Isoform-specific phosphorylation-dependent regulation of connexin hemichannels

Jette Skov Alstrøm, Daniel Bloch Hansen, Morten Schak Nielsen and Nanna MacAulay*

Department of Cellular and Molecular Medicine and Danish National Research Foundation Centre for Cardiac Arrhythmia and Department of Biomedicine, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.

*Correspondence to Nanna MacAulay, The Panum Institute, Blegdamsvej 3, bldg. 12.6, 2200 Copenhagen N, Denmark. Tel.: (+45)35327566; Email: macaulay@sund.ku.dk

Running head: Regulation of connexin hemichannels

Author contribution:
Conduction of experiments: JSA, DBH
Design of experiments: JSA, DBH, MSN, NM
Writing and critical reading of the manuscript: JSA, DBH, MSN, NM

Keywords: connexin, fluorescent dye, conductance, protein kinase C, hemichannel activity

ABSTRACT

Connexins (Cx) form gap junction channels made up of two connexons (hemichannels) from adjacent cells. Unopposed hemichannels may open towards the extracellular space upon stimulation by e.g. removal of divalent cations from the extracellular solution and allow isoform-specific transmembrane flux of fluorescent dyes and physiologically relevant molecules, such as ATP and ions. Cx43 and Cx30 are the major astrocytic connexins. Protein kinase C (PKC) regulates Cx43 in its cell-cell gap junction configuration and may also act to keep Cx43 hemichannels closed. In contrast, the regulation of Cx30 hemichannels by PKC is unexplored. To determine phosphorylation-dependent regulation of Cx30 and Cx43 hemichannels, these were heterologously expressed in Xenopus laevis oocytes and opened with divalent cation-free solution. Inhibition of PKC activity did not affect hemichannel opening of either connexin. PKC activation had no effect on Cx43-mediated hemichannel activity whereas both dye uptake and current through Cx30 hemichannels were reduced. We detected no PKC-induced connexin internalization from the plasma membrane indicating that PKC reduced Cx30 hemichannel activity by channel closure. In an attempt to resolve the PKC phosphorylation site(s) on Cx30, alanine mutations of putative cytoplasmic PKC consensus sites were created to prevent phosphorylation (T5A, T8A, T102A, T105A, T110A).
S222A, S225A, S239A, and S258A). These Cx30 mutants responded to PKC activation suggesting that Cx30 hemichannels are not regulated by phosphorylation of a single site. In conclusion, Cx30, but not Cx43, hemichannels close upon PKC activation, illustrating that connexin hemichannels display not only isoform-specific permeability profiles but also isoform-specific regulation by PKC.
INTRODUCTION

Gap junction channels are formed when two hemichannels (connexons) from neighboring cells dock in the extracellular space and create a connection allowing intercellular communication. Each hemichannel is a transmembrane channel composed of six connexins, of which 21 different human isoforms have been described and numbered according to their theoretical molecular mass in kDa (Nielsen et al., 2012). Gap junctions created from different connexin isoforms exhibit distinct selectivity profiles (Harris, 2007) and cell/tissue-specific and time-dependent isoform expression may thus determine the intercellular communication between specific cell types. In the case of astrocytes, intercellular communication is predominantly upheld by Cx30 and Cx43 (Wallraff et al., 2006; Gosejacob et al., 2011).

Gap junctions are regulated in part by phosphorylation, a post-translational modification most intensely studied for Cx43 gap junctional channels (Axelsen et al., 2013). Cx43 is highly phosphorylated in heart tissue under physiological conditions but becomes dephosphorylated under ischemia in the heart (Beardslee et al., 2000; Axelsen et al., 2006) as well as in astrocytes (Li et al., 1998). Phosphorylation of specific sites has been associated with direct regulation of Cx43 channel activity (e.g. Ser368 and Ser306 (Lampe et al., 2000; Procida et al., 2009)) as well as proper channel membrane localization (e.g. Ser325, 328 and 330 (Lampe et al., 2006; Remo et al., 2011)). In contrast, phosphorylation-dependent regulation of Cx30 gap junctional channels remains unexplored.

Connexins also act independently of their ability to directly couple neighboring cells: Unopposed connexin hemichannels may open to the extracellular space upon a variety of stimuli such as removal of divalent cations, metabolic inhibition, and inflammatory agents and thereby act as transmembrane transport routes (Contreras et al., 2002; Ye et al., 2003; Froger et al., 2010; Hansen et al., 2014a; for review, see Spray et al., 2006). We have recently demonstrated that open hemichannels, like cell-cell channels, display isoform-specific permeability profiles with Cx30 being permissive to a broad range of molecules and fluorescent dyes whereas Cx26, Cx36, and
Cx43, in their hemichannel configuration, are restricted to select permeants (Hansen et al., 2014a, 2014b).

Studies suggest that large transmembrane hemichannels composed of Cx30 or Cx43 are of (patho)physiological relevance (Anselmi et al., 2008; Wang et al., 2013; Chen et al., 2014) and since ischemia is associated with connexin dephosphorylation, it is of interest to evaluate the phosphorylation-dependent regulation of hemichannel activity. Currently, the phosphorylation-dependent regulation of Cx30 hemichannel activity remains completely unexplored and although reports suggest that protein kinase C (PKC) down regulates Cx43-hemichannels via Ser368 (Li et al., 1996; Bao et al., 2004b; Hawat and Baroudi, 2008), other researchers were unable to detect PKC-dependent phosphorylation of purified Cx43 hemichannels (Kim et al., 1999). We therefore set out to determine the PKC-dependent regulation of Cx30 and Cx43 in their hemichannel configuration.

