Phosphorylation of gap junction proteins, connexins, plays a role in global signaling events involving kinases. Connexin43 (Cx43), a ubiquitous and important connexin, has several phosphorylation sites for specific kinases. We appended an imaging reporter tag for the activity of the δ isoform of protein kinase C (PKCδ) to the carboxyl terminus of Cx43. The FRET signal of this reporter is inversely related to phosphorylation of serine 368 of Cx43. By activating PKC with the phorbol ester phorbol 12,13-dibutyrate (PDBu) or a natural stimulant, UTP, time lapse live cell imaging movies indicated phosphorylated Ser-368 Cx43 separated into discrete domains within gap junctions and was internalized in small vesicles, after which it was degraded by lysosomes and proteasomes. Mutation of Ser-368 to an Ala eliminated the response to PDBu and changes in phosphorylation of the reporter. A phosphatase inhibitor, calyculin A, does not change this pattern, indicating PKC phosphorylation causes degradation of Cx43 without dephosphorylation, which is in accordance with current hypotheses that cells control their intercellular communication by a fast and constant turnover of connexins, using phosphorylation as part of this mechanism.

Connexin43 (Cx43) is a ubiquitous and critical gap junction (GJ) protein, playing important regulatory and developmental roles in many tissues including heart, brain, and various endothelia, among others. Cx43 has a highly regulated life cycle during which several, hierarchical phosphorylation events occur at several specific serine residues in its C terminus and occur during all stages of the cell cycle. These events change which proteins interact with Cx43, the kinetics and/or localization of Cx43 trafficking, assembly, gating, and turnover in a cell cycle stage-specific manner that affects important biological processes, such as cell migration and proliferation. Cx43 contains at least 12 serines and 2 tyrosines in its C terminus are known to be phosphorylated at key steps in its life cycle (1); however, it is unknown how these specifically phosphorylated serines act as tags, gatekeepers, or signals to other proteins. During the cell cycle, the amount of gap junction-mediated cell-cell communication, gap junction morphology, and distribution of Cx43-containing structures can change dramatically. Trafficking of Cx43 is a highly coordinated process with different populations of Cx43 phospho-isoforms localized to specific cellular compartments. Of keen interest is where and when specific kinases phosphorylate Cx43 in a cell cycle-dependent manner. Several kinases phosphorylate Cx43 (protein kinase A, protein kinase C, Akt (protein kinase B), Src, CK1, and MAPK) at specific serines or tyrosines (2), however, the majority of these 12–13 phosphorylated residues have yet to be linked to specific kinase actions or signaling pathways.

It has been well established that protein kinase C (PKC) phosphorylates Cx43 at Ser-368 (3–9). The family of PKC enzymes controls the function of other proteins by phosphorylating specific Ser or Thr residues on these proteins (10). In Madin-Darby canine kidney cells or COS-7 cell lines, two predominant PKC isoforms, PKCδ and PKCe, both phosphorylate TPA, 12-O-tetradecanoylphorbol-13-acetate; FRET, Förster resonance energy transfer.

*This work was supported, in whole or in part, by National Institutes of Health Grants GM072881 (to G. E. S.) and GM043154 and P01 DK054441 (to A. C. N.). Most of the work presented here was conducted at the National Center for Microscopy and Imaging Research at San Diego, supported by National Institutes of Health Grant GM103412 (to M. Ellisman).

This article contains supplemental Movies S1–S4.

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2 The abbreviations used are: Cx43, connexin43; PKCδ, protein kinase Cδ isoform; ROI, region of interest; PDBu, phorbol 12,13-dibutyrate; NRK, normal rat kidney; GJ, gap junction; δCKAR, δ isoform C kinase activity reporter;

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**Background:** Connexin43, a ubiquitous gap junction protein, is phosphorylated by protein kinase C on serine 368.

**Results:** After PKCδ activation, phospho-Ser-368 Connexin43 channels segregated into the gap junction center and were subsequently internalized and degraded.

**Conclusion:** PKCδ phosphorylation triggered internalization and degradation of Connexin43 channels without dephosphorylation.

**Significance:** Differential phosphorylation events are used to sort and traffic Connexin43 channels within gap junctions and into the cytoplasm.
Ser-368 on the C terminus of Cx43 (5–7, 11–13). Ser-262 and Ser-255 are also phosphorylated by MAP kinase during the PKC response (13). Both PKCδ and PKCe isoforms are classified as “novel” that respond to increased levels of diacylglycerol. Novel PKCs serve signaling functions in apoptosis, cardioprotection from ischemia, heat shock response, and insulin exocytosis (14). Each isomser is believed to have a distinct role in cells; in early ischemic heart tissue, PKCδ and PKCe may regulate the opposite capabilities of apoptosis and cardioprotection, respectively. Studies of purified and reconstituted Cx43 hemichannels have shown that PKC phosphorylated Cx43 directly on Ser-368 (without other cellular factors), and that mutagenesis of Ser-368 to an Ala residue eliminated phosphorylation of Cx43 hemichannels preventing the decrease in dye and small molecule permeability (5). Ek-Vitorin et al. (6) demonstrated that early activation of Ser-368 through PKC phosphorylation was increased in ischemic hearts and that pSer368Cx43 remained predominantly at intercalated disks as intercellular channels and not hemichannels.

Here, we examined the spatio-temporal localizations after stimulation of PKCδ using a kinase reporter system (15) and a phospho-specific antibody for pSer368Cx43. Our goal was to determine the cellular locations of PKCδ-phosphorylated Cx43 as part of the synthetic, homeostatic, or degenerative pathways. Previously, Lampe et al. (7) showed that gap junction channels closed in response to treatment with TPA (tetradecanoyl phorbol 13-acetate), a phorbol ester, that stimulates PKC to phosphorylate its substrates specifically. We fused a genetically encoded fluorescence resonance energy transfer-based reporter for PKC δ activity, δ C kinase activity reporter (δCKAR) (15) to the C terminus of Cx43 (Cx43–δCKAR) and expressed it in COS-7 cells. This reporter tag provides a significantly better FRET readout than a Cx43 substrate and kinase, and specifically reports PKCδ activity, rather than its translocation to Cx43. The δCKAR reporter tag contains a monomeric CFP, a phospho-Thr-binding FHA2 domain, a substrate peptide specifically phosphorylated by PKCδ on a Thr residue, and monomeric YFP. In the unphosphorylated state, monomeric CFP and YFP are in close enough proximity and orientation to FRET (16). Once phosphorylated by PKCδ at the threonine within the substrate sequence, the FHA2 domain binds the phosphorylated sequence, resulting in a conformational change that decreases the FRET ratio. Cx43–δCKAR localized to GJ plaques with morphologies similar to wild type and gets phosphorylated at Ser-368. A strong FRET signal was observed at the GJ plaques with a subset of channels having a stronger signal within the plaque. Time-lapse FRET imaging of Cx43–δCKAR after stimulation by the PKC activator phorbol 12,13-dibutyrate (PDBu, a more water soluble version of TPA) caused a decrease in FRET within the GJ over time, with internalization and disappearance of pSer368Cx43 vesicles. Studies with PKC inhibitors showed that this is a specific response. Thus, phosphorylation by PKCδ at Ser-368 caused degradation of Cx43 channels and de-phosphorylation by phosphatases did not seem to be involved.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—For additional information about usage, below we provide antibody identification numbers in The Antibody Registry. Antibodies used for this study were: anti-pan-phospho-Cx43 (Sigma, catalog C6219, Antibody Registry ID AB_476857), pSer368Cx43 (R&D Systems Inc., Minneapolis, MN, catalog PPSO46, Antibody Registry ID AB_2110321). Unless specified otherwise, secondary antibodies for immunofluorescence were obtained from Jackson Laboratories.

