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Involvement of PKCα in PMA-induced facilitation of exocytosis and vesicle fusion in PC12 cells

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Abstract

Phorbol-12-myristate-13-acetate, a stable analogue of the important signaling membrane lipid diacylglycerol (DAG), is known to potentiate exocytosis and modulate vesicle fusion kinetics in neurons and endocrine cells. The exact mechanisms underlying the actions of PMA, however, is often not clear, largely because of the diversity of the DAG/PMA receptors involved in the exocytotic process, which include, most notably, various isoforms of protein kinase C (PKC). In the present study, the roles of PKCα in PMA-mediated regulation of exocytosis were investigated by over-expressing wild-type PKCα (wt-PKCα) or dominant negative PKCα (dn-PKCα). Amperometric measurements based on carbon fiber microelectrodes demonstrated that PKCα has a key role in the PMA-mediated facilitation of exocytosis and vesicle fusion in neuroendocrine PC12 cells.

Keywords: exocytosis; protein kinase C; amperometry; PMA; DAG

Regulated secretion, or exocytosis, of hormones and neurotransmitters is regulated by a plethora of proteins and lipids through their specific actions at distinct stages in the exocytotic cascade, involving vesicle trafficking, docking, priming, and final Ca^{2+}-dependent fusion [1]. A large body of experiments has demonstrated that exocytosis is regulated acutely by protein phosphorylation [2], most notably *via* protein kinase C (PKC) [3,4]. Several key secretory proteins, such as SNAP-25 [5,6], synaptotagmin [7], and Munc18 [8,9], are PKC substrates, suggesting the involvement of PKC in various aspects of exocytosis.

PKC functions are often examined using pharmacological tools, such as phorbol-12-myristate-13-acetate (PMA), a potent diacylglycerol (DAG) analogue that acts as a PKC activator, and bisindolylmaleimide (BIS), which blocks the ATP-binding sites in the catalytic domain of many PKCs and acts as a PKC inhibitor. PMA has been shown to potentiate exocytosis [10-13] and modulate vesicle fusion in various cell systems [14,15]. Many of these PMA effects are thought to be PKC-dependent, as they can be eliminated partially or completely by treatment with BIS. However, the exact mechanisms underlying the actions of PMA is often not clear, largely because of the diversity of PKC isoforms and the caveats of pharmacological assessment. The PKC family consists of conventional PKCs (α, β, and γ), novel PKCs (δ, ε, η, and θ), and atypical PKCs (ζ, τ, λ) [3]. To avoid these problems, over-expression of wild-type and dominant negative forms of individual PKC isoforms were used in this study to elucidate PKC-dependent PMA effects on exocytosis.

PKCα is expressed abundantly in neuron and neuroendocrine cells. In particular, it is the only conventional PKC expressed in bovine chromaffin cells [16], and it is the most

constitutively active PKC isoform in rat chromaffin cell-derived PC12 cells [17]. In this study, by over-expressing wild-type PKCα (wt-PKCα) and dominant negative PKCα (dn-PKCα), we demonstrated that PKCα is largely involved in the PMA-induced increase in the extent of exocytosis, the facilitation of fusion pore expansion, and the augment of quantal release in PC12 cells.

Materials and methods

Cell culture and transfection

PC12 cells (ATCC) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 5% horse serum and 1% penicillin-streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO_2 / 95% air. PC12 cells were transfected with plasmid DNA using ESCORT III transfection reagent (Sigma), 1-2 days prior to the experiments. Cells were planted on poly-L-lysine pre-coated coverslips and treated with 5 μM dexamethasone for 3-5 days as previously described [18,19] to enhance chromaffin cell phenotype.

Plasmids

The cDNA plasmids coding for wt- and dn-PKCα were obtained from Addgene Plasmid Collection. The pIRES2-EGFP vector was purchased from Clontech. The wt- or dn-PKCα-IRES-EGFP was constructed by subcloning cDNA of PKCα or its mutation into pIRES2-EGFP vector at the sites of Nhe I and Sma I.

