed in aliquots of the filtrate. The protein amount was estimated from the fluorescence and parallel Cx43 fluorescence/protein calibration curves using the BCA protein assay reagent from Bio-Rad with bovine serum albumin as standard.

Reconstitution of Wild-Type Cx43 and Cx43-S368A—The reconstitution procedure for Cx43 and Cx43-S368A was a modification of published techniques (28, 29). Briefly proteins were reconstituted in a mixture of phosphatidylcholine and phosphatidylserine at a 2:1 molar ratio. The lipids in chloroform stocks were mixed and lyophilized overnight under argon. The dry film was rehydrated in 75 μl of OG-containing washing buffer/mg of lipid and warmed to 37 °C until it became transparent. OG-solubilized proteins were added to the lipid-detergent mixture and dialyzed through a Spectra/Pro 6,000–8,000 molecular weight cut-off membrane (Spectrum Laboratories, Rancho Dominguez, CA) for 24 h at room temperature against 500 ml of detergent-free washing buffer containing 10 ml of a 50% (w/v) suspension of Biobeads SM-2 (Bio-Rad). Large unilamellar vesicles for the transport and proteolysis experiments (~100-nm diameter) were obtained by extrusion (Mini-Extruder, Avanti). For the sedimentation analysis, lissamine rhodamine B-labeled phosphatidylethanolamine was added (phosphatidylcholine:phosphatidylserine:phosphatidylethanolamine ratio of 2:1:0.05) to label the liposomes for easy identification. For these experiments, the buffer contained 459 mM urea.

Sucrose Uptake—Sucrose permeability of the proteoliposomes containing purified Cx43 GJHs was assessed by the transport-specific density shift technique (26, 30, 31) and by a rapid filtration assay. For the shift assay, the proteoliposomes containing lissamine rhodamine B-labeled phosphatidylethanolamine were layered onto a linear isoosmolar sucrose gradient (0–400 mM sucrose with a reverse urea gradient) in detergent-free buffer. The gradient was usually centrifuged on a swinging bucket Beckman SW55Ti rotor at 300,000 × *g* for 8 h at 4 °C, and the location of the proteoliposomes was determined in fractions collected from the top to the bottom of the gradient tubes. The fluorescence of samples excited at 560 ± 4 nm was measured at 590 ± 4 nm (Fluorolog-2, SPEX, Edison, NJ). In most of the experiments, the proteoliposomes were loaded with Lucifer Yellow by adding the fluorescent probe at a concentration of 2 mM during the extrusion procedure. The non-trapped Lucifer Yellow was removed by filtration through a PD-10 column (Amersham Biosciences). Lucifer Yellow fluorescence was measured in the sedimentation gradient fractions at an excitation of 430 ± 4 nm and an emission of 540 ± 4 nm. To measure sucrose uptake by rapid filtration, proteoliposomes were incubated at 25 °C in washing buffer containing 2 mM sucrose and 10 nCi/μl [^{14}C]sucrose (American Radiolabeled Chemicals, specific activity of 7.5 mCi/mmol). Filtration was through 0.2-μm nitrocellulose filters (Whatman). Radioactivity of the filters was measured by liquid scintillation after washing with 45 ml of ice-cold washing buffer containing 10 μM Gd^{3+}, a blocker of gap-junctional channels and GJHs (32, 33).

Tryptophan Fluorescence Measurements—Tryptophan fluorescence emission spectra of OG-solubilized Cx43 and Cx43-S368A were obtained on a SPEX CMT1 with excitation at 295 ± 4 nm. Data were obtained at protein concentrations between 0.1 and 0.3 μM in 10 mM KCl, 0.1 mM EDTA, 2.3% OG, and 10 mM potassium phosphate, pH 7.4, in 1-ml quartz cuvettes. The spectra shown were subtracted from the background (buffer alone).

Trypsin Digestion—Proteoliposomes were incubated with sequencing grade modified trypsin (V5111, Promega, Madison, WI) at a protein:trypsin ratio of 200:1 (w/w). Typically proteoliposomes containing 20 μg of Cx43 or Cx43-S368A were incubated with trypsin at 37 °C, and the reactions were stopped at varying times by adding SDS gel sample buffer and increasing the temperature to 95 °C. In the control experiments shown, 1 mM PMSF was added before trypsin.

Other Techniques—Protein concentrations were determined using the BCA protein assay reagent from Bio-Rad using bovine serum albumin as standard. To detect possible contaminants, the highly sensitive protein gel stain SYPRO Ruby (Molecular Probes) was used according to the manufacturer's instructions.

Statistics—Data shown are means ± S.E. Statistically significant differences were assessed by the Student's *t* test for unpaired data or one-way analysis of variance as appropriate.

