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Diacylglycerol levels modulate the cellular distribution of the nicotinic acetylcholine receptor

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ABSTRACT

Diacylglycerol (DAG), a second messenger involved in different cell signaling cascades, activates protein kinase C (PKC) and D (PKD), among other kinases. The present work analyzes the effects resulting from the alteration of DAG levels on neuronal and muscle nicotinic acetylcholine receptor (AChR) distribution. We employ CHO-K1/A5 cells, expressing adult muscle-type AChR in a stable manner, and hippocampal neurons, which endogenously express various subtypes of neuronal AChR. CHO-K1/A5 cells treated with dioctanoylglycerol (DOG) for different periods showed augmented AChR cell surface levels at short incubation times (30 min–4 h) whereas at longer times (18 h) the AChR was shifted to intracellular compartments. Similarly, in cultured hippocampal neurons surface AChR levels increased as a result of DOG incubation for 4 h. Inhibition of endogenous DAG catabolism produced changes in AChR distribution similar to those induced by DOG treatment. Specific enzyme inhibitors and Western blot assays revealed that DAGs exert their effect on AChR distribution through the modulation of the activity of classical PKC (cPKC), novel PKC (nPKC) and PKD activity.

1. Introduction

Diacylglycerol (DAG) is involved as a second messenger in different cell signaling cascades and cellular events (Carrasco and Merida, 2007; Merida et al., 2010). It has also been demonstrated that proteins responding to DAG, such as PKCs, PKDs and other kinases, have at least one copy of the conserved domain 1 (C1) in their amino acid sequence. In addition, DAGs' conical shape and small polar head group make them especially apt to move in slow flip-flop oscillations in the membrane, favoring vesicle budding, fusion and fission (Huttner and Zimmerberg, 2001; Lev, 2006). DAG therefore has a dual role in mammalian cells as both a modulator of membrane dynamics and a second messenger, tightly regulating DAG levels. The interrelationship between DAG metabolism and signaling functions is crucial to ensure correct maintenance of homeostasis during cell growth and development (Joshi et al., 2013; Liu et al., 2014; Meguro et al., 2006). Modification of membrane DAG levels and other non-membrane DAG pools, in combination with certain intracellular responses, is crucial for specifying the nature, intensity and duration of a given signal in the modulation of cellular processes.

The Golgi complex is the only organelle with relatively stable DAG levels in the absence of any stimuli. In contrast, DAGs located in the plasma membrane are generated and quickly degraded in response to extracellular signals through e.g. PKC/phospholipase C (PLC) and sphingomyelin synthase 2 (SMS2) pathways (Gallegos et al., 2006; Huitema et al., 2004; Lev, 2006).

Intracellular DAG accumulation in muscle triggers the activation of novel PKCs with subsequent impairment of insulin signaling (Schmitz-Peiffer, 2000, 2013). Although lipid accumulation has been thoroughly studied in relation to the insulin pathway, little is known about the consequences of this condition on the muscle-type nicotinic acetylcholine receptor (AChR), a key molecule in neuromuscular transmission.

The AChR is an integral membrane protein that belongs to the ligand-gated ion channel superfamily. It is composed of five homologous subunits organized pseudo-symmetrically around a central

Abbreviations: 4α-PMA, 4α-phorbol 12-myristate 13-acetate; AChR, muscle-type nicotinic acetylcholine receptor; aPKC, atypical protein kinase C; BTX, α-bungarotoxin; cPKC, classical protein kinase C; CPT, choline phosphotransferase; DAG, diacylglycerol; DAGK, diacylglycerol kinase; DOG, dioctanoylglycerol; GGOH, geranylgeraniol; nPKC, novel protein kinase C; PC, phosphatidylcholine; PDBu, phorbol 12-13-di butyrate; PKD, protein kinase D; PVDF, polyvinylidene fluoride; ROT, rottlerin; TTBS, Tween–Tris buffer solution.

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pore (Karlin, 2002). The binding of the natural neurotransmitter acetylcholine (ACh) to the AChR produces a conformational change, which opens the channel, thus allowing positively charged ions to enter the cell (Karlin, 2002). Previous research from our laboratory has shown that AChR is extremely sensitive to its lipid environment (reviewed in Barrantes (2004, 2010)). Furthermore, we have demonstrated that cholesterol and sphingomyelin are particularly important for correct AChR distribution and trafficking (Baier and Barrantes, 2007; Borroni et al., 2007; Borroni and Barrantes, 2011; Gallegos et al., 2008; Pediconi et al., 2004; Roccamo et al., 1999). Additional work from our laboratory also demonstrated that ceramides differentially modulate AChR trafficking to the plasma membrane in a concentration-dependent manner (Gallegos et al., 2008). A precise lipid balance is therefore required for AChR to reach the plasma membrane in a functional way (Baier and Barrantes, 2007; Gallegos et al., 2008; Roccamo et al., 1999).

The purpose of the present work was to study the modulation of muscle-type AChR distribution and function by DAGs in CHO-K1/A5 cells, a cell line model system that heterologously expresses adult-type muscle AChR (Roccamo et al., 1999). To this end, different experimental strategies were implemented to set cellular DAG levels. We demonstrate that AChR distribution in the cell line expressing adult muscle-type receptor is modified in a time-dependent manner by DAG oversupply acting through conventional and novel PKC (cPKC and nPKC) and PKD. These results are further confirmed using hippocampal neurons in primary culture.

2. Materials and methods

2.1. Materials

All chemicals were from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise specified. [^{125}I]iodine was from Perkin Elmer (Wellesley, MA, USA). 1-Palmitoyl-2-oleoyl-sn-glycerol (POG) was from Avanti Polar Lipids (Alabaster, AL, USA). Nile red and Alexa Fluor^{488}-conjugated BTX (Alexa Fluor^{488}-BTX) were from Molecular Probes (Eugene, OR, USA).

Rabbit polyclonal antibodies anti-PKD, anti-pPKD (Ser^{916}), anti-pPKCα/βII (Thr$^{638/641}$) and anti-pPKCδ/θ (Ser$^{643/676}$) were from Cell Signaling (Beverly, MA, USA). Mouse monoclonal antibodies anti-PKCβ, anti-PKCη and anti-PKCδ were from BD Biosciences (San Jose, CA, USA). Rabbit polyclonal antibody anti-pPKCη (Thr^{655}) was from Life Technologies (Grand Island, NY, USA). HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.2. Cell culture and protein content

CHO-K1/A5 cells were grown in Ham's F12 medium supplemented with 12% fetal bovine serum as previously described (Roccamo et al., 1999). Dissociated neuronal cultures were prepared from hippocampi of embryonic day 19 rats, as previously described (Brocco et al., 2003). When required, lipids and enzyme

