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MOL #46250

Regulation of Neutral Sphingomyelinase-2 (nSMase2) by Tumor Necrosis Factor-α involves Protein Kinase C-δ in Lung Epithelial Cells

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ABBREVIATIONS

N-SMase	Neutral sphingomyelinase
PKC	Protein kinase C
PM	Plasma membrane
PMA	Phorbol 12,13-myristoylacetate
SMase	Sphingomyelinase
TNF	Tumor necrosis factor-α

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ABSTRACT

Neutral sphingomyelinases (N-SMases) are major candidates for stress-induced ceramide production, but there is still limited knowledge of the regulatory mechanisms of the cloned N-SMase enzyme – nSMase2. Previously, we reported that p38 MAPK was upstream of nSMase2 in tumor necrosis-α (TNF)-stimulated A549 cells (Clarke et al. (2007) *J. Biol. Chem.* 282, 1384-1396). Here, we report a role for protein kinase C (PKC) in mediating TNF-induced translocation of nSMase2 from the Golgi to the plasma membrane (PM). Pharmacological inhibition of PKCs prevented TNF-stimulated nSMase2 translocation to the PM in A549 cells. Utilizing phorbol esters (PMA) as a tool to dissect PKC responses, it was found that PMA induced nSMase2 translocation to the PM in a time- and dose-dependent manner. Pharmacological inhibitors and specific siRNA implicated the novel PKCs, specifically PKC-δ, in both TNF and PMA-stimulated nSMase2 translocation. However, PMA did not increase *in vitro* N-SMase activity and PKC-δ did not regulate TNF-induced N-SMase activity. Furthermore, PKC-δ and nSMase2 did not co-immunoprecipitate suggesting other signaling proteins may be involved. PMA-stimulated nSMase2 translocation was independent of p38 MAPK and neither PKC inhibitors nor siRNA had significant effects on TNF-stimulated p38 MAPK activation indicating that PKC-δ does not act through p38 MAPK in regulating nSMase2. Finally, downregulation of PKC-δ inhibited induction of VCAM and ICAM, previously identified as downstream of nSMase2 in A549 cells. Taken together, these data implicate PKC-δ as a regulator of nSMase2 and, for the first time, identify nSMase2 as a point of crosstalk between the PKC and sphingolipid pathways.

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Ceramide is well-established as a bioactive lipid involved in the cellular responses to stress. The sphingomyelinase (SMase)-mediated hydrolysis of sphingomyelin has emerged as a major pathway of stress-induced ceramide production and the Mg^{2+}-dependent neutral SMases (N-SMases) are considered strong candidates for mediating this pathway (Hannun and Obeid, 2002). Currently, two cloned proteins with *in vitro* and *in vivo* N-SMase activity have been identified: nSMase2 and nSMase3 (Hofmann et al., 2000, Krut et al., 2007). Previous research has reported the translocation of nSMase2 to the plasma membrane (PM) in response to confluence (Marchesini et al., 2004), H_2O_2 (Levy et al., 2006) and TNF (Clarke et al., 2007). Importantly, ceramide production in response to H_2O_2 or confluence, and the upregulation of adhesion proteins induced by TNF were all prevented by downregulation of nSMase2 (Clarke et al., 2007; Levy et al., 2006; Marchesini et al., 2004). Moreover, in hepatocytes, nSMase2 is located constitutively at the PM where it plays a role in interleukin-1β-stimulated activation of JNK (Karakashian et al., 2004). Taken together, these results suggest that nSMase2 localization or translocation to the PM is necessary for its signaling functions but the regulation of this process is still poorly understood. Previously, we reported that p38 MAPK was involved in regulation of nSMase2 translocation (Clarke et al., 2007). However, this regulation was indirect suggesting that other signaling proteins could be involved.

Protein kinase C (PKC) is a family of serine/threonine kinases important in modulating a variety of biological responses including cell growth, differentiation and apoptosis. PKCs consist of eleven isoforms separated into three classes according to their regulation. The classical PKCs (α, βI and II, γ) are activated by diacylglycerol, calcium and phosphatidylserine; the novel (δ, ε, η, θ) are activated by DAG and PS, whereas the atypical isoforms (ζ and λ/ι) are independent of both DAG and calcium but can be regulated by PS, arachidonic acid, and ceramide. The classical and novel PKCs are also robustly activated by phorbol esters (PMA), tumor-promoting compounds that mimic the action of DAG

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(reviewed in Parker and Murray-Rust, 2004; Musashi and Shiroshita, 2000). Currently, there is accumulating evidence for isoform-specific roles of PKCs. For example, PKC-δ has been implicated in regulation of apoptosis of U937 cells (Jang et al., 2003) whereas PKC-ε is involved in UV-induced carcinoma development (Aziz et al., 2007). A role for PKCs in p38 MAPK activation has also been suggested in response to TNF and hyperglycemia (Nomiyama et al., 2007; Woo et al., 2005), and PMA induces p38 MAPK activation in many cell types including MCF-7, A549, and A172 glioblastoma cells (Kitatani et al., 2007; Nomura et al., 2007; Chang et al., 2005). Thus, some of the downstream effects of PKC can be attributed to its subsequent activation of p38 MAPK.

Considerable evidence suggests crosstalk between the sphingolipid and PKC pathways. Ceramide is reported to have regulatory effects on activity and localization of PKC-α, -δ, -ε, and –ζ (Fox et al., 2007; Kajimoto et al., 2004; Lee et al., 2000). Moreover, PKC can act upstream of ceramide production as PMA activates acid SMase and the ceramide salvage pathway in MCF-7 cells (Kitatani et al., 2007; Zeidan and Hannun, 2007). PKCs can also regulate N-SMase activity. In mesangial cells, the cytokine-induced activation of N-SMase activity was inhibited by PMA (Kaszkin et al., 1998) and N-SMase activity stimulated by chemotherapeutic drugs in U937 cells was inhibited by PKC-ζ overexpression (Bezombes et al., 2002). Also, both PKC-α and –δ were implicated in regulation of the Mg^{2+}-independent cytosolic N-SMase in response to interferon-γ and vitamin D (Visnjic et al., 1999). Thus, PKC-mediated regulation of N-SMases may be an important point of crosstalk between the two pathways. However, to date no study has examined the regulation of the cloned N-SMases by PKC.

In this study, we investigated the role of PKC in nSMase2 regulation. We find that PKCs mediate the effects of TNF on nSMase2 translocation. Additionally, PMA induces nSMase2 translocation to the PM in a time- and dose-dependent manner but does not increase endogenous N-SMase activity or overexpressed nSMase2 activity. The use of PKC inhibitors and siRNA implicated

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PKC-δ in both PMA and TNF responses but this was not through activation of p38 MAPK. Together, these data offer further insight into nSMase2 regulation and also implicate nSMase2 as a point of crosstalk between PKC and sphingolipid pathways in the cellular response to TNF-α.

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MATERIALS AND METHODS

Cell Culture and siRNA. A549 cells were maintained in 10% fetal bovine serum in DMEM (Gibco) at 37C in 5% CO_2. For siRNA experiments, cells were seeded in 60mm dishes (50-75k/dish) or 35mm confocal dishes (15K/dish). After 24h, cells were transfected with scrambled (Scr) or specific siRNA (20nM) using Oligofectamine according to manufacturer's protocol. Cells were allowed to grow for 48-60h prior to experiments or transfection with 3'V5-tagged nSMase2. siRNA for PKC-δ was as described previously (Zeidan & Hannun, 2007); prevalidated siRNA for PKC-ε (# 02622088) and –θ (# 03571148) were from Qiagen.

Immunofluorescence and confocal microscopy. A549 cells (2.5×10^4) were seeded in 35mm confocal dishes, and, 48h later, cells were transiently transfected with 0.3μg 3'V5-tagged-nSMase2 using Effectene (Qiagen) according to manufacturer's protocol. After 12-15h, media were replaced and cells allowed to grow for a further 3-6h prior to stimulation with PMA (100nM) or TNF-α (50ng/ml) as indicated. For PKC inhibitor experiments, A549 cells were preincubated with 2μM bisindolylmalemide, 3μM Go6976, or 10μM rottlerin for 1h prior to stimulation. Cells were fixed, permeablized and stained as described previously (Clarke *et al.*, 2007) and viewed on a Zeiss LSM 510 Meta Confocal Microscope.

Total Cell Lysate Preparation. Cells were washed in ice-cold PBS, scraped in 1ml PBS and phosphatase inhibitors (Pierce Halt Cocktail) and pelleted (5 min, 1000xg). Cells were resuspended in 100μl PBS and 2 x 3μl aliquots removed for protein estimation by Bradford assay (Bradford, 1976). Forty μl x 2 Laemmelli buffer was added to an equal volume of cell suspension and vortexed x 3 times for 10s to lyse cells. Samples were boiled for 10 min for immediate use, or storage at -20ºC.

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Immunoblotting. Protein samples were separated on 4-20% gradient gels (Biorad Criterion) at 60-100V before transfer to nitrocellulose membrane in tris/glycine buffer (100V, 30 min, 4°C). Membranes were blocked (5% milk, >30 min) and probed with primary antibody overnight (4°C). Membranes were washed (x 3, 0.1% Tween TBS), probed with HRP-conjugated secondary antibody (1:5000 Mouse or Rabbit in 5% Milk) for 30-45 min at room temp, and washed (x3, 0.1% Tween TBS). Proteins were visualized by enhanced chemiluminescence (Pierce).

In Vitro Sphingomyelinase Assay. Neutral sphingomyelinase assay was assessed *in vitro* using [*choline-methyl*-^{14}C]sphingomyelin as described previously (Clarke et al., 2007; Marchesini et al. 2004)

Immunoprecipitation. A549 cells were mock- or transiently transfected with 3'V5-nSMase2 prior to stimulation with PMA (100nM) or TNF-α (50ng/ml) as indicated. Cells were washed (x 2 cold PBS) and lysed in immunoprecipitation buffer (50mM Tris pH 7.4, 150mM NaCl, 0.5% Triton X-100, 5mM NaF, 5mM Na_3VO_4, 2mM EDTA, 1mM PMSF, and protease inhibitors) by sonication (4 x 10s). V5 and PKC-δ were immunoprecipitated from 100μg lysate using Protein A/G agarose beads (Santa Cruz) as previously described (Clarke et al., 2007)

Statistical Analysis. Comparisons between two groups were analyzed by Student's t-test. $P<0.05$ was considered statistically significant with n=number of experiments as indicated.

Materials. Monoclonal anti-V5 antibody was from Invitrogen (USA); polyclonal giantin was from Covance; polyclonal phospho-p38 MAPK was from Promega (Madison, WI); polyclonal anti-p38

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MAPK (clone C-20), polyclonal PKC-δ (C-20), monoclonal ICAM-1 (G-5) and polyclonal VCAM-1 (H-276) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal PKC-ε, PKC-θ, PKC-η and polyclonal p230 antibodies were from BD Biosciences. Anti phospho-PKC-δ, -ε, and –θ antibodies were from Cell Signaling (Boston, MA). Polyclonal anti TGN46 came from Novus Biologicals. Fluorescent secondary antibodies were from Jackson Laboratories (USA). PMA, bisindolylmalemide, rottlerin, Go6976 and rottlerin were from Calbiochem (CA, USA). Human recombinant TNF was purchased from Preprotech Inc. (NJ, USA). [*choline-methyl*-^{14}C]-sphingomyelin was provided by Dr. Alicja Bielawska (Medical University of South Carolina, Charleston, SC). All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Scintillation mixture Safety Solve was from Research Products International. All other products were from Sigma Aldrich unless stated.