Amperometry measurement

The bath solution contained (in mM): 150 NaCl, 5.5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES (titrated to pH 7.2 with NaOH); the high K^+ stimulation solution contained (in mM): 37 NaCl, 105 KCl, 6 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES (titrated to pH 7.2). In some experiments, cells were treated with 0.1 μM PMA for 10 minutes or / and 2.5 μM BIS for 30 minutes.

Exocytosis was induced by perfusing the high K^+ solution through a glass micropipette (tip size of 2~3 μm) positioned 5~10 μm away from the cell. Amperometric spikes were recorded by a 5 μm-in-diameter carbon fiber electrode (ALA Scientific Instruments, USA) gently positioned onto the cell membrane. The microelectrode was biased at 700 mV and the amperometry signals were sampled at 4 kHz and filtered at 1 kHz using an EPC-10 double patch-clamp amplifier (HEKA Electronik, Germany).

The parameters of amperometric spikes were obtained using an Igor program, Amperometric Spike Analysis [20]. Only spikes with amplitudes larger than 2 pA (5 times of the background noise) were considered as true amperometric events. Statistics of the spike parameters is represented by mean ± SEM. Statistical significance is evaluated with Student's *t*-test or Kolmogorov–Smirnov test.

Results and discussion

In comparison to electrophysiological measurement of membrane capacitance [21,22], amperometry using carbon fiber microelectrodes is instrumental in the direct detection of exocytosis with single-vesicle sensitivity and revealing vesicle fusion kinetics with millisecond resolution. Upon Ca^{2+} triggering, vesicle fusion starts with formation of a nanometric aqueous fusion pore through which secretory compounds escape from the

vesicular lumen into extracellular space, sometimes giving a small foot signal preceding the amperometric spike. The fusion pore then expands quickly to discharge the secretory compounds, causing a rapid increase in the amperometric signal. Finally, expansion of the fusion pore expansion may end in full collapse, or terminate prematurely in a so called kiss-and-run mode, which gives a more transient amperometric signal [23,24].

PMA modulates both the extent and the fusion kinetics of vesicle exocytosis

It has long been recognized that PMA enlarges the extent of Ca^{2+}-dependent exocytosis [12-14]. Fig. 1A presents typical amperometric recordings from PC12 cells in response to a membrane-depolarizing high-K^+ solution under different conditions. Each current spike corresponds to a single vesicle fusion event. PMA increased the number of exocytotic events per stimulus significantly (133.3 ± 12.1%, 11 cells, with respect to the average response from 22 control cells), and this enhancement was eliminated completely by BIS (90.5 ± 10.2%, 11 cells). Furthermore, BIS alone inhibited exocytosis severely compared to the control (38.1 ± 5.5%, 19 cells). Therefore, it is reasonable to conclude that the PMA enhancement is PKC-dependent and the basal activities of PKCs are important to support exocytosis.

The averaged amperometric spikes obtained from more than 400 fusion events in at least 11 cells are shown in Fig. 1B. In contrast to that from the control cells, the average amperometric signal from the PMA-treated cells has a steeper rising phase, indicating more rapid opening of the fusion pore. The rate of catecholamine release or the rate of pore expansion can be quantified by the slope of the rising amperometric signal between 35% and 90% of the peak amplitude. As shown in Fig. 1C, the average rise slope in the PMA-treated cells is significantly greater than that in the control cells (10.1 ± 0.3 pA/ms

