Mechanism of regulation of the gap junction protein connexin 43 by protein kinase C-mediated phosphorylation

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Bao, Xiaoyong, Guillermo A. Altenberg, and Luis Reuss. Mechanism of regulation of the gap junction protein connexin 43 by protein kinase C-mediated phosphorylation. Am J Physiol Cell Physiol 286: C647–C654, 2004. First published November 5, 2003; 10.1152/ajpcell.00295.2003.—Phosphorylation of the gap junction protein connexin 43 (Cx43) by protein kinase C (PKC) decreases dye coupling in many cell types. We report an investigation of the regulation by PKC of Cx43 gap junctional hemichannels (GJH) expressed in Xenopus laevis oocytes. The activity of GJH was assessed from the uptake of hydrophilic fluorescent probes. PKC inhibitors increased probe uptake in isolated oocytes expressing recombinant Cx43, indicating that the regulatory effect occurs at the hemichannel level. We identified by mutational analysis the carboxy-terminal (CT) domain sequences involved in this response. We found that 1) Ser368 is responsible for the regulation of Cx43 GJH solute permeability by PKC-mediated phosphorylation, 2) CT domain residues 253–270 and 288–359 are not necessary for the effect of PKC, and 3) the proline-rich CT region is not involved in the effect of phosphorylation by PKC. Our results demonstrate that Ser368 (but not Ser372) is involved in the regulation of Cx43 solute permeability by PKC-mediated phosphorylation, and we conclude that different molecular mechanisms underlie the regulation of Cx43 by intracellular pH and PKC-mediated phosphorylation.

protein kinase C blocker; dye loading; hemichannel

GAP JUNCTIONAL CHANNELS are aqueous channels formed by the docking of two connexons or gap junctional hemichannels (GJH) from adjacent cells (4, 17, 49). Each connexon is composed of six connexin (Cx) molecules (48, 49). Connexins have four transmembrane domains and intracellular amino and carboxy termini (reviewed in Ref. 18).

Cx43 is expressed in several tissues and organs, including brain, myocardium and kidney, as well as in capillary endothelial cells (1–3, 31, 40, 44, 52). Phosphorylation of specific serine or tyrosine residues of Cx43 has been shown to disrupt intercellular dye transfer, an effect that is abolished by deletion of the carboxy-terminal (CT) domain or by mutations of specific serine and tyrosine residues in this domain (reviewed in Refs. 10, 18, 25, 27). Relevant to our work, protein kinase C (PKC)-mediated phosphorylation decreases intercellular dye coupling (see Ref. 25 for review). The regulation of Cx43 by changes in intracellular pH also depends on the CT domain (10, 14). These observations underscore the importance of this domain in the regulation of gap junctional channels.

The presence of Cx43 nonjunctional precursors in the plasma membrane was first established by biochemical techniques (38), and the existence of functional hemichannels was demonstrated by studies in Xenopus oocytes expressing native Cx38 (13) or recombinant Cx46 (39), as well as in horizontal cells of the catfish retina (11). More recently, the existence of Cx43 GJH on the plasma membrane of mammalian cells has been strongly suggested from measurements of dye uptake stimulated by lowering external Ca2+, metabolic inhibition, or connexin dephosphorylation, both in native cells and in cell lines (8, 21, 28, 53). However, the physiological and pathophysiological significance of these channels is not well defined and their regulation is poorly understood. Unlike gap junctional channels, which enable communication between adjacent cells with identical or similar composition, GJH communicate the cell interior with the extracellular fluid, and therefore their activation may result in cell injury, including cell death, because of water and solute fluxes that cannot be compensated by the normal membrane transport mechanisms. This notion is supported by experimental data on cardiomyocytes (21), astrocytes (8), and renal proximal tubule cells (53), strongly suggesting that Cx43 GJH are activated by ATP depletion and that the mechanism may involve dephosphorylation. The proposal that Cx43 hemichannels play a critical role under certain physiological and pathophysiological conditions underscores the importance of understanding if and why Cx43 hemichannels undergo activation by dephosphorylation of PKC target residues.

