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REGULATION OF CONNEXIN 43 CHANNELS BY PKC-MEDIATED PHOSPHORYLATION.

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ABSTRACT.

Gap-junctional channels communicate adjacent cells electrically and metabolically. They are formed by 4-transmembrane helix proteins called connexins; six connexins form a connexon (or gap-junctional hemichannel) and two of these, one from each neighboring cell, dock head-to-head to form the gap-junctional channel. The permeability of gap-junctional channels is regulated by voltage, intracellular calcium activity, intracellular pH and phosphorylation by various kinases. This review focuses on the mechanism of the regulation of connexin 43 by protein kinase C. We discuss results obtained largely from studies of hemichannels that demonstrate that the channel pore is narrowed down, but not closed, by protein kinase C-mediated phosphorylation of a single site, serine 368, located 14 residues away from the C-terminal end of connexin 43. The effect of protein kinase C involves a large conformational change of the protein and requires phosphorylation of all six subunits. The precise mechanism of the decrease in pore cross-sectional area has not been established.

INTRODUCTION.

Gap-junctional channels (GJC) are formed by the head-to-head docking of two connexons or gap-junctional hemichannels (GJH) from adjacent cells (25). Each connexon is formed by six connexin (Cx) molecules (21, 22, 24, 25). Connexins have four transmembrane ? helices and intracellular amino and carboxy termini (reviewed in 11). A scheme depicting connexins, GJH and GJC is shown in Fig. 1. More than 20 connexin isoforms exist in vertebrates (12). GJC are permeable to molecules of M_r up to about 1,000 Da and have low ionic selectivity. They allow ion and hydrophilic-solute fluxes between adjacent cells, thus coupling them electrically and metabolically. Gap junctions are important in embryonic development and in the function of adult tissues and organs, cell growth and differentiation, and tissue homeostasis (reviewed in 23).

Cx43 is an isoform expressed in many tissues and organs, including brain, myocardium, kidney and capillary endothelial cells. In the heart, Cx43 is expressed in atrial and ventricular myocytes, providing the path for spread of electrical excitation. Phosphorylation of certain serine or tyrosine residues of Cx43 inhibits gap-junctional communication, an effect that is prevented by C-terminal domain (CTD) deletion or by mutations of certain CTD serine or tyrosine residues (reviewed in 6, 14, 15, 16, 17). The effect of PKC-

mediated phosphorylation of Cx43 is to reduce GJC dye permeation and single-channel conductance (8, 16, 20). In contrast, the effect of MAP kinase- and tyrosine kinase-mediated phosphorylation of Cx43 is to decrease the single-channel open probability, an effect shared by intracellular acidification (6, 14, 17).