MATERIALS AND METHODS

In vitro transcription

cDNA encoding mouse Cx30 (mCx30 obtained from Klaus Willecke, Bonn University) and rat Cx43 (rCx43 obtained from Zealand Pharma, Denmark) was subcloned into the expression vector pXOOM and their sequence verified. Mutations and C-terminal truncations were introduced with Quick Change site-directed mutagenesis kit (Stratagene, Santa Clara, CA) and verified with DNA sequencing. The cDNAs encoding WT and mutant versions of Cx30 and Cx43 were linearized downstream from the poly-A segment and transcribed with T7 mMessage Machine according to manufacturer’s instruction (Ambion, Austin, TX). The cRNA was extracted with MEGAclear (Ambion, Austin, TX) prior to microinjection into defolliculated Xenopus oocytes (10 ng RNA/oocyte). We have previously demonstrated that, in our hands, the endogenous Xenopus Cx38 does not significantly contribute to divalent cation free solution-induced dye uptake and membrane current (Hansen et al., 2014a). Cx38 antisense has therefore not been co-injected in the present study.

Oocyte preparation

Oocytes were surgically removed from Xenopus laevis frogs (Nasco, USA or National Center for Scientific Research, France) according to the European Community guidelines for the use of
experimental animals and performed under a license issued for the use of experimental animals by
the Danish Ministry of Justice (Dyreforsøgstilsynet). The oocytes were prepared as previously
described (Fenton et al., 2010) and subsequently kept in Kulori medium (in mM: 90 NaCl, 1 KCl, 1
MgCl₂, 1 CaCl₂, 5 HEPES, Tris ((HOCH₂)₃CNH₂) ≈ 2, pH 7.4) at 18°C for around 24 hours prior to
cRNA injection. The oocytes were kept at 18°C in Kulori medium for 3-5 days before the
experiments were performed.

Ethidium uptake
Five oocytes were washed in the respective test solution and subsequently placed in a 24-well cell
culture plate containing 500 µl test solution with 50 µM ethidium bromide. Two test solutions were
used: Control solution (in mM: NaCl 100, KCl 2, CaCl₂ 1, MgCl₂ 1, HEPES 10, Tris
((HOCH₂)₃CNH₂) ≈ 4, pH 7.4) and a solution in which divalent cations were substituted with
equiosmolar NaCl; DCFS (divalent cation free solution) (in mM: NaCl 103, KCl 2, HEPES 10, Tris
((HOCH₂)₃CNH₂) ≈ 4, pH 7.4). The uptake was performed under mild agitation for 1 hour at room
temperature after which the oocytes were washed twice in control solution. We have previously
demonstrated that the ethidium uptake was a linear function of time within this time frame (Hansen
et al., 2014a). Oocytes were placed individually in wells of a 96-well plate containing 50 µl water
(Mili-Q) and lysed by repeated pipetting. Emission of each well was determined with a Synergy HD
plate reader (Biotek, Vermont, USA) and Gen5 software (BioTek, Vermont, USA) with 340/11 and
590/35 nm filters for excitation and emission, respectively. The obtained fluorescence is indicated
as arbitrary units (a.u.).

Electrophysiology
Xenopus oocytes were placed in a circular recording chamber of around 3 mm diameter and 1 mm
depth (~7 µl) and continuously superfused (at a rate of 12 µl/s) (Zeuthen et al., 2006) with control
solution or DCFS at room temperature. Electrodes were pulled from borosilicate glass capillaries to
a resistance of 1-2 MΩ when filled with 1 M KCl. The recordings were performed with a Dagan
Clampator interfaced to a PC with a Digidata 1320 A/D converter and pClamp 9.2 (Both Axon
Instruments, CA, USA). Currents were low pass-filtered at 500 Hz and sampled at 2 kHz. I/V
curves were obtained from the steady-state current levels and recorded, before and after 60 sec
exposure to DCFS (with or without 200 nM PMA present 5 min prior to the exposure to DCFS),
from a holding potential of -50 mV by application of 100 ms voltage steps from -140 to +60 mV in
steps of 20 mV.
Membrane purification and Western Blot

