CHAPTER 5: INSIGHTS INTO THE MOLECULAR MECHANISM OF GATING OF CX43 HEMICHANNELS BY PROTEIN KINASE C (PKC)-MEDIATED PHOSPHORYLATION

INTRODUCTION

As mentioned in the general **INTRODUCTION**, Cx43 gap-junctional channels mediate the electrical coupling of cardiomyocytes, and opening of hemichannels due to dephosphorylation is likely to contribute to cell damage during ischemia. Opening of Cx43 hemichannels contributes to cell injury and death under ATP-depletion (myocardial infarction, stroke, ischemic renal tubule necrosis). Dephosphorylation of serine 368 (PKC target) is likely involved in hemichannel opening. Activation of PKC decreases cell-to-cell movement of hydrophilic permeability probes, such as fluorescent dyes (dye coupling), while electrical communication between cells persists (Lampe *et al*., 2000a). These effects are independent of the effects of phosphorylation on trafficking, assembly and/or degradation (Solan *et al*., 2005). Single-channel studies have shown that stimulation of PKC decreases the frequency of the dominant (~100 pS) conductance state, favoring a lower conductance state (~50 pS) of Cx43 gap-junctional channels (Lampe *et al*., 2000a). The presence of PKC-phosphorylated gap-junctional channels permeable to small ions, and possible increases in the channel open probability (Kwak *et al*., 1995), may explain the lack of consistent effects of changes in PKC activity on cell-to-cell electrical communication (Lampe *et al*., 2000a, Bao *et al*., 2004a, Bao *et al*., 2004b). However, the mechanism accounting for the apparent dissociation between the

effects of phosphorylation by PKC on the permeabilities to "large" hydrophilic solutes and small inorganic ions is unknown.

Phosphorylation of Cx43 and intracellular acidification close gap-junctional channels and hemichannels, but the underlying molecular mechanisms have not been elucidated (Harris, 2001). It is believed that this closure occurs through a ball-and-chain mechanism, where the cytoplasmic C-terminal domain (Fig. 1 in the general **INTRODUCTION**), acting as the ball, moves and plugs the channel pore (Homma *et al*., 1998, Liu *et al*., 2006). Deletion of the C-terminal domain abolishes the effect of PKC and produces a large decrease in pH sensitivity. The wild-type pH sensitivity is restored by coexpression of truncated Cx43 with the C-terminal domain. It has also been shown that the C-terminal domain can interact with peptides corresponding to fragments of the intracellular loop. Based on these observations, the simplest explanation for the gating of hemichannels by phosphorylation is that PKC phosphorylates Ser368 in the C-terminal domain, producing a conformational change that results in the movement of the C-terminal domain, which plugs the hemichannel pore. A ball-and chain mechanism was first proposed by Armstrong and Bezanilla (1973) to explain the inactivation of voltage-sensitive channels in excitable cells. A similar mechanism to explain the block of gap-junctional channels by intracellular acidification and tyrosine kinase phosphorylation was proposed by Delmar and co-workers (Homma *et al*., 1998).

In the previous chapters, I presented the methodology for the generation of purified and reconstituted hemichannels of controlled subunit composition and the principles and use of LRET to determine the subunit composition and to measure distances between selected residues. In this chapter, I present an application of those methodologies to study the molecular mechanism of regulation of Cx43 hemichannels by PKC-mediated phosphorylation. The objectives for these studies are: 1) to determine the

effects of varying PKC-phosphorylation subunit stoichiometries on hydrophilic solute permeability of Cx43 hemichannels, and 2) to determine whether the decrease in hemichannel permeability produced by PKC-mediated phosphorylation is mediated by a simple ball-and-chain mechanism.

MATERIALS AND METHODS

Plasmid engineering for C383 insertion mutant

The single-Cys mutant with the Cys inserted at the end of the Cx43 sequence, in the pFastBac transfer plasmid, was performed by site-directed mutagenesis using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), as described in previous chapters. The other single-Cys mutants employed were some of the M3 mutants described in Chapter 4.