It has been established that PKC-mediated phosphorylation reduces and dephosphorylation enhances Cx43-mediated dye coupling, whereas the effects on gap junctional conductance are variable (see Ref. 25). The dissociation between the effects of PKC activity on macroscopic conductance and dye coupling may be related to the different biophysical mechanisms involved. The dwell time of the channel in a low-conductance state increases with PKC-mediated phosphorylation and decreases when phosphorylation by PKC is reduced (23, 26, 35, 36, 43). This is in contrast with the effects of acidification and v-Src- and MAPK-mediated phosphorylation, which decrease gap junctional conductance and dye coupling in parallel, without changes in single-channel conductance, probably through a decrease in open probability (see Refs. 9, 19, and 29).

Both Ser368 and Ser372 are phosphorylated by PKC in vitro, and there is also evidence for Ser368 phosphorylation in vivo (26). Substitution of Ser368 with Ala abolishes the effect of PKC stimulation (26), but it is not clear whether Ser372 plays a role in this process (i.e., whether phosphorylation of the 2 residues is required). Cx43 pH gating requires interaction between the CT domain of Cx43 and other regions of the molecule. A ball-and-chain regulatory mechanism has been proposed on the basis of the following observations (see Ref. 25).
10. 1) CT domain truncation impairs acidification- and v-Src-mediated gating (32, 55), and these gating effects are rescued by coexpressed CT domain (37, 55). 2) Recombinant CT domain and intracellular loop peptides interact in vitro (12). The CT domain contains four (PXX)₄ proline repeats (see Fig. 1) between residues 271 and 287. This is a well-known peptide-peptide interaction motif necessary for the reduction in Cx43 permeability elicited by lowering intracellular pH (7, 37).

Our studies aim to expand our knowledge of the mechanisms of regulation of GIH, entities important by themselves, and to help better understand the regulation of gap junction channels by investigating a simpler system in which the coupling of two connexons cannot contribute to the observed effects. We undertook the present experiments to identify CT domain residues and domains that are critical for hemichannel inhibition by PKC-mediated phosphorylation.

**MATERIALS AND METHODS**

**Plasmid engineering.** A schematic representation of the constructs studied is shown in Fig. 1. The full-length rat Cx43 was cut with XbaI and SpeI from a plasmid generously provided by Dr. Scott John (21) and subcloned into the oocyte expression vector pOcyt7 (34) cut with XbaI. We obtained the Ser368Ala mutant in the same plasmid by site-directed mutagenesis (Quick Change Multisite site-directed mutagenesis kit; Stratagene, La Jolla, CA) with the following mutagenic primer:

5'CGACCTTCAGCAGGAGGCCGAGGCTTGCTCGGCTATGTCCTCCTCCTGGGTACACAGG-3'/H11032.

For each Ser to Ala substitution, we changed the underlined bases to GCC, and made the corresponding complementary changes in the reverse adaptors. To obtain the deletion mutant Cx43D2 we replaced the 165 bp XcmI/NheI fragment of the Cx43D1 DNA with a 66-bp oligonucleotide adaptor. The adaptor sequence was: forward, 5'-CTAGGCTTGTTTCCAGCAGGACTCAGGCTTGCTCGGCTATGTCCTCCTCCTGGGTACACAGG-3'/H11032, and reverse 5'-CTAGGCTTGTTTCCAGCAGGACTCAGGCTTGCTCGGCTATGTCCTCCTCCTGGGTACACAGG-3'/H11032. The resulting DNA had a new unique SpeI site (underlined), right before the proline-rich region of the CT domain, used to generate the Cx43D3 deletion mutant (see Fig. 1). The insertion of the SpeI site resulted in the addition of the coding sequence for two foreign amino acids in front of Cys271 (Thr-Ser). To obtain the Cx43D3 DNA, we removed the SpeI/NheI 57-bp fragment of the Cx43D2 DNA and performed a self-ligation of the plasmid. To generate Cx43D2-RC, we replaced the SpeI/NheI fragment of Cx43D2 DNA with the following oligonucleotide adaptor sequence: forward, 5'-CTAGGCTTGTTTCCAGCAGGACTCAGGCTTGCTCGGCTATGTCCTCCTCCTGGGTACACAGG-3'/H11032, and reverse 5'-CTAGGCTTGTTTCCAGCAGGACTCAGGCTTGCTCGGCTATGTCCTCCTCCTGGGTACACAGG-3'/H11032. DNA seq-