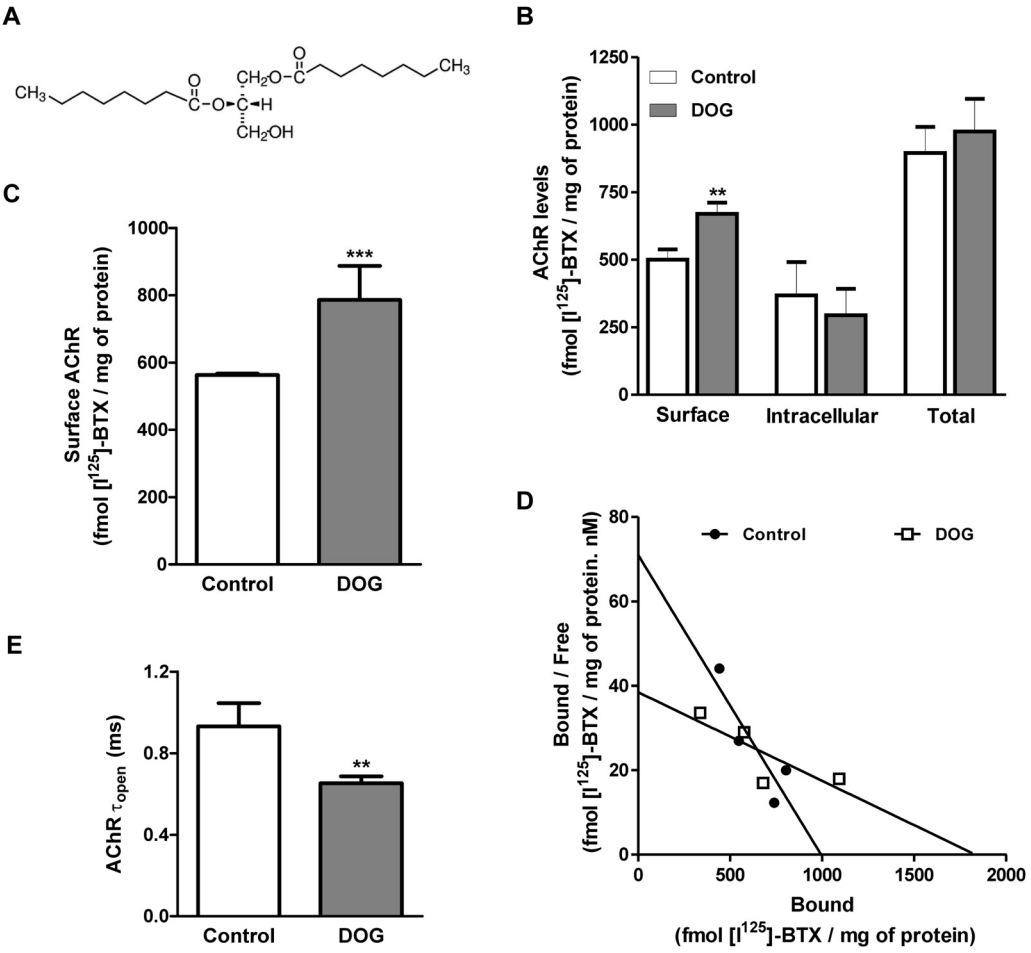

Fig. 1. Exposure to DOG from 30 min to 4 h increases surface AChR levels and alters ligand binding affinity and channel properties. (A) Schematic representation of DOG chemical structure. (B) CHO-K1/A5 cells were exposed to 300 μM DOG for 4 h at 37 °C and at the end of the incubation period cells were probed for surface, intracellular and total [^{125}I]-BTX binding. (C) CHO-K1/A5 cells were incubated with 300 μM DOG for 30 min at 37 °C and at the end of the incubation period surface [^{125}I]-BTX binding was quantified. (D) Scatchart-plots obtained from control and treated cells exposed to DOG for 30 min. (E) Histogram showing the mean open time of AChR activated with ACh after incubation of CHO-K1/A5 cells for 30 min at 37 °C with 300 μM DOG. Data represent average ± SD from $n = 4$. $^{**}p < 0.01$, $^{***}p < 0.001$.

inhibitors were added to the culture medium dissolved in dimethyl sulfoxide (DMSO). DMSO concentration was kept below 0.5%. Protein content was determined by Lowry method (1951).

2.3. BTX radioiodination and equilibrium studies of [^{125}I]-BTX binding

[^{125}I] α-bungarotoxin ([^{125}I]-BTX) was obtained in our laboratory following the protocol of Pediconi et al. (2004). Surface, intracellular and total AChR levels were determined by [^{125}I]-BTX binding as in Pediconi et al. (2004).

2.4. Lipid droplet labeling

After incubation with DOG, CHO-K1/A5 cells were fixed with 2% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 during 5 min at room temperature. Samples were subsequently stained with 1.5 μg/ml of Nile red solution for 10 min, washed thrice with medium 1 (M1: 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM KCl in 20 mM HEPES buffer, pH 7.4) and mounted for microscope examination as in Baier and Barrantes (2007).

2.5. Western blot

After each treatment, CHO-K1/A5 cells were washed with M1 and lysed at 4 °C during 1 h with cell lysis buffer (20 mM HEPES; 10 mM EGTA; 5 mM β-glycerol phosphate, 1% Nonidet P-40; 2.5 mM $MgCl_2$) containing protease inhibitors (2 μg/ml leupeptin; 1 μg/ml aprotinin; 1 μg/ml pepstatin; 0.1 mM PMSF). Proteins (40 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% polyacrylamide gels and subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were blocked and probed with primary antibodies overnight at 4 °C followed by incubation with the corresponding secondary antibodies at room temperature for 2 h. Immunoreactive bands were detected by enhanced chemiluminescence reagent (ECL, Amersham Biosciences) using standard X-ray film (Kodak X-Omat AR).

2.6. Patch clamp recording

Single-channel currents were recorded at a membrane potential of −70 mV and 20 °C using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Inc., CA) as in Vallés et al. (2007).

2.7. Data analysis

Quantification analyses of the immunoreactive bands were carried out using ImageJ 1.38 software. Data were analyzed using GraphPad Prism program from GraphPad Software Inc., CA, USA. Statistically significant differences were determined by Student's *t*-test (two-tailed) or one way ANOVA followed by Tukey post-test, when appropriate.

3. Results

3.1. Incubation of CHO-K1/A5 cells with exogenously added DAG analogs changes AChR distribution

In order to determine the effects of exogenous DAG on AChR levels, cells were incubated with a short-chain DAG, sn-1,2-dioctanoylglycerol (DOG, Fig. 1A). DOG is a synthetic lipid which mimics the action of endogenous DAGs and is widely used in lipid research mainly because of its good solubility in aqueous media (Davis et al., 1985; Kearns et al., 1997). DOG is not metabolized