Insect cell culture, generation of baculovirus, single-Cys Cx43 mutant expression and purification, dephosphorylation and PKC-mediated phosphorylation of purified connexins and reconstitution of purified hemichannels in liposomes

See Chapter 3, **METHODS**.

Hydrophilic Solute Transport

For details see Chapter 3, **METHODS**. The radiolabels used were ^{14}C-sucrose and ^{14}C-maltose (Amersham Biosciences, St. Louis, MO) and ^{14}C-ethyleneglycol (American Radiolabeled Chemicals, Piscataway, NJ). Background from probe trapped into compartments inaccessible for transport was assessed by permeabilization with 0.1%

DMSO as described (Bevans *et al*., 1998). For the assays of carboxyfluorescein transport, the experiments were performed as with the radiolabeled compunds, but 1 mM of carboxyfluorescein was used for loading during extrusion. Fluorescence retained after gel filtration was measured at excitation and emission wavelengths of 488 and 525 nm, respectively, after lysis with 0.1% of Triton X-100.

LRET experiments

See **METHODS** in Chapters 3 and 4.

RESULTS

Effect of phosphorylation of all six Cx43 subunits by PKC on the effective cross-sectional area of the hemichannel pore.

Phosphorylation by PKC of all 6 Cx43 subunits at Ser368 abolishes sucrose permeability of reconstituted hemichannels (Bao *et al*., 2004b). The absence of sucrose transport through hemichannels formed by PKC-phosphorylated Cx43 (Cx43-P) was confirmed by sucrose retention measurements (Fig. 5.1). Fig. 5.1 also shows that maltose permeability is abolished by PKC-mediated phosphorylation, as expected because both disaccharides have the same molecular weight (M_r 342). In contrast, the smaller probe ethyleneglycol (M_r 62) was permeant through hemichannels formed by either dephosphorylated Cx43 (Cx43-dP) or Cx43-P, indicating that a hemichannel pore of significant size remains when all 6 Cx43 molecules are phosphorylated by PKC. In other words, phosphorylation of all the subunits by PKC produces a decrease of the cross-

sectional area of the Cx43 hemichannel pore, but not its closure, i.e., the hemichannels remain permeable to ethyleneglycol.

Figure 5.1: Effects of PKC-mediated phosphorylation of hemichannels on their permeability to hydrophilic solutes.

The proteoliposomes were loaded with radiolabeled sucrose, and the percent retention of the permeability probe 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 (statistically indistinguishable from the value measured in liposomes without hemichannels, 112 ± 9%, n = 7), after subtraction of the background measured in dimethylsulfoxide-permeabilized liposomes. 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. From Bao *et al*. (2007).

Effects of the number of PKC-phosphorylated subunits on the sucrose permeability of Cx43 hemichannels.

To determine the number of hemichannel subunits that have to be phosphorylated to abolish sucrose permeability, liposomes containing an average of 0.8 hemichannels were used, and the percentage of sucrose-impermeable liposomes was determined. Under these conditions, very few liposomes will have more than one hemichannel and a fraction will not have hemichannels (background probe retention). If there is random exchange of connexin subunits, the distribution of the hemichannels composition will follow the binomial distribution, e.g., for a 3/3 mixture the most frequent hemichannels will contain 3 Cx43-dP and 3 Cx43-P subunits (~31%), but there will be decreasing frequencies of 2/4 and 4/2 (~23% each), 1/5 and 5/1 (~9% each), and 0/6 and 6/0 (~2% each). By comparing the percentage of sucrose-permeable proteoliposomes (those with one permeable hemichannel) with the liposomes with hemichannels with at least *x* number of phosphorylated subunits, it may be possible to determine the number of subunit that have to be phosphorylated to abolish sucrose permeability.