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RESULTS

PKCs are involved in TNF-stimulated translocation of nSMase2 to the plasma membrane. Previous studies have suggested cross-talk between PKC and N-SMase pathways (Bezombes et al., 2002; Visnjic et al., 1999; Kaszkin et al., 1998). As nSMase2 translocation to the PM is important for nSMase2-mediated signaling (Clarke et al., 2007; Levy et al., 2006; Marchesini et al., 2004), the role of PKCs in this process was investigated initially by a pharmacological approach. A549 cells overexpressing V5-nSMase2 were preincubated with the PKC inhibitors bisindolylamide (2μM) or Go6976 (3μM) for 1h prior to stimulation with TNF (50ng/ml, 30m). Localization of nSMase2 was investigated by immunofluorescence and confocal microscopy as described in "Materials and Methods." Preincubation of A549 cells with either inhibitor had no significant effect on basal nSMase2 localization with V5-nSMase2 co-localizing with the Golgi marker giantin consistent with our previous studies (Clarke et al., 2007). However, bisindolylamide inhibited the TNF-stimulated nSMase2 translocation whereas Go6976 had no effect (**Fig. 1A**). Taken together, this indicates a role for PKC in nSMase2 translocation with the differential effects of bisindolylamide (classical and novel PKC inhibitor) and Go6976 (classical PKC inhibitor) suggesting that the novel PKCs are involved.

PMA induces nSMase2 translocation to the plasma membrane. To further define the role of PKCs in nSMase2 translocation, PMA (a direct activator of novel and classical PKCs) was utilized. As before, V5-nSMase2 co-localized with giantin in unstimulated cells. Stimulation with PMA (100nM) induced a rapid (10-20m) and sustained translocation of nSMase2 to the PM (**Fig. 1B)**. To further characterize this effect, a dose response at 30m of stimulation was performed. At concentrations of 1 and 10nM PMA, nSMase2 was intracellular, mainly localizing to the Golgi. However, concentrations of 50nM PMA and higher were sufficient to induce PM translocation of nSMase2 (**Fig. 1C**).

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The higher doses of PMA required to induce nSMase2 translocation again suggested that the novel PKCs are involved, as they are reported to require higher PMA concentrations for activation (Kazanietz et al. 1993). Accordingly, the inhibitors bisindolylamide and Go6976 were utilized. Consistent with the effects seen with TNF, preincubation with both inhibitors had no effect on basal nSMase2 localization. However, whereas bisindolylamide inhibited nSMase2 translocation to the PM, Go6976 had no effect (**Fig. 1D**), supporting the evolving hypothesis that novel PKCs play a role in regulating nSMase2.

Investigation of the novel PKC isoform involved in nSMase2 translocation. To identify the novel PKC involved in regulation of nSMase2, it was first necessary to confirm which novel PKCs were detectable in A549 cells. Accordingly, total cell lysate was immunoblotted for PKC-δ, -ε, -η and –θ. As can be seen, the isoforms PKC-δ, -ε and –θ were readily detected (**Fig 2A**). However, we were unable to detect PKC-η.

To further explore the novel PKC role in nSMase2 translocation, we initially utilized rottlerin, an inhibitor shown to have some specificity for the novel PKC-δ (Geschwendt et al., 1994). Whereas preincubation of cells with rottlerin (10μM, 1h) had no effect on basal nSMase2 localization, translocation of nSMase2 in response to both PMA (**Fig. 2B**) and TNF (**Fig. 2C**) was prevented, further consistent with a role for the novel PKCs in regulating nSMase2 translocation.

To consolidate this data and avoid non-specificity issues of pharmacological inhibitors, siRNA for each novel isoform detected was utilized. To validate the efficacy and specificity of each siRNA, A549 cells were transfected with 20nM PKC-δ, -ε, -θ or a non-specific targeting (Scr) siRNA as control. After 48-60h, total protein was extracted and immunoblotted for all novel PKCs. Each siRNA was found to strongly and specifically downregulate the respective isoform with a minimal effect on the levels of

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the other novel PKC isoforms (**Fig. 3A**). Utilizing these siRNAs, the involvement of each novel PKC in regulation of nSMase2 was investigated. A549 cells were treated with siRNA for 48h prior to transient transfection with V5-nSMase2. Cells were stimulated with PMA (100nM, 30m) or TNF (50ng/ml, 30m), and nSMase2 localization was investigated by immunofluorescence and confocal microscopy. In all cases, basal localization of nSMase2 was unaffected by siRNA treatment (**Fig. 3B**). However, on PMA stimulation, PKC-ε siRNA had no effect on nSMase2 translocation. In contrast, PKC-δ downregulation markedly inhibited nSMase2 translocation with a predominantly intracellular localization of V5-nSMase2 observed (**Fig. 3B**). Interestingly, PKC-θ siRNA had a small effect such that both PM and intracellular localization of nSMase2 could be seen.

Given the above results, it became critical to determine if PKC-δ plays a key role in mediating the effects of TNF on nSMase2. Therefore, the effects of the siRNAs were evaluated as above. As before, no effect of the siRNA on basal nSMase2 localization was observed (**Fig. 3C**). However, as with PMA, PKC-δ downregulation markedly prevented relocalization of nSMase2 to the PM whereas PKC-ε and –θ siRNA were without significant effect (**Fig. 3C**). As an additional control, PKC-α siRNA was utilized and with both TNF and PMA stimulation, no significant effect on nSMase2 localization was observed (data not shown). These studies reveal a critical role for PKC-δ in mediating the effects of TNF on nSMase2 translocation.

The role of PKC-δ in regulation of N-SMase activity. In addition to inducing PM translocation of nSMase2, we have also reported that TNF rapidly and transiently increases nSMase2 activity peaking at 5 min of stimulation (Clarke *et al.*, 2007). Therefore, we were interested to see if PMA also stimulated nSMase2 activity in A549 cells. Accordingly, N-SMase activity both endogenously and in cells overexpressing nSMase2 was measured *in vitro* as described above (**Fig. 4A**). Results indicated that,

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unlike with TNF, PMA stimulation had no significant effect on endogenous N-SMase activity between 0-60m of stimulation. In contrast, PMA decreased activity of overexpressed nSMase2 at 30 and 60m of stimulation. This is consistent with previous studies reporting a PMA-induced reduction of N-SMase activity (Kaszkin et al., 1998) and suggests that PKCs may not be involved in post-translational activation of nSMase2 (see "Discussion").

To further explore this in response to TNF, the effects of PKC-δ and PKC-ε siRNA on TNF-stimulated endogenous N-SMase activity were investigated (**Fig. 4B**). A time point of 5 min was utilized as this was previously shown to be the peak stimulation time in A549 cells (Clarke *et al.*, 2007). As can be seen, neither PKC-δ nor PKC-ε siRNA had effects on basal activity. Moreover, neither siRNA prevented TNF stimulation of N-SMase activity. This is consistent with the lack of effect of PMA on endogenous N-SMase activity in A549 cells. Furthermore, this suggests that PKC-δ is important for nSMase2 translocation, but not activation in A549 cells.

***Co-immunoprecipitation studies of nSMase2 and PKC-δ*.** Translocation and regulation of proteins can often occur through direct interactions with other proteins at the membrane**.** To determine if PKC-δ and nSMase2 directly interact with each other in cells, co-immunoprecipitation studies were performed. Mock or V5-nSMase2-transfected A549 cells were stimulated with either PMA (100nM; 0, 10, 30m) or TNF (3nM; 0, 10, 30m), lysates were immunoprecipitated with V5 or PKC-δ antibody and immunoblotted for PKC-δ and V5-nSMase2 respectively. With both TNF (**Fig. 5A**) and PMA (**Fig. 5B**), there was no co-immunoprecipitation of V5-nSMase2 and PKC-δ either in basal or stimulated conditions. Together, this suggests that PKC-δ may function as an indirect regulator of nSMase2 translocation in both PMA and TNF responses.

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p38 MAPK does not play a role in PMA-stimulated nSMase2 translocation. In a previous study, we identified p38 MAPK as an indirect regulator of nSMase2 translocation and activation in response to TNF (Clarke et al., 2007). Previous studies have shown that PMA can induce p38 activation (Kitatani et al., 2007; Nomura et al., 2007; Chang et al., 2005) and implicated PKC upstream of p38 MAPK (Nomiyama et al., 2007; Woo et al., 2005). Given that PKC-δ and nSMase2 did not appear to interact, it became important to determine the role of PKC in respect to p38 MAPK in regulating nSMase2 translocation. To investigate this with PMA, the p38 MAPK inhibitor SB202190 was utilized. A549 cells overexpressing V5-nSMase2 were pre-treated with 10μM SB202190 for 1h prior to stimulation with PMA (100nM, 30m) and the effects on nSMase2 localization investigated. Preincubation with the inhibitor alone had no effect on basal nSMase2 localization consistent with our previous study (Clarke et al., 2007). Furthermore, inhibition of p38 MAPK had no effect on the translocation of nSMase2 stimulated by PMA but inhibited the TNF response on nSMase2 (**Fig. 6A**). These results suggest that p38 MAPK does not act downstream of PKC-δ in inducing translocation of nSMase2.

To consolidate these results, the effects of PKC inhibitors on PMA-stimulated p38 MAPK activation, as assessed by phosphorylation, were investigated. As can be seen, both bisindolylamide I and Go6976 inhibited p38 MAPK phosphorylation in response to PMA (**Fig. 6B**), suggesting a classical PKC-mediated activation of p38 MAPK. Consistent with this, rottlerin also had no effect on PMA-stimulated p38 phosphorylation (**Fig. 6C**). Taken together, these results suggest that activation of p38 MAPK by PMA is independent of the novel PKC-mediated regulation of nSMase2.

Novel PKCs do not act upstream of p38 MAPK in response to TNF. Having found that p38 MAPK and novel PKCs appeared to act independently in response to PMA, it was important to further explore this relationship in response to TNF. Initially, A549 cells were treated with bisindolylamide 1 or Go6976

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and p38 MAPK phosphorylation was assessed. As with PMA, neither inhibitor had a significant effect on basal p38 MAPK phosphorylation. However, neither inhibitor had a significant effect on TNF-stimulated p38 MAPK phosphorylation (**Fig. 7A**) suggesting that, unlike with PMA, classical PKC is not upstream of p38 MAPK activation in response to TNF. To consolidate these data, the effect of rottlerin was determined. Although a small effect of rottlerin on TNF-stimulated p38 MAPK phosphorylation was observed (20%), this was not statistically significant (p >0.1) (**Fig. 7B**). Thus, as with PMA, the TNF-stimulated activation of p38 MAPK is independent of novel PKCs, but unlike with PMA, it is also independent of classical PKCs.

