Effect of PMA on Phosphorylation of Cx43: A Quantitative Evaluation Using Blotting With Multiplex Fluorescent Detection

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Introduction

Cardiac action potentials are normally transmitted through intercellular gap junctions, which consist primarily of the phosphoprotein connexin 43 (Cx43). Cx43 has a relatively short half-life of less than 3 hours, which facilitates rapid changes in cell-to-cell coupling in response to various stimuli (Beardslee et al., 1998). Downregulation of myocardial Cx43 is observed following ischemia, resulting in reduced dissemination of potentially harmful factors via gap junctions (Saffitz et al., 2007). Protein kinase C (PKC) is a well-documented stress sensor, and PKC-mediated phosphorylation of Cx43 reduces gap junction permeability and flags the Cx43 molecule for internalization and degradation following ischemia (Girao and Pereira 2003, Laird 2005, Lampe et al., 2000). Phorbol 12-myristate 13-acetate (PMA) is a potent activator of PKC and is utilized in this study to simulate a stress response and induce phosphorylation of Cx43 in the murine cardiomyocyte cell line HL-1 (Claycomb et al., 1998, Liu and Heckman 1998). The phosphorylation status of Cx43 at serine 368 (Ser^{368}) as a response to PMA treatment was evaluated.

In this study, changes in Cx43 levels and phosphorylation were quantitatively evaluated using western blotting methodology with fluorescent detection. Data demonstrate the ability to detect both protein standards and sample proteins on a blot in a single image capture session using fluorescent signals from multiple color channels. This fluorescent multicolor imaging approach provides a simplified and robust western blotting workflow that allows a shorter protein detection process and results in high-quality quantitative data, including molecular weight (MW) estimation of sample proteins directly from a blot.

Methods

HL-1 cells were maintained in Claycomb medium (Sigma-Aldrich Co.), supplemented with 10% fetal bovine serum (Invitrogen Corporation), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen), 0.1 mM norepinephrine (Sigma-Aldrich), and 2 mM L-glutamine (Invitrogen), and maintained at 37°C, 5% CO_2, 95% air. Cells were cultured in 100 mm cell culture dishes (Corning, Inc.), coated with gelatin and fibronectin (Sigma-Aldrich). Confluent monolayers of cells were treated with 1 µM PMA (Sigma-Aldrich) for 15, 30, 45, and 60 min. Control cells were treated with vehicle (DMSO, Fisher Scientific) for 60 min, and cells were sampled at the end of each treatment, starting from time 0. During sampling, cells were washed with 5 ml Dulbecco's phosphate buffered saline (PBS) (Invitrogen) on ice, lysed in 150 µl RIPA lysis buffer (Pierce), scraped, and transferred to Eppendorf tubes. Lysates were sonicated and centrifuged at 13,000 rpm at 4°C. Protein concentrations were determined using the *DC*™ protein assay (Bio-Rad Laboratories, Inc.).

Proteins were resolved at a concentration of 30 µg/well using SDS-PAGE and transferred to FluoroTrans PVDF low-fluorescence membranes (Pall Corporation). Membranes were rinsed in TNT buffer (0.1 M Tris-HCl, pH 7.6, 0.15 M NaCl, 0.05%, Tween 20) twice, blocked for 1 hr at room temperature (RT) in TNT buffer containing 5% nonfat dried milk, washed twice in TNT, and incubated overnight at 4°C with rabbit anti-phospho Cx43 Ser^{368} (Cell Signaling Technology, Inc.; 1:500 in TNT containing 5% BSA). After incubation, membranes were washed 3 x 5 min in TNT to remove unbound antibody and probed with mouse total anti-Cx43 (Sigma-Aldrich; 1:1,000) and rat anti-tubulin (Abcam Inc.; 1:1,000) for 2 hr at RT in TNT buffer containing 5% nonfat dried milk. Unbound antibody was removed by rinsing twice and washing 3 x 5 min in TNT. Membranes were incubated in the dark with secondary antibodies: goat anti-rabbit Alexa Fluor 488, goat anti-rat Alexa Fluor 555, and goat anti-mouse Alexa Fluor 633 (Invitrogen; 1:1,000 in TNT buffer containing 5% nonfat dried milk) for 1 hr at RT. Unbound secondary antibody was removed by washing 4 x 5 min in TNT. Membranes were soaked in 100% methanol for 2 min and allowed to air dry in the dark prior to detection using the Molecular Imager® VersaDoc™ MP 4000 imaging system (Bio-Rad). Quantitative analyses of blots were performed with Quantity One® 1-D analysis software (Bio-Rad).