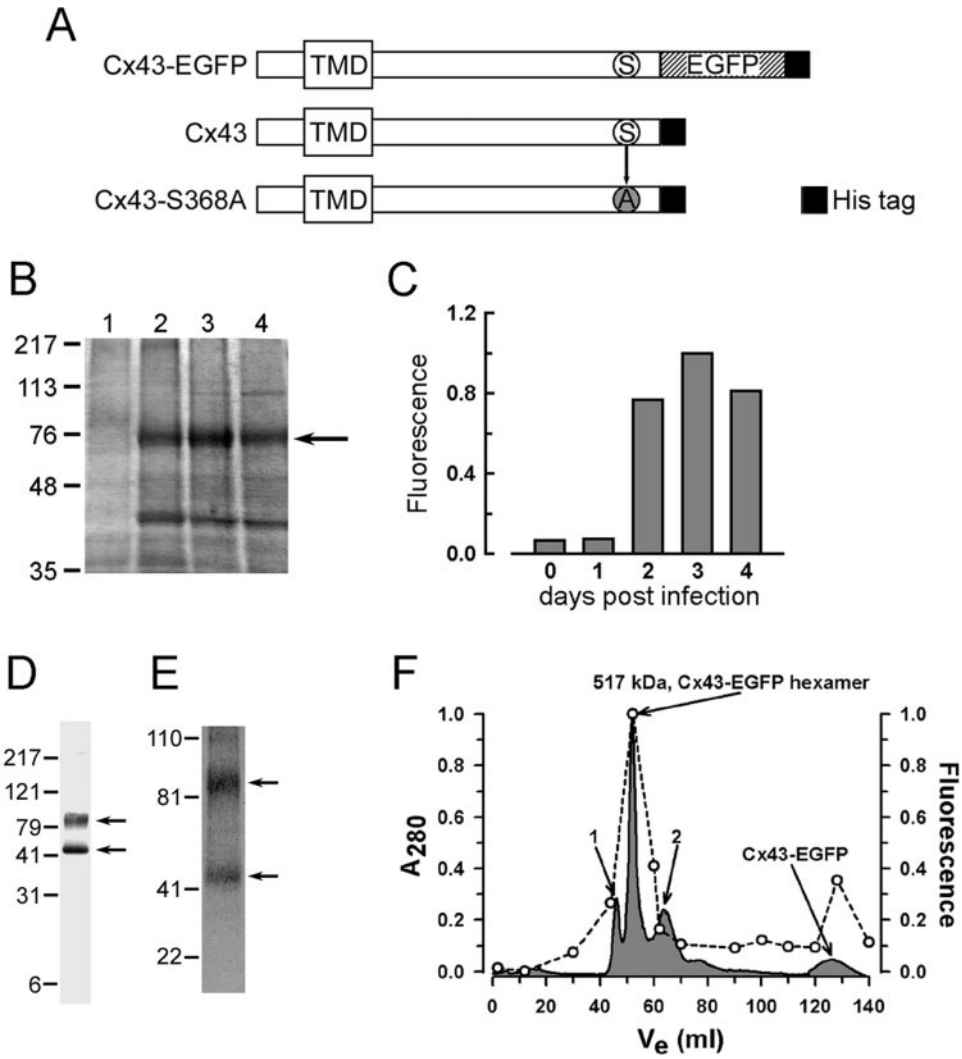

FIG. 1. **Purification and reconstitution of Cx43 overexpressed in insect cells.** *A*, schematic representation of the engineered Cx43 proteins. *TMD* represents the four membrane-spanning segments. *B*, time course of Cx43-EGFP expression in infected High-Five cells. *Lanes 1–4* correspond to alkali-stripped membranes from equal culture volumes (3×10^5 cells) at 1, 2, 3, and 4 days after infection, respectively. Samples were run on a 10% NuPage gel. The *arrow* points to Cx43-EGFP. *C*, time course of Cx43-EGFP expression assessed by cell fluorescence measurements. *D*, Coomassie Blue-stained gel (16% Tris/glycine) of purified Cx43 (14 μg) solubilized in OG. *E*, SYPRO Red-stained gel (10–20% Tris/glycine) of purified Cx43 (10 μg) in OG. The *arrows* in *D* and *E* point to the Cx43 monomer and dimer. *F*, gel filtration analysis chromatography. Purified Cx43-EGFP in 2.3% OG, 150 mM NaCl, 0.1 mM EDTA, and 10 mM HEPES/NaOH, pH 7.4, was run as described under "Experimental Procedures." Thyroglobulin and ferritin (669 and 440 kDa, respectively) were mixed with the Cx43-EGFP before injection into the FPLC system, and their peaks are labeled *1* and *2*, respectively. The *shadowed area* denotes the absorbance measured at 280 nm (A_{280}), and the *dotted line* joining *open circles* shows the fluorescence of some combined neighboring fractions (EGFP fluorescence). A_{280} and fluorescence were normalized to the corresponding Cx43-EGFP hexamer peak values.

RESULTS

Purification of Cx43 Overexpressed in Insect Cells—Our general strategy was to take advantage of the fluorescence of Cx43-EGFP to determine the best conditions for expression, solubilization, and purification and then to assess whether those conditions were also the best ones for the non-fluorescent Cx43 and Cx43-S368A (see Fig. 1*A* for schematics of these proteins). We found that initial trials with Cx43-EGFP indeed facilitated the initial screening and that the biochemical and functional properties of Cx43-EGFP and Cx43 were essentially identical in terms of detergent solubility, oligomerization, reconstitution efficiency, and permeability regulation by PKC-mediated phosphorylation. In this section, we summarize the main results of the expression, purification, and reconstitution of the Cx43s. Fig. 1, *B* and *C*, shows that a good expression level of Cx43-EGFP in High-Five cells is reached 2–3 days after infection. This time course of Cx43 expression was similar to that previously published for the expression of Cx32 (23). There was a good correlation between the expression assessed by SDS-PAGE (Fig. 1*B*) and the cell fluorescence (Fig. 1*C*). The latter method has the advantages that it can be used in real time and is simpler. The results for the expression of Cx43 and Cx43-S368A were similar (data not shown). Fig. 1 also shows that the His-tagged Cx43 can be easily purified based on its affinity for Ni^{2+}. The Coomassie Blue- and SYPRO Ruby-stained gels (Fig. 1, *D* and *E*, respectively) show that the preparation of Cx43 is highly purified, *i.e.* no contaminants were detected. The yield of Cx43 and Cx43-EGFP was 5–10 mg/liter of cells, whereas that of Cx43-S368A was ~70% of that of Cx43 (data not shown). The purified preparations obtained from membranes that were not alkali-extracted (see "Experimental Procedures") also appeared homogeneous, but the yield was somewhat lower.