The following antibodies were used as markers of subcellular compartments: anti-Rab4 (BD catalog number 610888, Antibody Registry ID AB_398205), anti-Clathrin (BD catalog number 610499, Antibody Registry ID AB_397865), anti-p47a/AP3M1 (BD catalog number 610890, AB_10015260), anti-LAMP1 (BD catalog number 611043, Antibody Registry ID AB_398356), and anti-26 S Proteasome antibody (AbCam catalog number AB58115, Antibody Registry ID AB_942116). Pharmacological agents PDBu, G66983, calyculin A, and bisindolylmaleimide (Bis IV) used in this study were obtained from EMB/Millipore (Calbiochem Division, Billerica, MA).

Plasmids—δCKAR plasmids were constructed in the mammalian expression vector pcDNA3.0 as described in Ref. 17. We cloned Cx43 into the pcDNA3.0 δCKAR using a BamHI restriction site and incorporated a 9-amino acid linker consisting of GSAAASFAT between the end of Cx43 and the beginning of the CFP. Site-directed mutagenesis was done with the QuikChange II XL Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) using PfuUltra High Fidelity DNA polymerase. Complete methods for site-directed mutagenesis are described in Ref. 18.

Cell Culture and Transfection—COS-7 cells were incubated in a 5% CO₂ incubator with regular medium (low glucose DMEM with 10% fetal bovine serum). They were transfected roughly 48 h after plating in MatTek dishes using FuGENE 6 (Promega) and TransfectaGRO Medium (Invitrogen). Twenty-four hours post-transfection with Cx43–δCKAR or Cx43–δCKAR and GFP–PKCe constructs, COS–7 cells were refreshed in Opti-MEM without phenol red (Invitrogen, catalog number 11058-021) and then imaged. All other cell lines were cultured at 10% CO₂ with regular medium.

Immunolabeling with pSer368Cx43 Antibodies and Subsequent Confocal Imaging—Cells were plated on poly-d-lysine-coated coverslips and allowed to grow for ~48 h in regular medium before being fixed with 4% paraformaldehyde for 15 min or, for transiently transfected cells, 100% cold methanol for 15 s followed by 1% paraformaldehyde for 15 min. After being rinsed in 0.1% Triton X-100 and then blocking solution (2% NDS, 1% glycine, 5% bovine serum albumin, final concentrations), the cells were exposed to two primary antibodies: anti-pSer368Cx43 to target serine 368–phosphorylated Cx43, and Sigma pan-phospho-Cx43 that labels all Cx43 phospho-forms present in the cell. Because both the pSer368Cx43 and the pan-phospho-Cx43 antibodies are rabbit polyclonal species, we used the Zenon Tricolor Rabbit IgG Labeling Kit 1 (Invitrogen, catalog number Z-25360) to label them with fluorophores with different spectral properties. The Zenon reagent is a ligand...
derived from a secondary antibody that is then conjugated to a fluorophore. The Zenon reagents were directly attached to their respective primary antibodies according to the manufacturer’s protocol. The cells were incubated with the labeled anti-pSer368Cx43 primary overnight at 4 °C, labeled with antipan-phospho-Cx43 for 1 h at room temperature, and then post-fixed with 2% paraformaldehyde. The cells were rinsed with PBS, some were stained with DAPI, rinsed again with PBS, and then mounted on slides using Gelvatol.

In the case of correlated immunolabeling of various organelles with the anti-pSer-368 antibody, the same process of fixation and rinsing was performed. However, after initial overnight labeling with anti-pSer368 attached to Zenon 568, coverslips were exposed to primary antibodies targeting proteins in degradation and synthesis pathways for 1 h at various concentrations. The primary mouse antibodies were secondarily detected by incubating with donkey anti-mouse CY5 secondary antibody for 1 h, whereas the rabbit primary was directly detected using the Zenon 647 reagent. The coverslips were rinsed in PBS, and then mounted on microscopy slides using Gelvatol.

Confocal immunofluorescence images were acquired on the Olympus Fluoview 1000 microscope using a ×60 oil immersion objective with numerical aperture 1.42. The pixel size is 0.069 μm/pixel. Acquired images were 1024 by 1024 pixels for a field size of 70.6 by 70.6 μm. For normal rat kidney (NRK) cells labeled with a double Zenon kit, the pSer368Cx43 antibody was bound to Zenon-Alexa 568, whereas the Zenon-Alexa 488 was attached to the pan-Cx43 antibody. Specimens were imaged with 568 nm laser and a 488 nm laser, respectively. COS-7 cells transfected with Cx43-ΔCKAR were labeled with anti-pSer368Zenon-Alexa 568 and another Ab against various cell markers secondarily detected using donkey anti-mouse-Cy5 (Jackson Laboratories) or Zenon-Alexa 647 (kit used on rabbit anti-LAMP1) and imaged with 488 nm laser (YFP/CFP), 568 nm laser (pSer368Cx43), and 633 nm laser (various cell markers).

Western Blots—Cell lysates of NRK cells endogenously expressing Cx43 or COS-7 cells transiently transfected with the Cx43-ΔCKAR construct were made following published methods (19). Electrophoresis was performed on 10% and 4–20% PAGE gels for Cx43 and Cx43-ΔCKAR, respectively. Transfer was performed using iBlot (Invitrogen). Western blots were made using the Sigma pan-phospho-Cx43, pSer368Cx43 antibodies, and Luminata Forte (EMD-Millipore) was used as ECL substrate.