Fig. 1. Schematic representation of connexin 43 (Cx43) and the Cx43 mutants used in these experiments. The proline-rich region and the sequences deleted in Cx43D1 (D1), Cx43D2 (D2), and Cx43D3 (D3) are shown. The residues relevant to the engineering of the mutants are marked with asterisks, and the mutated residues are underlined.
quencing of the constructs was performed at the Protein Chemistry Core Laboratory of the University of Texas Medical Branch.

**Oocyte preparation and cRNA injection.** For T7-directed capped cRNA synthesis (mMessage machine, Ambion, Austin, TX), we linearized the Cx43 plasmids in pOcyt7 with XhoII and used them as templates. Xenopus laevis oocytes were isolated and prepared for cRNA injection as described previously (6). Oocytes were injected with 7.4 ng of antisense Cx38 oligonucleotide (to reduce endogenous expression of Cx38; Ref. 13) alone or with 10–28 ng of wild-type or mutant Cx43 cRNA. Oocytes expressing Cx43 with mutations at position 368 underwent lysis in control solution at a much faster rate than control oocytes (data not shown). Oocyte lysis by expression of Cx46 GH was observed previously (39), although it has not been documented with the expression of constitutively active Cx43 GH in oocytes or mammalian cells (see, e.g., Refs. 14, 26). We do not have a definitive answer for the apparent discrepancy, but cell lysis in our experiments could be due to relatively higher expression levels of GH compared with other oocyte expression systems (e.g., our pOcyt7-based plasmid contains a β-globin untranslated region that boosts expression in oocytes; see Ref. 34) and/or the use of isolated cells. During the generation of mammalian cell lines, expression of Cx43-3368A could result in undetected lysis of some cells, but the selected clones should have most constitutively active connexons formed after injection of Cx43 cRNA. Oocyte lysis was prevented by elevating the Ca²⁺ concentration ([Ca²⁺]i) in Barth’s solution from 1 to 5 mM from the time of injection until immediately before the dye-loading test. High [Ca²⁺]i is known to block Cx43 GH expressed in mammalian cells (as e.g., Ref. 28), as well as other GH expressed in Xenopus oocytes (13), but in one study, putative Cx43 GH were found to be insensitive to external [Ca²⁺]i (8).

**PKC activity assay.** We measured total PKC activity in groups of 10 oocytes per experiment. The oocytes were lysed in 100 μl of (in mM) 20 Tris-HCl, pH 7.4, 2 EDTA, 0.5 EGTA, 5 β-mercaptoethanol, and 250 sucrose, with protease inhibitor cocktail (1/500 dilution of P8340, Sigma) and 1% Triton X-100. Cells were incubated in this solution for 60 min on ice, with continuous mixing. After centrifugation at 100,000 g for 60 min at 4°C, the PKC activity of the supernatant was assayed with the PepTag PKC assay kit (Promega, Madison, WI), using 5 μl of sample per assay, following the manufacturer’s instructions. Reactions proceeded at 30°C for 30 min and were stopped by heating at 95°C for 10 min. To subtract peptide phosphorylation independent of PKC, we carried out parallel measurements under each condition in the presence of 5 μM calphostin C added to the reaction in vitro. This concentration of the inhibitor is sufficient to block PKC activity completely (22). The phosphorylated peptide substrate was separated by agarose gel electrophoresis and measured by fluorescence emission at 592 ± 4 nm, with an excitation at 568 ± 4 nm on a SPEX Fluorolog-2 (SPEX Industries, Edison, NJ), using the substrate peptide as a standard.