Purified plasma membranes were obtained from 10 oocytes per condition and total membranes obtained from 3 oocytes per condition, as previously described in detail (Moeller et al., 2009). Briefly, to obtain the pure plasma membrane fraction, the oocyte vitelline envelope was partly enzymatically digested and polymerized to the plasma membrane with sequential incubation with subtilisin A, ludox and polyacrylic acid (all from Sigma-Aldrich). The oocytes were subsequently homogenized in homogenization buffer A (HbA) containing (in mM): 5 MgCl₂, 5 NaH₂PO₄, 1 EDTA, 80 sucrose, 20 Tris, pH 7.4 with protease inhibitors leupeptin (8 µM) and pefabloc (400 µM) and then exposed to a series of centrifugation steps (14 g for 30 s, 22 g for 30 s, 31 g for 30 s, and 42 g for 30 s). At each stage the pellet was kept, resuspended, and centrifuged at the higher speed. A final centrifugation at 17,000 g for 20 min was done to pellet the purified plasma membranes. The samples were analyzed by SDS-PAGE (12% precast gels, Bio-Rad) and Western blot using anti-Cx30, 1:125 (Invitrogen 71-2200) and anti-Cx43, 1:8000 (Sigma Aldrich C6219) followed by anti-rabbit HRP secondary antibody 1:3,000 (Bio-Rad 170-6515 or Dako P0448). Protein staining was visualized by chemiluminescence (SuperSignal West Pico, Thermo Scientific, BioSpectrumAC Imaging System (UVP)) and quantification in the linear range of exposure.

Chemicals

All activators and inhibitors were obtained from Sigma Aldrich, Denmark. Bisindolylmaleimide II (BIM) (5 µM final concentration, 1 mM stock in DMSO), chelerythrine (CHEL) (10 µM final concentration, 10 mM stock in DMSO), gadolinium (Gd³⁺) (50 µM final concentration, 100 mM stock in H₂O), phorbol 12-myristate 13-acetate (PMA) (200 nM final concentration, 200 µM stock in DMSO), and ethidium bromide (50 µM final concentration, 25 mM stock in H₂O). Where applicable, DMSO was included in control solutions to obtain identical vehicle concentrations.

Statistics

All experiments were performed with oocytes from at least three different animal donors. For uptake experiments, n refers to number of experiments (each carried out with 5 oocytes per experimental condition) whereas for electrophysiological experiments, n refers to number of oocytes obtained from at least three different animal donors. Statistical analysis was performed with GraphPad Prism 5 or SAS Enterprise Guide 6.1 for mixed model analysis of variance and the
statistical test as indicated in the figure legends. As not all data in the paired designs could be tested on each experimental day, the use of ANOVA was precluded for these data sets and mixed model analysis of variance employed instead. The significance of both main effects and interaction terms was tested and when interaction was not significant, data was pooled to reduce the model and increase statistical power. Data are expressed as average±SEM and P<0.05 is considered statistically significant.

RESULTS

Functional expression of Cx30 and Cx43 in *Xenopus laevis* oocytes

Functional expression of connexin hemichannels in *Xenopus* oocytes microinjected with cRNA encoding mouse Cx30 or rat Cx43 was determined by fluorescent dye uptake. These two connexins open in their hemichannel configuration upon removal of divalent cations from the extracellular solution (Valiunas and Weingart, 2000; Orellana et al., 2011; Hansen et al., 2014a). We, accordingly, exposed the oocytes to ethidium in the presence (control) or absence (DCFS) of divalent cations (Fig. 1A white bars). Removal of divalent cations significantly increased ethidium uptake in Cx30-expressing oocytes (~4.7 fold from 108±22 a.u. to 506±164 a.u, n=5, P<0.001) and Cx43-expressing oocytes (~3.3 fold from 114±32 a.u. to 378±113 a.u., n=5, P<0.05). In contrast, divalent cation removal had no effect in uninjected oocytes (from 68±13 a.u. to 62±17 a.u., n=6, P=1). Cx30 and Cx43 are thus functionally expressed in the *Xenopus* oocytes and the regulatory pattern of PKC inhibition and activation can be determined in this heterologous expression system.

Effect of PKC inhibition on hemichannel-mediated dye uptake

It has previously been reported that PKC inhibition induces hemichannel-mediated dye uptake in Cx43-expressing oocytes (Bao et al., 2004a). We therefore exposed Cx30- and Cx43-expressing oocytes to the membrane-permeable PKC inhibitors bisindolylmaleimide (BIM, 5 µM, grey bars) and chelerythrine (CHEL, 10 µM, black bars) during the ethidium uptake procedure. In the presence of divalent cations (control), uninjected as well as Cx30- and Cx43-expressing oocytes displayed slightly increased fluorescence upon exposure to PKC inhibitors. Mixed model analysis of variance (see Materials and Methods) demonstrated that there was no significant difference between the groups (P=0.53 for the interaction term between the inhibitors and the connexin expression, Fig. 1A), suggesting the PKC inhibitor lead to an increased fluorescence in all oocytes.
whether or not connexins were expressed in their membranes. Since the presence of connexins did
not affect the PKC inhibitor-induced fluorescence, the data for uninjected, Cx30- and Cx43-
expressing oocytes in control solution were pooled for further statistical analysis: On average,
fluorescence increased from 95±13 a.u. in vehicle solution to 134±17 a.u. in BIM containing
solution (P<0.001) and 130±19 a.u. in CHEL containing solution (P<0.01), n=16 for all groups. The
same statistical approach was employed for the dye uptake in DCFS where there were also no
significant difference between the groups (P=0.94 for the interaction term between the inhibitors
and the connexin expression, Fig. 1A). An increase in PKC inhibitor-mediated fluorescence was not
observed in DCFS (P=0.90 for BIM and P=0.86 for CHEL). However, any small increase in
fluorescence due to inhibitor addition, as observed in the control solution, could likely be masked
by the robust DCFS-induced uptake in the Cx-expressing oocytes. Testing the inhibitor effect in
uninjected oocytes in DCFS alone, did, however, show a significant increase (62±17 a.u. in vehicle
versus 92±19 a.u. in BIM, P<0.01 and 87±15 a.u. in CHEL, P<0.01, n=6 for all groups).