Structural, biochemical, and functional studies have shown that gap-junctional hemichannels are oligomers composed of six connexin molecules (for a review, see Ref. 1). To assess the

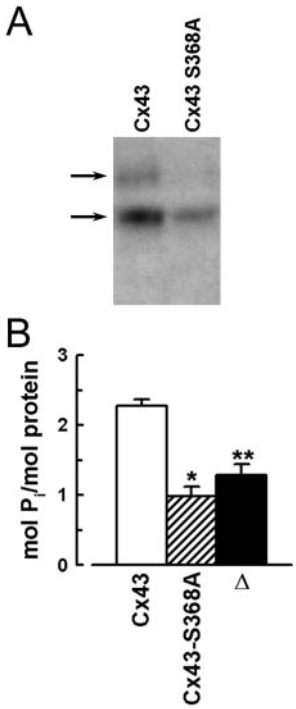

FIG. 2. **Phosphorylation of Cx43 by PKC.** *A*, autoradiography of purified Cx43 (*lane 1*) and Cx43-S368A (*lane 2*) phosphorylated by PKC *in vitro* in the presence of [γ-^{32}P]ATP. The proteins (4 μg each) were subjected to electrophoresis on a 16% Tris/glycine gel. The *arrows* point to the Cx43 monomer and dimer. *B*, phosphorylation stoichiometry. The moles of P_i incorporated into Cx43 and Cx43-S368A were determined as described under "Experimental Procedures." Δ refers to the difference between the phosphorylation of Cx43 and Cx43-S368A. Data from four experiments are shown. *, $p < 0.05$ compared with Cx43; **, $p < 0.05$ compared with 0.

oligomerization state of the purified Cx43 we performed gel filtration experiments on Cx43-EGFP solubilized in 2.3% OG. Fig. 1*F* shows that the major fluorescence peak (calculated molecular mass of 517 kDa) is compatible with a Cx43-EGFP hexamer with ~18% of the complex apparent weight in bound detergent. The absorbance peaks labeled *1* and *2* correspond to the 669- and 440-kDa markers (which did not affect Cx43 elution) added to the Cx43-EGFP sample. The smaller fluorescence peak is compatible with monomeric Cx43-EGFP. Similar results were obtained with Cx43 in 0.3% decyl-maltopyranoside (not shown). The predominance of connexons in the detergent-solubilized preparation was confirmed by determination of the sedimentation coefficients on a sucrose gradient (data not shown), a technique routinely used for the assessment of connexin quaternary structure (see Ref. 25). From the results in this section, we conclude that Cx43 in our preparation contains predominantly Cx43 assembled as connexons (70–80% of the total Cx43 from the gel filtration experiment data).

Ser-368 of Cx43 Is Phosphorylated by PKC—To test Cx43 function, we compared the permeability of GJHs reconstituted in liposomes in the dephosphorylated and PKC-phosphorylated states. We dephosphorylated the purified proteins with alkaline phosphatase (see "Experimental Procedures"). Dephosphorylated protein was then phosphorylated by PKC. Thus, we studied dephosphorylated and PKC-phosphorylated Cx43s in the absence of endogenous phosphorylation by other kinases. Pilot time course experiments using radiolabeled ATP showed that, under the conditions of our experiments, phosphorylation of Cx43 by PKC was complete by 1 h. Fig. 2*A* shows a representative autoradiography of Cx43 and Cx43-S368A phosphorylated by PKC in the presence of radiolabeled ATP. It is clear that the phosphorylation by PKC was substantially reduced when Ser-368 was substituted with Ala. The results of quantitative P_i incorporation studies show a maximal incorporation of P_i of ~2 and 1 mol/mol of Cx43 and Cx43-S368A, respectively (Fig. 2*B*). In control experiments performed in the absence of PKC Cx43 phosphorylation was <8% of the value in the presence of PKC. The calculated decrease in phosphorylation by the S368A mutation (~60 and ~75% from the stoichiometry and densitometry analyses, respectively) is consistent with previous observations that indicate that Ser-368 is the major phosphorylation target of PKC and that phosphorylation also occurs at Ser-372 (16, 17). We have recently shown that changes in the phosphorylation state of Ser-372 are not involved in the activation of Cx43 GJH carboxyfluorescein permeability by PKC blockers (33). The observation that the decrease in P_i incorporation in Cx43-S368A is ~1 mol/mol of protein suggests that under the conditions of these experiments all Cx43 Ser-368 residues are phosphorylated.

Phosphorylation of Ser-368 by PKC Reduces the Permeability of Cx43 GJHs—Previous studies using mutagenesis and pharmacological agents that affect the PKC signaling pathway suggested that phosphorylation of Ser-368 reduces the permeability of Cx43 gap-junctional channels to small hydrophilic solutes (16). However, it is not known whether the effects of PKC-mediated phosphorylation are direct (*i.e.* due to phosphorylation of Cx43) or indirect (*i.e.* via other signaling pathways affected by the PKC activation or involving regulatory factors or phosphorylation by PKC of one or more cytosolic factors). To determine whether or not the regulation of Cx43 GJH permeability is direct, we carried out functional studies with purified Cx43 GJHs reconstituted into liposomes. Purified Cx43 was reconstituted in phosphatidylcholine and phosphatidylserine liposomes by dialysis/extrusion as described under "Experimental Procedures." At protein:lipid ratios of 1:25, 1:60, 1:100, and 1:280 (w/w) essentially all the protein was incorporated into the liposomes. The reconstitution efficiency for Cx43-EGFP and Cx43-S368A was also >90%. In all experiments Cx43 amounts were measured in the proteoliposomes (*i.e.* after reconstitution, not inferred from the reconstitution efficiency).