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vs. 6.8 ± 0.2 pA/ms, $P < 0.001$). The acceleration of pore expansion likely relies on PKC activation, because BIS eliminated this effect of PMA. Application of BIS alone, however, did not cause apparent reduction in the fusion rate. It seems that basal PKC activities are not required for opening the fusion pore. Similar PMA facilitation of fusion pore opening has been found in chromaffin cells [14,15], in which PMA also causes premature closure of the fusion pore, leading to kiss-and-run fusion with small quantal sizes [9,14]. By contrast, the quantal size of vesicle release in PC12 cells, or the total charge (Q) from integration of the amperometric current signal, was enlarged in response to treatment with PMA. Since the distribution of Q is known to be severely skewed, we used the cube-root of the spike charge ($Q^{1/3}$) for analysis. Student's *t*-test can be applied to determine statistically significant differences, since $Q^{1/3}$ has a Gaussian distribution, and is proportional to the radius of the fusion vesicles [25]. As demonstrated in Fig. 1D, treatment with PMA increased $Q^{1/3}$ appreciably. The ability of BIS to reverse this PMA-mediated enlargement again suggests the involvement of PKC. BIS application alone decreased the quantal size significantly (Fig. 1D), implying that basal PKC activities are important for maintaining the normal quantal size.

It is clear from the data presented here and as well as that reported by others that PMA plays multiple regulatory roles in the Ca^{2+}-dependent exocytotic process, usually through activation of PKCs. We further hypothesize that PKCα, which is the abundant and most active conventional PKC isoform in PC12 cells activated by DAG and Ca^{2+}, has an important role in PMA-mediated regulation of Ca^{2+}-dependent exocytosis.

PKCα contributes to PMA-induced enhancement of exocytosis

The wt- or dn-PKCα created by a K368N mutation to permanently inactivate the ATP-binding site of PKCα catalytic domain was over-expressed in PC12 cells. The cDNA of PKCα is constructed together with enhanced green fluorescence protein (EGFP) in the same plasmid *via* an internal ribosome entry site (IRES) [26], which links two open reading frames. Thus, EGFP is co-expressed with PKCα as reporter.

Fig. 2 depicts the average time-courses and statistics of the amperometric responses to 60 s of high-K^+ stimulation of PC12 cells transfected with different plasmids, and with or without treatment with PMA. Only bright green cells, in which EGFP (hence PKCα) was amply expressed, were chosen for recording. Over-expression of dn-PKCα largely reduced the extent of exocytosis by 34% ($P < 0.01$) in comparison with over-expression of the empty vector (pIRES2-EGFP), indicating that competitive inhibition of endogenous PKCα activities impairs exocytosis. This is analogous to the effect of treatment with BIS. Compared to dn-PKCα, over-expression of dn-PKCε was much less inhibitory (12.9% reduction, $P < 0.05$, 25 cells). PKCε is a novel PKC isoform expressed in PC12 cells [17], and was found to be implicated in activity-dependent potentiation of exocytosis in chromaffin cells [27].

The over-expression of wt-PKCα gave only a marginal increase in exocytosis. Apparently, the abundance of PKCα is not sufficient to elevate exocytosis significantly. PMA together with PKCα over-expression, however, caused pronounced enhancement, greater than its potentiation in the vector cells (35% *vs.* 26%). On the other hand, PMA failed to rescue exocytosis in the dominant-negative cells. These observations provide strong support for the notion that PKCα plays a key role in PMA potentiation.

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PMA increases the rate of fusion pore expansion and enlarges the fusion quantal size in a PKCα-dependent manner

Fig. 3 presents the average amperometric spikes and statistical analysis for different experimental conditions. As expected, in the empty vector-expressing cells, PMA increased the speed of pore expansion as evidenced by the steeper amperometric rising phase. In contrast, such PMA-induced acceleration was absent from the cells expressing dn-PKCα; i.e., dn-PKCα, just like BIS, is able to abolish this PMA effect. This observation suggests the minor involvement of other PMA targets and the importance of PKCα activation in this phenomenon. Also similar to the BIS effect demonstrated in Fig. 1, over-expression of dn-PKCα without PMA did not affect the slope of the rising phase. Therefore, basal PKCα activities are probably not critically involved in normal fusion pore opening. The observations that over-expression of wt-PKCα gave a sharper rise in the amperometric signal, and that PMA could not further strengthen this effect in wt-PKCα over-expressing cells, suggest that the abundance of PKCα, but not that of DAG, is the limiting factor in activating the acceleration mechanism for fusion.