As both PKC inhibitors displayed autofluorescence in the ethidium emission spectrum (data
not shown), the small increase in fluorescence obtained in the presence of the PKC inhibitors could
be due to accumulation in and/or binding to the oocytes of these membrane-permeable inhibitors.
To determine a possible contribution of autofluorescence by BIM and CHEL to the total
fluorescence count, oocytes were exposed to the PKC inhibitors in control solution or DCFS in the
absence of ethidium (Fig. 1B). In the presence (control) and absence (DCFS) of divalent cations,
uninjected as well as Cx30- and Cx43-expressing oocytes displayed increased fluorescence upon
exposure to PKC inhibitors. Mixed model analysis of variance demonstrated that there was no
significant difference between the groups (P=0.38 for the interaction term between the inhibitors
and the connexin expression and P=0.23 for the interaction term between the inhibitors and the
solution, Fig. 1A). This lack of significance indicates that the fluorescence directly due to the
presence of the PKC inhibitors (no ethidium present) was similar for all oocytes, independently of
whether they expressed connexins or not. Since neither the presence of connexins nor the removal
of divalent cations affected the PKC inhibitor-induced fluorescence, the data for uninjected, Cx30-
and Cx43-expressing oocytes in both solutions were pooled for statistical analysis: BIM increased
the fluorescence to 20±2 a.u. (P<0.001) and CHEL to 17±2 a.u. (P<0.001), n=20 for each group.
The BIM/CHEL autofluorescence thus contributes to the BIM/CHEL-induced increase in total
fluorescence of oocytes observed in Fig.1A. Interestingly, neither the presence of Cx30 or Cx43,
nor the removal of divalent cations altered the responses to the PKC inhibitors (Fig. 1B), thus
precluding detectable hemichannel-mediated uptake of these inhibitors. Taken together, Cx30- and Cx43-mediated dye uptake was not affected by PKC-inhibition in the oocyte expression system.

**Effect of PKC activation on hemichannel-mediated dye uptake**

The lack of effect of PKC inhibitors on the hemichannel-mediated dye uptake might be due to low levels of basal PKC activity. Instead we measured DCFS-induced ethidium uptake in the presence of the membrane-permeable PKC activator phorbol 12-myristate 13-acetate (PMA, 200 nM). As PMA-induced PKC activation can be transient (Muthusamy et al., 2012), ethidium uptake was measured as a function of time and found linear during 60 minutes (Fig. 2). PKC-activation reduced the DCFS-induced Cx30-mediated dye uptake from 262±44 a.u. to 113±11 a.u. after 60 minutes PMA exposure (n=5, P<0.001), a level statistically indistinguishable from that obtained in the presence of the connexin hemichannel inhibitor gadolinium (Gd³⁺, 50 µM; 65±8 a.u., n=5, Fig. 2A, data summarized in panel C). Whereas Gd³⁺ significantly reduced the DCFS-induced ethidium uptake in Cx43-expressing oocytes (157±28 a.u. versus 36±2 a.u. with Gd³⁺, n=5, P<0.001, Fig. 2B, summarized in panel C), the uptake was unaffected by PMA (157±28 a.u. versus 178±26 a.u., n=5, Fig. 2B, summarized in panel C). The DCFS-induced dye uptake in uninjected oocytes was not affected by PMA or Gd³⁺ (n=6, Fig. 2B inset, summarized in panel C). The data show that DCFS-induced Cx30-mediated dye uptake, in contrast to that of Cx43, was down-regulated by PKC activation.

**Effect of PKC activation on Cx30 hemichannel-mediated membrane currents**

A previous study in cardiomyocytes demonstrated that gap junction permeability to dyes (larger molecules) and atomic ions (current) were differentially regulated by PKC (Kwak et al., 1995). We therefore measured DCFS-induced membrane currents in uninjected and Cx30-expressing oocytes by two-electrode voltage-clamp before and after 5 min PMA pre-treatment in control solution, Fig. 3. We detect no Cx43-mediated hemichannel currents (Hansen et al., 2014a, 2014b), even after prolonged removal of divalent cations (15 min, data not shown); and thus omitted Cx43 from this experimental series. PKC activation had no significant effect on the membrane currents of the uninjected oocytes (352±43 nA in DCFS vs. 232±44 nA in DCFS PMA at Vₘ= +60 mV, n=8), Fig. 3A, left panels and Fig. 3B, whereas the DCFS-induced membrane current of Cx30-expressing oocytes was significantly reduced by PKC activation (1943±240 nA in DCFS vs. 1232±181 nA in DCFS PMA at Vₘ= +60 mV, n=12, P<0.001), Fig. 3A, right panel and
Fig. 3C. The effect of PKC-activation was not increased by prolonged exposure to PMA (10 min, data not shown) and time control experiments illustrated that the DCFS-induced Cx30 current in the absence of PMA was stable over the experimental duration (Fig. 3C, insert). Cx30-mediated hemichannel activity, resolved with both dye uptake and membrane currents, was thus down-regulated by PKC activation.