Measuring FRET/CFP Ratios from Live COS-7 Cells—Images were acquired using a Zeiss Plan-Apochromat ×100 objective with numerical aperture 1.4 on a Zeiss Axiovert microscope (Carl Zeiss Microimaging, Inc.) with a MicroMax digital camera (Roper-Princeton Instruments, Trenton, NJ). The imaging itself was done through the MetaFluor Program (Molecular Devices, Sunnyvale, CA). Every 15 s, an image was acquired through a 10% neutral density filter using three channel settings. CFP and FRET images were obtained through a 420/20-nm excitation filter, a 450-nm dichroic mirror, and either a 475/40-nm emission filter (CFP) or a 535/25-nm emission filter (FRET). To control for photobleaching, YFP images were obtained through a 495/10-nm excitation filter, a 505-nm dichroic mirror, and a 535/25-nm emission filter. RFP images, used to visualize the movement of PKCδ, were obtained through a 560/25-nm excitation filter, a 593-nm dichroic mirror, and a 629/53-nm emission filter. Integration times were 200 ms for CFP and FRET and 100 ms for YFP and RFP.

The imaging process was done at room temperature in a darkened room. During the experiment, a baseline time of ~15 min was established before treatment of the COS-7 cells. After this time, various stimuliants (UTP, PDBu) or inhibitors (Gö6983, calyculin A, Bis IV) were added. The cells were then observed for ~25 min after treatment, unless another drug was to be added several minutes later. We used Fiji, a bundled distribution package of ImageJ (20) to construct regions of interest (ROIs) around key areas of the COS-7 cells to measure both CFP and FRET intensities. Using ROIs drawn in Fiji, the intensities of separate channels were measured and stored in a Microsoft Excel spreadsheet. After several rounds of calculations and analysis, individual experiments were averaged together to produce a final set of graphs. Sample sizes were typically taken for 4–6 runs that could each contain multiple gap junction ROIs. Animations for each experiment were made using Fiji to convert the image stack into ~10-s time lapse movies, showing the change of FRET/CFP ratio in the cell. Supplemental movies were made by combining individual animations using Fiji. Text for movies were made in a black frame using Photoshop and overlaid in Fiji. AVI movies were converted to Quicktime format using Quicktime Pro (Apple, Sunnyvale, CA).

Statistical Analysis—ROIs were drawn on raw image stacks to encompass four particular areas of the cell (gap junction plaques, Golgi, cytoplasm, and nucleus). To obtain a CFP/FRET ratio for graphing, the following equation was implemented,

\[
\text{CFP/FRET Ratio} = \frac{\text{Average ROI pixel intensity (CFP)}}{\text{Average ROI pixel intensity (FRET)}}
\]

(Eq. 1)

except for the UTP experiments for which background-subtracted CFP intensities were divided by background-subtracted FRET intensities. ROIs were drawn on regions of the images where no cells were present for background measurements. In the equation, the average ROI intensity of a particular fluorophore channel is represented by the average pixel intensity within all ROIs of the same type in one frame. The ratio was calculated, and then the same process was applied to every frame of the time lapse movie. The baseline and slope of these values were then calculated and the resulting data were plotted in Excel. Finally, to average different runs together, the timelines of each experiment were synchronized together and their values were averaged accordingly. Mean ± S.E. was calculated to generate error bars for each data point.

Although Cx43 is primarily found in the Golgi and at the plasma membrane, another important localization during its life cycle is within cytoplasmic vesicles. The phosphorylation of Cx43 at Ser-368 while trafficking through the cytoplasm in vesicles was graphed separately as a histogram. After thresholding the images, the Fiji Particle Analysis tool was used to automatically draw hundreds of ROIs around vesicles in every frame of...
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the time lapse data. This allowed the acquisition of the pixel intensities in these vesicles across the entire movie. Vesicles from 20 adjacent frames (5 min) were analyzed together to have significant numbers to measure, creating a snapshot within the dataset. Vesicles were measured before treatment, at treatment, and after treatment (see inset, Fig. 7A). This analysis utilized data from 60 frames of each data set to give 3 distinct snapshots of the experiments. The FRET ratio values of the vesicles in each snapshot were binned, counted, normalized to the average FRET ratio of all vesicles before treatment, and graphed as a histogram. Some datasets contained fewer vesicle ROIs than others. To normalize and average the different runs, the number of vesicles in each bin is shown as a percentage of all the vesicles in the run. Error bars are S.E.

To quantify complex co-localization patterns between antibodies against two different proteins, Pearson coefficients were calculated as a measure of pixel-by-pixel cross-correlation coefficients scaled from 0 (no correlation) to 1 (complete overlap). Pearson coefficients were calculated utilizing the Coloc2 tool in FIJI (21, 22). Multiple image sets were taken from similarly prepared slides and were inputted into Coloc2. Once numerous Pearson coefficients were generated, a bar graph with an average value and S.E. bar was created.

GJ Dye Coupling Assays—Parental HeLa cells were transiently transfected with a Cx43-CKAR construct using FuGENE 6 reagent (Promega). A solution of Alexa 568 dye (2 mM in 200 mM KCl, Invitrogen) was injected into cells expressing each of the constructs as gap junctions (as first identified based on the YFP fluorescence at cell appositional areas). Parental HeLa cells (gap junction deficient) were also injected in each experiment to serve as negative controls. Dye injections were performed on a BX50WI Olympus infrared differential interference contrast/epifluorescent microscope (Olympus, Melville, NY) equipped with a ×40 water immersion objective. A fluorescein isothiocyanate filter set was used before injection to image the Cx43-CKAR expression, and a rhodamine filter set was used after injection to image the Alexa 568. Epifluorescent images of the Alexa 568 dye were captured after each injection with a Nikon D300 digital camera.

RESULTS

Although we first started our experiments with intermolecular FRET (YFP-PKCδ and Cx43-CFP) to examine the recruitment of PKCδ to Cx43, we found the interaction of PKC with Cx43 occurred too quickly and too transiently for good spatio-temporal resolution. This was not unexpected, as the agonist-evoked interaction of PKCδ with Cx43 would be a fast and temporary event. Rather, we used the approach of a “biosensor,” a FRET reporter that would permanently remain tethered to the Cx43, optimized to give a localized signal, reflecting not only the phosphorylation of the reporter, but mimicking the phosphorylation of Ser-368. However, as with any genetically appended tag, we first compared the agonist-dependent phosphorylation of Cx43 in an endogenously expressing (“native”) system to that of the tagged Cx43 species to make sure they trafficked similarly.