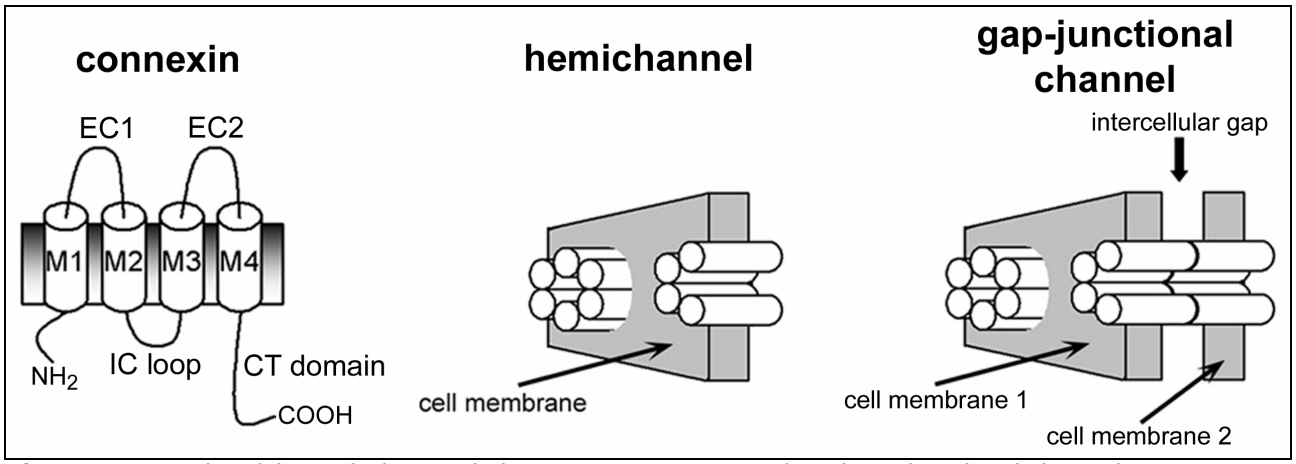

Figure 1: *Cx43, hemichannels (connexin hexamers or connexons) and gap-junctional channels. M1, M2, M3 and M4 represent the four transmembrane helices. The intracellular sequences (N-terminal region (NH_2), intracellular loop (IC loop), and the C-terminal domain (CT domain) are involved in channel gating and regulation, and the two extracellular loops (EC1 and EC2) are essential for docking with hemichannels of the neighboring cell. From J Biol Chem 280: 8647-8650, 2005.*

GJH traffic to the plasma membrane in vesicles, are incorporated as such, and then diffuse laterally to reach plaques where they dock with neighboring-cell GJH to form GJC (9, 18). Endogenous GJH have been studied by dye uptake or electrophysiological measurements in native cells. They have been also obtained by heterologous expression of recombinant Cx in *Xenopus laevis* oocytes and other cells. GJH are non-selective or mildly cation-selective and have relatively high conductances and are permeable to larger hydrophilic solutes (7, 19).

GJH are "closed" in normal cells, but can be activated by lowering external [Ca^{2+}] (19), by membrane depolarization (7) or by metabolic inhibition (5, 13, 26). The use of GJH as an experimental model has been well validated. In many respects, demonstrated experimentally, a GJC is two GJH in series, with compatible, predictable basic properties such as dye permeability and electrophysiological features (4, 5, 27). Electrophysiological studies of single-channel or ensemble currents are much easier in GJH than in GJC, single-channel studies have been performed in oocytes and mammalian cells (11), and bilayer reconstitution is possible with GJH but no success has been reported with GJC (11). The regulatory mechanisms of GJH and GJC overlap well (*e.g.*, 27). Therefore, information obtained from GJH studies will facilitate the understanding of GJC function; in addition, GJH are physiologically (10, 28) and pathophysiologically (5, 13, 26) significant in their own right.

A role for dephosphorylation of GJH (inserted in the plasma membrane but not docked with those in adjacent cells) has been suggested to exist in cardiac muscle, brain astrocytes and renal proximal-tubule cells (5, 13, 26). The dephosphorylation of the PKC-phosphorylated sites is thought to "open" the channels, which communicate the cytoplasm and the extracellular fluid, allowing ion and "large" hydrophilic solute fluxes conducive to cell damage (5, 13, 26). In this article we review the mechanism of Cx43 GJH regulation by PKC-mediated phosphorylation. These studies shed light on the mechanisms of regula-

tion of GJH and GJC function, as well as on the role that GJH activation may have in the cell damage elicited by metabolic depletion.

THE TARGET OF PKC IS Ser368.

Lampe et al. (16) showed that PKC stimulation in cells phosphorylates Ser368 of Cx43, reduces GJC hydrophilic-dye fluxes and reduces the amplitude of single-channel currents. All these effects were prevented by mutating Ser368 to Ala. We confirmed and extended these results studying Cx43 GJH expressed in *Xenopus* oocytes (1) and Cx43 GJH formed by purified Cx43 reconstituted in liposomes (3).

In the oocyte studies we found that Ser372 (which is also phosphorylated by PKC *in vitro*) had no role in the effects of PKC. In addition, several partial deletions of the CTD did not influence the effect of PKC, including deletions of domains necessary for the effects of intracellular acidification and mitogen-activated protein kinases (MAP kinases) on Cx43 (1). The main conclusion from these studies was that the required CTD sequences for the effects of pH and MAP kinases, on the one hand, and PKC, on the other, are different.

Our studies with purified Cx43 required the identification of an overexpression system, the development of a purification strategy and the use of appropriate biophysical techniques to test the function and regulation of reconstituted GJH (3). We found that rat Cx43 is well expressed in High-Five insect cells in suspension and that a highly-purified preparation can be obtained based on the affinity of a C-terminal poly-His tag for Ni^{2+}. The purified protein is stable and forms functional GJH when reconstituted in liposomes. This functionality can be assessed by measuring solute fluxes (*e.g.*, ^{14}C-sucrose). Dephosphorylated Cx43 GJH are sucrose-permeable, whereas GJH formed by PKC-phosphorylated GJH are impermeable to sucrose (Fig. 2). When Ser368 was mutated to Ala the GJH were constitutively active (permeable to sucrose) and insensitive to PKC. These studies demonstrated that the regulation of Cx43 hemichannels by PKC does not involve molecules other than Cx43 itself (3).