We assessed sucrose permeability by measuring the migration of the proteoliposomes on a linear isoosmolar sucrose/urea gradient, a method developed by Harris and co-workers (30). This assay has been used previously to evaluate sucrose permeability of proteoliposomes containing immunopurified Cx32 and Cx43 from rat and mouse liver (26, 30, 31), and it is based on the higher density of sucrose-loaded (permeable) compared with urea-loaded (impermeable) liposomes under isoosmolar conditions. For these experiments, we loaded the proteoliposomes with Lucifer Yellow (M_r 445), a probe that permeates connexin hemichannels. This allowed us to assess transport of sucrose and Lucifer Yellow in opposite directions and also to rule out migration of sucrose-impermeable proteoliposomes due to aggregation (31, 34). Aggregates of liposomes containing phosphorylated Cx43 would migrate as permeable proteoliposomes but would also retain Lucifer Yellow. The liposomes containing untreated Cx43 GJHs showed a bimodal distribution with most of them remaining closer to the top of the gradient tube (not shown). The proteoliposomes containing PKC-phosphorylated or dephosphorylated Cx43 GJHs showed unimodal distribution with the former closer to the top and the latter closer to the bottom of the gradient tube (Fig. 3*A*). The absence of unshifted sucrose-impermeable proteoliposomes in these experiments is because essentially all liposomes contained at least one connexon (different from the experiments in Fig. 3*C*, see "Experimental Procedures"). The tube image in Fig. 3*A*, *right*, shows a sharp proteoliposome band (see *arrow*) as expected for sucrose-permeable proteoliposomes (30, 31).