As anticipated from the earlier experiments (Fig. 1), PMA enlarged the quantal size in the vector cells. Again, analogous to BIS actions, dn-PKCα over-expression eliminated this PMA-induced augmentation and led to notably smaller quantal size in the absence of PMA. These observations clearly highlight the importance of PKCα activation in supporting normal vesicle fusion and PMA enhancement. Endogenous PKCα is adequate to ensure PMA-induced enhancement, as over-expression of wt-PKCα did not boost the effect of PMA further.

The results of this study are contrast to those of a recent study on rat chromaffin cells [28], in which the authors, mainly on the basis of pharmacological assessments, reported that it is novel PKCθ and not conventional PKC isoforms that is important in maintaining normal quantal size and the normal extent of exocytosis. The discrepancy between their findings and those presented here may be attributable to differences in the cell types used, and the stimulation protocols. Firstly, PC12 cells do not express PKCθ [29]. Secondly, we recorded the amperometric responses during 60 s of high-K^+ stimulation, while they recorded the responses after only a brief (3 s) high-K^+ stimulation. In addition, unlike chromaffin cells, which typically have hundreds of releasable pre-docked vesicles, the amperometric responses from PC12 cells are essentially resulted from the release of newly arrived vesicles [30].

We cannot completely rule out the possible involvement of other PKC isoforms in PMA regulations of vesicle fusion, due partly to cross-reactivity in the enzyme–substrate binding. To partially address this issue, we examined the effects of PKCε on vesicle fusion and found that PKCε was not able to modulate the rate of fusion pore expansion: wt (14.40 ± 0.60 pA/ms, 28 cells) *vs.* dn (12.63 ± 0.75 pA/ms, 25 cells), $P > 0.05$. Further, opposite to the effect of nt-PKCα, nt-PKCε enlarged the quantal size ($Q^{1/3}$): wt (3.55 ± 0.02 fC$^{1/3}$) *vs.* dn (3.74 ± 0.03 fC$^{1/3}$), $P < 0.001$.

PKC facilitation of vesicle fusion can be achieved by modulating the fusion protein machinery or by enzymatic alteration of the lipidic structures of the fusion pore. Phospholipase D (PLD), which can be activated by PKC [31], produces phosphatidic acids (PAs) at the inner leaflet of the cell membrane at the fusion site. Cone-shaped PAs energetically favor the formation of a negatively curved fusion pore and promote its

expansion [32]. We investigated the effects of 1-butanol, which blocks formation of PA by competitive binding to PLD [33], on the fusion kinetics. It was found that 1-butanol attenuated the spike slope from 6.81 ± 0.20 pA/ms (22 untreated cells) to 4.14 ± 0.35 pA/ms (11 treated cells) $P < 0.001$. But the application of PMA was able to rescue the amperometric rise slope of the cells treated with 1-butanol (6 cells) to a level even faster than that of the control (8.70 ± 0.85 pA/ms $vs.$ 6.81 ± 0.20 pA/ms; $P < 0.001$). So, PLD is unlikely to be the target of PMA (thus PKC) in facilitating fusion pore expansion.

Foot probability is lowered by PMA through PKC activities

Fusion between vesicular and plasma membrane must overcome substantial energy barriers. When the driving force provided by the protein fusion machinery barely overwhelms these energy barriers, a small pre-spike foot in the amperometric signal occurs due to slow expansion of the fusion pore [34]. PMA accelerates fusion pore expansion, likely by lowering certain energy barriers of the fusion process. In line with this view, the probability of recording a foot signal was found to be reduced by treatment with PMA (Fig. 4). Foot probability is obtained by calculating the percentage of the events that have a preceding foot among all the recorded exocytotic events that have amplitude > 20 pA (foot can be resolved definitely only for events with relatively large amplitude [35]). Not surprisingly, this PMA effect is also PKC-dependent, because BIS was able to inhibit it, while BIS application alone increased the foot probability. With reference to the vector-expressing cells, over-expression of dn-PKCα behaved just like BIS to increase the foot probability, whereas wt-PKCα decreased it. These experiments demonstrated that PKC activities, including, but maybe not exclusive to, the PKCα

isoform, lowered the fusion energy barriers leading to a decrease in the number of footed events.