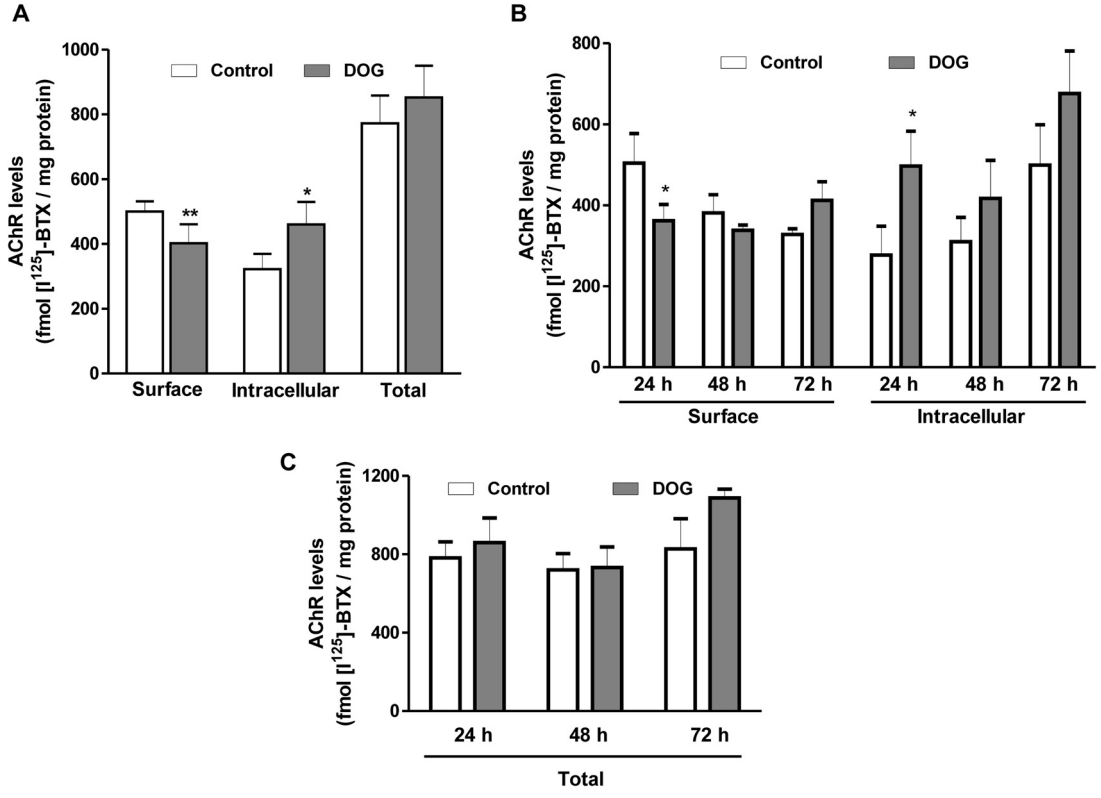

Fig. 2. Exposure to DOG for 18 h changes AChR cellular distribution. (A) CHO-K1/A5 cells were treated with 300 μM DOG for 18 h at 37 °C and at the end of the incubation period cells were probed for surface, intracellular and total [^{125}I]-BTX binding. (B, C) CHO-K1/A5 cells were treated with 300 μM DOG for 24, 48 and 72 h at 37 °C and at the end of the incubation period cells were probed for surface, intracellular (B) and total (C) [^{125}I]-BTX binding. Data represent average ± SD from $n = 4$. $^{*}p < 0.05$, $^{**}p < 0.01$.

to phosphatidic acid (PA) and does not enter into phospholipid biosynthetic pathways (Henneberry et al., 2001). Since it was planned to carry out the experiments at a high concentration (300 μM) of DOG, the possibility that this treatment may induce cell damage was first explored. Incubation of CHO-K1/A5 cells with DOG modified neither cell morphology nor protein levels, even at the longest incubation time (72 h), thus indicating that cell viability was not compromised (Supplementary Fig. S1).

In mammals, lipid excess is stored in lipid droplets (Guo et al., 2009; Thiele and Spandl, 2008). In order to investigate whether DOG or its metabolites were directed to such lipid droplets, cells were stained with Nile-red and the amount of lipid droplets per cell was measured after incubation with DOG for 4 h and 18 h. No changes in the number of lipid droplets per cell after a 4 h treatment with DOG were observed. However, after 18 h of DOG treatment, the number of cells with 2 or more lipid droplets was significantly higher (Supplementary Fig. S2).

Changes in AChR levels and distribution induced by DOG treatment for different incubation times were analyzed next. For this purpose [^{125}I]-BTX binding assays were performed. An increase in AChR surface levels was measured after a 4 h treatment with 300 μM DOG (Fig. 1B). These changes were observed even after incubation times as short as 30 min (Fig. 1C). Scatchard plots obtained from control and treated cells showed that 30 min incubation with DOG not only increased surface binding sites but also reduced AChR affinity for the antagonist BTX (Fig. 1D). Interestingly, electrophysiological recordings showed that AChR mean open time was shortened by 30% after incubation with DOG, demonstrating that AChR ion channel function was also affected (Fig. 1E).

We continued studying the effects of longer incubations with 300 μM DOG. To this end, CHO-K1/A5 cells were incubated at 37 °C for 18, 24, 48 and 72 h. After 18 h incubation with DOG, surface AChR levels decreased with respect to the control condition with a concomitant increase in the amount of intracellular receptors, although total AChR levels remained unaffected (Fig. 2A). The same changes in AChR distribution were observed after 24 h incubation (Fig. 2B). However, after longer incubation times surface and intracellular AChR levels returned to control values (Fig. 2B). None of the experimental conditions studied altered AChR total levels (Fig. 2A and C).

We further tested the effects of treating CHO-K1/A5 cells with a physiological DAG, palmitoyl-2-oleoyl-sn-glycerol (POG). For this purpose we incubated CHO-K1/A5 cells for 4 h at 37 °C with 300 μM POG and analyzed changes in surface-, intracellular- and total AChR levels by [^{125}I]-BTX binding assay. Fig. 3A shows that the incubation with POG increased AChR surface expression. However, this was accompanied by an increase in total AChR levels without affecting intracellular AChR levels. These results indicate that DOG and POG exert similar effects on AChR surface levels.

3.2. Endogenous modification of DAG levels affects AChR distribution

3.2.1. Inhibition of diacylglycerol kinase (DAGK)

One of the main routes for DAG metabolization is its transformation into PA through the enzyme diacylglycerol kinase (DAGK) (Shulga et al., 2011). A strong inhibitor of DAGKs, R59949 (Chianale et al., 2007; Jiang et al., 2000; Sato et al., 2013), was thus used in order to induce an endogenous increase in DAG levels.