In order to further support inhibitor data, avoid inhibitor specificity issues, and clarify individual roles of novel PKCs in p38 MAPK activation or not, the effects of novel PKC siRNA (δ, ε, θ) on p38 MAPK phosphorylation were determined. As can be seen, siRNA downregulation of novel PKCs had no significant effect on basal or TNF-stimulated p38 MAPK phosphorylation (**Fig. 7C**), confirming that PKC-δ is not upstream of p38 MAPK in A549 cells. Importantly, these data are also consistent with the lack of a role for PKC-δ in regulating endogenous N-SMase activity in A549 cells, as we have previously shown that this activity is p38 MAPK dependent (Clarke *et al.*, 2007). Taken together, this confirms that novel PKCs do not act upstream of p38 MAPK in regulating nSMase2 in response to TNF.

Regulation of novel PKCs by TNF in A549 cells. Although previous studies have reported that TNF increases PKC activity as early as 10 min in A549 cells (Chen et al., 2001), there is currently little information as to the PKC isoforms regulated by TNF in A549 cells. Therefore, the specific novel PKCs regulated by TNF in our system were investigated. To this end, antibodies specific to phosphorylated forms of novel PKCs were employed. These sites were previously reported to correspond to active forms of the enzymes and were Thr-505 for PKC-δ (Le Good et al., 1998), Ser-729 for PKC-ε (Cenni et al.,

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2002) and Thr-538 for PKC-θ (Liu et al., 2002). A549 cells were stimulated with TNF for various time between 0 - 60min, and phosphorylation state was probed by immunoblot (**Fig. 8**). For normalization, blots were re-probed for total PKC levels. As can be seen, PKC-δ was basally phosphorylated to some extent at Thr-505, as reported previously (Le Good et al., 1998). Stimulation with TNF increased this phosphorylation peaking at 30 min at 1.47±0.12-fold over basal with levels were returning towards that of unstimulated cells by 60 min (**Fig. 8A**). In contrast to this, phosphorylation of PKC-δ at Tyr-311, known to be regulated by H_20_2 (Konishi et al., 2001) was not observed at any time point (data not shown). Interestingly, phosphorylation of PKC-ε at Ser-729 was not detected either basally or on stimulation (**Fig. 8B, upper**). Additionally, whereas basal phosphorylation of PKC-θ at Thr-538 was observed, this was not significantly affected by TNF at any of the time points investigated (**Fig. 8B, lower**). Taken together, these data suggest that PKC-δ is the major novel PKC regulated by TNF in A549 cells, consistent with its role in regulating nSMase2.

PKC-δ is upstream of VCAM and ICAM induction in A549 cells. The results above suggest that PKC-δ is an upstream regulator of nSMase2. Previously, nSMase2 was reported as upstream of TNF-stimulated VCAM and ICAM induction in A549 cells (Clarke *et al.*, 2007). Therefore, as a functional readout, the effects of PKC siRNA on VCAM and ICAM induction were investigated (**Fig. 9**). A549 cells treated with siRNA to PKC-δ and –ε for 48-60h were stimulated with TNF (3nM, 3h) and VCAM and ICAM level analyzed by immunoblot (**Fig. 9A**). As can be seen, siRNA to PKC-δ resulted in significant reduction of VCAM and ICAM induction at 3h of TNF stimulation (41 ± 2% for ICAM, $p<0.04$; 31 ± 5% for VCAM, $p<0.01$). This is consistent with the effects of nSMase2 reported previously (Clarke *et al.*, 2007) and further supports a role for PKC-δ as an upstream regulator of nSMase2. In contrast, PKC-ε siRNA had no effect on ICAM levels, but did have a modest effect on

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VCAM levels (25 ± 4%, $p<0.05$). As PKC-ε did not appear to be activated at early time points (**Fig. 8B**), it is possible that this isoform may play a regulatory role at later time points of TNF stimulation.

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DISCUSSION

In this study, PKC-δ was implicated as an upstream regulator of nSMase2 translocation to the PM in PMA- and TNF-stimulated A549 cells. Moreover, this regulation does not occur through activation of p38 MAPK, previously identified as a regulator of nSMase2 (Clarke *et al.*, 2007). A schematic of the major conclusions from the results obtained is shown (**Fig. 10**). Taken together, these data shed light on the regulation of nSMase2 in TNF responses and implicate nSMase2 as a point of crosstalk between the PKC and sphingolipid signaling pathways.

Using a combination of pharmacological inhibitors and direct activation of PKC by PMA, nSMase2 was identified as a PKC-regulated enzyme. The effects of specific inhibitors together with the higher doses of PMA required for nSMase2 translocation suggested this was mediated by a novel PKC. Upon further investigation with specific siRNA, PKC-δ was identified as the novel PKC isoform involved in regulating nSMase2 translocation to the PM in response to both PMA and TNF. Previous research has suggested that nSMase2 translocation to the PM is important for its signaling functions (Clarke et al., 2007; Levy et al., 2006; Karakashian et al. 2004; Marchesini et al., 2004). Consistent with this, siRNA downregulation of PKC-δ decreased TNF-stimulated induction of VCAM and ICAM in A549 cells, matching what we have previously reported for nSMase2 (Clarke *et al.*, 2007). Taken together, these data implicate PKC-δ as a positive regulator of nSMase2 in A549 cells.

While previous studies have reported regulation of N-SMase by PKC, these have focused solely on endogenous N-SMase activities without explicit identification of the specific enzyme involved owing to a lack of molecular tools. Interestingly, PMA-sensitive PKC isoforms inhibited N-SMase activation by interleukin-1β in mesangial cells (Kaszkin et al., 1998), and PKC-δ negatively regulated N-SMase activity in HL-60 cells. In that study, pretreatment of cytosol extracts with PMA suppressed vitamin-D_3 stimulation of N-SMase activity but depletion of the novel PKC-δ from the cytosol using specific

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antibodies prevented this. Interestingly, similar depletion of the classical PKC-α prevented vitamin-D_3 stimulation of N-SMase activity (Visnjic et al., 1999), suggesting it functions upstream of N-SMase. These differences could be attributed to both cell type and stimuli used in these studies as well as possible divergent roles of PKC isoenzymes. Moreover, as both studies investigated endogenous (generic) N-SMase activities and the latter study in HL-60 cells focused on the cytosolic Mg^{2+}-independent N-SMase activity, the contribution of nSMase2 to these effects is not clear.

The current study also clarifies the mutual roles of p38 MAPK and PKC-δ in regulation of nSMase2. As we have previously reported that p38 MAPK acted upstream of nSMase2 translocation in TNF responses (Clarke *et al.*, 2007), the relationship between PKC, p38 MAPK and nSMase2 was further explored. Interestingly, PKC-δ did not appear to regulate nSMase2 through activation of p38 MAPK as indicated by a number of lines of evidence: 1) p38 MAPK was not required for PMA-induced nSMase2 translocation of nSMase2; 2) classical PKCs act upstream of p38 MAPK in PMA-stimulated A549 cells, consistent with this pathway being distinct from the novel PKC-mediated regulation of nSMase2; 3) Neither inhibition nor siRNA downregulation of novel PKCs have significant effects on basal or TNF-stimulated p38 MAPK activation; 4) TNF stimulation of N-SMase activity was PKC-δ-independent, but was previously shown to require p38 MAPK (Clarke *et al.*, 2007). Taken together, this is strong evidence that p38 MAPK activation in TNF-stimulated A549 cells is independent of both novel and classical PKCs, agreeing with studies in neutrophils (Konishi et al., 2001) and monocytes (Nguyen et al., 2006). However, this is contrary to previous research in A549 cells, where inhibitors and antisense oligonucleotides implicated PKC-δ upstream of p38 MAPK in TNF responses (Woo et al., 2005). This might be due to the different TNF concentrations used (10ng/ml vs 50ng/ml here). Although these results suggested that PKC-δ and p38 MAPK may regulate nSMase2 independently, the possibility that p38 MAPK may be upstream of PKC-δ in regulating nSMase2 cannot be ruled out.

PKC-δ was the major novel PKC regulated by TNF in A549 cells, as evidenced by increased phosphorylation at Thr-505. This agrees with previous work in A549 cells (Woo et al., 2005) and in bronchial epithelial cells, where TNF increased PKC-δ activity (Page et al., 2003). Additionally, the lack of effect of TNF on both PKC-ε and –θ provides additional evidence against a role of these isoforms in regulating nSMase2. As phosphorylation of these residues on –ε and -θ is reported to correlate with the active enzyme (Cenni et al., 2002; Liu et al., 2002), this also suggests that TNF does not activate either isoform in A549 cells.

Despite the identification of PKC-δ (here) and p38 MAPK (Clarke *et al.*, 2007) as regulators of nSMase2 translocation, the mechanism by which nSMase2 translocates remains unclear. However, nSMase2 is a membrane-associated protein with two hydrophobic segments and thus, fundamental principles indicate that it must move as part of membrane trafficking. Moreover, nSMase2 is palmitoylated as previously reported by our laboratory (Tani & Hannun, 2007), but the palmitoylation status of nSMase2 does not change on PMA or TNF stimulation (Tani & Hannun, unpublished observation). Therefore, regulated palmitoylation does not play a role in nSMase2 translocation. The basal localization of nSMase2 to the Golgi complex suggests a role for Golgi-derived vesicles in an exocytic pathway. Consistent with this, V5-nSMase2 colocalizes with both p230 and TGN46 (Supplementary Figure 1), markers of the trans-Golgi network and TGN-derived vesicles. Furthermore, p38 MAPK has been implicated in Golgi to PM transport of the bile salt export pump in HepG2 cells and rat hepatocytes (Kubitz et al., 2004). Although PKCs have yet to be implicated in transport of proteins from Golgi to PM, studies have suggested they play a regulatory role in sphingolipid transport to the apical PM of hepatoma cells (Zegers and Hoekstra, 1997). Thus, we speculate that PM translocation of nSMase2 occurs through trans-Golgi network -derived vesicles. Interestingly, nSMase2 also contains two putative recycling endosome motifs (Marchesini, N., Truong T-G., and Hannun Y.A.,

unpublished observations), suggesting that the recycling endosomes may be important in regulating nSMase2 localization. Current studies in our laboratory are investigating this mechanism further.

Interestingly, although implicated in nSMase2 translocation, PKCs were not necessary for the observed increase in N-SMase activity in A549 cells. Direct activation of PKC by PMA had no effect on endogenous N-SMase activity, consistent with previous research in MCF-7 cells (Zeidan and Hannun, 2007) but disagreeing with studies in mesangial cells (Kaszkin et al., 1998). This is likely due to cell specific differences, either in PKC isoenzymes or in N-SMase isoforms present. Surprisingly, persistent PMA stimulation decreased activity of overexpressed nSMase2. As the observed decrease (30-60m) is temporally distinct from PMA-induced translocation (5-10m), this suggests that nSMase2 translocation and activation are distinct processes. This hypothesis is further supported by the role of PKC-δ in TNF-induced translocation, but not activation, of nSMase2. However, it should be noted that 'activation' in this case most likely denotes a post-translational modification since it persists following cell lysis (which would usually dissociate allosteric regulators). This 'activation' by TNF might not be necessary for nSMase2 activation in cells. Indeed, translocation itself may be a sufficient mechanism of cellular activation by relocating the enzyme to contact and act on substrate.