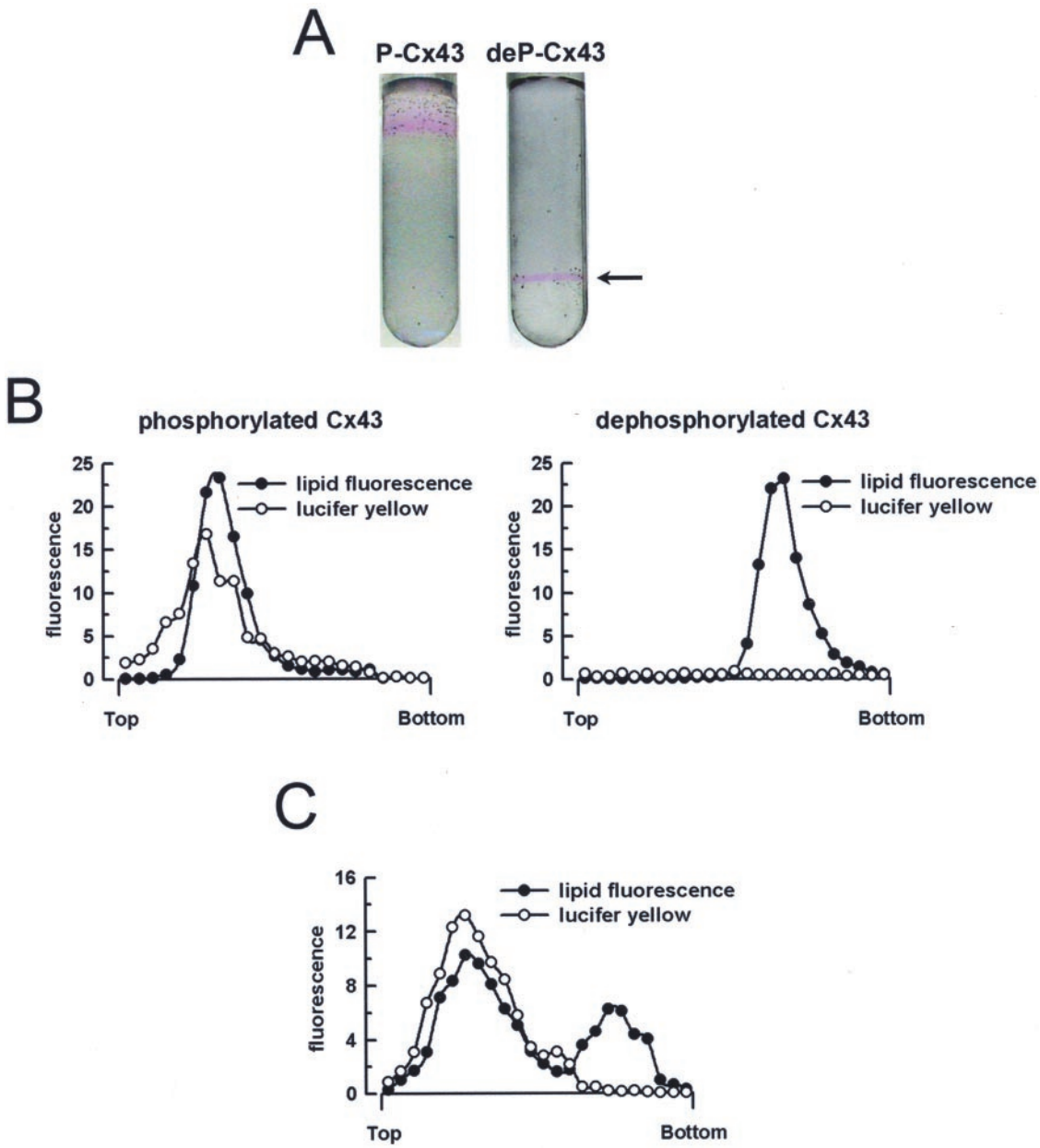

FIG. 3. **Effects of PKC-mediated phosphorylation on the sucrose permeability of Cx43 hemichannels.** *A*, migration of Cx43 proteoliposomes analyzed in an isoosmolar linear sucrose density gradient. Images showing the postcentrifugation position of the rhodamine B-labeled proteoliposomes containing PKC-phosphorylated Cx43 (*P-Cx43*) or dephosphorylated Cx43 (*deP-Cx43*) are shown. The *arrow* points to the sharp band containing the sucrose-permeable proteoliposomes. The images acquired under visible room fluorescent light are from representative experiments using proteoliposomes containing a calculated average of ~2.3 connexons/liposome (1:100 protein:lipid ratio, w/w). *B*, distribution of the rhodamine B and Lucifer yellow fluorescence in the gradients. The graphs are from representative experiments performed with the same protocol as those in *A*. The rhodamine B-labeled proteoliposomes containing Cx43 were preloaded with Lucifer Yellow and analyzed in an isoosmolar linear sucrose density gradient by measuring the fluorescence from rhodamine B and Lucifer Yellow in fractions from the gradient. *C*, data obtained using proteoliposomes containing dephosphorylated Cx43 incorporated at a 1:280 protein:lipid ratio (average of 0.8 connexons/liposome). See *A*, *B*, and "Experimental Procedures" for additional details.

Fig. 3*B* shows that the proteoliposomes containing phosphorylated Cx43 also contain Lucifer Yellow, indicating that these proteoliposomes are not leaky. The absence of Lucifer Yellow in the proteoliposomes containing dephosphorylated Cx43 indicates that they were not only loaded with sucrose during the centrifugation but also lost the Lucifer Yellow (during the gel filtration step prior to the overlay of the liposomes onto the gradient tube). From these results we conclude that purified Cx43 reconstituted in liposomes forms functional sucrose- and Lucifer Yellow-permeable channels and that phosphorylation of GJHs by PKC decreases sucrose and Lucifer Yellow fluxes (and thus permeabilities).

To determine the fraction of Cx43 molecules that form sucrose-permeable GJHs, we again used the transport-specific density shift technique. In the experiments shown in Fig. 3, *A* and *B*, we reconstituted Cx43 at a protein:lipid ratio of 1:100 (w/w) to assure that essentially all liposomes contained at least one connexon (2.3 connexons/liposome). For the experiments in Fig. 3*C* we used dephosphorylated Cx43 reconstituted at a ratio of 1:280, which for 100-nm-diameter liposomes yields an average of 0.83 connexons/liposome, assuming that all Cx43 molecules form connexons, that the bilayer thickness is 4 nm, and that the surface area of a phospholipid molecule is 0.7 nm^2 (see Ref. 34). According to Poisson's distribution, 56% of the proteo-

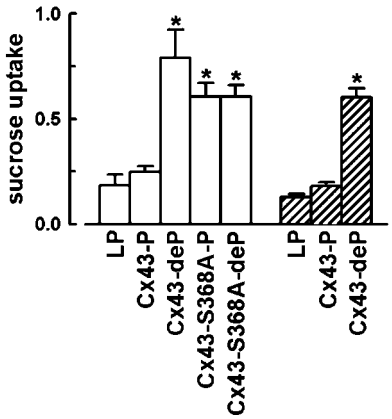

FIG. 4. **Effect of phosphorylation on sucrose uptake by proteoliposomes containing Cx43 or Cx43-S368A.** The effect of PKC-mediated phosphorylation was assessed from the steady-state (30-s) sucrose uptake expressed in nmol of sucrose/mg of lipid (3.6 μg of protein in the proteoliposomes). *LP* represents liposomes, and *-P* and *-deP* represent PKC-phosphorylated and dephosphorylated Cx43, respectively. The *open* and *hatched bars* correspond to data obtained in the absence and presence of 459 mM urea, respectively. The data are presented as means ± S.E. of triplicate measurements from at least three independent experiments. * indicates $p < 0.05$ compared with liposomes alone.

liposomes should be sucrose-permeable and migrate faster along the gradient. The experiment in Fig. 3*C* shows that ~32% of the proteoliposomes are sucrose-permeable. Therefore, ~60% of the reconstituted Cx43 forms functional connexons. Since ~75% of the solubilized Cx43 is present as connexons (see Fig. 1*F*), it appears that most or all of the Cx43 that appeared as connexons in OG are reconstituted as functional GJHs.