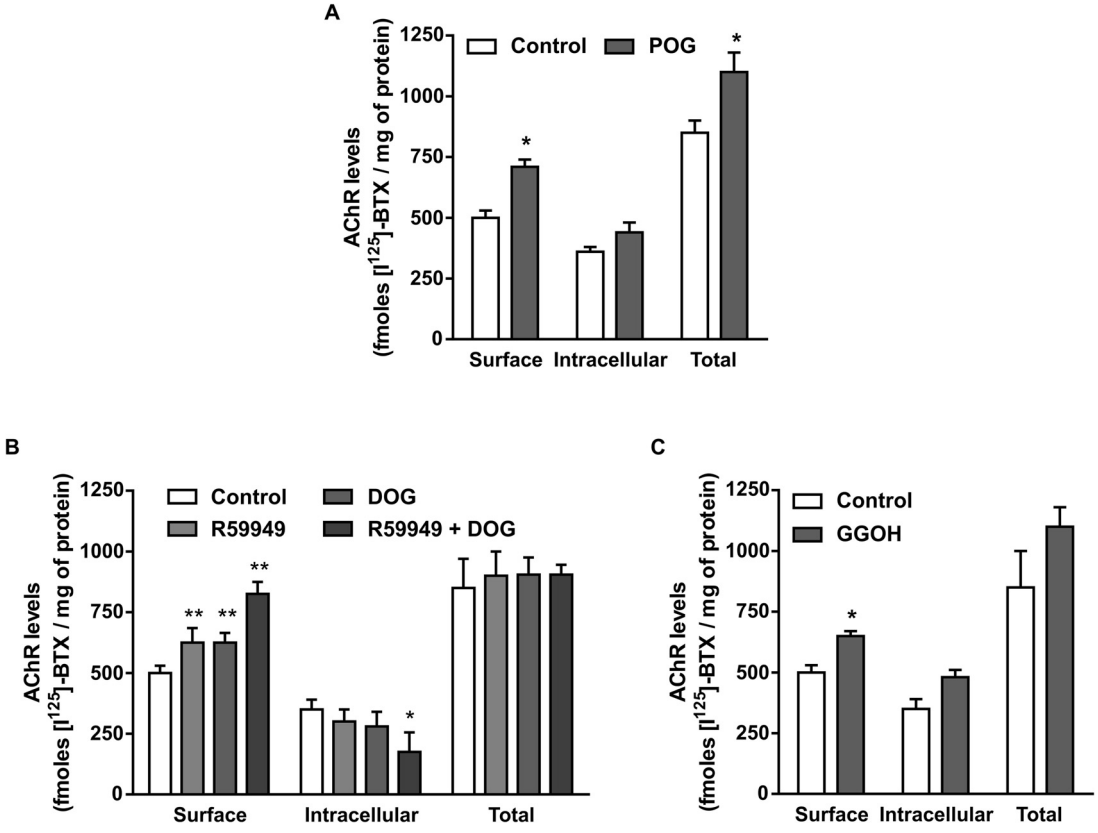

Fig. 3. Exposure to POG and endogenous modification of DAG levels affects AChR cellular distribution. (A) CHO-K1/A5 cells were treated with 300 μM POG for 4 h at 37 °C and at the end of the incubation period cells were probed for surface, intracellular and total [^{125}I]-BTX binding. (B) Surface, intracellular and total AChR levels in CHO-K1/A5 cells, were determined by [^{125}I]-BTX binding assay, after 4 h at 37 °C treatment with vehicle (control), 300 nM R59949, 300 μM DOG or DOG 300 μM plus 300 nM R59949. (C) CHO-K1/A5 cells were treated with 80 μM GGOH for 4 h at 37 °C, and at the end of the incubation period cells were probed for surface, intracellular and total [^{125}I]-BTX binding. Data represent average ± SD from $n = 4$. $^{*}p < 0.05$.

CHO-K1/A5 cells were incubated for 4 h at 37 °C with 300 nM R59949 and surface, intracellular and total AChR levels were analyzed by [^{125}I]-BTX binding assay. As shown in Fig. 3B, the endogenous increase in DAG induced by the DAGK inhibitor augmented surface AChR levels without significantly affecting intracellular and total AChR amounts, thus mimicking the effect previously seen with DOG. Furthermore, co-incubation of the cells with DOG and R59949 produced a greater increase in surface AChR than that obtained with either of the drugs alone. Interestingly, a concomitant decrease in intracellular AChR levels was also observed (Fig. 3B).

3.2.2. Inhibition of cholinephosphotransferase (CPT)

In the last step of the Kennedy pathway of phosphatidylcholine (PC) synthesis (Kennedy and Weiss, 1956), PC is generated by the enzyme CPT which transfers a molecule of choline from CDP-choline to a molecule of DAG (Weiss et al., 1958). CPT can be inhibited by isoprenoids (Gibellini and Smith, 2010; Weiss et al., 1958), such as geranylgeraniol (GGOH) (Miquel et al., 1998). Thus, we inhibited this route, with the aim of increasing cellular DAG levels. CHO-K1/A5 cells were incubated with 80 μM GGOH for 4 h and AChR levels were then quantified as described above (Fig. 3C). GGOH treatment increased surface AChR levels whereas intracellular and total AChR levels were not significantly affected (Fig. 3C). In addition, no alterations in AChR amounts or distribution were observed after 18 h treatment (Supplementary Fig. S3).

3.3. DAG effects on AChR cellular distribution are mediated by PKCs

3.3.1. Modulation of PKC activity

We next sought to identify the precise DAG-modulated molecular mechanisms that affect AChR cellular distribution. Given that most of DAG actions are PKC-mediated, we turned our attention to this kinase family (Antal and Newton, 2013; Newton, 2010; Wu-Zhang and Newton, 2013), which includes conventional (cPKC), novel (nPKC) and atypical (aPKC) PKCs, all of which are classified according to the second messengers required for the activation process. cPKC and nPKC require DAG to be activated. In addition, cPKC also requires Ca^{2+} (Zeng et al., 2012).

In order to determine whether cPKC and nPKC are involved in DAG effects exerted on AChR localization, cells were treated with phorbol 12-13-di butirate (PDBu), which is known to activate cPKC and nPKC (Carrasco and Merida, 2007; Dougherty et al., 2014; Wang et al., 2012; Xiao et al., 2013). An inactive phorbol, 4α-phorbol 12-myristate 13-acetate (4α-PMA), was used as a control treatment (Rahm et al., 2012). A 4 h treatment with 20 μM PDBu increased surface AChR levels but affected neither intracellular nor total AChR levels (Fig. 4A). The affinity of the AChR for [^{125}I]-BTX was also unaffected (Fig. 4B). After 18 h incubation with PDBu, surface AChR levels decreased with a concomitant increase in the amount of intracellular receptors. Total AChR levels were not altered either (Fig. 4C).

Changes in AChR localization induced by PDBu were qualitatively and quantitatively similar to those produced by DOG (Figs. 1 and 2), thus suggesting that cPKC and/or nPKC are involved in the redistribution of AChR observed after DOG treatment. In addition, the inactive 4α-PMA did not modify AChR localization confirming the specificity of this treatment (Fig. 4A).