Crosstalk between different signaling pathways is often important for amplification and specificity of agonist responses and there has been considerable study on the crosstalk between PKC and sphingolipid pathways. This study provides further evidence that PKC-δ is a primary point of crosstalk between these two pathways. Previous studies have found roles for PKC-δ in regulation of acid SMase activation, and subsequent ceramide production in MCF-7 cells (Zeidan and Hannun, 2007). Additionally, ceramide is also known to have effects on PKC-δ. In leukemia cells, ceramide treatment or generation by SMase caused PKC-δ translocation to the cytosol whereas in HeLa cells, ceramide caused PKC-δ activation subsequent to Golgi translocation (Kajimoto et al., 2004; Lee et al., 2000).

Interestingly, membrane translocation of PKC-δ in interferon-γ-stimulated HeLa cells and on chemotherapeutic stimulation of C6 glioma cells required N-SMase activation (Peng et al., 2006; Kajimoto et al., 2001). When considered with data presented here, this raises the interesting possibility that N-SMases might function both upstream and downstream of PKC-δ; this is currently the subject of ongoing studies. Finally, by identifying the connection between PKC-δ and nSMase2, this offers insight into the mechanisms by which some PKC-δ-mediated functions may occur.

In conclusion, this study has identified a role for PKC-δ as a regulator of nSMase2 translocation to the PM in TNF-stimulated A549 cells. This offers further insight into the regulation of nSMase2 and, for the first time, identifies a specific N-SMase as a point of crosstalk between PKC and sphingolipid pathways.

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REFERENCES

Aziz, M.H., Manoharan, H.T, and Verma, A.K. (2007) Protein kinase Cε, which sensitizes skin to sun's UV radiation-induced cutaneous damage and development of squamous cell carcinomas, associates with Stat3. *Cancer Res.* **67**: 1385-1394.

Bezombes, C., de Thonel, A., Apostolou, A., Louat, T., Jaffrezou, J.P., Laurent, G., and Quillet-Mary, A. (2002) Overexpression of protein kinase Cζ confers protection against antileukemic drugs by inhibiting the redox-dependent sphingomyelinase activation. *Mol. Pharmacol.* **62**: 1446-1455.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.

Cenni, V., Doppler, H., Sonnenburg, E.D.,Maaldi, N., Newton, A.C., and Toker, A. (2002) Regulation of novel protein kinase C ε by phosphorylation. *Biochem. J.* **363**: 537-545.

Chang, M.S., Chen, B-C., Yu, M-T., Sheu, J-R., Chen, T-F., and Lin, C.H. (2005) Phorbol 12-myristate 13-acetate upregulates cyclooxygenase-2 expression in human pulmonary epithelial cells via Ras, Raf-1, ERK, and NF-kappaB, but not p38 MAPK, pathways. *Cell Signal*. **17**: 299-310.

Chen, C-C., Chou, C-Y., Sun, Y-T., and Huang, W-C. (2001) Tumor necrosis factor α-induced activation of downstream NF-kB site of the promoter mediate epithelial ICAM-1 expression and monocyte adhesion: involvement of PKCα, tyrosine kinase, and IKK2, but not MAPKs, pathway *Cell. Signal*. **13**: 543-553.

Clarke, C.J., Truong, T-G., and Hannun, Y.A. (2007) Role for neutral sphingomyelinasee-2 in tumor necrosis factor α-stimulated expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 in lung epithelial cells: p38 MAPK is an upstream regulator of nSMase2. *J. Biol. Chem.* **282**: 1384-1396.

Fox, T.E., Houck, K.L., O'Neill, S.M., Nagarajan, M., Stover, T.C., Pomianowski, P.T., Unal, O., Yun, J.K., Naides, S.J., and Kester, M. (2007) Ceramide recruits and activates protein kinase C zeta (PKC zeta) within structured membrane microdomains. *J. Biol. Chem.* **282**: 12450-12457.

Gschwendt, M, Muller, H.J., Kielbassa, K., Zang, R., Kittstein, W., Rincke, G., and Marks, F. (1994) Rottlerin, a novel protein kinase inhibitor. *Biochem. Biophys. Res. Comm.* **199**: 93-98.

Hannun, Y.A., and Obeid, L.M. (2002) The Ceramide-centric universe of lipid-mediated cell regulation: Stress encounters of the lipid kind. *J. Biol. Chem.* **277**: 25847-25850.

Hofmann, K., Tomiuk, S., Nix, M., Zumbansen, M., and Stoffel, W. (2000) Cloning and characterization of the mammalian brain-specific, Mg^{2+}-dependent neutral sphingomyelinase. *Proc. Nat. Acad. Sci. USA.* **97**: 5895-5900.

Jang, B.C., Choi, E.S., Im, K.J., Baek, W.K., Kwon, T.K., Suh, M.H., Kim, S.P., Park, J.W., and Suh, S.I. (2003) Induction of apoptosis by Se-MSC in U937 human leukemia cells through release of cytochrome c and activation of caspases and PKC-delta: mutual regulation between caspases and PKC-delta via a positive feedback mechanism. *Int. J. Mol. Med.* **12**: 733-739.

Kajimoto, T., Ohmori, S., Shirai, Y., Sakai, N., and Saito, N. (2001) Subtype-specific translocation of the δ subtype of protein kinase C and its activation by tyrosine phosphorylation induced by ceramide in HeLa cells. *Mol. Cell. Biol.* **21**: 1769-1783.

Kajimoto, T., Shirai, Y., Sakai, N., Yamamoto, T., Matsuzaki, H., Kikkawa, U., and Saito, N. (2004) Ceramide-induced apoptosis by translocation, phosphorylation and activation of protein kinase C-δ in the Golgi complex. *J. Biol. Chem.* **279**: 12668-12676.

Karakashian, A.A., Giltiay, N.V., Smith, G.M., Nikolova-Karakashian, M.N. (2004) Expression of neutral sphingomyelinase-2 (NSMase-2) in primary rat hepatocytes modulates IL-beta-induced JNK activation. *FASEB J.* **18**: 968-70.

Kaszkin, M., Huwiler, A., Scholz, K., Van den Bosch, H., and Pfeilschifter, J. (1998) Negative regulation of interleukin-1β-activated neutral sphingomyelinase by protein kinase C in rat mesangial cells. *FEBS Lett.* **440**: 163-166.

Kazanietz, M., Areces, L.B., Bahador, A., Mischak, H., Goodnight, J., Mushinski, J.F., and Blumberg, P.M. (1993) Characterization of ligand and substrate specificity for the calcium-dependent and calcium-independent protein kinase C isozymes. *Mol. Pharmacol.* **44**: 298-307.

Kilpatrick, L.E., Sun, S., Mackie, D., Baik, F., Li, H., and Korchak, H.M. (2006) Regulation of TNF mediated antiapoptotic signaling in human neutrophils: role of delta-PKC and ERK1/2. *J. Leukocyte Biol.* **80**: 1512-1521.

Kitatani, K., Idkowiak-Baldys, J., Bielawski, J. Taha, T.A., Jenkins, R.W., Senkal, C.E., Ogretman, B.E., Obeid, L.M., and Hannun, Y.A. (2007) Protein kinase C-induced activation of a ceramide/protein phosphatase 1 pathway leading to dephosphorylation of p38 MAPK. *J. Biol. Chem.* **281**: 36793-36802.

Konishi, H., Yamauchi, E., Taniguchi, H., Yamamoto, T., Matsuzaki, H., Takemura, Y., Ohmae, K., Kikkawa, U., and Nishizuka Y. (2001) Phosphorylation sites of protein kinase C δ in H_2O_2-treated cells and its activation by tyrosine kinase in vitro. *Proc. Nat. Acad. Sci. USA.* **98**: 6587-592.

Krut, O., Wiegmann, K., Kashkar, H., Yazdanpanah, B., and Kronke, M. (2006) Novel tumor necrosis factor-responsive mammalian neutral sphingomyelinase-3 is a C-tail-anchored protein. *J. Biol. Chem.* **81**: 13784-13793.

Kubitz, R., Sutfels, G., Kuhlkamp, T., Kolling, R., and Haussinger, D. (2004) Trafficking of the bile salt export pump from the Golgi to the canalicular membrane is regulated by the p38 MAP kinase. *Gastroenterology* **126**: 541-553.

Lee, J.Y., Hannun, Y.A., and Obeid, L.M. (2000) Functional dichotomy of protein kinase C in tumor necrosis factor-α (TNF-α) signal transduction in L929 cells. *J. Biol. Chem.* **275**: 29290-29298.

Le Good, J.A., Ziegler, W.H., Parekh, D.B., Alessi, D.R., Cohen, P., and Parker, P.J. (1998) Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* **281**: 2042-2045.

Levy, M., Castillo, S.S., and Goldkorn, T. (2006) nSMase2 activation and trafficking are modulated by oxidative stress to induce apoptosis. *Biochem. Biophys. Res. Commun.* **344**: 900-905.

Liu, Y., Graham, C., Li, A., Fisher, R.J., and Shaw, S. (2002) Phosphorylation of the protein kinase C-theta activation loop and hydrophobic motif regulates its kinase activity, but only activation loop phosphorylation is critical to in vivo nuclear-factor-kB induction. *Biochem. J.* **361**: 255-265.

Marchesini, N., Osta, W., Bielawski, J., Luberto, C., Obeid, L.M., and Hannun, Y.A. (2004) Role for mammalian neutral sphingomyelinase 2 in confluence-induced growth arrest of MCF7 cells. *J. Biol. Chem.* **279**: 25101-25111.

Musashi, M., Ota, S., and Shiroshita, N. (2000) The role of protein kinase C isoforms in cell proliferation and apoptosis. *Int. J. Hematol.* **72**: 12-19.

Nguyen, J., Gogusev, J., Knapnougel, P., and Bauvois, B. (2006) Protein tyrosine kinase and p38 MAP kinase pathways are involved in stimulation of matrix metalloproteinase-9 by TNF-alpha in human monocytes. *Immunol. Lett.* **106**: 34-41.

Nomiyama, Y., Tashiro, M., Yamaguchi, T., Watanabe, S., Taguchi, M., Asaumi, H., Nakamura, H., and Otsuki, M. (2007) High glucose activates rat pancreatic stellate cells through protein kinase C and p38 mitogen-activated protein kinase pathway. *Pancreas.* **34**: 364-372.

Nomura, N., Nomura, M., Sugiyama, K., and Hamada, J.I. (2007) Phorbol 12-myristate 13-acetate (PMA)-induced migration of glioblastoma cells is mediated via p38MAPK/Hsp27 pathway. *Biochem. Pharmacol.* **74**: 690-701.