Endogenously Expressing NRK Cells Showed an Increase and Segregation of Phospho-Ser-368 in Response to PDBu—NRK cells represent an excellent system for ensuring that tagged forms of Cx43 behave similarly. NRK cells are a canonical Cx43 endogenously expressing cell line with expression patterns similar to those seen in tissue. Quiescent, unstimulated NRK cells contain a “necklace” of ~0.5-μm gap junctions between apposing cells. Fig. 1 shows co-localization of pan-phospho-Cx43 (all phospho-forms, shown in red) and pSer368Cx43 (green) by immunofluorescence. In unstimulated, quiescent cells, pSer368Cx43 is typically found at low levels interspersed within gap junction plaques as seen in Fig. 1A. After activation by 200 nM PDBu for 15–30 min, pSer368Cx43 segregated into “islands” or subdomains within gap junction plaques as evidenced by the appearance of yellow co-labeling (Fig. 1, B and D). Most plaques in NRK cells were small and in general, we did not see systematic increases in plaque size after PDBu treatment. However, the larger plaque in the representative image selected for Fig. 1B (shown at higher magnification in Fig. 1D) was chosen because it clearly showed the segregation of pSer-368 labeling. A Western blot analysis with these two antibodies revealed differences in phosphorylation before and after PDBu treatment (Fig. 1, E and G). As established first as described in Ref. 23, Cx43 in NRK cell lysates were separated on Western blots into three macroscopic phospho-bands (termed P0, P1, and P2) when probed with an antibody whose epitope is not part of the C-terminal domain (Fig. 1E). An antibody that recognizes the sequence of a phosphorylated Ser-368 peptide only detected one band on these Western blots (Fig. 1G). After treatment with PDBu, the pan-phospho-Western blot had similar intensities of the P0, P1, and P2 bands, but a specific pSer368Cx43 band increased ~5–6-fold in intensity after PDBu incubation. These results serve as positive controls for the comparison of untagged versus tagged Cx43.

The Cx43-δCKAR Biosensor Was Phosphorylated at Ser-368 and Maintained a Basal FRET Level That Decreased with PDBu Stimulation—The CKAR reporters (nonspecific and δCKAR version) have previously been appended to amino acid sequences that target to specific organelles such as mitochondria, the Golgi apparatus, nuclei, and the plasma membrane (15, 17, 24) as well as to specific proteins such as AKAP79 (25) and PSD95. Here, we appended δCKAR to the C terminus of Cx43. A schematic of this construct is shown in Fig. 2A. Cx43 (displayed in gold) is depicted with the x-ray atomic model of the highly homologous Cx26 (26) connecting to the NMR solution structure of a C terminus peptide (27). The δCKAR domain is depicted with two Richardson diagrams of the atomic structure of GFP colored cyan and yellow for CFP and YFP, respectively. Connecting these is the atomic model of the FHA2 domain (28) and the PKCδ substrate peptide shown as an orange cylinder. When phosphorylated, the two fluorescent proteins move apart and the FRET signal is decreased (16).

We compared immunofluorescence images and Western blots of COS-7 cells transfected with Cx43-δCKAR to ascertain that the δCKAR reporter does not significantly alter gap junc-
tion trafficking. COS-7 cells are typically flat and thus, useful for imaging experiments. Cx43-δCKAR gap junctions (Fig. 2B) are larger than those found in endogenously expressing cell lines, which is consistent with previous studies showing that C-terminal tags eliminate ZO-1 binding and plaque size control, but otherwise exhibit normal trafficking (29, 30). This larger plaque size was helpful in our analysis of imaging changes, within gap junctions, which would be much harder to observe in normal size gap junctions such as those in NRK cells (Fig. 1A). In the COS-7 cells we used for our experiments, there is a small amount of endogenous Cx43. We have found that this promotes more efficient trafficking of the Cx43-δCKAR to gap junctions than Cx43-deficient COS-7 cells. When probed with a pSer368Cx43 antibody, COS-7 cells transfected with Cx43-δCKAR showed similar rearrangements into subdomains as NRK cells after PDBu treatment. Fig. 2B shows the pSer368Cx43 antibody labeling overlaid on the CFP fluorescence from the δCKAR. Western blots of non-treated and PDBu-treated cell lysates were probed with anti-GFP antibodies or anti-pSer368Cx43 antibodies (Fig. 2C). Here, we used an anti-GFP antibody to examine the full-length protein. The Cx43-δCKAR is 1071 amino acids with a molecular mass of ~117 kDa. The anti-GFP antibody Western blots showed multiple bands as before, although there were more variations in the bands, possibly due to our introduction of an additional phosphorylation site in the δCKAR reporter. Similar to NRK cells, anti-pSer368Cx43 immunoblots showed a significant increase in band intensity after PDBu incubation (Fig. 2D). Lysates were also probed for tubulin levels as a loading control, to ensure that differences in intensities were not due to different amounts of loaded protein.

To confirm that macroscopic coupling of the Cx43-δCKAR-transfected cells was similar to Cx43 endogenously expressing cells, we used dye injection assays in non-Cx43 expressing HeLa cells. The particular clone of HeLa cells we used in these experiments was verified using immunolabeling as a non-Cx43 expressing cell line (31). Cells expressing Cx43-δCKAR gap junctions were identified first using the CFP/YFP fluorescence of the δCKAR reporter and one cell was injected with Alexa 568 (Fig. 2E). Images were recorded after 1 min to track progress and 8 min after injection. The longer time period was chosen, because dye transfer would be most likely completed. In Fig. 2E, which represents a typical injection out of five independent
injections, showed that the dye spread to three adjacent cells coupled by Cx43-δCKAR gap junctions. Thus, gap junctions formed by Cx43-δCKAR are functional.

Next, we analyzed FRET images of Cx43-δCKAR-transfected cells. Because Cx43 is constantly turning over with a short half-life (32), giving rise to separate intracellular and plasma membrane pools of Cx43, we used several ROIs in cells for our analysis. Cx43 pools in the Golgi are part of the trafficking of new protein to the membrane. A change in FRET ratio in this area would indicate that PKCδ phosphorylates at the Golgi and serves to stimulate movement of connexons to the plasma membrane, however, this was not observed. Indicated on the YFP image shown in Fig. 3A are ROIs for gap junctions, cytoplasm, nucleus, Golgi apparatus, and several vesicles. An ROI for the nucleus served as a negative control, because the expectation is that there is minimal FRET signal in this organelle. A typical FRET ratio image displayed on a spectral color table is shown in Fig. 3B. As expected, unstimulated Cx43-δCKAR-transfected COS-7 cells showed expression and high fluorescence in the Golgi region and gap junction plaques, however, the CFP/FRET ratio, the indicator of increased PKCδ-mediated phosphorylation, in Fig. 3C remained constant for averages of all four categories of ROIs. Because the sizes of the ROIs were variable, we calculated and graphed the average FRET and CFP signals per pixel. In our images, the color table is displayed as FRET/CFP (red/yellow colors indicating less phosphorylation with higher FRET ratio), whereas in our graphs, we calculated the percent change in CFP/FRET. The latter indicated an increase in phosphorylation of δCKAR and thus, Ser-368. Without
PDBu treatment, the percent change in CFP/FRET signal did not significantly change over the imaging time, even after dimethyl sulfoxide was added (“mock”), to serve as a negative control (Fig. 3C, supplemental Movie S1). To summarize and emphasize our results, we showed, both through imaging and biochemical correlation with phospho-specific antibodies, that the Cx43-δCKAR represented an excellent reflection of PDBu-mediated phosphorylation of Cx43 at Ser-368, whereas maintaining previously observed trafficking patterns and functionality.