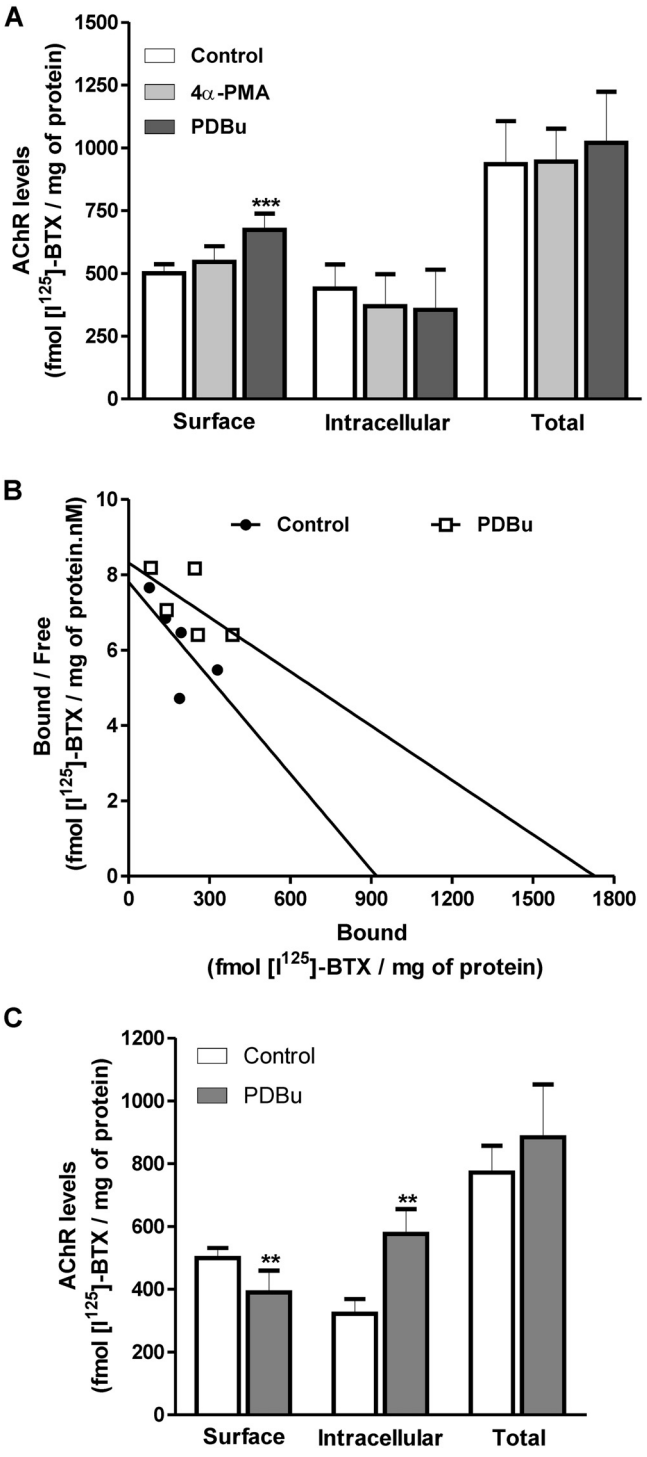

Fig. 4. DAG effects on AChR distribution are mimicked by PDBu treatment. (A) Surface, intracellular and total AChR levels in CHO-K1/A5 cells, were determined by [^{125}I]-BTX binding assay, after 4 h at 37 °C with the vehicle, 20 μM 4α-PMA or PDBu. (B) Scatchart-plots obtained from control and PDBu treated cells. (C) Surface, intracellular and total AChR levels in CHO-K1/A5 cells as determined by [^{125}I]-BTX binding assay after treatment with 20 μM PDBu for 18 h at 37 °C. Data represent average ± SD from $n = 4$. $^{**}p < 0.01$, $^{***}p < 0.001$.

3.3.2. Inhibition of cPKC

To determine whether cPKCs are modulated by DOG treatment, the selective cPKC inhibitor Gö 6976 was used (Martiny-Baron et al., 1993). The inhibition of cPKC activity with 1 μM Gö 6976 applied for 4 h induced similar changes in AChR distribution (i.e. increase in surface AChR levels and no modification of intracellular and total AChR levels) to those induced by DOG (Fig. 5A), suggesting that inhibition of cPKC mediates DOG actions. The co-incubation of CHO-K1/A5 cells with both drugs yielded no additional effect on AChR distribution (Fig. 5A). In contrast, 18 h treatment with either Gö 6976 or DOG alone had different effects. While DOG decreased surface AChR levels and increased intracellular AChR levels, Gö

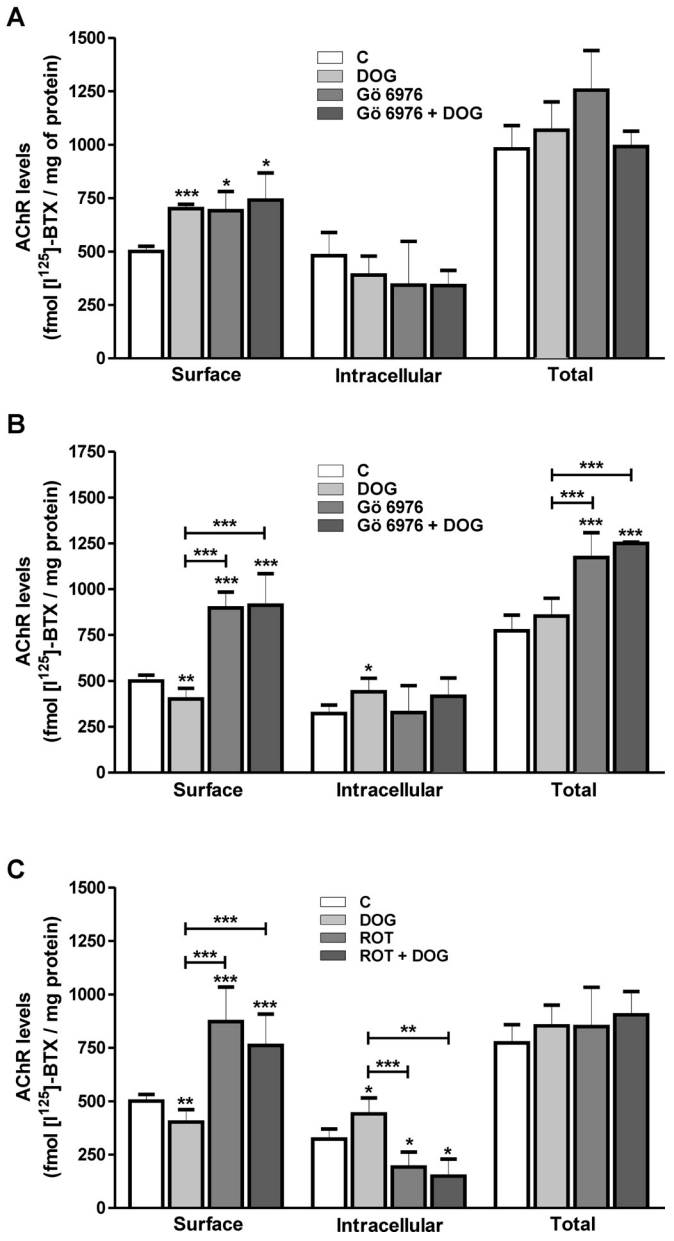

Fig. 5. DAG effects on AChR distribution are mediated by inhibition and activation of cPKC and nPKC. (A, B) Surface, intracellular and total AChR levels in CHO-K1/A5 cells were determined by [^{125}I]-BTX binding assay after 4 h (A) and 18 h (B) treatment at 37 °C with the vehicle, 300 μM DOG, 1 μM Gö 6976 or 300 μM DOG + 1 μM Gö 6976. (C) Surface, intracellular and total AChR levels in CHO-K1/A5 cells were determined by [^{125}I]-BTX binding assay after 4 h at 37 °C with the vehicle, 300 μM DOG, 3 μM ROT, or 300 μM DOG + 3 μM ROT. Data represent average ± SD from $n = 4$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

6976 increased surface and total AChR levels, without affecting intracellular AChR levels (Fig. 5B). Interestingly, 18 h co-incubation precluded DOG actions, suggesting that cPKC activation is involved in DOG effects on AChR distribution after 18 h treatment (Fig. 5B).

3.3.3. Inhibition of nPKC

We next studied the effect of PKCδ (a member of nPKC family) inhibition, which requires DAG as activator. Rottlerin (ROT), a PKCδ inhibitor, was used at 3 μM, which is the lowest concentration at which this inhibitor is selective (Gschwendt et al., 1994; Soltoff, 2007). Furthermore, since nPKC effects are seen after incubation times longer than those required for cPKC inhibition, the effect of ROT on DAG actions was analyzed after 18 h treatment (Antal and Newton, 2013; Gallegos and Newton, 2008). Incubation of CHO-K1/A5 cells with 3 μM ROT for 18 h increased surface AChR levels and decreased the intracellular receptor pool (Fig. 5C), in marked contrast to those obtained with DOG treatment for the same period (Fig. 5C). In addition, ROT prevented DOG modulation of AChR distribution (Fig. 5C), demonstrating that PKCδ activation also mediates DOG actions after 18 h. In contrast, ROT alone did not modify AChR distribution in CHO-K1/A5 cells after 4 h (Supplementary Fig. S4).