PKC-MEDIATED PHOSPHORYLATION CAUSES A CONFORMATIONAL CHANGE.

In our studies with purified Cx43, wild-type and the Ser368Ala mutant, we tested for changes for the effects of PKC-mediated phosphorylation on intrinsic Trp fluorescence (in detergent) and sensitivity to proteolysis with trypsin (in liposomes). Phosphorylation increased tryptophan fluorescence intensity, with a displacement of the peak fluorescence to a longer wavelength (3). These effects were absent in the Ser368Ala mutant (3). Phosphorylation of wild-type Cx43 by PKC, but not of the Ser368Ala mutant, protected the CTD from limited trypsin proteolysis, indicating that the conformational change in response to phosphorylation also occurs when the protein is present in the phospholipid bilayer (3). These studies indicate that phosphorylation of Cx43 Ser368 by PKC produces a detectable conformational change.

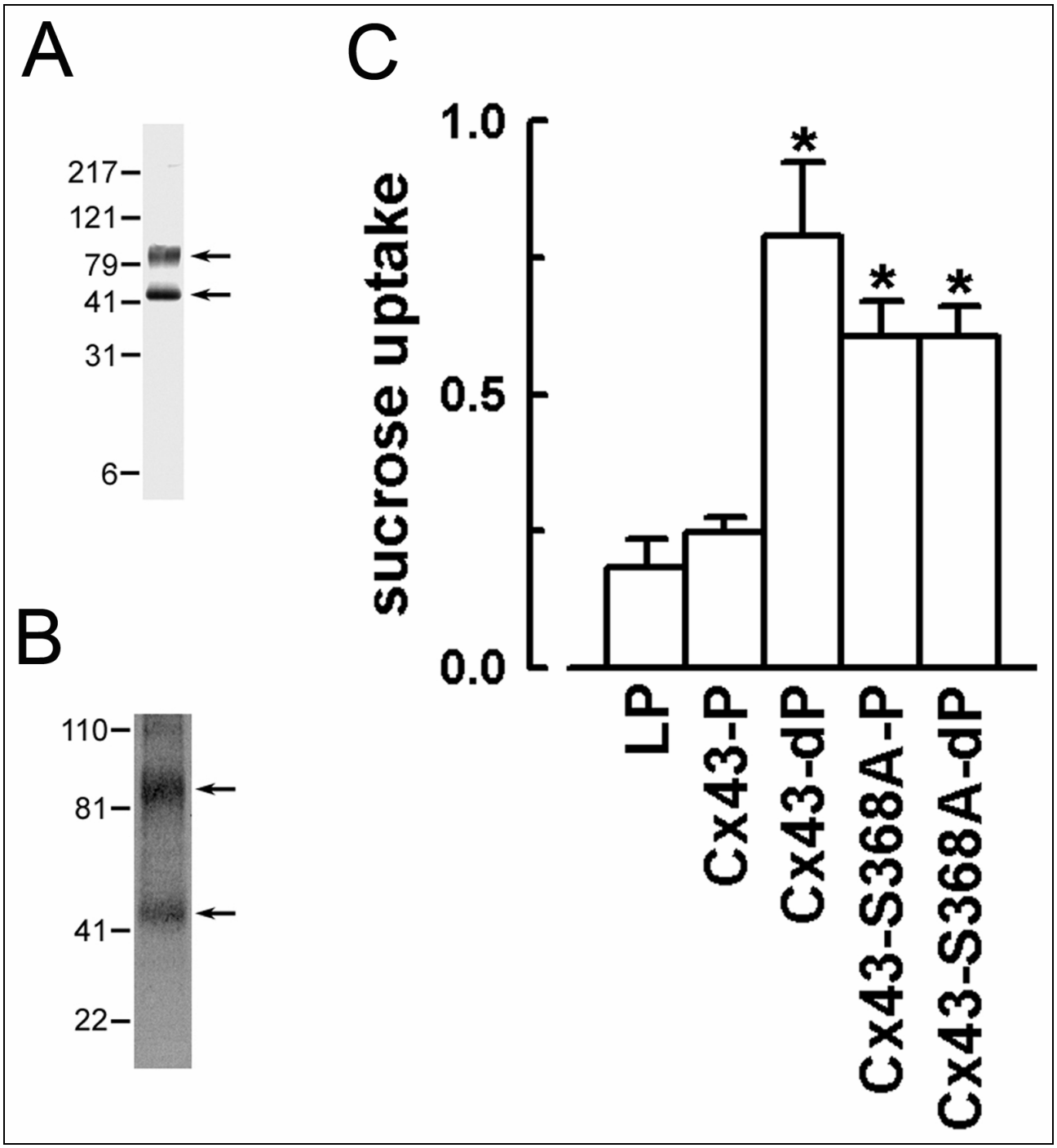

Figure 2: *Purification and functional reconstitution of Cx43 overexpressed in insect cells. A. Coomassie Blue-stained gel of purified Cx43. B. SYPRO Red-stained gel of purified Cx43. The arrows in panels A and B point to the Cx43 monomer and dimer. C. Effect of phosphorylation on sucrose uptake by proteoliposomes containing Cx43 or Cx43-S368A. The effect of PKC-mediated phosphorylation was assessed from the steady-state (30 s) sucrose uptake, expressed in nmol sucrose per mg lipid. LP represents liposomes, and -P and -dP represent