Page, K., Li, J., Zhou, L., Iasvoyskaia, S., Corbit, K.C., Soh, J-W., Weinstein, I.B., Brasier, A.B., Lin, A., and Hershenson, M.B. (2003) Regulation of airway epithelial cells NF-kB-dependent gene expression by protein kinase Cδ. *J. Immunol.* **170**: 5681-5689.

Parker, P.J., and Murray-Rust, J. (2004) PKC at a glance. *J. Cell Sci.* **117**: 131-132.

Peng, C-H., Huang, C-N., Hsu, S-P., and Wang, C-J. (2006) Penta-acetyl geniposide induced apoptosis in C6 glioma cells by modulating the activation of neutral sphingomyelinase-induced p75 never growth factor receptor and protein kinase Cδ pathway. *Mol. Pharmacol.* **70**: 997-1004

Tani, M, and Hannun, Y.A. (2007) Neutral sphingomyelinase 2 is palmitoylated on multiple cysteine residues. Role of palmitoylation in subcellular localization. *J. Biol. Chem.* **282**: 10047-10056.

Visnjic, D., Batinic, D., and Banfic, H. (1999) Different roles of protein kinase C alpha and delta isoforms in the regulation of neutral sphingomyelinase activity in HL-60 cells. *Biochem. J.* **344**: 921-928.

Woo, C.H., Lim, J.H., and Kim, J.H. (2005) VCAM-1 upregulation via PKCδ-p38 kinase-linked cascade mediates the TNF-α-induced leukocyte adhesion and emigration in the lung airway epithelium. *Am. J. Physiol. Lung Cell Mol. Physiol.* **288**: L307-L316.

Zegers, M.M.P. and Hoekstra, D. (1997) Sphingolipid transport to the apical plasma membrane domain in human hepatoma cells is controlled by PKC and PKA activity: A correlation with cell polarity in HepG2 cells. *J. Cell Biol.* **138**: 307-321

Zeidan, Y.H., and Hannun, Y.A. (2007) Activation of acid sphingomyelinase by protein kinase Cδ-mediated phosphorylation. *J. Biol. Chem.* **282**: 11549-11561.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. **PKC is involved in regulation of nSMase2 translocation in response to TNF and PMA.** A549 cells (20-25K) were seeded in 35mm-confocal dishes and 48h later were transiently transfected with 3'V5-tagged nSMase2 (0.25μg/dish) for 12h, media was changed and cells were allowed to grow for 6-12h before stimulation as indicated. Cells were fixed and stained with anti-V5 (green) and anti-giantin (red) as described in "Materials and Methods." (**A**) Effect of PKC inhibitors on TNF-stimulated nSMase2 translocation. Cells were preincubated in bisindolylamide I (2μM) or Go6976 (3μM) for 1h prior to stimulation with TNF (50ng/ml) for 30m. (**B**) Time course of PMA stimulation (100nM) from 0-60m. (**C**) Dose response of PMA stimulation - 0-200nM for 30m. (**D**) Effect of PKC inhibitors on PMA-stimulated nSMase2 translocation. Cells were preincubated as in (**A**) prior to stimulation with PMA (100nM) for 30m. Pictures are representative of >5 fields taken from at least 4 independent experiments.

Figure 2. Rottlerin inhibits nSMase2 translocation in response to TNF and PMA. (**A**) Total lysate was prepared from A549 cells and immunoblotted for novel PKCs as described in "Materials and Methods." (**B**) & (**C**) A549 cells (20-25K) were seeded in 35mm-confocal dishes and 48h later were transiently transfected with 3'V5-tagged nSMase2 (0.25μg/dish) for 12h, media was changed and cells were allowed to grow for 6-12h before stimulation as indicated. Cells were preincubated with rottlerin (10μM) for 1h prior to stimulation as indicated. Cells were fixed and stained with anti-V5 (green) and anti-giantin (red) as described in "Experimental Procedures" (**B**) Effect of rottlerin on PMA-stimulated nSMase2 translocation. After preincubation, cells were stimulated with PMA (100nM) for 30m. (**C**) Effect of rotterlin on TNF-stimulated nSMase2 translocation. After preincubation, cells were stimulated

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with TNF (50ng/ml) for 30m. Pictures are representative of >5 fields taken from at least 4 independent experiments.

Figure 3. siRNA downregulation implicates PKC-δ in PMA- and TNF-stimulated nSMase2 translocation. (**A**) Validation of novel PKC siRNA. A549 cells were seeded in 60mm dishes (65K) and 24h later were transfected with negative control (Scr) or novel PKC siRNA (20nM). After 48h, total protein was extracted and immunoblotted for novel PKCs as described in "Experimental Procedures." (**B**) & (**C**) Cells were seeded in 35mm-confocal dishes and, 24h later, were transfected with Scr, PKC-δ, -ε, or -θ siRNA. After 48h, cells were transfected with 3'V5-tagged nSMase2 (0.25μg/dish) for 12h, media was changed and cells were allowed to grow for 3-6h prior to stimulation as indicated. Cells were fixed and stained with anti-V5 (green) and anti-giantin (red) as described in "Materials and Methods." (**B**) Effect of PKC siRNA on PMA-stimulated nSMase2 translocation. Following siRNA downregulation and nSMase2 transfection, cells were stimulated with PMA (100nM) for 30m. (**C**) Effect of PKC siRNA on TNF-stimulated nSMase2 translocation. Following siRNA downregulation and V5-nSMase2 transfection, cells were stimulated with TNF (50ng/ml) for 30m. Pictures are representative of >5 fields taken from at least 3 independent experiments performed in duplicate.

Figure 4. PKC does not regulate *in vitro* nSMase2 activity in A549 cells. (**A**) A549 cells with (solid squares) and without (open circles) nSMase2 overexpression were stimulated with PMA (100nM) for 0-60m (* P <0.05, n=5); (**B**) A549 cells were transfected with negative control (Scr), PKC-δ or -ε siRNA (20nM) for 48-60h prior to stimulation with TNF for 5 min (* P <0.05, n=4). *In vitro* N-SMase activity was measured as described in "Materials and Methods"

Figure 5. Co-immunoprecipitation studies of PKC-δ and nSMase2. A549 cells (1.5-2 x 10^5) were seeded in 60mm dishes and 48h later were either mock-transfected (UTF) or transiently transfected with 3'V5-tagged nSMase2 (2μg/dish) for 12h, media was changed and cells were allowed to grow for 6-12h before (**A**) PMA (100nM) and (**B**) TNF (50ng/ml) stimulation as indicated. V5-nSMase2 and PKC-δ were immunoprecipitated from cell lysates as described in "Materials and Methods." Immunoprecipitates (IP) and supernatants (SUP) were analyzed for V5-nSMase2 and PKC-δ content by SDS-PAGE and immunoblotting. Shown are immunoblots of V5-nSMase2 and PKC-δ immunoprecipitates and supernatants, representative of at least 3 independent experiments. N = negative control.

Figure 6. p38 MAPK is not involved in PMA-stimulated nSMase2 translocation. A) A549 cells (200K) were seeded in 35mm-confocal dishes and 48h later were transiently transfected with 3'V5-tagged nSMase2 (0.25μg/dish) for 12h, media was changed and cells were allowed to grow for 3-6h prior to preincubation with SB202190 (10μM). Subsequently, cells were stimulated with PMA (100nM) for 30m, fixed and stained with anti-V5 (green) and anti-giantin (red) as described in "Materials and Methods." (**B**) & **(C**) A549 cells (2 x 10^5) were seeded in 60mm dishes and 48h later, media was changed for 1-2h. Cells were preincubated with (**B**) bisindolylamide I (2uM) or G06976, or (**C**) rottlerin (10uM) for 1h prior to stimulation with PMA (100nM) for 30m, total protein was extracted and immunoblotted for phospho-p38 MAPK and p38 MAPK as described in "Experimental Procedures." (* P <0.05, n=4).

Figure 7. **PKC is not upstream of p38 MAPK in response to TNF.** (**A**) & (**B**) A549 cells (200K) were seeded in 60mm dishes and, 48h later, were preincubated with (**A**) bisindolylamide I (2uM) or

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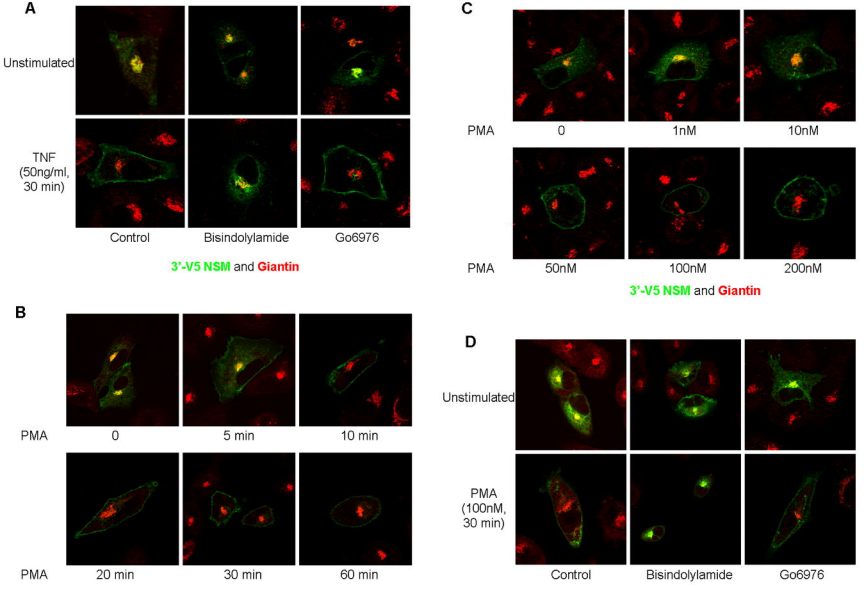
G06976 (3uM) or (**B**) rottlerin (10uM) for 1h prior to stimulation with TNF (50ng/ml, 10m). (**C**) A549 cells (65K) were seeded and 24h later, transfected with negative control (Scr) or novel PKC siRNA. After 48-60h, media was changed, cells incubated for 1-2h and stimulated with TNF (3nM, 10m). Total protein samples were prepared and analyzed for phospho-p38 MAPK and p38 MAPK as described in "Materials and Methods." (* P <0.05 vs unstimulated, n=5).

Figure 8. PKC-δ is the major novel PKC regulated by TNF in A549 cells. A549 cells (200K) were seeded in 60mm dishes and, 48h later, media was changed. Cells were incubated for 1-2h and then stimulated with TNF (50ng/ml) for 0-60m as indicated. Total protein samples were prepared in the presence of phosphatase inhibitors and analyzed for (**A**) phospho-PKC-δ (Thr-505), (**B**) phospho-PKC-ε (Ser-729) and phospho-PKC-θ (Thr-538) as described in "Materials and Methods." Blots were reprobed with total PKC antibodies as loading controls.