**Mechanism of PKC-mediated down-regulation of Cx30 hemichannels**

PKC-mediated down-regulation of Cx30 hemichannel activity could occur either by internalization of Cx30 or by channel closure. Consequently, we measured connexin surface expression following PMA-induced activation of PKC. Purified plasma membranes from Cx30- and Cx43-expressing oocytes pretreated for 60 minutes in either DCFS or PMA-containing DCFS were immunoblotted with Cx30 or Cx43 antibodies and quantified by densitometry. Representative Western blots are depicted in Fig. 4A. The plasma membrane abundance of the two connexins is illustrated as the fraction of Cx30 and Cx43 present in the plasma membrane after exposure to PMA compared to that obtained in the absence of PMA. The membrane fraction of both Cx30 and Cx43 was unperturbed by PMA exposure (1.0±0.2 of vehicle for Cx30 and 0.9±0.1 of vehicle for Cx43, n=5, data summarized in Fig. 4A). PMA-induced mobility shifts were observed for Cx43 but not for Cx30. Purified plasma membranes prepared from uninjected oocytes displayed no Cx30 or Cx43 antibody immune-reactivity, Fig. 4A. To verify that the overall Cx30 expression was unperturbed by PMA treatment (i.e. that no subcellular fractions of Cx30 were altered), we quantified the Cx30 content in preparations containing all cellular membranes. We did not discover PMA-induced alterations in the ratio between plasma membrane and subcellular fractions (1.0 ± 0.1% of vehicle, n=5, Fig. 4B). The lack of PMA-induced reduction of surface expression of Cx30 or Cx43 indicates that PKC-activation promotes Cx30 channel closure rather than connexin internalization.

**Mutational analysis of Cx30 PKC phosphorylation sites**

We identified seven intracellular PKC consensus sequences by the Group-based Prediction System (GPS 3.0: see diagram of Cx30 in Fig. 5A). The serines and threonines within these sequences were independently mutated to alanines to mimic a constitutive dephosphorylation to elucidate if either of these consensus sequences acts as the site of PKC phosphorylation: Cx30-T5A, -T8A, -T102A, -S222A, -T225A, -S239A, and -S258A. All mutant constructs displayed DCFS-induced ethidium uptake when expressed in *Xenopus* oocytes, indicating functional expression at
the plasma membrane (Fig. 5B, n=8-17 experiments with each construct). However, PKC-activation (60 min exposure to PMA) significantly reduced the DCFS-induced dye uptake in all mutant constructs, indicating that none of these putative PKC phosphorylation sites are solely responsible for the observed PKC-dependent regulation. A small but significant (P<0.01) PMA-dependent increase in ethidium uptake was observed in the uninjected oocytes (Fig 5B) which, however, is not of a magnitude where it would interfere with the results obtained with the mutant constructs.

To remove several putative PKC phosphorylation sites in combination, we generated C-terminally truncated Cx30 constructs which terminated after amino acid 215 (Cx30.215stop), 221 (Cx30.221stop), and 250 (Cx30.250stop), Fig. 5A. Expression of these truncated Cx30 constructs in Xenopus oocytes, however, failed to provide DCFS-induced ethidium uptake (Fig. 5C) or membrane current (data not shown), indicating that they were either non-functional or not expressed at the oocyte plasma membrane. Thus, abolishing individual PKC phosphorylation sites in Cx30 did not prevent PKC-induced down-regulation of Cx30-mediated dye uptake.

**Mutational analysis of a proposed Cx43 PKC phosphorylation site**

Cx43 PKC phosphorylation at Ser368 was suggested to decrease Cx43 hemichannel activity (Bao et al., 2004a, 2004b). As we were unable to detect PKC-mediated regulation of Cx43 hemichannel activity using inhibitors and activators of PKC (Fig. 1,2), we further pursued the possibility of phosphorylation-dependent gating of Cx43 in its hemichannel configuration by a mutational approach. Ser368 was mutated to an alanine (Cx43-S368A) to mimic dephosphorylation or to an aspartate (Cx43-S368D) to mimic phosphorylation. Oocytes expressing Cx43-S368A or Cx43-S368D displayed ethidium uptake similar to that of the WT Cx43-expressing oocytes in control solution (227±38 a.u. for WT Cx43, 207±39 a.u. for Cx43-S368A and 241±29 a.u. for Cx43-S368D, n=5, Fig. 6), indicating that the phosphorylation state of Ser368 does not directly regulate Cx43 hemichannel activity at physiological Ca\(^{2+}\) concentrations. The DCFS-induced hemichannel activity of Cx43-S368D was comparable to that of the WT Cx43 (compare 537±99 a.u. for Cx43-S368D with 497±57 a.u. for WT Cx43, n=5, Fig. 6), indicating that DCFS-induced activity was not inhibited by mimicking a Ser368 phosphorylation. We did, however, observe that mimicking the dephosphorylated form of S368 (Cx43-S368A) reduced the DCFS-induced ethidium uptake (compare 357±36 a.u. for Cx43-S368A with 497±57 a.u. for WT Cx43, n=5, P<0.05, Fig. 6) which is in contrast to the suggested *increase* in channel opening upon dephosphorylation of Ser368. Phosphorylation of Ser368 in the C-terminus of Cx43 therefore does not appear to decrease
hemichannel activity nor does dephosphorylation of Ser368 appear to increase hemichannel activity under our experimental conditions.