A validation experiment was performed to ensure that data from multiplexed fluorescent western blotting can be quantitated. Two proteins, actin (a housekeeping control protein whose concentration was kept constant) and human transferrin (with varied concentrations), were used for validation. Samples were loaded on a Criterion™ 4–20% gradient Tris-HCl gel (Bio-Rad), with actin at a concentration of 150 ng/lane and transferrin at 25, 12.5, and 5 ng/lane (n = 3 for each concentration). To determine MW and to assess transfer efficiency, 5 µl of Precision Plus Protein™* WesternC™ standards (Bio-Rad) were run alongside the sample proteins on the gel. Proteins were transferred to FluoroTrans PVDF membrane and blocked with BSA-PBS buffer for 1 hr at RT. Membrane was then incubated with two primary antibodies: rabbit anti-human transferrin (Dako; 1:1,000) and mouse anti-actin (Sigma-Aldrich; 1:3,000) for 1 hr at RT and washed 3 x 10 min in TBS buffer. The blot was incubated at RT with secondary antibodies — goat anti-rabbit Alexa Fluor 647 and goat anti-mouse Alexa Fluor 568 (Invitrogen; 1:1,000 in blocking buffer) for 1 hr in the dark before being washed in TBS wash buffer 3 x 10 min. The membrane was equilibrated in methanol for 2 min and air dried. Imaging was achieved using a Molecular Imager® PharosFX™ system (Bio-Rad). Alexa Fluor 568 and standards with MWs of 75, 50, and 25 were detected with a 532 nm laser and a 605 nm bandpass filter. A 635 nm laser and a 695 nm bandpass filter were used to detect Alexa Fluor 647, and standards with MWs of 150, 100, and 37. Images were viewed and analyzed using Quantity One software.

Results

Validation of Quantitative Fluorescent Western Blotting

Precision Plus Protein WesternC standards can be used to estimate MW directly from blots by plotting the log MW of the standard bands against the relative migration distance (Rf) of the standards and sample protein bands (for more information, see bulletin 5576).

Band analysis of actin indicated an apparent MW of 41 and mean trace quantity (intensity x mm) of 2,279 with a standard deviation of 159, giving a percent coefficient of variation (CV) of 6.98% (Figure 1A, C). Transferrin was detected at an apparent MW of 76. The mean trace quantities of transferrin were 1,253, 570, and 238 for each concentration. The CVs were 3.8%, 4.3%, and 24.7%, respectively (Figure 1B, C). The relative quantities of the transferrin loads were 1, 0.5, and 0.2, and the relative calculated quantities after western blotting were 1, 0.45, and 0.19. Data for this analysis are shown in Table 1.

Table 1. Quantitative analysis of fluorescent blotting.