Acknowledgements

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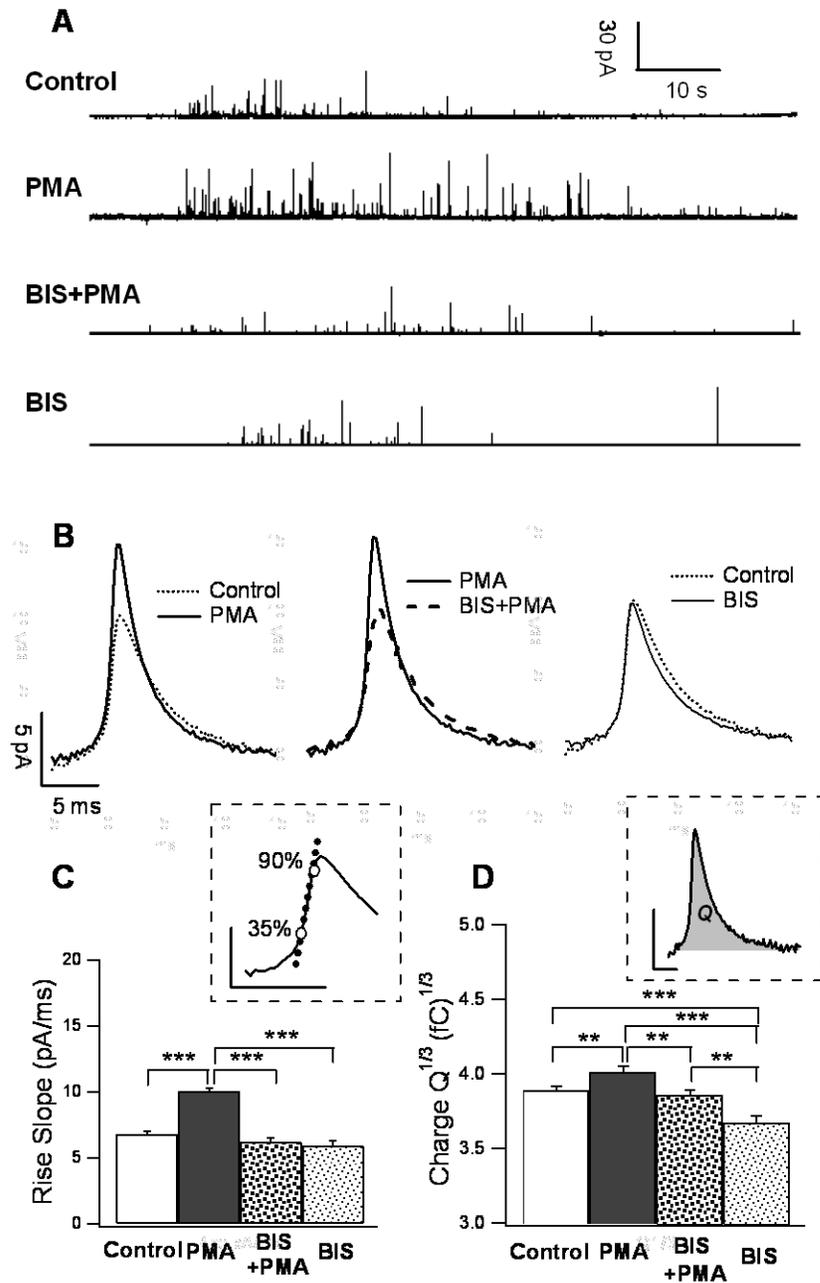
Fig. 1. Effects of PMA and BIS on exocytosis and vesicle fusion. **A**. Representative amperometric recordings from a control, PMA-treated, BIS+PMA-treated or BIS-treated cell, in response to 60 s of stimulation by a high-K^+ solution. **B**. Averaged amperometric spikes of all fusion events from 22 control cells, 11 PMA-treated cells, 11 BIS+PMA-treated cells or 19 BIS-treated cells. **C**. Statistics of the spike rise slope. The rise slope is