Sucrose retention by proteoliposomes reconstituted with a 3/3 Cx43-dP/Cx43-P ratio was 6 ± 11% of the value in sucrose-impermeable proteoliposomes containing Cx43-P hemichannels (Fig. 5.2), although the percentage of liposomes with hemichannels containing 3 to 6 Cx43-P subunits is ~66% according to the binomial distribution. This result indicates that the presence of 3 Cx43-P subunits *per* hemichannel yields sucrose-permeable hemichannels. Fig. 5.2 shows that at Cx43-dP/Cx43-P ratios ≤2/4, the percentage of sucrose retained was inversely proportional to the hemichannel content of Cx43-dP, but even for an average of 5 Cx43-P *per* hemichannel, sucrose retention was much less than that observed with proteoliposomes containing 6 Cx43-P hemichannels. For the Cx43-dP/Cx43-P reconstitution ratio of 1/5, only ~30% of the

proteoliposomes were sucrose permeable, although ~83% of the Cx43 is Cx43-P in the detergent mixture. If 5 Cx43-P *per* hemichannel were sufficient to render the hemichannels sucrose impermeable, then the expected sucrose-retention value would be ~74%, the sum of the proteoliposomes containing 5 (~40%) and 6 (~34%) Cx43-P subunits, according to the binomial distribution. This value is significantly larger than the

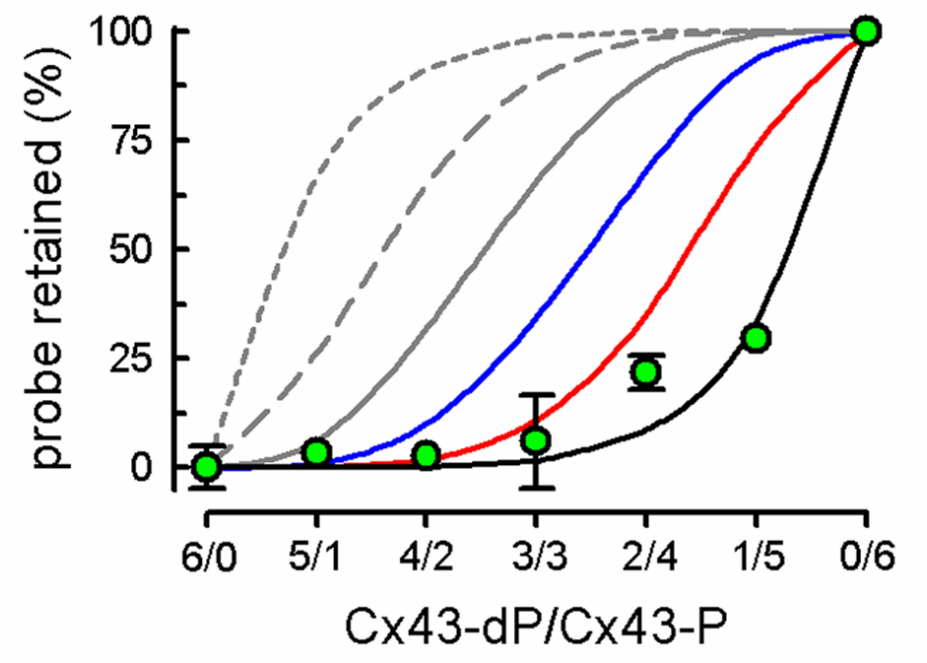

Figure 5.2: Effects of the number of PKC-phosphorylated subunits *per* hemichannel on sucrose permeability.

Effects of varying Cx34-dP/Cx43-P average ratios on the percentage of sucrose retained in proteoliposomes pre-loaded with the radiolabeled probe. Values were normalized as described in Fig. 1. 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 is ≥ 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. From Bao *et al*. (2007).

measured sucrose retention of 30 ± 1%, but is similar to the percentage of proteoliposomes containing 6 Cx43-P subunits (~34%). These results strongly suggest that all 6 hemichannel subunits must be phosphorylated to abolish sucrose permeability.