To determine the effects of phosphorylation on sucrose permeability we also measured sucrose uptake in proteoliposomes containing an estimated average of 0.8 connexons/liposome. Pilot time course experiments showed that equilibration of sucrose across the liposome bilayer containing dephosphorylated Cx43 occurs in less than 1–2 s. Since the expected equilibration time, based on the Cx43 gap-junctional channel data from Valiunas *et al.* (35), is much shorter (<0.1 ms), it is not possible to measure the initial influx rate using rapid filtration techniques. However, the experiments shown in Fig. 3 indicate that the effect of PKC-mediated phosphorylation on sucrose permeability is essentially an all or none phenomenon (the proteoliposomes are either impermeable or permeable). Therefore, we measured the steady-state liposome-associated sucrose (30-s uptakes) to evaluate the response of Cx43 to phosphorylation by PKC. Fig. 4 shows the uptake of sucrose/mg of lipid (3.6 μg of protein in the proteoliposomes) using Cx43 purified without alkali extraction of the membranes both in the absence (*open bars*) and presence (*hatched bars*) of 459 mM urea, the concentration of the chaotropic agent used in the transport-specific density shift technique. Incorporation of dephosphorylated Cx43 into liposomes produced a significant increase in the sucrose uptake as expected from the results shown in Fig. 3, whereas phosphorylated Cx43 did not elicit liposome permeability. These observations are entirely in agreement with the data shown in Fig. 3 and strongly support the conclusion that phosphorylated Cx43 GJHs are impermeable to sucrose. In addition, the data in Fig. 4 show that Cx43-S368A GJHs are permeable to sucrose independently of their phosphorylation status. Finally the presence of urea does not cause liposome leakage or affect the response to phosphorylation, validating the results obtained with the transport-specific density shift technique.

The orientation of the connexons in the proteoliposomes is not a concern for the interpretation of our transport experiments because dephosphorylation and phosphorylation were carried out in detergent-solubilized Cx43 and the transport of organic solutes of $M_r < 1{,}000$ occurs in both directions (influx and efflux) through GJHs (see Fig. 3). However, it is interesting to note that the sucrose uptake into proteoliposomes reconstituted with dephosphorylated Cx43 that was phosphorylated by PKC after reconstitution was only 10 ± 5% ($n = 3$) of that in proteoliposomes subjected to the same treatment but without PKC. This value is not different from that in proteoliposomes reconstituted with Cx43 phosphorylated in solution before reconstitution (shown in Fig. 4). In both cases the liposomes are impermeable to sucrose. Since the proteoliposomes are tight for sucrose and Lucifer Yellow (see Fig. 3) and therefore also must be PKC-impermeable, we conclude that in most Cx43 connexons Ser-368 is accessible to the external solution and thus that the hemichannels are inserted into the liposomes in an inside-out orientation. In these experiments, dephosphorylated Cx43 was reconstituted, and the phosphorylation was performed on an aliquot of the preparation. Therefore, these results also show that neither differences in protein handling before reconstitution nor the amount of reconstituted protein account for the decreased sucrose permeability elicited by PKC-mediated phosphorylation.

Phosphorylation of Ser-368 Elicits a Conformational Change of Cx43—The preceding results indicate that Ser-368 is critical for the regulation of Cx43 GJHs by PKC-mediated phosphorylation. In the next series of studies, we explored the possibility that phosphorylation of this residue by PKC produces a conformational change of Cx43. We tested this in purified Cx43 solubilized in detergent (Trp fluorescence experiments) or incorporated in liposomes (limited proteolysis experiments). Fig. 5*A*, *left*, shows that the Trp fluorescence emission spectrum of dephosphorylated Cx43 solubilized in OG is different from that of PKC-phosphorylated Cx43. The emission maximum of the dephosphorylated protein is decreased in intensity and shifted to a shorter wavelength (by ~10 nm). In contrast, phosphorylation of the Cx43-S368A mutant did not affect the Trp fluorescence spectrum (Fig. 5*A*, *right*). The changes in Cx43 Trp fluorescence suggest that one or more Trp residues of Cx43 are in a more hydrophobic environment when the protein is dephosphorylated. We then performed limited proteolysis experiments to determine whether the conformational changes elicited by phosphorylation occur when Cx43 is reconstituted in liposomes and not only in the detergent-solubilized protein. Fig. 5*B* shows that the phosphorylation state changes the conformation of Cx43 in liposomes. Cx43 and Cx43-S368A were digested with trypsin, and the digestion products were identified by immunoblotting using an antibody against the Cx43 C-terminal region (see "Experimental Procedures"). The results indicate that several Cx43 fragments containing the antibody epitope are less sensitive to trypsin digestion in the phosphorylated protein. Under the conditions of our experiments, after 30 min of digestion, we observed trypsin-resistant fragments containing the antibody epitope (Fig. 5*B*, *a*, *b*, and *c*) only in the digests of the proteoliposomes containing phosphorylated Cx43, whereas no resistant fragments were detected in the digests of dephosphorylated Cx43 (*right panel*) or Cx43-S368A independently of its phosphorylation state (*left panel*). Based on their mobility in SDS-PAGE, *a* corresponds to the full-length protein, and fragments *b* and *c* with apparent molecular masses of 26 and 18 kDa, respectively, could correspond to the C-terminal domain plus the last two transmembrane segments (calculated size of 29 kDa) and the C-terminal domain (calculated size of 19 kDa), respectively.