Western blots and immunoprecipitation. We prepared enriched oocyte plasma membranes by membrane biotinylation and streptavidin affinity-purification and performed Western blots with a rabbit anti-rat Cx43 polyclonal antibody against the Cx43 carboxy terminus (CT) (Zymed, South San Francisco, CA) and a horseradish peroxidase-labeled goat anti-rabbit secondary antibody. Detection was by chemiluminescence (ECL, Amersham Pharmacia Biotech, Cleveland, OH). For the surface membrane protein biotinylation, oocytes were incubated with a cell-impermeant biotinylation reagent (EZ-Link Sulfo-NHS Biotin, Pierce Biotechnology, Rockford, IL) for 30 min at 4°C. Enrichment of the biotinylated membrane proteins was performed as described previously (6). Immunoprecipitation of Cx43 was carried out as described previously (30) with minor modifications. Briefly, 100 oocytes were washed twice in ice-cold Barth’s solution before homogenization on ice (with a prechilled Dounce homogenizer) in 3 ml of lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.6 mM PMSF, 0.15 μM aprotinin, 2 μM leupeptin, 17 mM Nonidet P-40, and 12 mM sodium deoxycholate. The lysate was centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was immunoprecipitated by addition of 3.75 μg of anti-Cx43 monoclonal antibody (BD Biosciences, Lexington, KY). The antigen-antibody reaction was allowed to proceed overnight at 4°C, and then 50 μl of protein G was added and the incubation proceeded at 4°C for 4 h. The complexes were collected by centrifugation at 12,000 g for 20 s at 4°C and were washed twice. Western blot analysis of phosphoserines was performed on membranes blocked with 5% bovine serum albumin and 0.2% Tween 20 in Tris-buffered saline (TBS) for 2 h at room temperature. The rabbit anti-phosphoserine monoclonal antibody (Zymed) was used at a concentration of 1 μg/ml and detected by ECL.

**Uptake of (56)-carboxyfluorescein in single oocytes.** After cRNA injection, the oocytes were maintained in Barth’s solution containing either 1 or 5 mM CaCl₂ (to block constitutively active mutant GH), see Oocyte preparation and cRNA injection. For the (56)-carboxyfluorescein (CF) uptake measurements, the cells were incubated for 40 min at 16°C in ND96 solution (in mM: 96 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, and 5 HEPES-NaOH, pH 7.4) containing 2 μM CF with or without PKC inhibitors (calphostin C, chelerythrine, or bisindoylmaleimide at concentrations of 1.5, 10, or 5 μM, respectively), purinergic channel blockers (100 μM suramin or 50 μM pyridoxal-phosphate-6-azophenyl-2′-4′-disulfonic acid, PPADS) or gap junction channel blockers (100 μM octanol or 20 μM glycyrrhetinic acid (18'-GA)]. After this incubation period the extracellular CF was removed by extensive washing with ice-cold ND96 solution containing 10 μM Gd³⁺, Gd³⁺, a blocker of GH (Ebihara L, personal communication; Refs. 15 and 51), was used to minimize leakage of intracellular CF via GH during washing. Individual oocytes were lysed by sonication in 2 ml of 5 mM Tris-HCl, pH 9, and CF was measured on a spectrofluorometer (Fluorolog-2, SPEX Industries) at excitation and emission wavelengths of 488 ± 4 and 525 ± 4 nm, respectively.

**Statistics.** Data are expressed as means ± SE. Statistical differences were assessed by one-way ANOVA.

**RESULTS**

**PKC blockers activate Cx43 GH expressed in Xenopus oocytes.** To assess Cx43 hemichannel activity, we used the uptake of CF (M₄, 376). In preliminary experiments, we observed that PKC blockers had no effect on CF uptake in noninjected or water-injected oocytes. Because X. laevis oocytes express Cx38 hemichannels in the plasma membrane (13), these pilot studies suggest that these hemichannels have low basal activity and are not regulated by PKC-mediated phosphorylation. However, we found that oocyte injection with antisense Cx38 cRNA reduced the variability of CF uptake from oocyte to oocyte (SD = 0.29 and 0.02 for an equal number of water-injected and anti-Cx38-injected oocytes, respectively). Hence, we always injected antisense Cx38 cRNA, with or without concomitant injection of wild-type or mutant Cx43 cRNA. In pilot studies, we also found that functional expression was evident from day 1 after injection of Cx43 cRNA and continued for at least two additional days. We performed most experiments 2 days after injection.