Movement of the C-terminal ends of hemichannels in response to phosphorylation by PKC.

If the notion of the block of the hemichannels by a simple ball-and-chain mechanism is correct, and the C-terminal domain moves to plug the pore, then one would expect a shortening of the distance separating probes attached to a Cys residue near Ser368, the PKC-target residue (Bao *et al*., 2004b). Following this reasoning, our first studies were on a single-Cys mutant with the Cys introduced next to the last amino acid of the Cx43 sequence (C383 mutant, see Fig. 5.3). Cys383 is just 14 amino acids away from Ser368.

The results summarized in Table 5.1 clearly indicate that instead of the ends of the C-terminal domain approaching each other, they move away upon phosphorylation by PKC. The increased distances calculated from the Tb^{3+}/F-Mal (~3 Å) and Tb^{3+}/ATTO-Mal pairs (~9 Å, respectively), most likely represent changes in distances between diametrically-opposed and next to neighbor subunit C-terminal domain ends, suggesting that the movement of the C-terminal domains is not uniform, but depends on the relative position of the subunits in the hemichannel. Although the origin of the differences calculated from the Tb^{3+}-fluorescein and Tb^{3+}-ATTO 465 pairs was not explored further, it does not seem to arise from the properties of the donor-acceptor pairs, because there

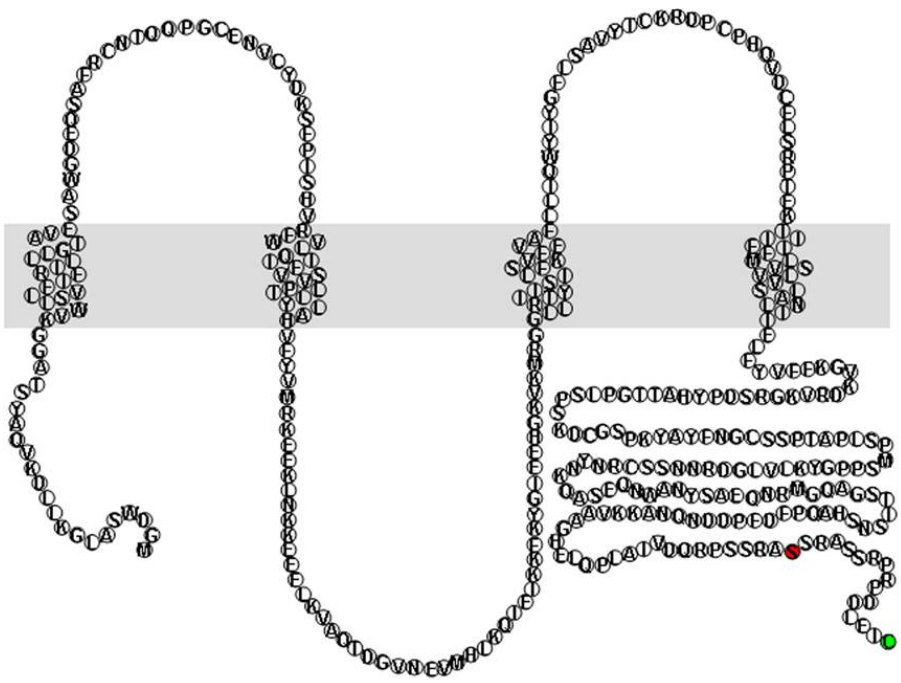

Figure 5.3: Schematic representation of the C383 Cx43 mutant.