defined as the slope of the linear fit between 35% and 90% of the peak amplitude of the spike, as shown in the inset. Kolmogorov–Smirnov test: *** P <0.001. **D**. Statistics of the cube-root of the spike charge, $Q^{1/3}$. The spike charge (Q) was defined as the integration of the amperometric spike current as shown in the inset. Student's t-test: ** P <0.01, *** P <0.001. **C** and **D**, the data are shown as mean ± SEM.
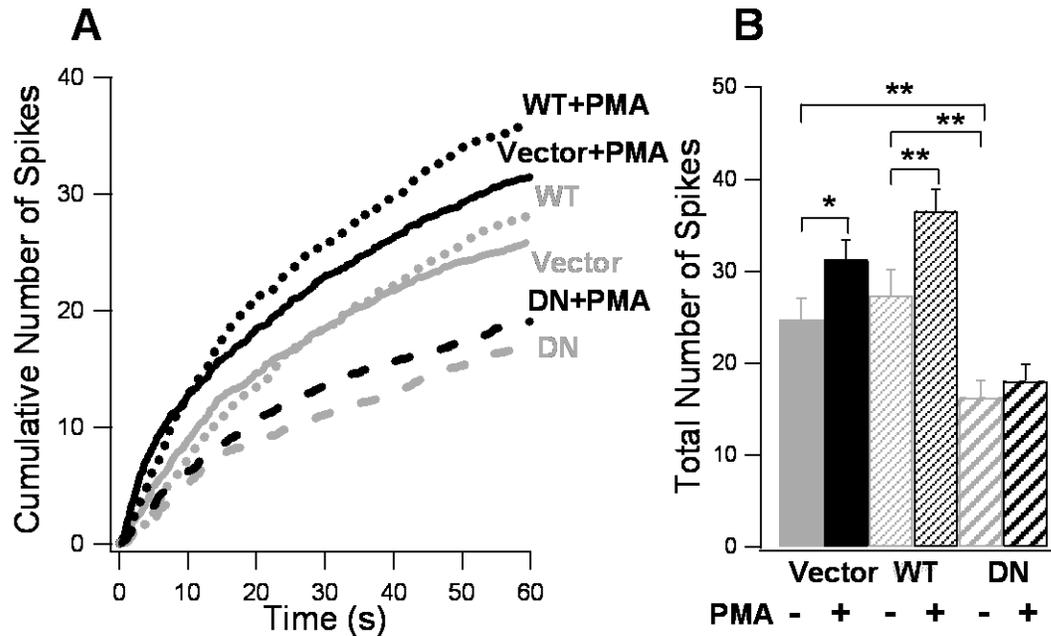
Fig. 2. Effects of PMA on exocytosis in PKCα over-expressing cells. Data were obtained by averaging the responses from 14 empty vector-expressing cells (Vector), 13 PMA-treated vector cells (Vector+PMA), 16 wt-PKCα-expressing cells (WT), 14 PMA-treated WT cells (WT+PMA), 21 dn-PKCα-expressing cells (DN) and 18 PMA-treated DN cells (DN+PMA), during 60 s high-K^+ stimulation. **A.