Fig. 9. PKC-δ is upstream of TNF-stimulated VCAM and ICAM induction in A549 cells. A549 cells were seeded in 60mm dishes (70K) and 24h later were transfected with negative control (Scr), PKC-δ or -ε siRNA (20nM). After 48-60h, cells were stimulated with vehicle or TNF (3nM) for 3h, total protein was extracted and analyzed for VCAM and ICAM levels by immunoblot. (**A)** Representative immunoblot; (**B**) Quantification of effect of PKC siRNA on ICAM (* P<0.05, n=4); (**C**) Quantification of effect of PKC siRNA on VCAM (* P<0.05, n=4).

Figure 10. PKC-δ is upstream of nSMase2 but is not upstream of p38 MAPK in TNF and PMA responses. TNF-induced translocation of nSMase2 from the Golgi to the plasma membrane (PM) requires both p38 MAPK and PKC-δ. However, although p38 MAPK is upstream of nSMase2 activation

by post-translational modification (*-nSMase2), PKC-δ does not play a role. The colored dashed lines represent three alternative possibilities: 1) TNF activates PKC-δ independently of p38 MAPK; 2) p38 MAPK may regulate nSMase2 through activation of PKC-δ; 3) p38 MAPK regulates nSMase2 independently of PKC-δ activation. With PMA, nSMase2 translocation is independent of p38 MAPK and activation of p38 MAPK is downstream of a classical PKC (cPKC) isoform in a pathway distinct from the PKC-δ-mediated regulation of nSMase2.


A
Unstimulated
TNF
(50ng/ml,
30 min)
Control
Bisindolylamide
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3'-V5 NSM and Giantin
B
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0
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PMA
50nM
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3'-V5 NSM and Giantin
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30 min)
Control
Bisindolylamide
Go6976

FIGURE 1

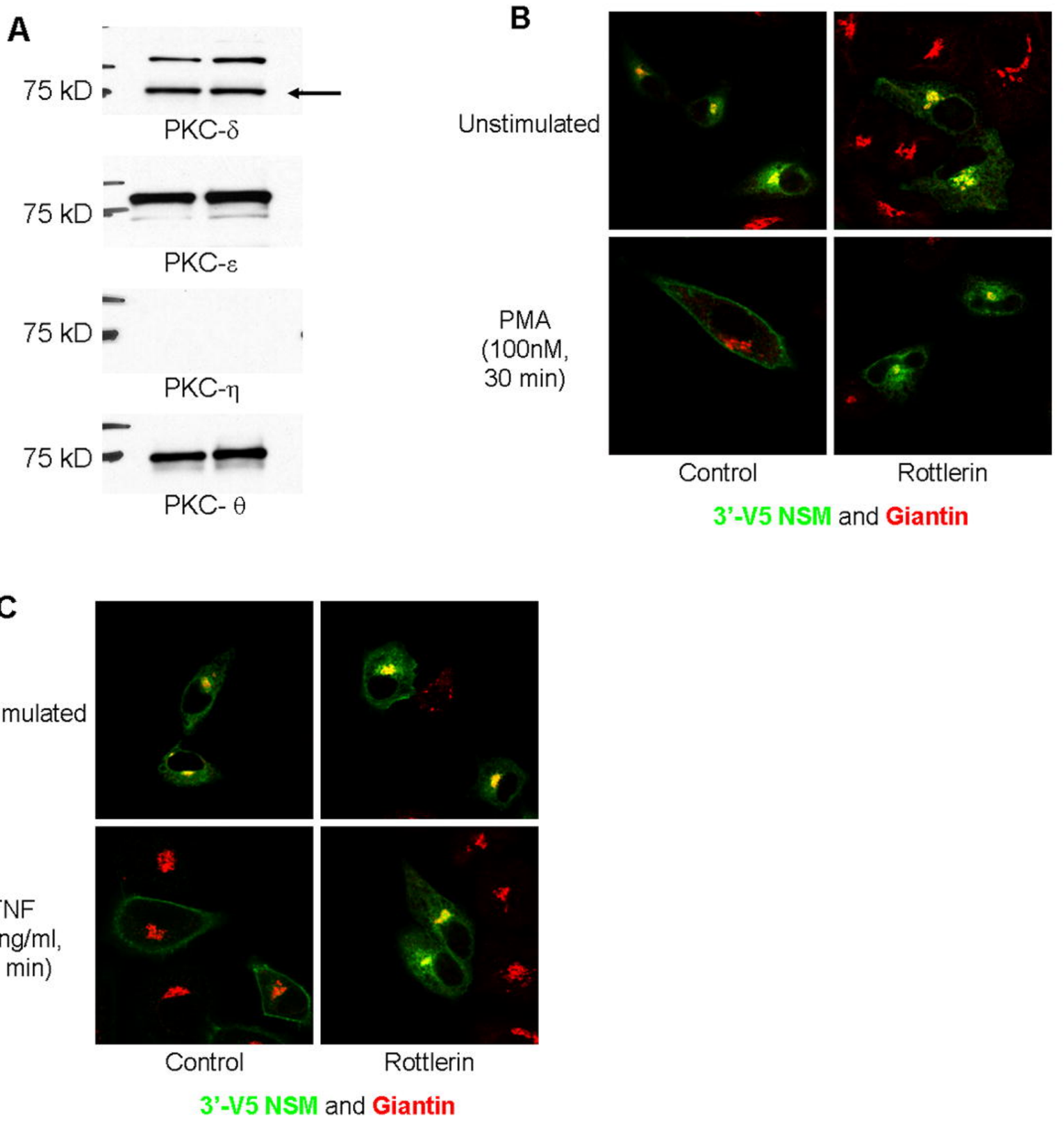
A
75 kD
PKC-δ
75 kD
PKC-ε
75 kD
PKC-η
75 kD
PKC- θ
B
Unstimulated
PMA
(100nM,
30 min)
Control
Rottlerin
3'-V5 NSM and Giantin
C
Unstimulated
TNF
(50ng/ml,
30 min)
Control
Rottlerin
3'-V5 NSM and Giantin

Figure 2

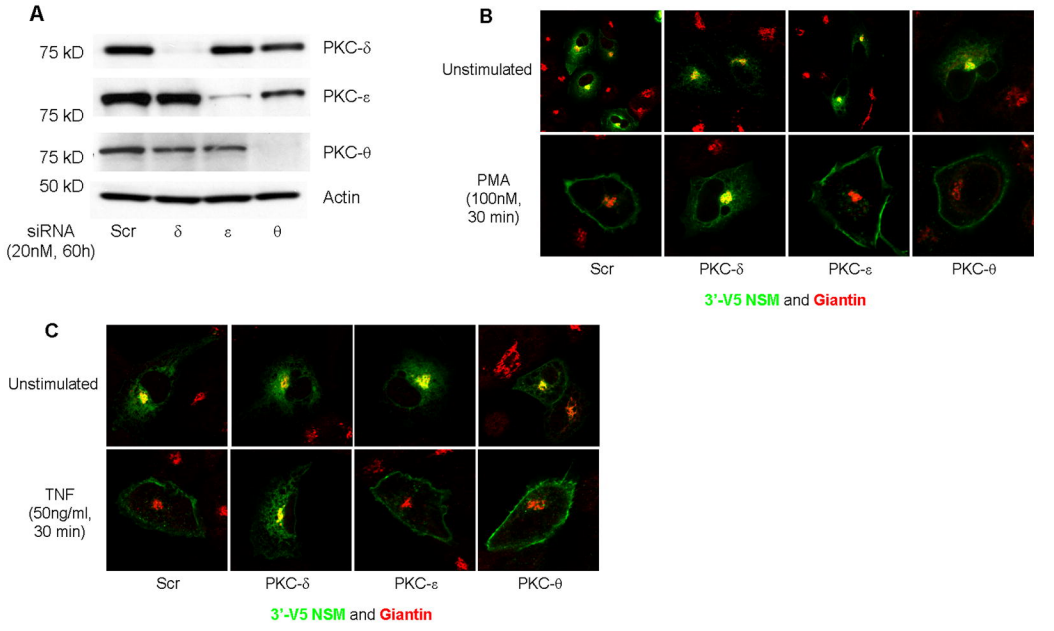
A
75 kD
PKC-δ
PKC-ε
75 kD
75 kD
PKC-θ
50 kD
Actin
siRNA
(20nM, 60h)
Scr
δ
ε
θ
B
Unstimulated
PMA
(100nM,
30 min)
Scr
PKC-δ
PKC-ε
PKC-θ
3'-V5 NSM and Giantin
C
Unstimulated
TNF
(50ng/ml,
30 min)
Scr
PKC-δ
PKC-ε
PKC-θ
3'-V5 NSM and Giantin

Figure 3

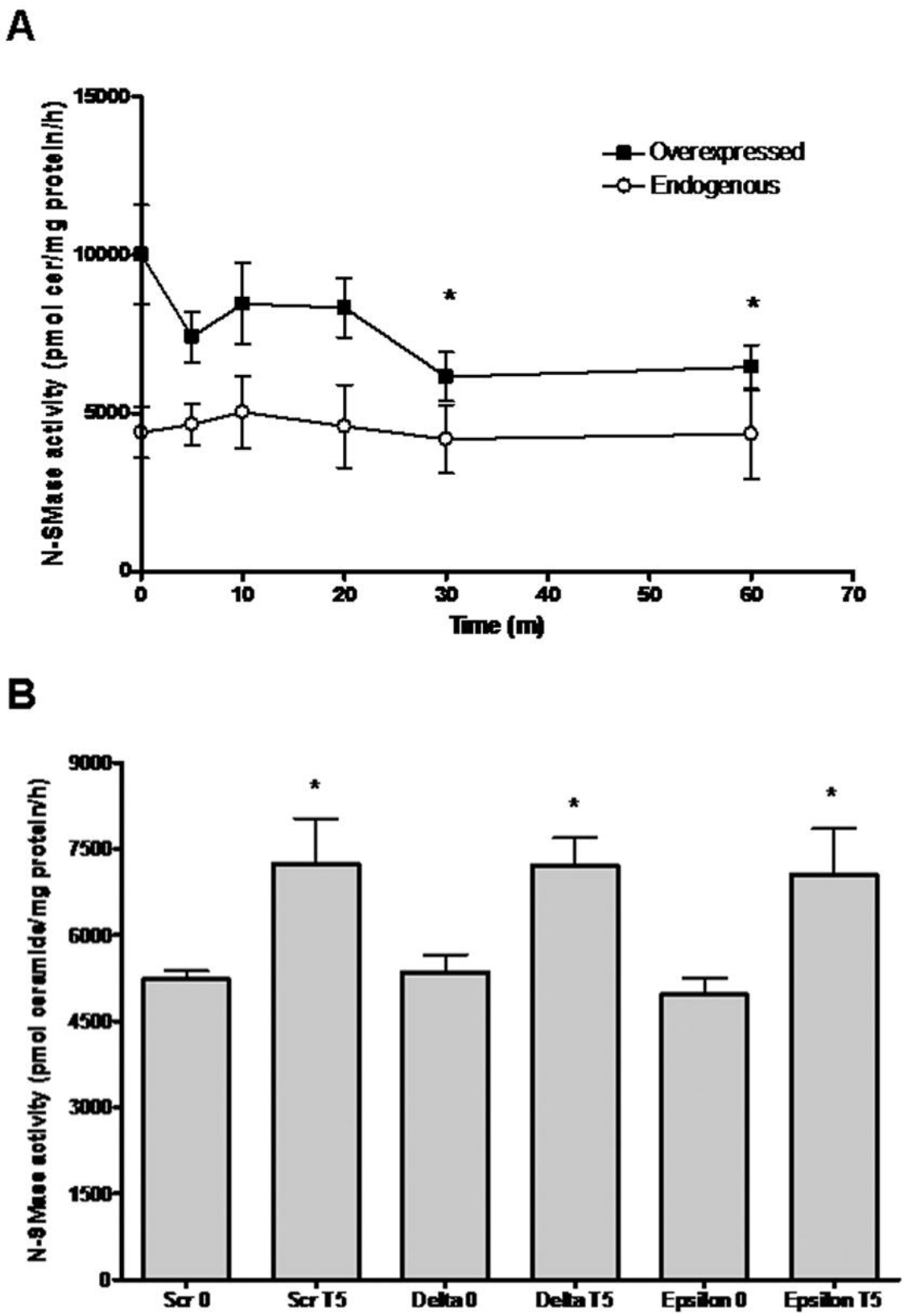
A
15000
10000
5000
0
N-SMase activity (pmol cer/mg protein/h)
Overexpressed
Endogenous
*
*
0
10
20
30
40
50
60
70
Time (m)
B
9000
7500
6000
4500
3000
1500
0
N-SMase activity (pmol ceramide/mg protein/h)
*
*
*
Scr 0
Scr T5
Delta 0
Delta T5
Epsilon 0
Epsilon T5