**Injection of Cx43 cRNA resulted in expression of Cx43 at the plasma membrane, as shown by immunoblots of oocyte membranes biotinylated with a membrane-impermeant reagent (Fig. 2A).** Because under the conditions of our assays there is a linear relationship between CF concentration and fluorescence, we determined the uptake from the CF fluorescence. An increase in CF fluorescence in response to calphostin C was apparent in oocytes injected with Cx43 cRNA (Fig. 2B), whereas the uptake of M₄, 3,000 dextran (M₄, range 1,500–4,500) labeled with Texas red (2 mM; protocol identical to that
able uptake of the labeled dextran rules out endocytosis as the mechanism of dye uptake in response to PKC inhibition, consistent with the homogeneous distribution of CF inside the oocytes (not shown). We excluded the possibility that CF uptake is via purinergic receptor channels such as those of the P2X family by experiments with two blockers of these channels (24, 42), suramin and PPADS. Figure 2C shows that the increase in CF uptake in response to calphostin C was still observed in the presence of 100 μM suramin or 50 μM PPADS. In addition, 200 μM ATP failed to increase CF uptake in Cx43-expressing oocytes (change = −8 ± 20% compared with untreated oocytes, n = 9 and 10 for control and ATP-treated oocytes, respectively; not shown). The well-known gap junction inhibitors octanol (1 mM) and 18β-GA (20 μM), however, did prevent the increase in CF uptake elicited by calphostin C (Fig. 2C). These data support the notion that CF permeates across Cx43 hemichannels.

One alternative interpretation for the increased CF permeability by calphostin C is that the PKC blocker produces a large increase in Cx43 GJH expression at the plasma membrane. However, Western blots with membranes from surface-biotinylated oocytes do not support this conclusion, i.e., exposure to calphostin C for 40 min did not produce a major increase in plasma membrane expression of Cx43 GJH (Fig. 2D).

In Cx43 cRNA-injected oocytes the selective PKC blockers calphostin C, bisindoylmaleimide, and chelerythrine, which are used to measure CF uptake) was undetectable and unaffected by Cx43 cRNA injection in the absence or presence of calphostin C (Fig. 2B). In oocytes injected with antisense Cx38 alone (no Cx43 expression) calphostin C did not affect CF uptake (see Fig. 3), and the uptake of Texas red-labeled M₈ 3,000 dextran was undetectable (not shown). These results indicate that the pathway responsible for hydrophilic probe loading has size selectivity consistent with that of functional gap junctions (permeation of solutes up to M₈ 1,000) and rule out nonspecific permeation pathways. The absence of measur-
chemically dissimilar, increased CF uptake six- to eightfold, whereas they had no effect on CF uptake in control oocytes (Fig. 3, A–C). The fact that the effects of the blockers occur only in oocytes injected with Cx43 cRNA (Fig. 3, A–C) strongly suggests that the increased CF uptake is mediated by changes in the permeability of Cx43 hemichannels. These results are consistent with the activation of Cx43 gap junctional communication by decreased phosphorylation of PKC site(s) (see Ref. 25 for review) and show that Cx43 hemichannels expressed in the plasma membrane of frog oocytes with basal PKC activity are essentially impermeable to CF.

Partial deletion of Cx43 CT does not affect response to PKC blockers. The two candidate consensus sites for PKC-mediated phosphorylation of Cx43 are Ser368 and Ser372 (see Fig. 1), and the former was shown to be necessary for the effect of PKC agonists on gap junctional channels (26). To facilitate the generation of mutants at these sites with oligonucleotide adaptors, we engineered a Cx43 without residues 307–360 (Cx43D1; see Fig. 1). As shown in Fig. 3C, exposure to the PKC inhibitors calphostin C or bisindoylmaleimide elicited similar increases in CF uptake in oocytes injected with wild-type Cx43 or Cx43D1 cRNAs. As expected, CF uptake by oocytes expressing Cx43D1 was blocked by the nonselective GJH blocker Gd3+ (Fig. 3D). These results indicate that the sequence encompassed by residues 307 and 360 is not required for the expression of Cx43 or for the effect of PKC blockers on CF permeability.