**Cx43-δCKAR Was Internalized from Gap Junctions after PDBu Stimulation of PKC—**Next we proceeded to study the effects of activating or inhibiting PKC-mediated phosphorylation by changes in the FRET/CFP ratio. Fig. 4, A–D, shows a panel of four sets of live cell images from one experiment before (Fig. 4A) and after (Fig. 4, B–D) addition of 200 nM PDBu. For these sets of experiments, we also transfected RFP-PKCδ to see changes in the kinase distribution and increase in phosphorylation as compared with endogenous levels of PKCδ (Fig. 4, E and F, supplemental Movie 2). Within the YFP channel imaging the Cx43-δCKAR (center image), gap junctions, the Golgi area, and several vesicles were highly fluorescent. In the FRET/CFP channel, mainly the gap junction areas showed a significant signal, whereas in the cytoplasm and Golgi there was almost no FRET/CFP signal. On the other hand, the RFP-PKCδ image contained a broad distribution of the kinase, before PDBu treatment, which changed dramatically after PDBu treatment. Within the live cell movie, the RFP-PKCδ moved toward the plasma membrane (supplemental Movie S2). Concurrently, we saw a decrease in FRET/CFP at the gap junctions, coincident with decreases in FRET intensity and vesicles breaking from the gap junction (arrow in Fig. 4, A–D, left image) and moving into the cytoplasm. With time, the signal on these vesicles disappeared, suggesting that they were degraded.

The graphs in Fig. 4, E and F, indicated a quantitative analysis of these time-lapse images. Fig. 4E revealed the change in phosphorylation of the reporter with PDBu stimulation of endogenous PKCδ, whereas Fig. 4F are graphs of image experiments such as the one displayed in Fig. 4, A–D, where RFP-PKCδ was co-expressed. Note that there is a significant increase in phosphorylation in gap junction ROIs that were enhanced with co-transfection of exogenous PKCδ. The exogenous PKCδ amplifies the signal by enhancing the rate of phosphorylation of Cx43 in accordance with Michaelis-Menten enzyme kinetics. Other regions (cytoplasm, nuclei, and Golgi ROIs) rose, but at much lower levels. These epifluorescence images collect light from a deep focal plane and therefore, we get a superposition of all cellular components, including Golgi appara-
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tus and cytoplasmic vesicles above and below nuclei and vesicles contained within the cytoplasm. PDBu is a super-stimulator of PKC and when cells are co-transfected with exogenous RFP-PKC and Cx43-H9254/CKAR, we do see an increase in non-junctional areas that we hypothesize is due to the imaging conditions we used. However, non-gap junction areas statistically change less than in the gap junctions.

A key question that we embarked on to answer was whether the FRET readout from the Cx43-H9254/CKAR reporter was coupled to the phosphorylation of Cx43-H9254/CKAR. COS-7 cells were transfected with the Cx43-H9254/CKAR construct and underwent live cell imaging 24 h post-transfection. A solution of 200 nM PDBu was introduced after baseline measurements were established (normalized to time = 0 min in the graphs and indicated by the arrow on the graphs). A–D, time sequence of images (see supplemental Movie S2). Each panel contains the RFP-PKC image (left), the YFP image from Cx43-H9254/CKAR (center), and FRET/CFP image indicating a change in phosphorylation (right). Here, a decrease in this ratio (red indicates high FRET/CFP and blue indicates low FRET/CFP, inset = color table) indicates increased phosphorylation. E and F, the percent change in CFP/FRET ratios were calculated as a function of time using the intensities from the original image channels. E, graph of change in phosphorylation from cells transfected with Cx43-H9254/CKAR. This data were averaged from 4 separate experiments utilizing the same protocol. Note the increase in phosphorylation (decrease in FRET/CFP) at GJs after addition of PDBu. The average graphs calculated from other areas rise slightly, but not at the same levels at the GJ centers. F, graph of percent change in CFP/FRET from cells co-transfected with Cx43-H9254/CKAR and RFP-PKCα. This data were averaged from 6 individual experiments. The addition of exogenous PKCα enhanced this increase in the FRET changes at the GJs, but not in other areas.

FIGURE 5. Mutagenesis of the PKC phosphorylation site, Ser-368, eliminated the response of Cx43-H9254/CKAR to PDBu. Cx43-deficient HeLa cells were transfected with the Cx43-H9254/CKAR construct or its phosphorylation deficient mutant, S368A. Live cell imaging was performed 24 h post-transfection. 200 nM PDBu was introduced after baseline measurements were established (normalized to time = 0 min in the graphs and indicated by the arrow on the graphs). In paired experiments, A and C, S368ACx43-H9254/CKAR gap junctions showed no response to PDBu, unlike B and D, the wild-type construct. An example set of images for S368ACx43-H9254/CKAR gap junctions and wild-type gap junctions are shown in A and C, respectively. A YFP image of the entire field of view is shown at the top left. The box indicates a gap junction between two cells. A ratio image of the boxed area before addition of PDBu (left) and a frame after PDBu treatment from the end of the imaging series (right) are shown at higher magnification. Corresponding graphs for the time lapse imaging series in A (S368A mutant) and B (wild-type) are shown in C and D, respectively. Three independent experiments were averaged to produce the graph in C, whereas the D graph was the average of 4 independent imaging runs. Error bars represent the S.E.
phorylation at Ser-368. We proceeded to generate a site-specific mutant incapable of being phosphorylated at position 368 by replacing Ser with an Ala residue. This mutant, S368A-Cx43, was transfected into Cx43-deficient HeLa cells so no endogenous Cx43 could be phosphorylated. As seen in the graph of Fig. 5A, the S368A-Cx43 did not increase in phosphorylation in response to PDBu, whereas a parallel transfection using "wild type" Cx43 in these HeLa cells showed a similar response (Fig. 5B) to that of Fig. 6A in COS-7 cells (both cell lines having only endogenous PKC). However, it should be noted that the percent change in CFP/FRET values for the plateau in the Fig. 6A graph was ~6% versus the Fig. 5D graph plateau that was ~5%. Therefore, there was not much difference between the plateaus of the two wild type graphs but because the HeLa cell gap junctions are smaller than COS-7 gap junctions, the ROIs are smaller and the graphs were much noisier than the graphs from our COS-7 cell gap junctions. Surprisingly, PKCδ activity did not increase upon phorbol ester stimulation when the phospho-acceptor site on connexin was mutated to Ala. One possible explanation is that phosphorylation of this site is required to dock PKCδ near connexin. Alternatively, PKCδ may bind to S368A-Cx43, but does not dissociate, because it is unable to phosphorylate Ser-368 and gets locally absorbed. The important implication here is that, whereas we read out FRET signals from the covalently attached biosensor, it is the phosphorylation of Ser-368 that controls and regulates the movement of the protein and that if PKC does not phosphorylate Cx43, it also does not phosphorylate the δCKAR as well.