Figure 4

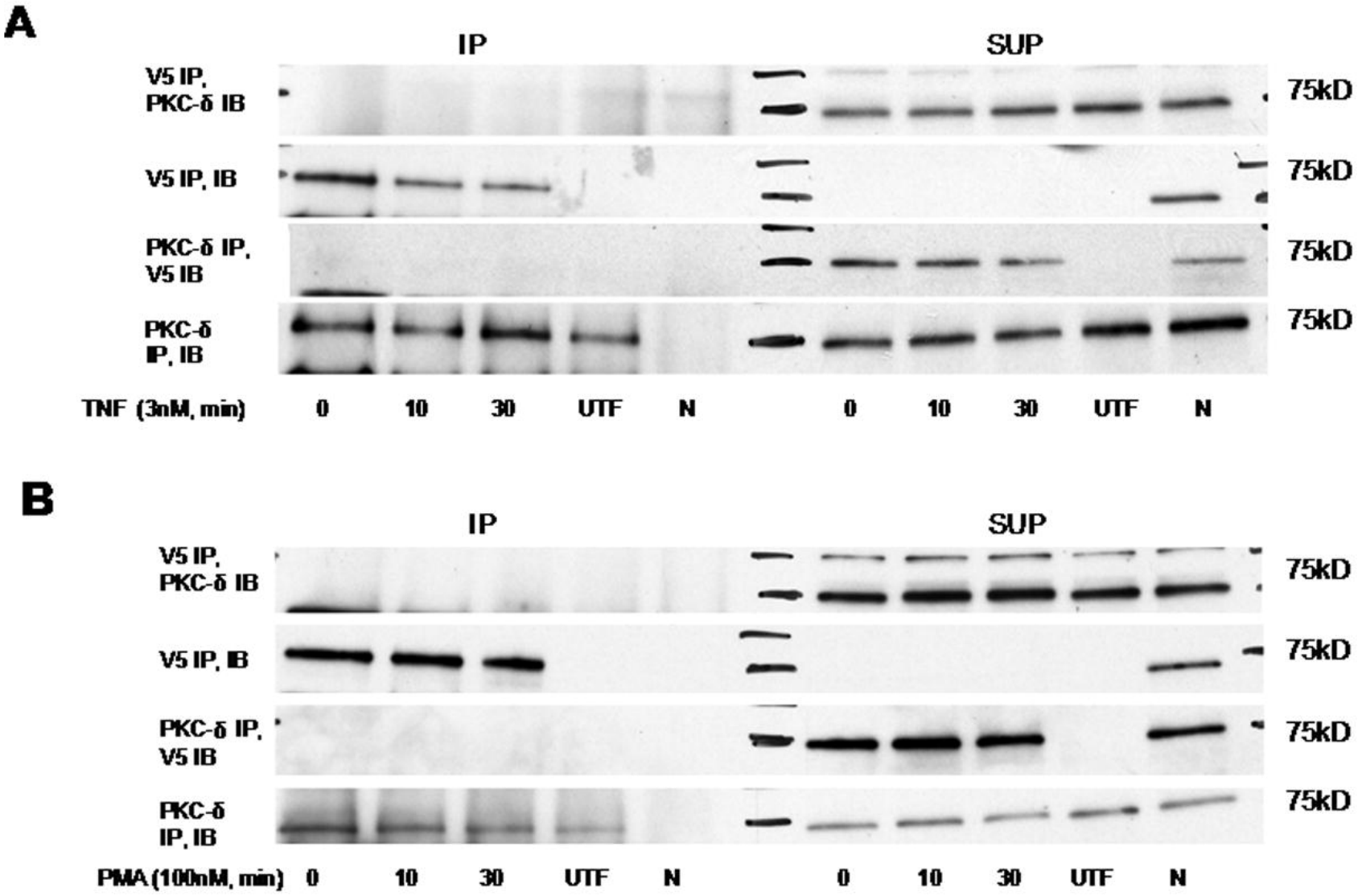
A
IP
SUP
V5 IP, PKC-δ IB
V5 IP, IB
PKC-δ IP, V5 IB
PKC-δ IP, IB
75kD
75kD
75kD
75kD
TNF (3nM, min)
0
10
30
UTF
N
0
10
30
UTF
N
B
IP
SUP
V5 IP, PKC-δ IB
V5 IP, IB
PKC-δ IP, V5 IB
PKC-δ IP, IB
75kD
75kD
75kD
75kD
PMA (100nM, min)
0
10
30
UTF
N
0
10
30
UTF
N

Figure 5

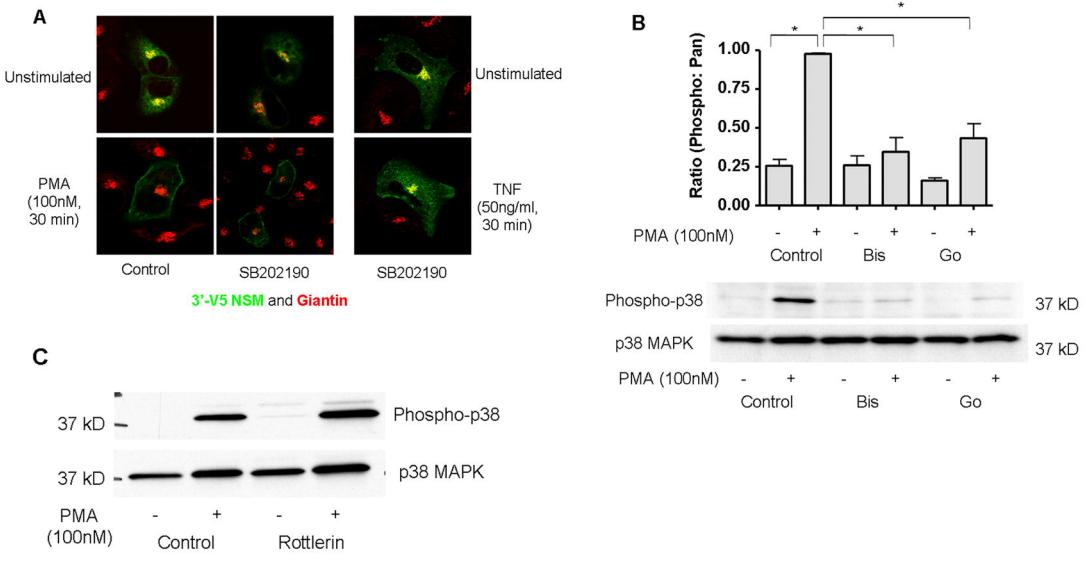
A
Unstimulated
PMA (100nM, 30 min)
Unstimulated
TNF (50ng/ml, 30 min)
Control
SB202190
SB202190
3'-V5 NSM and Giantin
B
Ratio (Phospho: Pan)
1.00
0.75
0.50
0.25
0.00
PMA (100nM)
- + - + - +
Control
Bis
Go
Phospho-p38
37 kD
p38 MAPK
37 kD
PMA (100nM)
- + - + - +
Control
Bis
Go
C
37 kD
Phospho-p38
37 kD
p38 MAPK
PMA (100nM)
- + - +
Control
Rottlerin

Figure 6

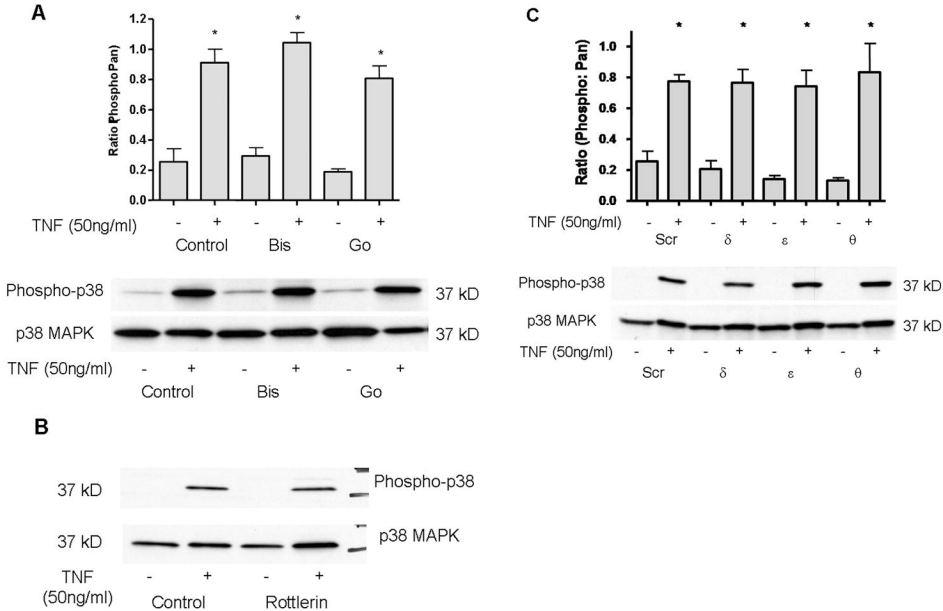
A
Ratio PhosphoPan)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
TNF (50ng/ml) - + - + - +
Control Bis Go
Phospho-p38
37 kD
p38 MAPK
37 kD
TNF (50ng/ml) - + - + - +
Control Bis Go
C
Ratio (Phospho: Pan)
1.0
0.8
0.6
0.4
0.2
0.0
TNF (50ng/ml) - + - + - + - +
Scr δ ε θ
Phospho-p38
37 kD
p38 MAPK
37 kD
TNF (50ng/ml) - + - + - + - +
Scr δ ε θ
B
37 kD
Phospho-p38
37 kD
p38 MAPK
TNF (50ng/ml) - + - +
Control Rottlerin

