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

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Submitted 11 July 2003; accepted in final form 4 November 2003

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
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 GJH, 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'-H11032-CGACCTTCCACGACCAAGCCGCCGCCGCCACGCCAGGCTCCTCGG-3' 
5'-H11032-CCTGCTGCTGGCGCGCTGCTGCTCGCTCTGGAGAAGGTCGTTGGG-3'

We used the same methodology to generate a Cx43 mutant in which Pro277 and Pro280 were substituted with Ala. The mutagenic primer sequence was 5'-H11032-CCACAACGGCTGCACTCTCGGCTATGTCTCCTCC-3'. In the deletion mutant Cx43D1, we removed base pairs coding for residues Glu307 to Asp360 by replacing the NheI/StuI fragment of pOcyt-Cx43 with a 50-bp NheI/StuI adaptor coding for residues 361–374. We used adaptors with the Cx43 wild-type sequence or mutated sequences in which Ser368 and/or Ser372 was replaced with Ala to generate Cx43D1 and its mutants. The adaptors for the wild-type sequence were: forward, 5'-CTAGCGACCAACGACCTTCCACGAGCCGCCGCCGCCGCCAGGCTCCTCGG-3' and reverse, 5'-CTAGCGACCAACGACCTTCCACGAGCCGCCGCCGCCGCCAGGCTCCTCGG-3'. 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 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'-CTAGCGACCAACGACCTTCCACGAGCCGCCGCCGCCGCCAGGCTCCTCGG-3' and reverse, 5'-CTAGCGACCAACGACCTTCCACGAGCCGCCGCCGCCGCCAGGCTCCTCGG-3'. DNA se-

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**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) or wild-type or mutant (antisense) Cx38 cRNA. Oocytes expressing Cx34 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 GJH was observed previously (39), although it has not been documented with the expression of constitutively active Cx43 GJH in mammalian cells. During the generation of mammalian cell lines, expression of Cx46 was observed previously (13), but in one study, putative Cx34 GJH were found to be insensitive to external 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 GJH 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 connexins forming functional channels, with mutations at position 368 preventing lysis by elevating the Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]_i) in Barth’s medium from 1 to 5 mM from the time of injection until immediately before the dye-loading test. High [Ca\(^{2+}\)]_i is known to block Cx43 GJH expressed in mammalian cells (see, e.g., Ref. 28), as well as other GJH expressed in *Xenopus* oocytes (13), but in one study, putative Cx34 GJH were found to be insensitive to external [Ca\(^{2+}\)]_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 assessed 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, 10 oocytes were used as functional channels, with few or no GJH. In any case, the membrane proteins were separated by SDS-Page 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 polyclonal antibody (Zymed) was used at a concentration of 1 μg/ml and detected by ECL.

**Uptake of 5(6)-carboxyfluorescein in single oocytes.** After cRNA injection, the oocytes were maintained in Barth’s solution containing either 1 or 5 mM CaCl\(_2\) (to block constitutively active mutant GJH, see Oocyte preparation and cRNA injection). For the 5(6)-carboxyfluorescein (CF) uptake measurements, the cells were incubated for 40 min at 16°C in ND96 solution (in mM: 96 NaCl, 2 KCl, 1 MgCl\(_2\), 1.8 CaCl\(_2\), and 5 HEPES-NaOH, pH 7.4) containing 2 mM 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 (1 μM octanol or 20 μM glyccyrhetic acid (18)-GA). After this incubation period the extracellular CF was removed by extensive washing with ice-cold ND96 solution containing 10 μM Gd\(^{3+}\), Gd\(^{1+}\), a blocker of GJH (Ebihara L., personal communication; Refs. 15 and 51), was used to minimize leakage of intracellular CF via GJH 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 GJH expressed in Xenopus oocytes.** To assess Cx43 hemichannel activity, we used the uptake of CF (Mf3, 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 M:\_f3, 3,000 dextran (Mf3, 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, bisindolylmaleimide, 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 (no Cx43 expression) calphostin C did not affect CF uptake (see Fig. 3), and the uptake of Texas red-labeled Mts 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 Mts 1,000) and rule out nonspecific permeation pathways. The absence of measurable 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.

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In Cx43 cRNA-injected oocytes the selective PKC blockers calphostin C, bisindolylmaleimide, and chelerythrine, which are
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 bisindoyl maleimide elicited similar increases of 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 Ser368A 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 P1-oocyte−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 PKC blockers.

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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 in 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 poly-glycine sequence (Cx43D2-RC, 10 glycines, a segment expected to be unstructured. The resulting Cx43 GJH (Cx43D2-RC) did respond to calphostin C, resulting in a Cx43 GJH with a permeability of 67% 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. 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 $5 \times 10^9$ molecule/s, and assuming a CF flux per Cx43 channel of 1,560 molecule/s (based on Ref. 50), we estimated the number of GJH per oocyte at 3.2–6.4 $\times 10^3$. Assuming a GJH conductance of 150 pS, the maximum GJH-dependent oocyte conductance would be $<1 \mu$S, 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 $\sim 16$-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
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.

ACKNOWLEDGMENTS

We thank Dr. Scott John (Cardiovascular Research Laboratory, University of California at Los Angeles) for the Cx43 DNA and Drs. Lisa Ebihara and Nalin Kumar for comments on the manuscript.

GRANTS

This work was supported in part by American Heart National Grant-in-Aid 0050353N.

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