		Transferrin, ng/lane		
	Actin, 150 ng/lane	25	12.5	5
Mean trace quantity	2,279	1,253	570	238
Standard deviation	159.1	47.4	24.4	58.7
CV, %	7.0	3.8	4.3	24.7

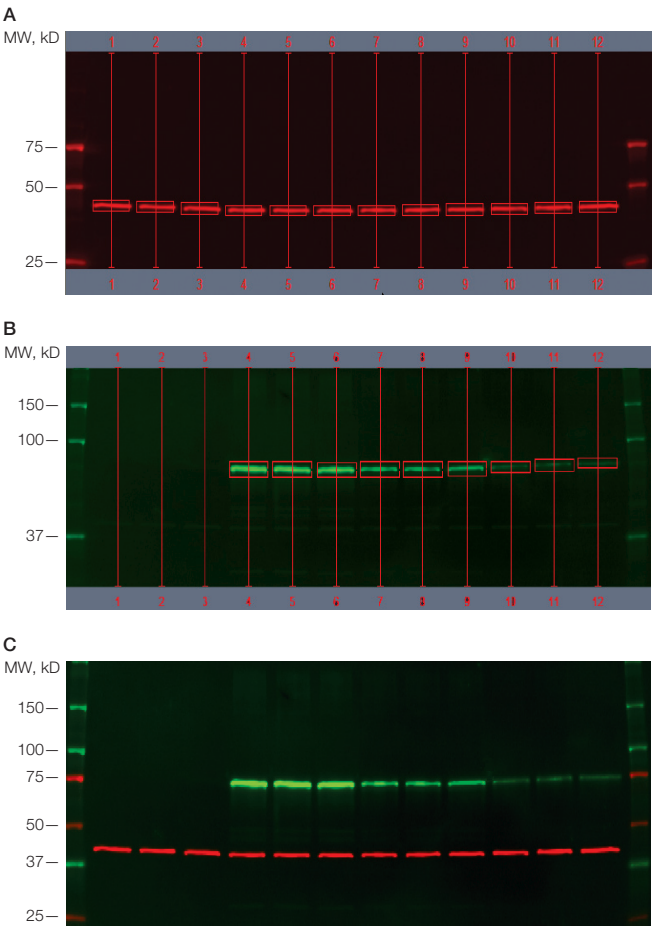

Fig. 1. Validation of quantitative fluorescent blotting. A, fluorescence image of blot probed with anti-actin; all lanes had equal protein loads (150 ng/lane); **B**, fluorescence image of blot probed with anti-human transferrin; amount of protein/lane varied (lanes 1–3, 0 ng; lanes 4–6, 25 ng; lanes 7–9, 12.5 ng; lanes 10–12, 5 ng); **C**, merged image of A and B.

Effect of PMA on Phosphorylation Status of Cx43

An increase in phospho Cx43 Ser^{368} (green) was detected at 15 min postincubation with 1 µM PMA (Figure 2A, D). This induction of Cx43 phosphorylation was followed by a reduction in total Cx43 levels (red) at 30 min (Figure 2B, D), consistent with the model of PKC regulation of Cx43 degradation through phosphorylation at Ser^{368}. Quantitative results were normalized to tubulin (purple), which served as an internal control (Figure 2C, D). Phosphorylation of Cx43 was sustained for the duration of the experiment, relative to the total levels of Cx43, which remained significantly reduced (Figure 2E).