3.3.4. DOG treatment mediates PKCs phosphorylation

PKCs are constitutively modified by phosphorylation to form a competent mature enzyme (Antal and Newton, 2013; Griner and Kazanietz, 2007; Lipp and Reither, 2011). Once in this mature conformation, they are activated by interaction with their effectors (Antal and Newton, 2013; Freeley et al., 2011). Thus, the amount of phosphorylated PKC is an indicator of the relative amount of competent and ready-to-be-activated PKCs available in the cell. The state of phosphorylation of cPKC (PKCα and PKCβ) and nPKC (PKCη and PKCδ) in CHO-K1/A5 cells after the above-mentioned treatments was further analyzed by WB. Incubation of CHO-K1/A5 cells for 4 h with DOG or Gö 6976 did not change the cPKC phosphorylation ratio (phosphorylated PKC (p-cPKC)/total cPKC) although total cPKCβ levels were found to increase (Fig. 6A). However, the co-incubation of cells with both drugs dramatically reduced the amount of p-cPKC without changes in enzyme expression (Fig. 6A). In addition, DOG either added alone or with ROT for 4 h significantly reduced PKCδ/θ phosphorylation (Fig. 6B), thus suggesting an inhibition of these enzymes. ROT alone did not change p-PKCδ/θ levels (Fig. 6B). In contrast, treatment with DOG for 18 h increased p-cPKC levels while treatment with Gö 6976 for the same period was found to decrease them (Fig. 6C). Furthermore, co-incubation with both drugs precluded DOG action on cPKC phosphorylation and total enzyme levels (Fig. 6C). p-PKC$_{\delta/\theta}$/PKC$_{\delta}$ ratio was not altered by DOG treatment whereas ROT, either alone or with DOG, diminished it (Fig. 6D). The relative amount of p-PKCη underwent no changes after 4 h or 18 h treatment with DOG (Fig. 6E).

Our results, together with the fact that PKC must be phosphorylated in order to be activated suggest that (i) 4 h-DOG treatment produces inhibition of cPKC and nPKCδ and activation of nPKCη, and (ii) after 18 h treatment with DOG, the activation of cPKC mediates DOG effects on AChR distribution.

3.4. PKD is activated by DOG treatment

Another downstream effector of DAG signaling is the enzyme PKD, whose activation requires DAG and nPKC-mediated phosphorylation (Rozengurt, 2010; Sinnett-Smith et al., 2009; Young et al., 2012). We therefore analyzed PKD phosphorylation status by WB. PKD was, in fact, poorly activated in resting CHO-K1/A5 cells (Fig. 7). However, incubation of CHO-K1/A5 cells with DOG for 4 h or 18 h significantly increased pPKD levels without affecting PKD expression (Fig. 7). This indicates that incubation with DOG induces PKD activation, and this activation is higher after 4 h than after 18 h incubation.

3.5. Neuronal AChR expressed in hippocampal neurons in primary cultures is modulated by DOG treatment

We next studied the effect of DOG treatment on neuronal AChR endogenously expressed in hippocampal neurons. As shown in Fig. 8, DOG applied for 4 h increased surface AChR levels. Moreover, cPKC inhibition with Gö 6976 and PKCδ inhibition with ROT both increased surface AChR levels, thus suggesting the involvement of these two kinds of protein kinases in this phenomenon. Taken