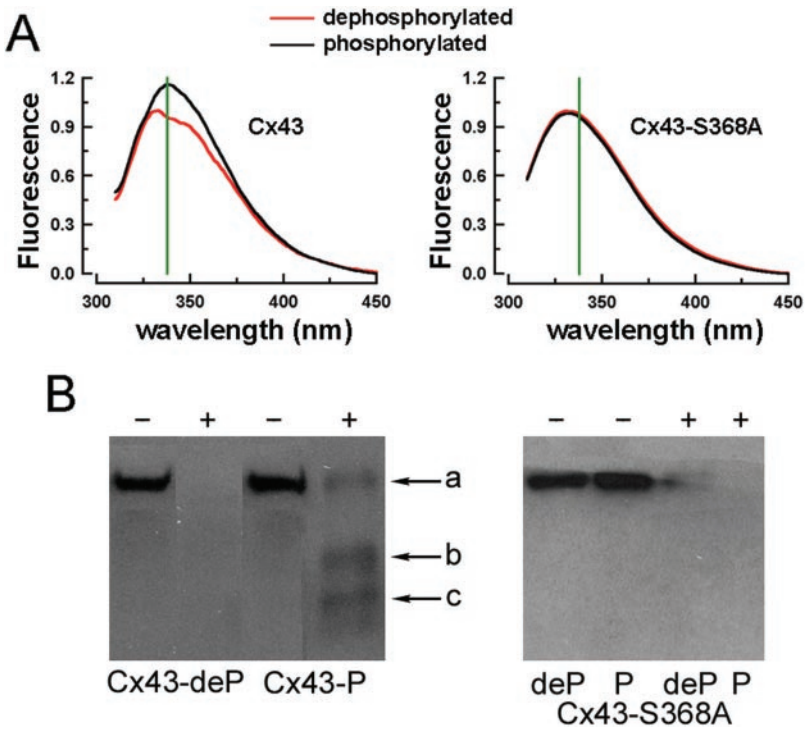

FIG. 5. **Conformational changes of Cx43 elicited by PKC-mediated phosphorylation.** *A*, effects of phosphorylation state on intrinsic tryptophan fluorescence of Cx43 and Cx43-S368A. Trp fluorescence of the purified proteins in OG was measured at an excitation wavelength of 295 nm. The *vertical green lines* mark the wavelength of the maximum fluorescence obtained with phosphorylated Cx43. The data shown are representative of four (Cx43) and three (Cx43-S368A) separate experiments on two different preparations. *B*, effects of phosphorylation by PKC on the sensitivity of Cx43 and Cx43-S368A to trypsin digestion. Proteoliposomes containing dephosphorylated or PKC-phosphorylated Cx43 or Cx43-S368A were subjected to proteolysis with trypsin as described under "Experimental Procedures." Protein amount/lane was 4 μg, and the Western blots were probed with an anti-C terminus Cx43 antibody. − and + indicate the absence (PMSF added before trypsin) and presence of active trypsin, respectively. The *arrows* point to clearly identifiable proteolytic fragments (*a*, *b*, and *c*) of phosphorylated Cx43 containing the antibody epitope after a 30-min digestion. The blots shown are representative of seven and three experiments on proteoliposomes containing Cx43 and Cx43-S368A, respectively. *-P* and *-deP* represent PKC-phosphorylated and dephosphorylated Cx43, respectively.

DISCUSSION

In this work, we showed that phosphorylation of purified Cx43 GJHs by PKC produces a conformational change evident in both detergent-solubilized and liposome-reconstituted protein and reduces the sucrose permeability of GJHs in proteoliposomes. Our data strongly suggest that Ser-368 is the critical target residue for these effects because the S368A mutant is constitutively active and PKC-insensitive. Finally the phosphorylation effect is direct on Cx43 without the need for cytosolic regulatory factors because it is present in highly purified Cx43 preparations.

Although the primary function of connexins is to form the gap junctions that communicate with neighboring cells, recent evidence indicates that hemichannels are present at the plasma membrane of a variety of cells where they may have important physiological and pathophysiological roles (7–9, 12, 36–39). Independently of the importance of GJHs *per se*, it is clear that they are a simpler system useful for studies of the basic properties of gap-junctional channels as well as their regulation (see Ref. 1).

Due to the complex interactions between cell signaling pathways, it is important to use functional, purified, and reconstituted Cx43 to dissect direct from indirect regulatory effects of phosphorylation. Inasmuch as overexpression of Cx32 and Cx43 in insect cells has been obtained using a baculovirus expression system (23, 40), we chose the same system for overexpression of Cx43. We also took advantage of previous information on purification of Cx32 and Cx43, including alkali extraction of the crude membranes (23) and use of *n*-dodecyl-β-D-maltoside and OG for solubilization (23, 26) and phosphatidylcholine/phosphatidylserine for reconstitution (26). Using the available information plus a rapid screening of conditions in pilot experiments using Cx43-EGFP, we were able to develop a modified method that allows us to obtain milligram amounts of a homogeneous preparation of His-tagged Cx43. The sucrose permeability of Cx43 reconstituted in proteoliposomes is regulated by phosphorylation of Ser-368 by PKC, indicating that our expression-purification-reconstitution system is useful to elucidate the properties of Cx43. There is no effect of the His tag near the end of Cx43 on its response to phosphorylation because our data show the same regulation by PKC-mediated phosphorylation of Cx43 and Cx43-EGFP. In the latter, the His tag is >700 amino acids away from the end of the Cx43 primary sequence. More importantly, the permeability of Cx43 GJHs to sucrose and Lucifer Yellow is essentially abolished by phosphorylation of Ser-368 in the His-tagged Cx43, a result consistent with our recent data on the PKC-regulated permeability to carboxyfluorescein of Cx43 GJHs expressed in *Xenopus* oocytes (33). In those experiments, we used Cx43 without a His tag, and phosphorylation of Ser-368 by PKC also rendered the hemichannels impermeable to the hydrophilic solute.

In the analysis of the filtration experiments, we assume that the difference between the sucrose associated with the proteoliposomes and liposomes represents sucrose uptake. Several observations support this assumption including the following. (*a*) Phosphorylation/dephosphorylation of Cx43 produced large changes in proteoliposome-associated sucrose. (*b*) The amounts of sucrose associated with liposomes and with proteoliposomes containing PKC-phosphorylated Cx43 were indistinguishable and very small compared with that associated with proteoliposomes containing dephosphorylated Cx43. (*c*) Sucrose is a hydrophilic solute that is not expected to permeate pure lipo-

somes or partition significantly into lipids, which is consistent with the very low level of sucrose association to protein-free liposomes. (*d*) The direct estimation of changes in proteoliposome density on an isoosmolar sucrose gradient indicates that the phosphorylation status of Cx43 affects the sucrose uptake.