Cx43-δCKAR Is Similarly Trafficked from Gap Junctions by UTP Activation of PKC—Similarly, we investigated changes in PKC-mediated phosphorylation with UTP, a G protein-coupled receptor agonist. In COS-7 cells, UTP treatment results in activation of phospholipase C, which results in the production of diacylglycerol and thus activation of PKC (15). Unlike PDBu, UTP is metabolized and so its effect is short-lived. As shown in Kajimoto et al. (15), UTP had a smaller effect on PKCδ-mediated phosphorylation at the plasma membrane. We saw a small increase in phosphorylation of Cx43-δCKAR with endogenous PKC. We observed a similar change in the CFP/FRET ratio compared with Kajimoto et al. (15) where an initial increase of 2–3% is seen upon UTP stimulation without the addition of exogenous PKCδ. When expressing exogenous PKCδ in the cells, we see an increase in Cx43-localized PKCδ activity at gap junction plaques and very little activity in the nuclear, cytoplasmic, or Golgi ROIs similar to PDBu treatment (Fig. 6, A–F, supplemental Movie S3). The internalization is less pronounced but still occurs (arrows in Fig. 6, B–D).

Spatio-temporal Patterns of Cytoplasmic Cx43-δCKAR Containing Vesicles—Using an algorithm in FIJI that allows us to inscribe vesicles within an ROI (see example in supplemental Movie S4), we analyzed the data sets used in Figs. 3 and 4 to determine the time course of the vesicle population based on their FRET intensity. This analysis allows us to investigate the spatio-temporal patterns of internalization versus synthetic pathways as well as a steady state condition ("before" treatment) as opposed to after kinase stimulation. We examined intervals before treatment (blue), and 10 min after treatment (red) as shown in the inset of Fig. 7A. Consistent with the images described in Figs. 4 and 5, we observed a shift in the vesicle populations toward increased phosphorylation after PDBu stimulation (Fig. 7B) as compared with the mock treatment (Fig. 7A). This shift was not seen with UTP incubations as UTP is a less potent stimulator of PKC and is metabolized within these time frames. Note that the X position of the peaks were the same for all three graphs, but the height of the peaks were proportional to the number of vesicles in each data set. The latter varies with the data sets.

Inhibitors for PKC and Phosphatases Indicate That Cx43-δCKAR Was Not Dephosphorylated by Phosphatases, but Rather Was Degraded—To ensure that the PDBu-stimulated phosphorylation of the reporter was mediated by PKC, cells were pretreated with the PKC inhibitor Gö6983 before PDBu treatment. This effectively prevented the PDBu-dependent phosphorylation of the reporter (Fig. 8A). This inhibition of PKCδ activation was also seen using Bis IV, a substrate-competitive inhibitor for the ATP binding site of PKC (25). Previous studies using the δCKAR reporter showed a decrease in phosphorylation caused by addition of Gö6983 after PDBu treatments such that FRET read-out returned to approximately baseline levels (15), reflecting inactivation of PKC by the inhibitor and dephosphorylation of the sensor. In contrast, here we observed that phosphorylation levels of the reporter stayed at a constant level after the initial action of PDBu and subsequent treatment with the PKC inhibitor Gö6983 (Fig. 8B). Although the graphs shown in Fig. 8, A and B, contain experiments from only endogenous PKCδ, we observed the same inhibition in cells co-transfected with RFP-PKCδ. It has been previously shown that PKC activity is refractory to active site inhibitors when bound to protein scaffolds (25). Thus, one possible explanation is that PKCδ is scaffolded on or next to Cx43 channels such that they are insensitive to the active site inhibitor Gö6983.

However, because we did not see a decrease in phosphorylation after incubations with PKCδ inhibitors, we hypothesized Cx43 was being degraded rather than dephosphorylated. In addition, we wanted to directly compare our results with that of Kajimoto et al. (15) where they demonstrated that adding a phosphatase inhibitor to cells co-transfected with organelle-targeted δCKAR and RFP-PKCδ did have an effect on dephosphorylation. As per Kajimoto et al. (15), we added the phosphatase inhibitor calyculin A, which is a potent inhibitor with high specificity for the PP-1 and PP2A classes of protein serine/threonine phosphatases (33). As shown in Fig. 8C, addition of calyculin A had no immediate effect on the FRET ratio but caused cell rounding within 8–10 min, which is typical of the effects of calyculin A. When stimulated with PDBu, post-treatment with calyculin A has little effect on gap junctions and other cellular compartments (Fig. 8D). The inset in Fig. 8D shows a post-calyculin A time period that has adjusted for a baseline after PDBu addition but before calyculin A was applied. Thus, dephosphorylation of Cx43-δCKAR does not occur and rather the Cx43 is degraded.

Co-localization of pSer368Cx43 with Cellular Compartments—As part of this study on the protein trafficking dynamics of pSer368Cx43, we examined co-localization patterns of this phospho-form with trafficking organelles involved in pro-
Spatio-temporal Analysis of PKCδ-phosphorylated Connexin43
tein degradation. Gap junction degradation involves complex pathways that include lysosomal and proteasomal pathways in ways that are not well understood and are most likely dependent on physiological and pathophysiological stimuli (34, 35). We labeled components for proteasomes (26 S proteasome subunit), early endosomes (Rab4), lysosomes (Lamp1), clathrin vesicles (clathrin), and degradation vesicles derived from the Golgi (p47A, also known as AP3M1). The latter is a rat homolog of clathrin-associated adaptor proteins that interact with the tyrosine-based sorting signal in the trans-Golgi network (36). The monomeric GTPase Rab4 is associated with early endosomes, regulates recycling vesicle formation, and provides for vesicle sorting, before it reaches lysosomes for degradation (37, 38). Clathrin also has been shown to play a role in endocytosis of clathrin-coated Cx43 containing vesicles internalized from gap junctions (39).