The illustration shows the PKC target residue (Ser368, red), and the Cys inserted for labeling the C-terminal domain end (C383, turquoise) 14 amino acids away.

was agreement between distances calculated from these pairs for other residue positions (see below). These results indicate that phosphorylation by PKC does not elicit a large movement of the C-terminal domain ends towards the center of the pore. Interestingly, the calculated distances between the probes attached to Cys383 are quite similar to those between residues of the pore (compare data from Tables 4.1, 4.3 and 5.2), suggesting that the ends of the C-terminal domains are normally positioned under the pore. Additional experiments are needed to determine the distances of the C-terminal domain ends relative to the bilayer.

Table 5.1: Effect of PKC on distances between the ends of the C-terminal domains

C383				
	τ (μs)		R (Å)	
	deP	P	deP	P
ATTO-465	338 ± 15	922 ± 89*	22.2 ± 0.2	31.0 ± 2.6*
Fluorescein	306 ± 27	430 ± 26[§]	36.2 ± 0.8	39.1 ± 0.6[#]

n = 8-10, [#] P< 0.02, [§] P< 0.01 and * P< 0.001 vs. corresponding deP value.

Effect of PKC-mediated phosphorylation of the hemichannels on the M3 helices

Since the measurements between C-terminal domain ends cannot explain the decreased permeability of the PKC-phosphorylated hemichannels, studies were performed to determine whether phosphorylation elicits a significant rearrangement of pore-lining helices. The focus was on M3 because it was the first pore-lining helix that we identified (see Chapters 2, 3 and 4). For these experiments, the single-Cys mutants used were I156C, V164C and V167C (see Chapter 4).

The data from the 3 single-Cys M3 mutants studied are summarized in Table 5.2. During gating by PKC-mediated phosphorylation, diametrically-opposed M3 helices

approach each other by ~4 Å. The results obtained from the LRET data using the Tb^{3+}-ATTO 465 donor-acceptor pair suggest a larger change near the cytoplasmic side (I156C) compared to that near the extracellular side (V167C) of the pore (6 *vs*. 3 Å). An asymmetry in the movement in the response to phosphorylation (next-to-neighbor *vs*. diametrically-opposed C-terminal domain ends; cytoplasmic *vs*. extracellular ends of M3) seems possible, but additional experiments will be required to determine whether it is a true phenomenon.

Table 5.2: Effect of PKC on M3-M3 distances

I156C				
	τ (μs)		R (Å)	
	deP	P	deP	P
ATTO-465	798 ± 40	340 ± 24*	27.9 ± 0.7	22.2 ± 0.4*
Fluorescein	424 ± 19	300 ± 11*	38.9 ± 0.4	36.2 ± 0.3*

n > 16, * P< 0.001 vs. corresponding deP value.

V164C				
	τ (μs)		R (Å)	
	deP	P	deP	P
Fluorescein	455 ± 42	268 ± 8*	39.5 ± 0.9	35.4 ± 0.2*

n = 7, * P< 0.001 vs. corresponding deP value.

V167C				
	τ (μs)		R (Å)	
	deP	P	deP	P
ABD	1211 ± 118	429 ± 29*	22.6 ± 2.6	13.0 ± 0.2*
ATTO-465	756 ± 59	466 ± 46*	27.3 ± 0.1	23.9 ± 0.6*
Fluorescein	483 ± 39	284 ± 8*	40.1 ± 0.8	35.8 ± 0.2*

n = 5-10, * P< 0.001 vs. corresponding deP value.