Ser368 is essential for modulation of CF permeability of Cx43 hemichannels by PKC blockers. Two residues in PKC consensus sequences near the end of the CT, Ser368 and Ser372, have been shown to be phosphorylated in vitro. Mutagenesis studies strongly suggest that phosphorylation of Ser368 is necessary for the downregulation of gap junctional channels by PKC-mediated phosphorylation (26). However, it is unresolved whether both Ser368 and Ser372 must be phosphorylated or the phosphorylation of Ser368 alone is sufficient and whether the CT domain regions involved in the effect of pH also play a role in the effect of PKC-mediated phosphorylation.

To quantify CF uptake, we increased the extracellular [Ca2+] from 1 to 5 mM, from the time of cRNA injection until the CF uptake experiments (see MATERIALS AND METHODS). This maneuver prevented oocyte lysis in the cells expressing Cx43 with substitution of Ser368 and allowed us to test for changes in CF loading by PKC inhibition in all experimental groups. For these experiments, the cells were washed three times with 1 mM Ca2+ buffer immediately before the exposure to CF. As shown in Fig. 4, calphostin C increased CF loading significantly in oocytes injected with wild-type Cx43 and Cx43D1, indicating that these oocytes had low CF permeability before treatment with the PKC blocker and that dephosphorylation activated the hemichannels. In contrast, oocytes expressing Cx43-S368A/S372A had a constitutively high CF permeability that was not modified by the PKC blocker.

The results in Fig. 4 also show that Cx43-S368A is constitutively active and unresponsive to PKC inhibition whereas Cx43-S372A behaves like Cx43D1. We conclude that phosphorylation of Ser368 by itself is responsible for the modulation of Cx43D1 CF permeability by PKC. To ascertain whether this conclusion holds for the full-length protein, we performed the Ser368(A) mutation in full-length Cx43. Expression of this mutant in oocytes also yielded constitutively high CF permeability and insensitivity to PKC block (Fig. 4). The results of these mutagenesis studies indicate that Ser368 is necessary for the regulation of the solute permeability of Cx43 GJH by PKC and that Ser372 has no role in the response of Cx43 GJH to PKC blockers.

Because our conclusions are based on the assumption that PKC inhibitors reduce the level of Cx43 phosphorylation, we decided to test whether calphostin C did in fact decrease the PKC activity in oocytes and whether there was a reduction in the Cx43 phosphorylation level. As expected, a 40-min exposure to 1.5 μM calphostin C in ND96 at 16°C reduced total oocyte PKC activity from 3.8 to 0.95 pmol P/oocyte h−1 min−1. The change in Cx43 migration secondary to PKC-mediated phosphorylation at Ser368 varies among cell types (45), and it was difficult to detect in the oocytes (data not shown). Instead, we took advantage of the fact that PKC phosphorylates only Cx43 serine residues (26, 43) and assessed the effect of calphostin C on Cx43 phosphorylation by immunoblotting with an anti-phosphoserine antibody. The Western blot shown in Fig. 5 shows that the phosphoserine level is clearly higher in oocytes expressing Cx43-S368A/S372A than in those expressing wild-type Cx43, indicating that PKC inhibits Cx43 phosphorylation.

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Fig. 5. Effects of calphostin C treatment on serine phosphorylation of Cx43 and Cx43-S368A. Lysates of 6 oocytes per condition were immunoprecipitated with an anti-Cx43 antibody, subjected to SDS-PAGE (10% Tris-glycine), and immunoblotted with anti-phosphoserine (anti-P-Ser) or anti-Cx43 (anti-Cx43) antibodies. The blot is representative of 3 similar experiments.
in the presence of the PKC blocker or in the Ser368Ala mutant with or without exposure to calphostin C. These results cannot be explained by differences in expression levels (Fig. 5) and therefore confirm that Ser368 is the main target of PKC-mediated phosphorylation of Cx43.