We specifically examined two time points, 5 and 25 min, after PDBu treatment, one where we would see large changes from PDBu and then one after most of the effect from PDBu had occurred, respectively. These two time points represent the start of vesicle trafficking and an end point of the PDBu effect. Note that after 5 min, in the graphs in Fig. 4, E and F, we see an intermediate level in phosphorylation, whereas by 25 min the curve plateaus. These were compared with homeostatic cells with no PDBu treatment, representing steady state (non-stimulated) trafficking patterns. Representative images from the non-PDBu condition are shown in Fig. 9, A–E, with anti-pSer368Cx43CKAR labeling displayed in green and anti-organellar labeling in red.

To quantitate the degree of overlaps and changes with time after PDBu treatment, we calculated Pearson coefficients for each image and obtained average and mean ± S.E. for each labeling condition (Fig. 9F). The Pearson coefficient served as a quantitative measure of spatial co-localization of the two immuno-labels for each image. Average Pearson coefficients were calculated for each set of images for the different conditions and we analyzed the change over time by the change in average Pearson coefficients. This is especially important as many of these cellular markers are localized to small vesicular structures that may be difficult to see in low magnification images. Except for the p47A co-staining where essentially no temporal change in Pearson coefficients was observed, we saw a decrease in the Pearson coefficient at 5 and 25 min after PCK stimulation as compared with unstimulated cells. We interpret these decreases in co-localization as breakdown of pSer368Cx43 by the protein degradation apparatus. The largest temporal co-localization changes were seen in the proteasome, lysosome, and clathrin vesicles. In particular, the disappearance of co-labeling was quick for lysosomes where the large change was seen in the 5-min time point, but remained at an almost equivalent level at 25 min. In contrast, a large decrease in proteasomal labeling occurred after 5 min and another significant decrease was observed after 25 min. Another pattern of successive decreases in the Pearson coefficient was observed in clathrin co-labeled specimens, whereas smaller, yet significant decreases in Rab4 labeled early endosomes. Taken together, these results indicate that the lysosomal pathway may have been activated first after PDBu stimulation of PKC, but that the proteasomal degradation pathways were also activated on a slightly slower time scale, as more pSer368Cx43 was endocytosed from gap junctions during this acute internalization process.

**DISCUSSION**

In this study, we examined spatio-temporal dynamics of Cx43 phosphorylation in various cell compartments upon PKC stimulation. In particular, using a reporter for the δ isoform of PKC, we were able to focus in and follow changes in four cellular compartments and vesicles using ROI image analysis software. Our goal was to address how PKC influences particular aspects of gap junction trafficking (synthetic versus degradation) and also to study where, within gap junctions, particular phospho-forms are found and how they move through the plaque. Here we showed that the responses of the reporter were coupled to the phosphorylation status of Ser-368. Importantly, elimination of phosphorylation at Ser-368 by replacement with Ala prevented the activation of Cx43-δ-localized PKCδ. Thus, phosphorylation of the sensor is an effective read-out of the phosphorylation of Ser-368. Because the reporter is tethered to Cx43, it is either specifically coupled to regulation of Cx43 or cannot dissociate and may be absorbed locally. This result brings an interesting question that there are other yet unknown cytoplasmic components involved in moving the activated PKC to the target Cx43 at the gap junction plaque, because PKC is a broad acting kinase with many target proteins.

These FRET-based biosensors, genetically appended to Cx43, are useful tools for not only dissecting the dynamics of specific kinase interactions, but potentially also to analyze the responses of other signaling pathways that are normally activated during unstimulated trafficking or in pharmacological agents or growth factor-stimulated kinase pathways. Stimulation of signaling pathways by fibroblast growth factor 2 resulted in a physical interaction of PKCδ with Cx43 during signaling in osteoblasts (40). Ruch et al. (41) found that the MAP kinase ERKs were activated by PKC in response to TPA treatment and phosphorylated Cx43 at Ser-255, Ser-279, and Ser-282 (42). These findings suggest that there is hierarchy to phosphorylation events at different serine residues in the Cx43 C terminus.

**Gap Junction Trafficking Dynamics after PKC Stimulation**—PKC directly phosphorylates Cx43 on Ser-368 (5–7). Ser-368 phosphorylation has been shown to change single channel behavior leading to a decrease in intercellular communication.

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**FIGURE 6. Changes in CFP/FRET ratios in various COS-7 cell compartments after UTP treatment.** COS-7 cells were transfected with Cx43-CKAR alone or Cx43-CKAR and RFP-PKCa constructs and underwent live cell imaging 24 h post-transfection. After baseline measurements were established, 200 μM UTP was introduced. On these graphs, the time of UTP addition was set to 0 min and the CFP/FRET (increased phosphorylation) was calculated. A–D, time sequence of images (see supplemental Movie S3). Each panel contains the RFP-PKCa image (left), the YFP image from Cx43-CKAR (center), and FRET/CFP image indicating a change in phosphorylation (right). Here, a decrease in this ratio (red indicates high FRET/CFP and blue indicates low FRET/CFP, inset = color table) indicates increased phosphorylation. E, graph of change in phosphorylation from cells transfected with Cx43-CKAR. This data were averaged from 4 separate experiments utilizing the same protocol. Although UTP is a natural stimulator of PKCδ, phosphorylation occurs at much lower levels unless additional PKCδ is added as in F. In F, the graph of change in phosphorylation from cells co-transfected with Cx43-CKAR and RFP-PKCa shows increases in gap junction phosphorylation but not in other cell areas. This analysis was performed on four independent experiments.
Spatio-temporal Analysis of PKCδ-phosphorylated Connexin43

A

Mock

B

PDBu

C

UTP
Bukauskas et al. (43) estimated that only 10% of gap junction channels are active within a gap junction plaque and combined with results from Gaietta et al. (44) demonstrating that channels are internalized from the center of the plaque, it is likely that the decrease in cell-cell communication is due to acute turnover in the number of channels. Here, we demonstrated that under conditions of PDBu stimulation and inhibition of PKC and phosphatases that pSer368Cx43 in gap junction plaques was found in highest concentrations in the middle of the plaques, where it was internalized. Although intermolecular FRET is possible in tight packing situations, typically the packing of channels within gap junction plaques in tissue culture cells is fairly uniform across a plaque (45) and thus, the changes and spatial distributions we see in FRET/CFP are most likely due to phosphorylation differences, not channel packing. Furthermore, we showed that pSer368Cx43 was not dephosphorylated.