The phosphorylation studies using radiolabeled ATP showed a clear difference in ^{32}P labeling between Cx43 and the Cx43-S368A mutant. This is consistent with the previous demonstration that phosphorylation of Cx43 by PKC occurs at Ser-368 (16, 17). It has been shown that PKC phosphorylates Ser-368 and Ser-372, and mutagenesis experiments suggest that phosphorylation of Ser-368 decreases the permeability of gap-junctional channels (16). However, the question remains as to whether the response to phosphorylation by PKC requires other factors. Extensive work indicates that a receptor-particle mechanism, somewhat analogous to the inactivation of some K^+ channels ("ball-and-chain mechanism," see Ref. 41), is involved in the regulation of Cx43 by intracellular pH and mitogen-activated protein kinase phosphorylation (20, 42). It has been proposed that the Cx43 C terminus, which is necessary for the regulation, acts as the particle (ball). The studies on the regulation of Cx43 permeability by intracellular pH strongly support the existence of this receptor-particle mechanism (18–20, 43), but the need for a cytosolic intermediary factor has been suggested for some connexins (21). It is therefore conceivable that one or more factors (*e.g.* a regulatory protein) are needed for the regulation by phosphorylation. Our observations using Cx43 GJHs purified and reconstituted into proteoliposomes indicate that phosphorylation by PKC directly reduces sucrose permeability without the need for a soluble regulatory protein or other cytosolic factors. Because of the high purity of our Cx43 preparation, we also ruled out the need for an associated regulatory integral membrane protein (which has not been proposed for the regulation by PKC-mediated phosphorylation). In addition, since we dephosphorylated Cx43 prior to the phosphorylation by PKC, the presence of other endogenously phosphorylated residues is not required for the response to phosphorylation by PKC. From these results, we conclude that the effect of phosphorylation of Cx43 GJHs by PKC is direct, *i.e.* it does not require other molecule(s) and is independent of phosphorylation by other kinases.

As mentioned above, a particle-receptor mechanism where the C-terminal domain is the particle may be involved in the reduction of Cx43 permeability by lowering intracellular pH and phosphorylation by mitogen-activated protein kinase and PKC. However, the subdomains involved are clearly different. A Pro-rich region in the C-terminal domain is required for the effect of pH and phosphorylation by mitogen-activated protein kinase (18, 42) but is not involved in the response to phosphorylation by PKC (33). The results presented here do not address in detail the mechanism by which PKC-mediated phosphorylation abolishes the permeability of Cx43 GJHs to organic hydrophilic solutes of $M_r < 1{,}000$. However, phosphorylation by PKC results in the presence of trypsin-resistant C-terminal fragments that contain the whole C-terminal domain as well as partial protection of the full-length protein. This is compatible with the notion that phosphorylation of Ser-368 results in physical interaction of the C-terminal domain with other regions of Cx43, perhaps the intracellular loop as in the case of acidification (see Ref. 43), reducing the protease sensitivity of the C-terminal domain and the whole protein.

An interesting fact is that the decrease in small organic solute permeability by PKC activation of gap-junctional channels is not necessarily accompanied by a decrease in gap-junctional electrical conductance (for a review, see Ref. 14). In fact, total Cx43 gap-junctional channel conductance can rise in parallel with the decrease in solute permeability (44). Single channel studies indicate that there is a decrease in average single channel conductance by an increase in the frequency of low conductance channel openings (45), which would account for the decreased permeability to organic solutes. As a consequence of the differential modulation of permeability to small solutes and ions, phosphorylation of Cx43 could reduce fluxes of organic hydrophilic solutes such as ATP, cAMP, and NAD^+ without affecting small ion fluxes and electric coupling significantly.

In this work, we obtained evidence for conformational changes by phosphorylation of detergent-solubilized Cx43 as well as liposome-reconstituted Cx43. Our observations indicate that PKC-mediated phosphorylation of Cx43, but not of Cx43-S368A, produces a conformational change that affects Trp fluorescence emission and resistance to trypsin digestion. PKC-mediated phosphorylation produces an increase in Trp fluorescence and a shift of the maximum to a longer wavelength. These effects are absent in Cx43-S368A whose Trp emission spectrum was unchanged by PKC-mediated phosphorylation and is similar to that of dephosphorylated Cx43. Since Cx43 has six Trp residues located in several regions of the molecule (extracellular, intracellular, and transmembrane), we cannot ascribe the structural changes to specific domains. However, the availability of large amounts of purified, functional Cx43 will allow us to carry out more detailed structural-functional studies to elucidate the mechanism of Cx43 regulation. We propose that phosphorylation of Ser-368 by PKC produces a conformational change in the C-terminal domain that leads to a decrease in Cx43 GJH permeability to small organic solutes.

Acknowledgments—We thank Dr. Scott John (Cardiovascular Research Laboratory, University of California at Los Angeles) for the Cx43 DNA, Dr. James Lee for advice during the course of the studies, and Dr. Nalin Kumar for comments on the manuscript.

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Regulation of Purified and Reconstituted Connexin 43 Hemichannels by Protein Kinase C-mediated Phosphorylation of Serine 368

Xiaoyong Bao, Luis Reuss and Guillermo A. Altenberg

J. Biol. Chem. 2004, 279:20058-20066.
doi: 10.1074/jbc.M311137200 originally published online February 17, 2004