PKC-phosphorylated and dephosphorylated Cx43, respectively. The data are presented as means ± SEM of triplicate measurements from at least 3 independent experiments. * indicates P<0.05 compared to liposomes alone. See reference 3 for details.*

PKC-MEDIATED PHOSPHORYLATION DECREASES THE EFFECTIVE CROSS-SECTIONAL AREA OF THE PORE.

PKC-mediated phosphorylation of reconstituted Cx43 GJH abolishes the sucrose permeability. This result would be consistent with either a fall in single-channel open probability (P_o) to zero or a reduction in the cross-sectional area of the pore, so that the sucrose flux is abolished by steric hindrance (*i.e.*, the sucrose molecule is too large to fit in the narrowed pore). It is clear that PKC-mediated phosphorylation reduces the ion conductance of Cx43 GJC (8, 16, 20), a result consistent with the second hypothesis. We tested this measuring the permeability of reconstituted GJH to sucrose (M_r 342) and to a smaller hydrophilic solute, ethylene glycol (M_r 62), expecting that the pore of phosphorylated GJH would become impermeable to sucrose but remain permeable to ethyleneglycol. This was exactly the result, shown in Fig. 3 (2), confirming the previous observation of a reduction in channel conductance (8, 16, 20), which could be explained by a "narrowing" of the permeation pathway. Whether this occurs at the cytoplasmic, transmembrane or extracellular regions of the GJH or GJC remains to be determined.