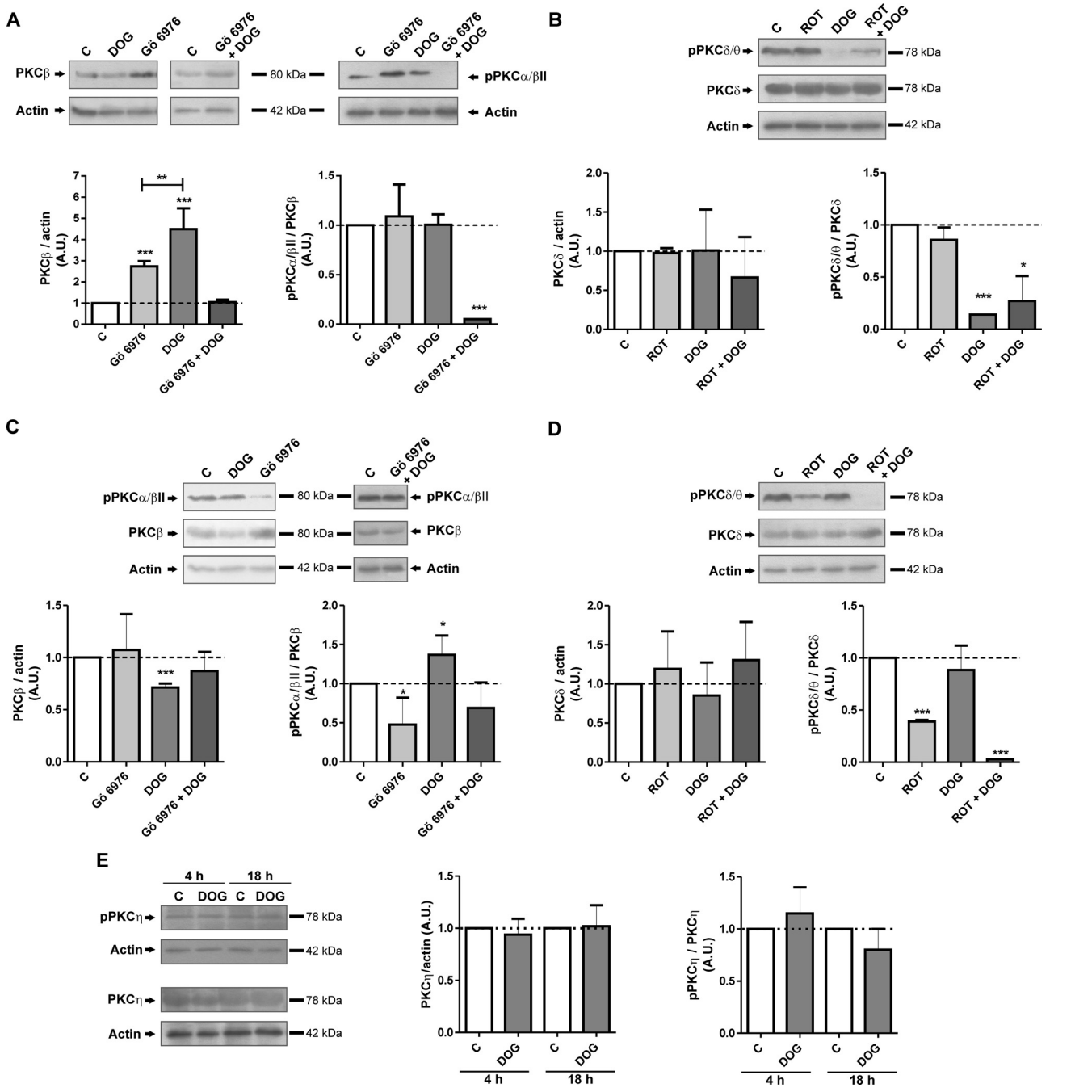

Fig. 6. DOG treatment changes phosphorylation status of cPKC and nPKC. (A) Representative Western Blot showing $pPKC_{\alpha\beta II}$ (right panel) and PKC_{β} (left panel) in CHO-K1/A5 cells treated with 1 μM Gö 6976, 300 μM DOG or 300 μM DOG + 1 μM Gö 6976 for 4 h at 37 °C. Bar graphs show the densitometry values of PKC_{β}/actin and $pPKC_{\alpha\beta II}/PKC_{\beta}$ expressed as arbitrary units (A.U.). (B) Representative Western Blot showing $pPKC_{\delta/\theta}$ (upper panel) and PKC_{δ} (middle panel) in CHO-K1/A5 cells treated with 3 μM ROT, 300 μM DOG or 300 μM DOG + 3 μM ROT for 4 h at 37 °C. Bar graphs show the densitometry values of PKC_{δ}/actin and $pPKC_{\delta/\theta}/PKC_{\delta}$ expressed as arbitrary units (A.U.). (C) Representative Western Blot showing $pPKC_{\alpha\beta II}$ (upper panel) and PKC_{β} (middle panel) in CHO-K1/A5 cells treated with 1 μM Gö 6976, 300 μM DOG or 300 μM DOG + 1 μM Gö 6976 for 18 h at 37 °C. Bar graphs show the densitometry values of PKC_{β}/actin and $pPKC_{\alpha\beta II}/PKC_{\beta}$ expressed as arbitrary units (A.U.). (D) Representative Western Blot showing $pPKC_{\delta/\theta}$ (upper panel) and PKC_{δ} (middle panel) in CHO-K1/A5 cells treated with 3 μM ROT, 300 μM DOG or 300 μM DOG + 3 μM ROT for 18 h at 37 °C. Bar graphs show the densitometry values of PKC_{δ}/actin and $pPKC_{\delta/\theta}/PKC_{\delta}$ expressed as arbitrary units (A.U.). (E) Representative Western Blot showing $pPKC_{\eta}$ (upper panel) and PKC_{η} (lower panel) in CHO-K1/A5 cells treated with 300 μM DOG for 4 h or 18 h at 37 °C. Bar graphs show the densitometry values of PKC_{η}/actin and $pPKC_{\eta}/PKC_{\eta}$ expressed as arbitrary units (A.U.). Actin was used as loading control in all experiments, numbers indicate molecular weights. Data represent average ± SD from at least $n = 4$. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

together, these experiments demonstrate that DOG and PKCs also modulate endogenous AChR distribution in hippocampal neurons.

4. Discussion

Previous research has demonstrated that DAG has a dual role as a modulator of membrane dynamics and as a second messenger in mammalian cells (Carrasco and Merida, 2007; Huttner and Zimmerberg, 2001; Lev, 2006; Merida et al., 2010). In this work we demonstrate that DAG oversupply affected AChR cellular distribution in CHO-K1/A5 cells and in hippocampal neurons. Incubation of CHO-K1/A5 cells with POG increased surface and total AChR levels (Fig. 3A). Similarly, 30 min incubation with DOG also increased surface AChR levels; these receptors

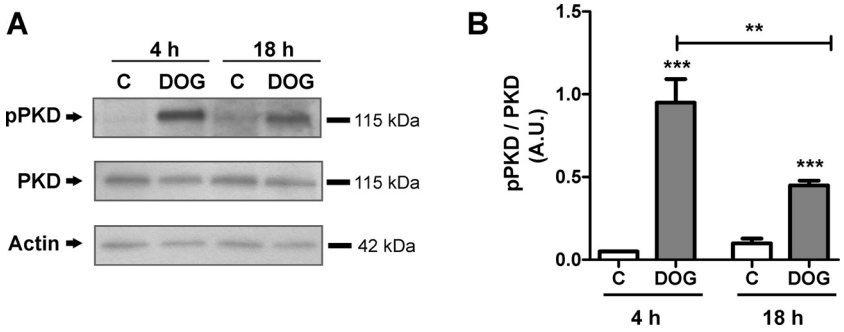

Fig. 7. Exposure to DOG activates PKD. (A) Representative Western Blot showing pPKD (upper panel) and PKD (lower panel) in CHO-K1/A5 cells treated with 300 μM DOG for 4 h or 18 h at 37 °C. (B) Bar graph shows the densitometry values of pPKD/PKD expressed as arbitrary units (A.U.). Actin was used as loading control and numbers indicate molecular weights. Data represent average ± SD from $n = 3$. $^{***}p < 0.001$.

exhibited a lower affinity for the antagonist BTX and a shorter channel mean open-time (Fig. 1). The fact that the lower affinity of AChR for BTX was not observed after incubation with PDBu indicates that this effect is not a consequence of PKC action (Fig. 4).

These findings can be explained by the fact that the DOG increase in CHO-K1/A5 cells after 30 min incubation occurs mainly in the plasma membrane, which is supported by several studies showing that DAG alters the physical state of membranes (Coorssen and Rand, 1990; Das and Rand, 1986; Heimburg et al., 1992; Jiménez-Monreal et al., 1998) and the fact that AChR is very sensitive to the composition and state of its lipid environment (Barrantes, 2010; Borroni et al., 2007; Borroni and Barrantes, 2011). Therefore, membrane physical alterations induced by DOG may reduce channel ability for ligand recognition and ion translocation. Similar findings were observed in neuronal AChRs expressed in PC12 cells, in which treatment with different DAGs, among them DOG, inhibited nicotinic-induced currents independently of PKC activity (Andoh et al., 2001, 2004).

After 4 h incubation with DOG, the increase in surface AChR levels was found to persist without changes in intracellular and total receptor levels (Fig. 1). However, POG treatment increased surface and total AChR (Fig. 3A). This means that the rise of total AChR levels after incubation with POG could be attributed to a POG metabolite that is not produced when cells are incubated with DOG. Furthermore, after 18 h exposure to DOG, both a decrease in surface AChR levels and an increase in intracellular AChR levels were observed (Fig. 2). These concomitant changes were found to persist for up to 24 h incubation, although at longer exposure times the same effect was no longer observed, probably as a result of DOG metabolization or its storage in lipid droplets (Supplementary Fig. S2).

The increase in endogenous DAG levels also produced changes in AChR distribution similar to those resulting from exogenously added DOG. DAGK inhibition with R59949 and CPT inhibition with GGOH were found to increase surface AChR levels after 4 h treatment (Fig. 3B, C). These treatments have been reported to generate DAG in different subcellular compartments, depending on the localization of the enzymes involved. CPT is located at the Golgi