**DISCUSSION**

The present study demonstrates isoform-specific regulation of Cx30 and Cx43 hemichannels, illustrated by regulation of Cx30 and not Cx43 hemichannels upon PKC activation with PMA. In order to obtain an experimental setting in which the connexin hemichannels could be investigated in isolation, the connexins were individually expressed in *Xenopus laevis* oocytes which have negligible intrinsic expression of other large-pore channels such as pannexins, purinergic P2X7 receptors, and volume-activated anion channels which display overlapping permeability profiles and inhibitor sensitivity with those of the connexin hemichannels (Spray et al., 2006; Hansen et al., 2014b). In addition, *Xenopus* oocytes are known to express the majority of known PKC isoforms (Rajagopal et al., 2008) although we cannot rule out some PKC isoform or activation pathway, specific to astrocytes, are absent in this heterologous expression system.

Oocytes expressing Cx30 or Cx43 displayed characteristic DCFS-induced Gd3+-sensitive fluorescent dye uptake in agreement with earlier reports (Stout et al., 2002; Bao et al., 2004a, 2004b; Orellana et al., 2011; Berger et al., 2014; Hansen et al., 2014a, 2014b). We recently demonstrated that Cx30 was permeable to a range of different fluorescent dyes (e.g. ethidium, Yo-Pro, and propidium iodide) whereas Cx43 displayed a preference towards ethidium (Hansen et al., 2014b). We therefore employed ethidium as our experimental read-out in the present series of experiments.

Inhibition of PKC with two different membrane-permeable PKC inhibitors at concentrations earlier used in oocytes (Bao et al., 2004a) did not augment the divalent cation free solution-induced ethidium uptake in Cx30- or Cx43-expressing oocytes, nor did they induce further hemichannel opening in divalent cation-containing solution. The latter results differ from a previous report by Bao and co-workers, who showed an increased uptake of 5(6)-carboxyfluorescein after PKC inhibition in Cx43-expressing oocytes at 16°C (Bao et al., 2004a). We did, however, observe a small increase in fluorescence in oocytes exposed to PKC inhibitors irrespective of whether they expressed connexins or not. The increase was reproduced in the absence of ethidium and thus represents autofluorescence of the employed PKC inhibitors in the emission spectrum of ethidium employed to determine hemichannel activity. This autofluorescence could potentially act as a
confounding factor in given experimental settings. The effect of PKC inhibitors relies on basal
intrinsic phosphorylation of Cx43 and Cx30 under control conditions, which may not be present in
our setting at optimal Xenopus oocyte experimental temperature (room temperature), and therefore
we also determined hemichannel activity after PMA-induced PKC activation. We observed a
significant reduction of the DCFS-induced Cx30-mediated hemichannel activity as measured by
ethidium uptake as well as membrane currents, indicative of phosphorylation-dependent
hemichannel regulation. The decreased activity was not due to changes in connexin surface
expression, as determined from western blotting of purified plasma membranes, indicating that
channel closure, rather than internalization, reduced the dye/ion permeability. SDS-PAGE mobility
shifts were observed for Cx43 but not for Cx30, as previously reported (Talhouk et al., 2005). This
observation aligns with some phospho-sites in Cx43 not being associated with mobility shifts, e.g.
S368) (Solan and Lampe, 2007) and therefore does not preclude PMA-dependent phosphorylation
of Cx30.

In an attempt to identify a putative PKC phosphorylation site in Cx30, we replaced the
serine/threonine of seven intracellular PKC consensus sequences to an alanine in order to mimic a
constitutive dephosphorylation. All mutant forms of Cx30 responded to PMA treatment, indicating
that none of the seven mutated sites alone are obligatory for the observed PMA-induced
hemichannel down regulation. It is possible that PMA may instead act through the ERK
(extracellular signal-regulated kinase) signaling pathway (Rivedal and Opsahl, 2001; Ruch et al.,
2001; Sirnes et al., 2009) or that PMA can work through a kinase-independent pathway (Shimada-
Shimizu et al., 2014). Alternatively, PKC-dependent regulation of Cx30 may rely on
phosphorylation at multiple sites. Along these lines, Yogo et al. (2002) showed that intercellular
coupling in Cx43-expressing HeLa cells was reduced by dephosphorylation of S365/368/369/373,
but only when all sites were affected. Such mechanisms may explain how PKC-dependent
regulation could persist despite mutation of single phosphorylation sites. To eliminate several
phosphorylation sites at once, we therefore created Cx30 constructs with truncations of the C-
terminus, in which the majority of the PKC consensus sites are located. Unfortunately, these
truncated Cx30 versions did not generate functional hemichannels in the plasma membrane, in
agreement with previous reports showing lack of functional plasma membrane expression of the C-
terminally truncated Cx30 (Ozawa et al., 2009; Pannasch et al., 2014), and the role of the C-
terminus PKC phosphorylation sites could thus not be evaluated by this experimental strategy.
Cx43-mediated ethidium uptake was, in our hands, unperturbed by PKC activation. These results contrast those of Bao and co-workers who previously observed that purified Cx43 reconstituted into liposomes could be phosphorylated by PKC on Ser368 (Bao et al., 2004b) and that all six Cx43 subunits had to be phosphorylated in order to limit hemichannel permeability towards larger molecules (Bao et al., 2007). Notably, a smaller permeant such as ethyleneglycol remained permeable even with fully phosphorylated Cx43 (Bao et al., 2007). A previous study did, however, not detect direct PKC-mediated phosphorylation of purified Cx43 (Kim et al., 1999). In the present study, mimic of a constitutive dephosphorylation of Cx43-Ser368 by mutation to an alanine did not increase hemichannel permeability to ethidium whether in the absence or presence of divalent cations. In contrast, a small decrease in DCFS-induced ethidium permeability was observed compared to Cx43 WT. Furthermore, mimic of a phosphorylation by mutating S368 to an aspartate did not affect the ethidium uptake in either control solution or DCFS. Together these results indicate that dephosphorylation of Ser368 alone is insufficient for increasing Cx43 hemichannel activity in a cell-system-based setting with physiologically occurring membrane potentials, experimental temperatures (for the *Xenopus* oocytes), extracellular solutions, and pH.