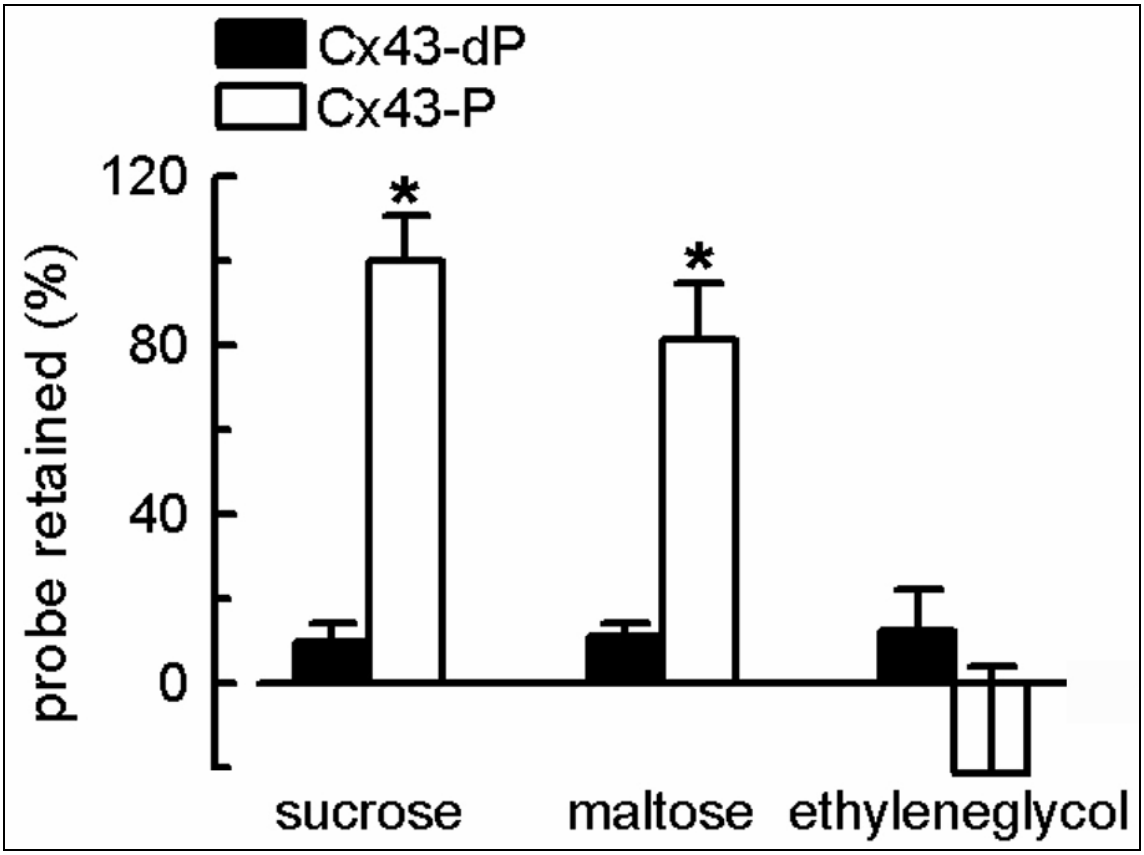

Figure 3: *Effects of PKC-mediated phosphorylation of hemichannels on their permeability to hydrophilic solutes. The proteoliposomes were loaded with radiolabeled probes, and the percent retention of the permeability probes was measured after gel filtration. Studies were performed in proteoliposomes containing hemichannels formed by fully-dephosphorylated Cx43 (Cx43-dP) or Cx43 fully phosphorylated by PKC (all 6 Ser368 residues phosphorylated, Cx43-P). Values were normalized to the amount of probe retained by liposomes formed by Cx43-P (same as that in liposomes without hemichannels), after subtraction of the background measured in dimethylsulfoxide-permeabilized liposomes. Ethyleneglycol values were normalized to those in liposomes without hemichannels. The average number of hemichannels per liposome was 2.3. Data are means ± SEM of 4-7 experiments. Asterisks denote P < 0.05 compared to proteoliposomes containing Cx43-dP hemichannels. See reference 2 for details.*

ALL SIX SUBUNITS OF THE Cx43 GJH NEED TO BE PHOSPHORYLATED TO ABOLISH SUCROSE PERMEABILITY.

To determine how many Cx43 GJH subunits need to be phosphorylated to abolish sucrose permeability, we carried out experiments on purified Cx43 that was either phosphorylated or dephosphorylated, and then produced GJH reconstituted in liposomes at know subunit composition ratios, *i.e.*, dephosphorylated/phosphorylated of 0/6, 1/5, 2/4, 3/3, 4/2, 5/1 and 6/0. We validated the production of GJH of controlled subunit composition by luminescence resonance energy transfer measurements (2), which demonstrated that the subunit composition of GJH follows the binomial distribution., *i.e.* the 3/3 mixture will yield a maximum number of 3/3 proteoliposomes, smaller number of 2/4 and 4/2, etc. (2). After reconstitution of GJH with varying numbers of phosphorylated and dephosphorylated subunits, we determined sucrose permeability by preloading the proteoliposomes with ^{14}C-sucrose and then passing them through a gel filtration column to remove extraliposomal sucrose, including that permeating the GJH. In Fig. 4 we compare the measured retention of the probe with that predicted by the subunit composition (and binomial distribution), if the number of subunits that must be phosphorylated is 1, 2, 3, 4, 5 or all 6. To our surprise, the results indicate that all six subunits must be phosphorylated, suggesting a highly cooperative effect of phosphorylation by PKC.