**FIGURE 7.** Analysis of the distribution of FRET ratios in populations of vesicles before and after activation of PKCα/H9254. Populations of vesicles were binned according to the inverse of their average normalized FRET intensities (CFP/FRET) and graphed as a histogram. The CFP/FRET corresponds to their levels of PDBu stimulation and inhibition of PKC and phosphatases that pSer368Cx43 in gap junction plaques was found in highest concentrations in the middle of the plaques, where it was internalized. Although intermolecular FRET is possible in tight packing situations, typically the packing of channels within gap junction plaques in tissue culture cells is fairly uniform across a plaque (45) and thus, the changes and spatial distributions we see in FRET/CFP are most likely due to phosphorylation differences, not channel packing. Furthermore, we showed that pSer368Cx43 was not dephosphorylated.

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FIGURE 9. Co-localization of pSer368Cx43 with markers for protein trafficking organelles. Immunofluorescence confocal images of cells labeled with an anti-pSer-368 antibody and various antibodies for organelles involved in protein trafficking and degradation. These example images were from the non-PDBu-treated cells (homeostatic) where there was maximum co-overlap of the three conditions we analyzed. Dual labeling of pSer368Cx43 and A, proteasomes, utilizing an anti-26 S proteasome subunit antibody; B, anti-Rab4 (early endosomes); C, anti-LAMP1 (lysosomes); D, anti-clathrin (clathrin-coated vesicles); and E, antibodies against p47A (vesicles headed to degradation pathways from the Golgi apparatus). F, a comparison of average Pearson coefficient values between pSer368Cx43 staining and staining for each of the five different organelle antibodies used. We calculated Pearson coefficients in each image to quantify the spatial overlaps between pSer368Cx43 labeling and cellular markers. The three bars represent averages and mean ± S.E. for each of the three different categories of cells used: cells that received no PDBu treatment, cells after 5 min of PDBu treatment before fixation, and cells after 25 min of PDBu treatment before fixation. The number of images in each measurement is shown above with its corresponding bar in the graph.
phorylated, but rather was most likely degraded by lysosomes and proteasomes.

Connexins have a short half-life and cells dynamically remodel their gap junction size and shape. The present view of the Cx43 life cycle involves synthesis in the ER, oligomerization in the Golgi, and insert of vesicles containing hemichannels (connexons) into the plasma membrane. These “new” hemichannels add to the edges of plaques and dock with hemichannels from the neighboring cell membrane. Older channels are found at the center of the plaque and are internalized (44, 46). Whether channels are internalized in endocytotic vesicles or circularized gap junctions (called annular junctions) may depend on cell line types and/or transfection conditions.

Multiple pathways for connexin degradation are differentially regulated in response to physiological conditions or outside stimuli (34). Lyosomal degradation is the most prevalent, although proteasomal degradation is another pathway for channel degradation (35, 47), as first shown by Laing and Beyer (48), who reported that pharmacological inhibition of proteasomal activity, using ALLN, resulted in increased levels and reduced turnover of Cx43 protein in Chinese hamster ovary (CHO) cells. An earlier report by Ren et al. (49) found that internalization of Cx43-containing gap junctions in communication competent WB-F344 rat liver epithelial cells stably expressing Cx43 occurred after TPA treatment and led to the formation of hyper-phosphorylated forms of Cx43. Here, under stimulation of PKC, we saw mainly endocytosis of vesicles from gap junctions and a reduction in co-localization with lysosomes and proteasomes. The latter is consistent with findings by Leithe et al. (50) that proteasomal degradation occurs after stimulation of PKC with TPA. Cx43 has been shown to co-localize with clathrin, AP-2, Dab2, and dynamin2 at gap junction plaques (51–53). Additionally, the loss of dynamin GTPase activity or the reduction of dynamin2, clathrin, AP-2, or Dab2 proteins inhibited the internalization of Cx43, as observed by a significant decrease in the number of annular junctions. The Cx43-interacting protein of 85 kDa, CIP85, is a Rab GTPase-activating protein that was found to co-localize with Cx43 at the plasma membrane (54, 55). In our experiments, where cells undergo acute turnover and connexin degradation by pharmacological stimulation of PKC, lysosomal degradation is followed by proteasomal degradation.

Physiological Effects of Phosphorylated Ser368Cx43 in More Complex Systems—PKC phosphorylation of Cx43 occurs in vivo, particularly in acute insults to organ systems. One well studied example is the change of Cx43 phosphorylation in ischemic hearts. Cx43 is dephosphorylated and degraded in early ischemia induced in rats, although no specific serines were identified (56). Ischemic pre-conditioning suppresses the decrease in phosphorylated Cx43 and an increase in dephosphorylated Cx43, whereas only the former was inhibited by the PKC inhibitor chelerythrin. The amount of pSer368Cx43 was increased in ischemic hearts in rats and, whereas pSer368Cx43 remained predominantly at intercalated disks, there was an increase in Cx43 localization to the lateral plasma membrane (6). Characterization of Cx43 remodeling, in the epicardial border zone of healing canine infarcts 5 days after coronary occlusion, was characterized by a decrease in Cx43 protein, lateralization, and increased Cx43 phosphorylation at Ser-368 (57).

Increases in pSer-368 due to PKC phosphorylation is also found in scratch wounding. Using HeLa cells as a model system, quantification of Western blots showed increasing pSer368Cx43 in Cx43-containing HeLa cells following scratch wounding of cultured monolayers (9). As shown in Ref. 3 in human foreskin and using a pSer368 antibody, 24 h after wounding of primary human basal keratinocytes, pSer368Cx43 phosphorylated by PKC was increased in cells adjacent to the scratch and was not evident in suprabasal layers adjacent to the wound. Increases were not seen at 6 or 72 h. Cells at the edge of the scratch transferred dye much less efficiently at 24 h. However, keratinocyte migration, to fill the scratch, required early (within less than 6 h) gap junctional communication. Taken together, these results point out that pSer368Cx43 plays an important role in setting up communication compartments that spatially regulate wound repair.

Concluding Remarks—The model systems and new probes we have used in this study help us gain a more mechanistic determination of the role that PKC plays in Cx43 phosphorylation and how this phospho-site acts as regulator of movement and degradation. This, in turn, will lead to understanding its role in more complex systems such as tissues and organs, such that increases in pSer368Cx43 effected by PKC can be better modulated during disease processes.

Acknowledgments—We thank Amy Walker, Junru Hu, Raime Shah, and Mark Kirzon for assistance in this project.

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