**Mechanism of regulation of Cx43 by PKC-mediated phosphorylation does not involve the proline-rich region of the CT domain.** The results described above indicate that the increased permeability of Cx43 GJH in response to calphostin C is present in molecules with a partial deletion of the CT domain (Figs. 3 and 4). Because it was shown previously that the decrease in Cx43 permeability elicited by lowering cytosolic pH involves the proline-rich region of the CT domain (Ref. 14; see Fig. 1), we performed additional experiments to determine whether mutations that alter the response of Cx43 to pH also change the response to PKC-mediated phosphorylation. The results of this series of experiments are summarized in Fig. 6. A deletion of the CT domain that retains the proline-rich region (Cx43D2) still responds normally to PKC inhibition, but deletion of the proline-rich region resulted in a Cx43 GJH with a constitutively high dye permeability (Cx43D3). This result suggested that the proline-rich region is essential for the block of the Cx43 GJH by PKC-mediated phosphorylation. However, the substitution of two proline residues in the proline-rich region with alanine (2P2A constructs) on either Cx43 or Cx43D2, which impairs the response to intracellular acidification (14), had no effect on the response to calphostin C. Therefore, the potential structured proline-rich subdomain is not needed for the response of Cx43 to PKC inhibition. Because Cx43D3 67% of the CT domain is deleted, there is the possibility that the absence of response to the PKC blocker is a consequence of the magnitude of the deletion, rather than of the primary sequence deleted. To test this hypothesis, we substituted 11 residues of the proline-rich region sequence with glycines, a segment expected to be unstructured. The resulting Cx43 GJH (Cx43D2-RC) did respond to calphostin C treatment with a decrease in CF permeability. Therefore, the response to calphostin C is maintained in Cx43 with deletion of 55% of its CT domain, and the proline-rich region of the CT domain is not involved in the response to PKC inhibitors.

**DISCUSSION**

In this work we confirm that Cx43 GJH solute permeability is regulated by PKC-mediated phosphorylation, as demonstrated by Li et al. (28), and we conclude that Ser368 is the PKC target residue for the effect and that the mechanism of regulation by PKC-mediated phosphorylation is different from that elicited by changes in intracellular pH (10, 14, 37). Extensive studies have been performed on the regulation of Cx43 gap junctional channel permeability by changes in intracellular pH. It has been shown that the decrease in permeability elicited by lowering intracellular pH and by MAPK-mediated phosphorylation responds to a ball-and-chain mechanism (reviewed in Ref. 10) that involves the proline-rich region of the CT domain and part of the intracellular loop (12). We found that the proline-rich region is not involved in the regulation of Cx43 by PKC-mediated phosphorylation. Therefore, we conclude that the mechanisms of Cx43 regulation by intracellular pH on the one hand (14, 37), and PKC-mediated phosphorylation on the other, are different from the point of view of the Cx43 subdomains involved.

The wild-type Cx43 hemichannels expressed in the plasma membrane of frog oocytes are essentially impermeable to hydrophilic probes but become permeable under the effect of PKC blockers. Our results also demonstrate that deletion of 55% of the residues of the CT domain has no effect on the activation of the hemichannels by PKC blockers. Understanding the molecular mechanism of the modulation of Cx43 GJH by phosphorylation of Ser368 will require additional studies.

We have been unable to detect currents that can be attributed to Cx43 expression in *Xenopus* oocytes, in agreement with previous data in oocytes (39, 54). We attribute this result to a low expression level. From a calculated CF influx per oocyte of 5–10 × 10^3 molecules/s and assuming a CF flux per Cx43 channel of 1,560 molecules/s (based on Ref. 50), we estimated the number of GJH per oocyte at 3.2–6.4 × 10^3. Assuming a GJH conductance of 150 pS, the maximum GJH-dependent oocyte conductance would be <1 μS, i.e., difficult to detect because of background currents. Hence, we conclude that in oocytes the channel density is insufficient for electrophysiological detection. Because the background signal in the CF uptake experiments is negligible and the signal-to-noise ratio can be increased by measuring CF influx for long periods of time, under the conditions of our experiments the fluorescence detection of Cx43 GJH activity is expected to be more sensitive than the whole cell current measurements.