Cx43, in its gap junction configuration, is well known to conduct current between pairs of cells. This transjunctional current is intricately regulated by PKC in a manner including reduction of single-channel unitary conductance but increased electrical coupling of cardiomyocytes, which must arise from an increased open probability (Kwak et al., 1995, for review see Alstrom et al., 2015). Nevertheless, Cx43 hemichannel-mediated divalent cation-sensitive currents are generally not detectable at negative membrane potentials whether Cx43 is expressed in *Xenopus* oocytes, HeLa cells or cultured rat astrocytes (Contreras et al., 2003; Valiunas, 2013; Hansen et al., 2014a, 2014b), even when recorded during prolonged periods of exposure to divalent cations (present study). Cx43 hemichannel-mediated currents may, however, arise at excessively positive membrane potentials in the range +40 to +100 mV (Contreras et al., 2003; Wang et al., 2012). Despite this requirement for positive potentials, ATP, cAMP, and ethidium readily penetrates the pore at physiologically relevant negative membrane potentials (Kang et al., 2008; Valiunas, 2013; Hansen et al., 2014a, 2014b). The molecular basis for the ability of Cx43 to selectively gate its permeation pathway is presently unidentified.

Although we are unable to explain the discrepancy in PKC-dependent regulation of Cx43 hemichannels observed by different researchers, we suggest that the high variability in experimental approaches may generate part of the variance: 1) purified and reconstituted Cx43 versus
heterologous expression in mammalian cells and oocytes, 2) different hemichannel permeants (sucrose / Lucifer Yellow / carboxyfluorescein / ethidium / ethylene glycol / atomic ions), 3) experimental temperatures ranging from 4°C to 37°C, 4) pH from 5.6 to 8, 5) Cx43 versus Cx43-GFP, 6) quantification of number of cells permitting dye uptake versus altered degree of permeation in all cells, and 7) possible differences in the expression of PKC isoforms (Li et al., 1996; Kim et al., 1999; Bao et al., 2004a, 2004b, 2007; De Vuyst et al., 2007; Hawat and Baroudi, 2008; Brokamp et al., 2012).

In summary, DCFS-induced Cx30 hemichannel activity was down regulated via channel closure after PKC activation through exposure to the membrane-permeable PMA. In contrast, ethidium uptake via Cx43 hemichannels was unperturbed by PKC activation in our experimental cell system. We propose that hemichannels composed of different connexins display isoform-specific phosphorylation-dependent regulation which may be of importance for the unique physiological function of each connexin hemichannel.

ACKNOWLEDGEMENTS

The assistance of Charlotte Goos Iversen and Catia Correa Goncalves Andersen is gratefully acknowledged. We are grateful to Klaus Willeck e, Bonn University, Germany and Zealand Pharma, Denmark for providing the cDNA encoding mCx30 and rCx43. This study was supported by the Danish Medical Research Foundation, FSS (to NM) and the Danish National Research Foundation (to MSN).

REFERENCES


FIGURE LEGENDS

Fig 1. Effect of PKC inhibition on hemichannel permeability to ethidium. A: Ethidium uptake was determined in uninjected oocytes and oocytes expressing Cx30 or Cx43 exposed to control solution or DCFS for 60 min in the presence or absence of the PKC-inhibitors BIM (5 µM) and CHEL (10 µM) (n=5 for Cx43- and Cx30-expressing oocytes and n=6 for uninjected oocytes). B: As in A but without ethidium present (n=3 for Cx43- and Cx30-expressing oocytes and n=4 for uninjected oocytes). For comparison, the dashed line indicates the fluorescence count obtained in the presence of ethidium in uninjected oocytes in the control solution from A. Statistical significance was tested using mixed model analysis of variance, with Bonferroni (A) or Dunnett’s (B) post-hoc tests. * refers to statistical significance versus corresponding group in control solution and # refers to statistical significance versus vehicle treated oocytes. Note, the statistics were carried out on pooled data for control solutions in (A) or for all groups in (B), please see explanation in Result section. For clarity the symbols in (B) are shown adjacent to the legends in the figure. *, P<0.05, ##, P<0.01, ***/###, P<0.001, ns; not significant.