DISCUSSION

For the transport studies I measured retention of permeability probes after gel filtration of proteoliposomes pre-loaded with radiolabeled or fluorescent probes (Bevans *et al*., 1998). The methodology (probe-retention studies) and experimental system (reconstituted purified hemichannels) have been previously described (Bao *et al*., 2004b and Bevans *et al*., 1998), and additional validation is presented in Chapter 4, **RESULTS**. Using carboxyfluorescein as a probe, all mutants tested (C383, I156C, V164C and V167C) display a permeability that was abolished by PKC-mediated phosphorylation of all hemichannel subunits (not shown). For the most relevant experiments, I used sucrose as permeability probe because its molecular mass is on the lower side of the range of the masses of second messengers of functional significance that permeate gap-junctional channels and hemichannels. Although an accurate estimation of the minimum hydrophilic pore size of Cx43-P hemichannels is complicated by the potential interaction of the permeability probes with pore-lining side chains (Weber *et al*., 2004), the hydrodynamic diameter of ethyleneglycol is 4.4 Å (Gong *et al*., 2001), i.e., significantly larger than that of hydrated K^+ and Cl^- (~3.3 Å) (Nightingale, 1959), the main ions carrying currents through gap-junctional channels. Therefore, the results presented in this chapter explain why activation of PKC reduces dye transfer (Bao *et al*., 2004a and Bao *et al*., 2004b), but has no substantial effect on cell-to-cell gap-junctional currents (Lampe *et al*., 2000b). The differential modulation of Cx43 permeability by PKC-mediated phosphorylation could reduce fluxes of organic hydrophilic solutes such as ATP, cAMP, IP3 and NAD^+ (300-700 Da molecular weight) without major effects on small-ion fluxes and electric coupling.

As mentioned in the **INTRODUCTION**, stimulation of PKC decreases the frequency of the dominant (~100 pS) conductance state of Cx43 gap-junctional channels,

favoring a lower conductance state (~50 pS) (Lampe *et al*., 2000a). It seems possible that these lower conductance channels are formed by the fully-phosphorylated hemichannels permeable to ethyleneglycol that are described here. However, the level of Cx43 phosphorylation in studies in cells is uncertain, and it is important to point out that there is no simple correlation between the single-hemichannel conductance and the permeability to large hydrophilic solutes of hemichannels formed by different connexin isoforms (Harris, 2001 and Beltramello *et al*., 2005).

It is important to consider that the equilibration time of transport probes such as sucrose across liposomes containing Cx43-dP hemichannels (<0.1 ms) is orders of magnitude shorter than the transit of the liposomes through the gel-filtration column. Therefore, our measurements provide steady-state information on the permeability cut-off, but not on permeation rates, needed to analyze detailed permeability changes (Bao *et al*., 2004b). Therefore, our results cannot rule out that partial phosphorylation of the Cx43 hemichannels decreases sucrose permeability, but show that complete phosphorylation is needed to abolish sucrose permeability.

Although dynamic exchange of subunits between detergent-solubilized hemichannels was directly demonstrated by the gel-filtration and LRET experiments described in chapter 3, the sucrose permeability studies confirmed this important observation using a different methodology. For example, in the absence of subunit exchange, 50% and 83% of the proteoliposomes with hemichannels reconstituted from 3/3 and 1/5 Cx43-dP/Cx43-P ratios would contain Cx43-P hemichannels impermeable to sucrose, respectively. However, the values measured were not statistically different from zero and ~30%, respectively (Fig. 5.2), which is incompatible with the absence of subunit exchange, and strongly suggests that hemichannels are reconstituted in the liposomes according to the connexin composition in detergent. The results in Fig. 5.2 strongly

suggest that for PKC to abolish permeability to larger hydrophilic solutes all 6 Cx43 hemichannel subunits must be phosphorylated.

Regarding the molecular mechanism of the partial closure of the Cx43 hemichannels by phosphorylation, the results presented in this chapter show that phosphorylation of Ser368, located near the end of the regulatory C-terminal domain, not only produces movements of the C-terminal domain, but also of transmembrane pore helices. To assess the magnitude of the narrowing of the pore produced by Ser368 phosphorylation and generate a model of the partially-closed hemichannel, experiments looking at the movement of M2, the main pore-lining helix, will be needed. The experiments in this chapter constitute the first direct attempt to show how domains of the hemichannel move in response to gating. Since LRET-based measurement can be performed in hemichannels in their native environment, they provide a powerful tool for structural studies, in particular complementing high-resolution structural data that are obtained on proteins in a non-native environment and frequently in a single conformation.