In untreated, isolated *Xenopus* oocytes, most Cx43 appears to be phosphorylated, keeping the hemichannels impermeable to hydrophilic dyes. This is consistent with the properties of Cx43 GJH expressed in isolated Novikoff and HeLa cells. The percentage of dye-loaded cells was decreased to <50% with PMA and increased ~16-fold with removal of external Ca^{2+}, suggesting that the untreated GJH have a low permeability to CF (28). In contrast, in confluent T51B rat liver epithelial cells calphostin C treatment did not increase dye coupling, whereas

**Fig. 6.** Effects of carboxy terminal (CT) domain deletions and proline mutations of the CT domain proline-rich region on the regulation of Cx43 gap junctional hemichannels (GJH) by PKC. Effect of calphostin C on CF uptake in oocytes expressing the deletion mutants Cx43D1 (n = 26 and 40), in the absence and presence of calphostin C, respectively), Cx43D2 (n = 55 and 64), and Cx43D3 (n = 56 and 62), mutants of critical P residues in the P-rich region (Cx43D2-P277/280A, n = 24 and 29; Cx43D2-P277/280A, n = 24 and 31), and a Cx43D2-based mutant in which the proline-rich sequence was replaced by a poly-glycine sequence (Cx43D2-RC, n = 21 and 22). Dye uptake protocol and data analysis were as described in Figs. 3 and 4; data were obtained from 2–3 separate experiments. *P < 0.05 compared with the value in the presence of calphostin C.
PMA reduced the number of dye-coupled cells significantly (20).

It is well known that changes in PKC activity modulate Cx43 gap junctional channel assembly, and therefore it is conceivable that the Cx43 GJH in oocytes are CF permeable even when phosphorylated but are not expressed at the plasma membrane; in this case, the increase in CF permeability by the PKC blockers would be the result of increased traffic of hemichannels to the plasma membrane. This explanation is not correct because 1) the changes in Cx43 GJH expression during the course of our acute experiments are not likely to be significant (in fact, we found that treatment with calphostin C for 40 min does not elicit significant changes in expression of Cx43 at the plasma membrane); 2) Cx43 GJH are expressed in the plasma membrane in the absence of exposure to PKC blockers; and 3) PKC-mediated phosphorylation directly blocks sucrose and dye permeability of proteoliposomes reconstituted with highly purified, detergent-solubilized Cx43 hemichannels (unpublished observations). Therefore, we conclude that phosphorylation of Ser368 by PKC reduces the permeability of Cx43 GJH to CF.

Although the primary function of connexins is to form the gap junctions that communicate neighboring cells (17, 33, 48), recent evidence indicates that functional hemichannels are present at the plasma membrane of a variety of cells, where they may have important physiological and pathophysiological roles (see, e.g., Refs. 5, 8, 11, 16, 21, 28, 41, 46, 47, 51, 53). Independently of the importance of the Cx43 GJH per se, we believe that the mechanisms of regulation of dye permeability in Cx43 gap junctional channels and GJH are similar. This view is supported by the following arguments: 1) Cx43 gap junctional channels and GJH show similar modulation of hydrophilic dye permeability by PKC-mediated phosphorylation; 2) phosphorylation of Ser368 is the initial event for the decrease in dye permeability in Cx43 gap junctional channels and GJH; and 3) lowering intracellular pH decreases dye permeability of Cx43 gap junctional channels and GJH. Therefore, it is reasonable to conclude that our results in Cx43 GJH can be extrapolated to gap junctional channels.

In summary, we have shown that regulation of Cx43 by PKC-mediated phosphorylation depends on phosphorylation of Ser368, does not need 55% of the CT domain residues, and does not involve the proline-rich region of the CT domain. Therefore, the molecular mechanisms of regulation of Cx43 dye permeability by changes in intracellular pH and PKC-mediated phosphorylation are different.

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