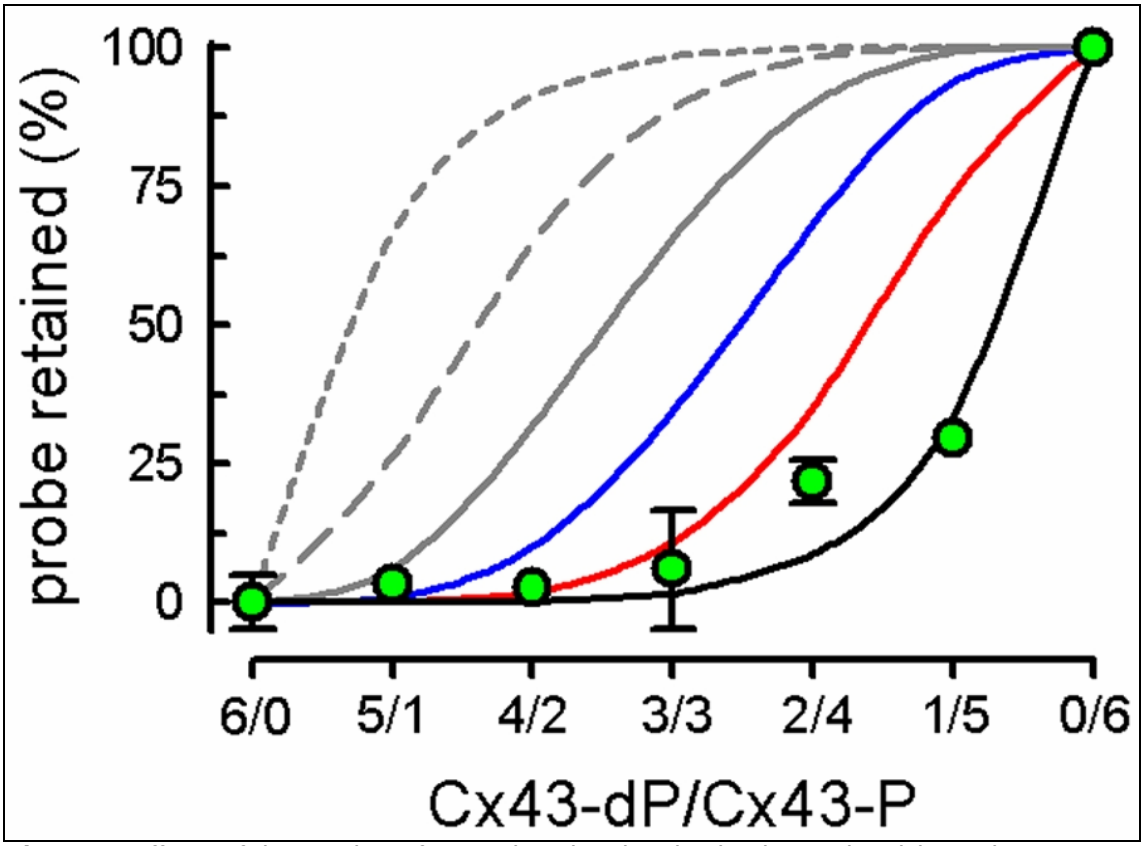

Figure 4: *Effects of the number of PKC-phosphorylated subunits per hemichannel on sucrose permeability. Effects of varying Cx34-dP/Cx43-P average ratios on the % of sucrose retained in proteoliposomes pre-loaded with the radiolabeled probe. Values were normalized as described in Fig. 3. The average number of hemichannels per liposome was 0.8, and data are means ± SEM of 4-7 experiments. The lines represent the % probe retained, expected if the number of Cx43-P subunits necessary to render the hemichannels impermeable to sucrose are = 1 (gray, short dash), = 2 (gray, long dash), = 3 (gray, solid), = 4 (blue), = 5 (red) or 6 (black). The lines were obtained by joining with spline lines the values, calculated from the binomial distribution, for each Cx43-dP/Cx43-P mixture. See reference 2 for details.*

CONCLUSIONS AND FUTURE STUDIES.

The studies summarized above demonstrate that phosphorylation of Ser368 is responsible for the effects of PKC on the permeability properties of Cx43 GJC and GJH. The phosphorylation causes a substantial change in conformation of the protein that eventually results in an apparent decrease in pore effective cross-sectional area, *i.e.*, the pore permeability for inorganic ions remains (at a reduced single-channel conductance), but the permeability to large hydrophilic solutes is abolished. All six subunits of the hemichannel must be phosphorylated for the latter effect to occur.

An issue that remains unresolved is whether partial hemichannel phosphorylation causes a decrease (but not abolishment) of large-solute permeability. To address this question it will be necessary to carry out kinetic measurements of solute permeability instead of the steady-state measurements reported so far.

The main question however, is the location of the pore narrowing and the portion(s) of the Cx43 molecule responsible. It has been proposed that the effect of acidification on Cx43 GJC permeability is mediated by a ball-and-chain mechanism involving association of the CTD and the intracellular loop. Structural studies under way will allow us to ascertain whether such a mechanism operates in response to PKC-mediated phosphorylation or whether the molecular bases of these two regulatory mechanisms are radically different.

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