Fig 2. Regulation of Cx30 but not Cx43 hemichannels by PKC activation. Ethidium uptake was determined after exposing Cx30-expressing oocytes (A) Cx43-expressing oocytes (B), or uninjected oocytes (B, insert) to DCFS for 10, 20, 40 and 60 min with vehicle, PMA, or Gd³⁺ present in the solution, n=5. C: Summary of the ethidium uptake after 60 min. Statistical significance was tested using repeated measures Two-way Anova with Bonferroni post-hoc test. ***, P<0.001, ns; not significant.

Fig 3. Regulation of Cx30-mediated currents by PKC activation. A: Membrane currents were recorded by application of 100 ms voltage steps from -140 to +60 mV in steps of 20 mV from a holding potential of -50 mV in oocytes exposed to control solution (top panels) followed by DCFS (1 min, middle panels) and finally to DCFS+PMA (5 min pretreatment with PMA-containing control solution and 1 min exposure to DCFS+PMA, lower panels), vehicle included when required. Left side illustrates traces obtained from a representative uninjected oocyte whereas right side illustrates representative traces obtained from a Cx30-expressing oocyte. Summarized I/V relations are illustrated for uninjected oocytes (n=8, panel B) and Cx30-expressing oocytes (n=12,
panel C). Insert: Cx30 exposed to DCFS for 1 min before and after 5 min in control solution. When symbols indicating the different experimental conditions are not visible, it is due to overlay of the I/V relations under those conditions. Statistical significance (see text for numbers) was tested using repeated measures Two-way Anova with Bonferroni post-hoc test.

Fig 4. Membrane expression of Cx30 and Cx43 after PMA stimulation. A: Uninjected oocytes and oocytes expressing Cx30 or Cx43 were exposed to DCFS with PMA or vehicle for one hour prior to plasma membrane purification. The purified plasma membrane fraction was subjected to SDS gel electrophoresis and subsequent immunoblotting with anti-Cx30 or anti-Cx43 antibodies. Representative western blots are shown for Cx30 (left panel) and Cx43 (right panel). Summarized densitometry illustrates the fractional connexin surface expression after PMA treatment relative to that obtained in control solution, n=5. B: Cx30-expressing oocytes were treated as above prior to total membrane purification and western blotting (representative blot illustrated in top panel and below is shown summarized densitometry illustrating the Cx30 abundance after PMA treatment relative to that obtained in control solution, n=5. Statistical significance was tested using one sample t-test against the hypothetical value of 1. ns; not significant.

Fig 5. PMA-mediated regulation of Cx30 does not occur via a single phosphorylation site. A: Diagram of the seven putative PKC consensus sites in Cx30 which were mutated to alanine (Cx30-T5A, -T8A, -T102A, -S222A, -T225A, -S239A, and -S258A) and the three truncated Cx30 versions (Cx30.L215stop, Cx30.R221stop and Cx30.D250stop). B: Ethidium uptake determined after one hour in control solution, DCFS, or DCFS with PMA for uninjected oocytes, wt Cx30, and the mutated Cx30 constructs, n=8-17. Statistical significance was tested using mixed model analysis of variance and Dunnett's post-hoc test with Bonferroni correction for nine comparisons. Levels of significance are given versus DCFS within each group. C: Ethidium uptake after one hour in control solution or DCFS for uninjected oocytes or oocytes expressing either wt Cx30 or one of the three truncated Cx30 constructs. Statistical significance was tested using repeated measures Two-way Anova and Bonferroni post-hoc test. *; P<0.05, **; P<0.01, ***; P<0.001, ns; not significant.

Fig 6. Mimic of (de)phosphorylation of S368 in the Cx43 C-terminus does not affect hemichannel activity. The Cx43-S368 residue was mutated to alanine (Cx43-S368A) or aspartate (Cx43-S368D). Ethidium uptake was quantified after one hour in control solution or DCFS. Statistical significance
was tested using Two-way Anova, repeated measurements with Bonferroni *post-hoc* test. *; P<0.05, ns; not significant.
Fig 1

A

Eth uptake a.u.

Control

DCFS

Vehicle

BIM

CHEL

Uninj  Cx30  Cx43

Uninj  Cx30  Cx43

B

Fluorescence a.u.

Control

DCFS

Vehicle

BIM

CHEL

Uninj  Cx30  Cx43

Uninj  Cx30  Cx43

***

*
Fig 2
Fig 3

A

Uninj

Control

DCFS

DCFS PMA

B

Vm (mV)

I (nA)

Uninj

Control

DCFS

DCFS PMA

C

Cx30

Vm (mV)

I (nA)
Figure 4
Fig 5

A

N-terminal
T5 T8
T102

L215
S222
S225

R221

Intracellular

D250
S239
S258

C-terminal

Extracellular

B

Eth uptake a.u.

Control
DCFS
DCFS + PMA

C

Eth uptake a.u.

Control
DCFS
Fig 6