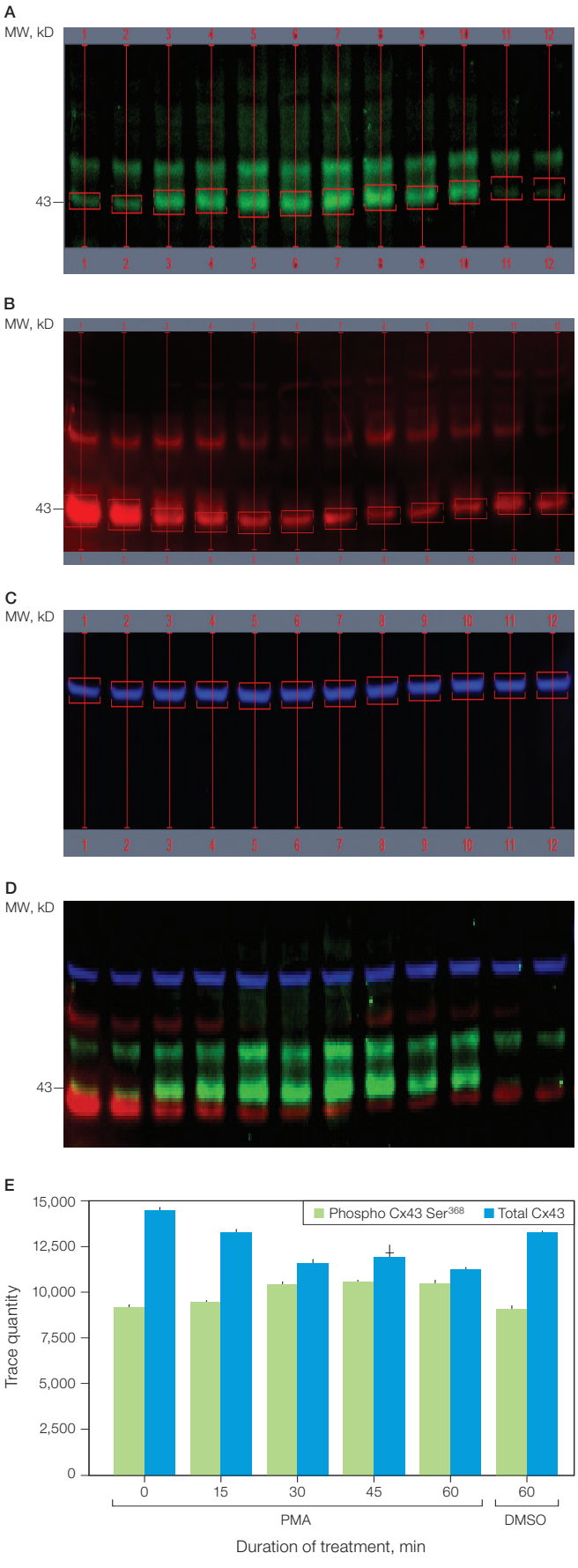

Table 2. Duration of drug treatment of HL-1 cells.

Lanes	Duration, min	Drug
1–2	0	PMA
3–4	15	PMA
5–6	30	PMA
7–8	45	PMA
9–10	60	PMA
11–12	60	DMSO

Conclusions

The loss of gap junctional intercellular communication as a result of altered expression/localization of Cx43 seriously impacts the function of the working myocardium in ischemic heart disease. Despite protective effects elicited by the body to contain the spread of potentially toxic factors, uncoupling of gap junctions prevents cardiomyocytes from contracting in a coordinated manner and can lead to pathologies, such as ventricular fibrillation. In this study, we illustrate that exposure of a cardiomyocyte cell line (HL-1) to PMA results in the rapid PKC-mediated phosphorylation of Cx43 at Ser^{368}. It is believed that phosphorylation of Cx43 not only reduces gap junction permeability, but also promotes internalization and degradation of the Cx43 protein. Consistent with this model, we observed a significant reduction in total Cx43 levels following induction of PKC-mediated phosphorylation at Ser^{368}, similar to that observed in ischemic heart disease. The function of cardiac PKC is being elucidated further and is emerging as an attractive candidate for therapeutic intervention in ischemic heart disease.

We also investigated the practicality of fluorescent western blotting for multiplex protein detection and demonstrated the method of quantitation using proteins of known concentrations. In addition, the use of high-quality MW standards such as Precision Plus Protein WesternC standards allows simultaneous estimation of sample protein MW directly from blots without additional steps. With multiplex blotting, a control “housekeeping” protein can be used as a loading reference and correction factor for more accurate quantitation of a second protein of interest, which may have varying levels of expression.

Fig. 2. Effect of 1 µM PMA on phosphorylation of Cx43 in the cardiomyocyte cell line HL-1. A, phosphorylated Cx43; **B**, total Cx43; **C**, tubulin; **D**, merged images of A, B, and C; and **E**, plot of trace quantity from blot probed for phospho Cx43 and total Cx43 against duration of drug treatment. Duration of treatment is described in Table 2.

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