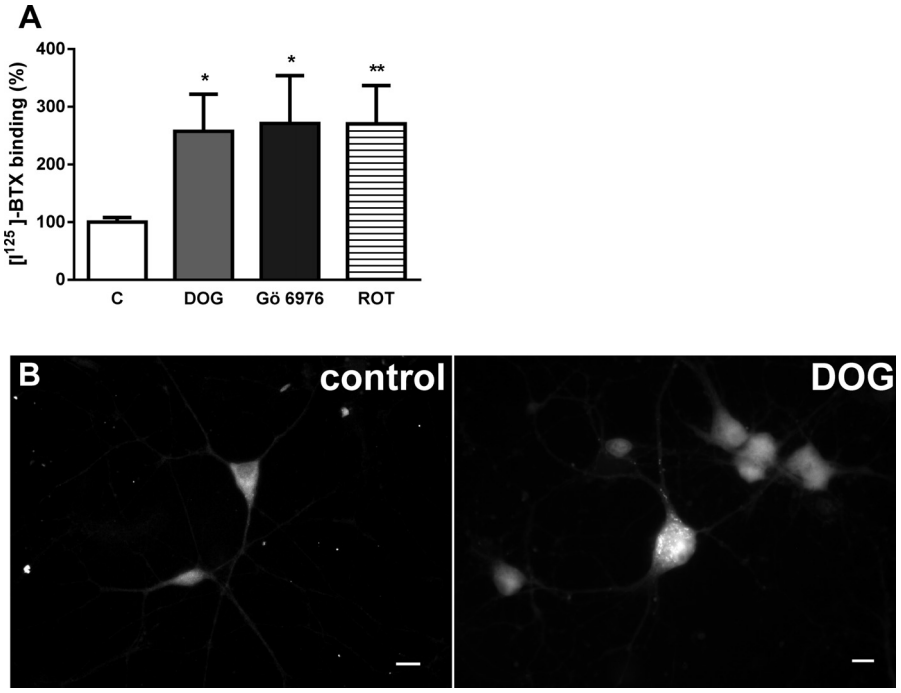

Fig. 8. DOG treatment increases neuronal AChR surface levels in culture hippocampal neurons. (A) Surface [^{125}I]-BTX binding in 14 days culture hippocampal neurons incubated with 300 μM DOG, 1 μM Gö 6976 or 3 μM ROT for 4 h at 37 °C. Bars represent average ± SD from $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$. (B) 14 days culture hippocampal neurons were incubated with 300 μM DOG for 4 h. At the end of the incubation period, neurons were fixed with paraformaldehyde, stained with Alexa^{488}-BTX and imaged. Scale bar 10 μm.

apparatus and its inhibition has been reported to increase DAG Golgi levels (Lagace and Ridgway, 2013; Miquel et al., 1998). Furthermore, among the DAGKs inhibited by R59949 treatment are DAGKκ and DAGKα, both tightly associated to the plasma membrane (Shulga et al., 2011). Thus, DAGs generated either at the plasma membrane or at the Golgi apparatus produce essentially the same effects on AChR distribution, although different mechanisms are probably involved.

The fact that DOG effects on AChR were reproduced by PDBu after 4 h and 18 h treatment, respectively, indicates that proteins containing C1 domains are involved in these effects (Fig. 4). After 4 h incubation, DOG-induced effects on AChR distribution in CHO-K1/A5 cells were mimicked by the cPKC inhibitor Gö 6976, thus suggesting that DOG may exert its effects through inhibition of this family of PKCs (Fig. 5A). These results are consistent with previous work showing that sustained incubations with PDBu induce PKC down regulation and inhibition (Carrasco and Merida, 2007; Dougherty et al., 2014; Wang et al., 2012; Xiao et al., 2013). Interestingly, 4 h treatment with DOG was found to significantly decrease p-PKCδ levels (Fig. 6B) and still the pharmacological inhibition of the enzyme with ROT was observed to produce no changes in AChR distribution (Supplementary Fig. S4), indicating that this nPKC activity is not related to DOG action on AChR – at least for the time window explored.

Incubation of CHO-K1/A5 cells with DOG for 18 h decreased surface AChR levels and increased intracellular receptor levels, both effects being abrogated by Gö 6976 treatment (Fig. 5B). Thus, cPKC activation appears to mediate AChR redistribution induced by 18 h incubation with DOG. Moreover, cPKC phosphorylation did, in fact, increase, although total PKCβ levels were observed to decrease (Fig. 6A). DAG analogs were found to promote not only PKC dephosphorylation but also a decrease in total enzyme levels, thus favoring the degradation process (Gould and Newton, 2008; Junoy et al., 2002; Leontieva and Black, 2004; Liu and Heckman, 1998; Newton, 2009; Parker et al., 1995). However, after prolonged exposure, phosphorylation was found to recover, probably by the action of chaperones that can rescue PKCs from the degradation process by promoting their rephosphorylation (Gao and Newton, 2006; Newton, 2010). It can therefore be concluded that the increase in cPKC phosphorylation levels augments the possibilities of cPKC-DOG interaction, and their subsequent activation.

DOG effects on AChR after 18 h were also blocked by ROT, a finding which was coincident with the decrease in pPKCδ levels (Figs. 5C and 6D). Furthermore, ROT and DOG produced opposite effects on AChR distribution after 18 h incubation. Since total and phosphorylated PKCδ levels did not change after 18 h treatment with DOG it can be concluded that DOG-mediated PKCδ inhibition observed after 4 h exposure is lost. Therefore, PKCδ activation could also be involved in DOG-induced changes in AChR levels after 18 h.

Previous research on AChR located at the neuromuscular junction (NMJ) demonstrated that cPKC inhibition with calphostin C increases surface AChR levels because AChR internalization decreases and recycling of previously internalized AChRs increases (Martinez et al., 2013). However, recycling does not significantly contribute to the maintenance of AChR surface levels in denervated muscle or in CHO-K1/A5 cells (Bruneau and Akaaboune, 2006; Kumari et al., 2008; Martinez et al., 2010). On the contrary, PKC activation by phorbol esters favors the decrease in AChR surface levels at the NMJ (Martinez et al., 2010) and in myotubes in culture (Lanuza et al., 2000; Nelson et al., 2003). Therefore, the increase in AChR surface levels observed in the present study after 4 h treatment could be partly explained by a decrease in AChR internalization rate resulting from the inhibition of cPKC by DOG oversupply. Activation of cPKC may, in turn, be responsible for the decrease in surface AChR after 18 h treatment with DOG. Interestingly, obesity and diabetes type II, two diseases in which accumulation of DAG in muscle has been reported, are associated with impairments in skeletal muscle function (Blaak, 2004; Lee et al., 2015; Nomura et al., 2007; Park et al., 2006).

Our results further show that PKD was not activated in resting CHO-K1/A5 cells, indicating that under basal conditions this kinase is not specifically involved in AChR export to the plasma membrane. However after treatment with DOG an increase in pPKD was observed, demonstrating the activation of the enzyme (Fig. 7). Recruiting cytosolic proteins is the limiting process in TGN vesicle biogenesis, a process which is also modulated by DAG. This neutral lipid is essential for PKD anchoring to TGN membranes where the enzyme regulates vesicle fission and protein export to the cell surface (Baron and Malhotra, 2002; Malhotra and Campelo, 2011). Thus, PKD activation after 4 h treatment with DOG may contribute to the observed increase in surface AChR levels at this incubation time. In contrast, after 18 h incubation PKD was still activated by DOG although the net effect of DOG treatment consisted in an increase in intracellular AChR levels (Fig. 2). This leads us to conclude that PKD activation does not significantly contribute to the redistribution of AChR after 18 h treatment with DOG.

Taking together the findings from the present study we can conclude that muscle AChR is a sensitive target of DAG misbalance. After short incubation times (4 h), AChR surface levels increase as a result of cPKC inhibition by DOG whereas at longer incubation times (18 h), DOG activates both cPKC and nPKC and the AChR distribution shifts, with accumulation of the protein in intracellular compartments. The results obtained with hippocampal neurons indicate that the phenomenon is also observed with neuronal AChRs; it remains to be established which subtype of receptor is involved.

5. Conclusion

AChR cellular distribution is modulated by DAG levels through modulation of cPKC and nPKC, and PKD activity. These events may have implications in muscle physiology and pathophysiology under conditions in which DAG muscle levels are altered, such as in obesity or diabetes type II.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2016.02.010.

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