Figure 7

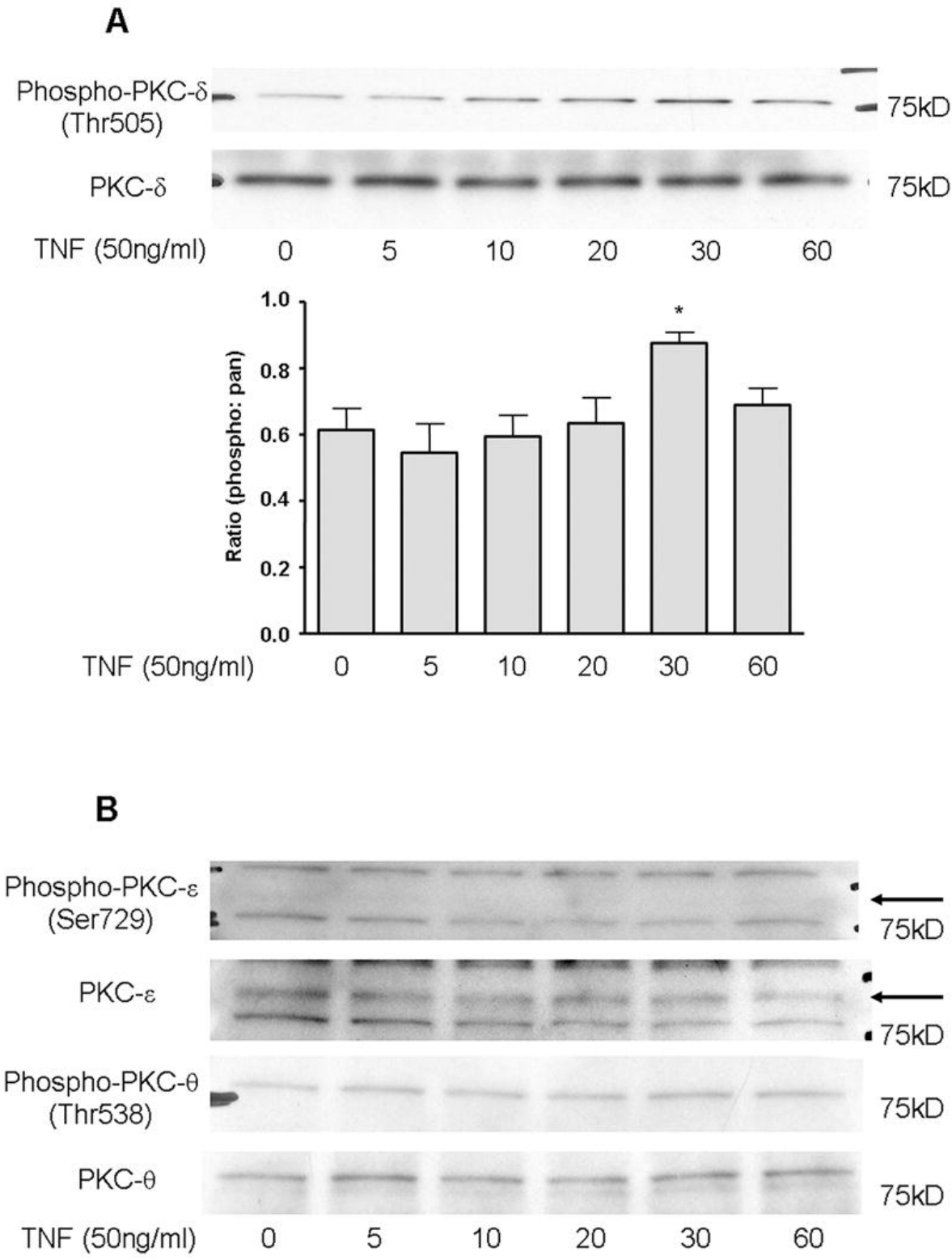
A
Phospho-PKC-δ
(Thr505)
75kD
PKC-δ
75kD
TNF (50ng/ml)
0
5
10
20
30
60
Ratio (phospho: pan)
1.0
0.8
0.6
0.4
0.2
0.0
*
TNF (50ng/ml)
0
5
10
20
30
60
B
Phospho-PKC-ε
(Ser729)
75kD
PKC-ε
75kD
Phospho-PKC-θ
(Thr538)
75kD
PKC-θ
75kD
TNF (50ng/ml)
0
5
10
20
30
60

Figure 8

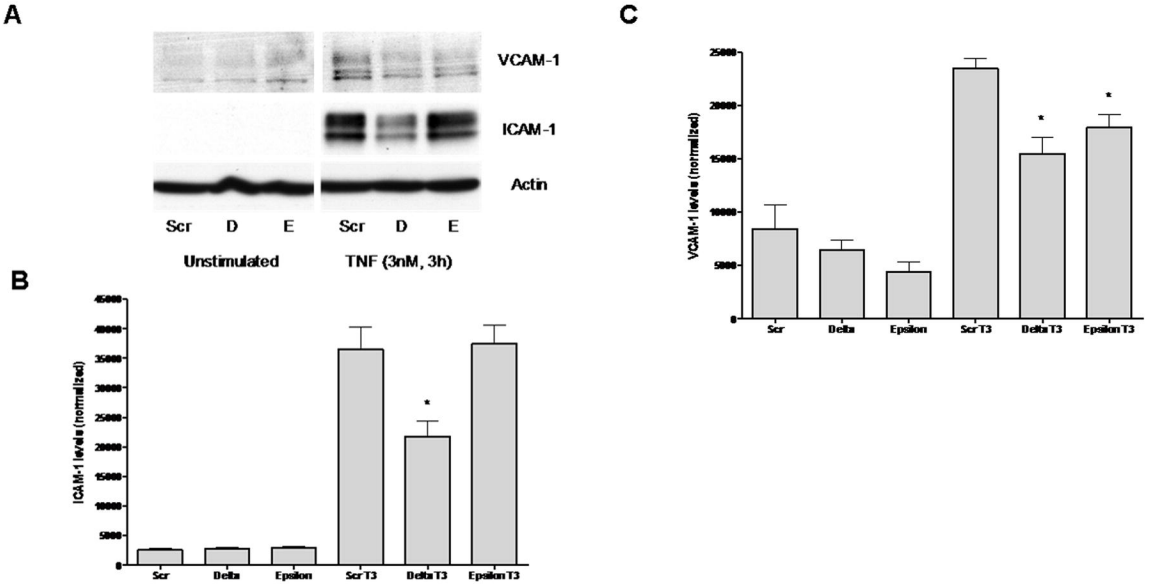
A
VCAM-1
ICAM-1
Actin
Scr D E Scr D E
Unstimulated
TNF (3nM, 3h)
B
ICAM-1 levels (normalized)
Scr
Delta
Epsilon
Scr T3
Delta T3
Epsilon T3
C
VCAM-1 levels (normalized)
Scr
Delta
Epsilon
Scr T3
Delta T3
Epsilon T3

Figure 9

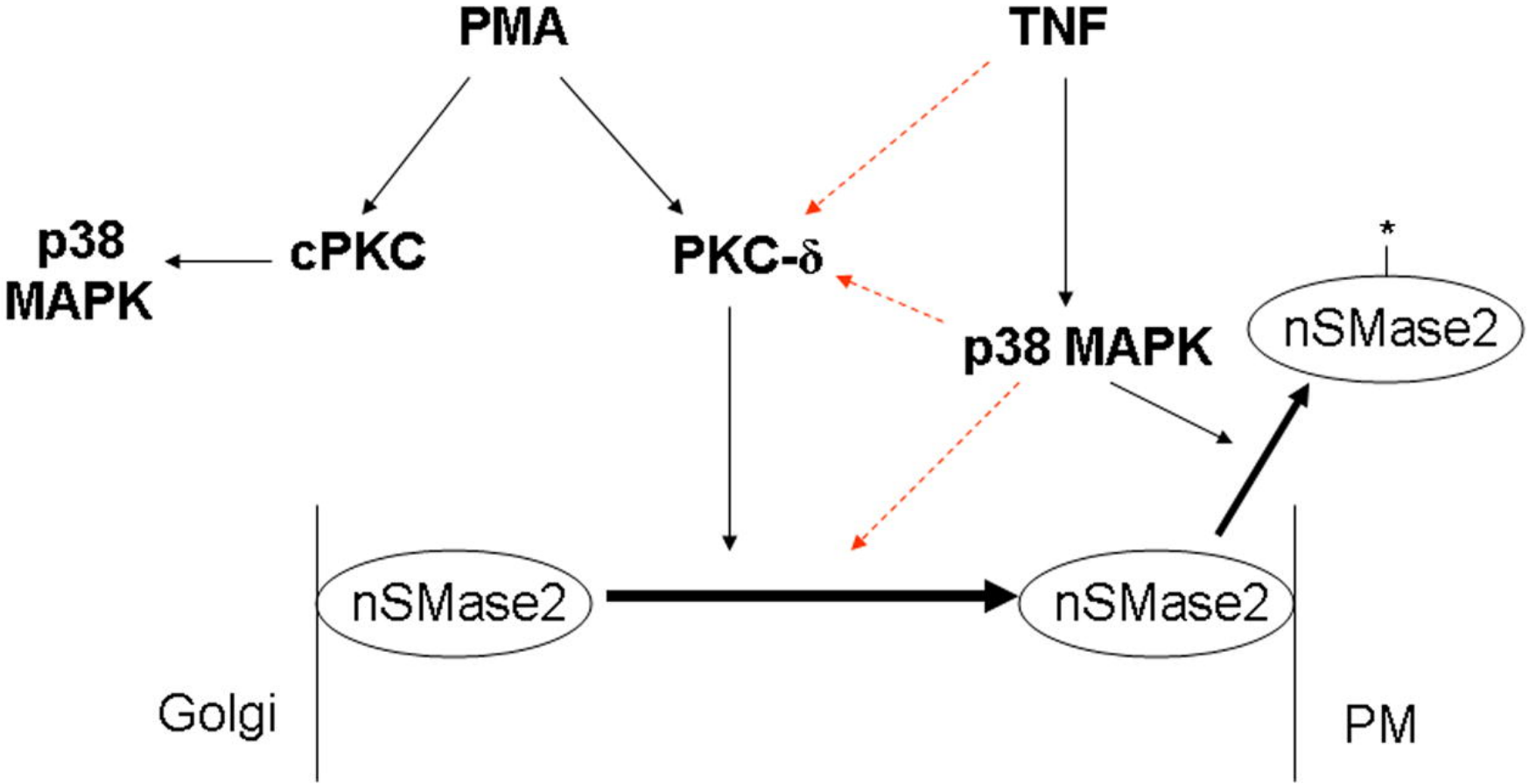
PMA
TNF
p38
MAPK
cPKC
PKC-δ
p38 MAPK
*
nSMase2
nSMase2
nSMase2
Golgi
PM

Figure 10