** The cumulative spike number is plotted against time. **B.** The total numbers of the resulting spikes are shown as mean ± SEM. Student's *t*-test: * $P <0.05$, ** $P <0.01$.
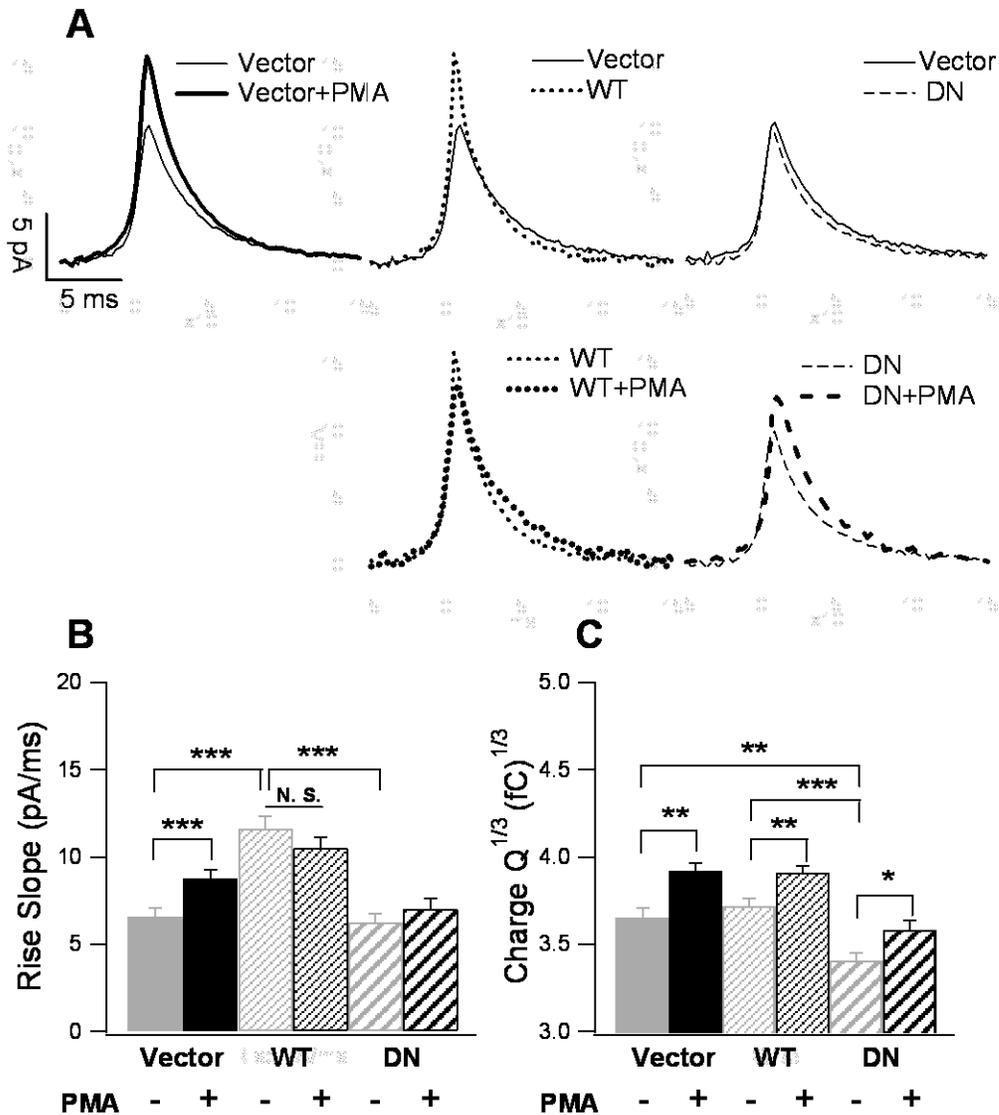
Fig. 3. PMA modulates vesicle fusion *via* PKCα. The analysis is based on the experiments presented in Fig. 2. **A.** The average amperometric spikes of all events from differently treated cells. **B.** The rise slopes are shown as mean ± SEM. Kolmogorov–Smirnov test: *** P <0.001, not significant (N. S.) for P >0.05. **C.** The cube-roots of spike charges ($Q^{1/3}$) are shown as mean ± SEM. Student's *t*-test: * P <0.05, ** P <0.01, *** P <0.001.
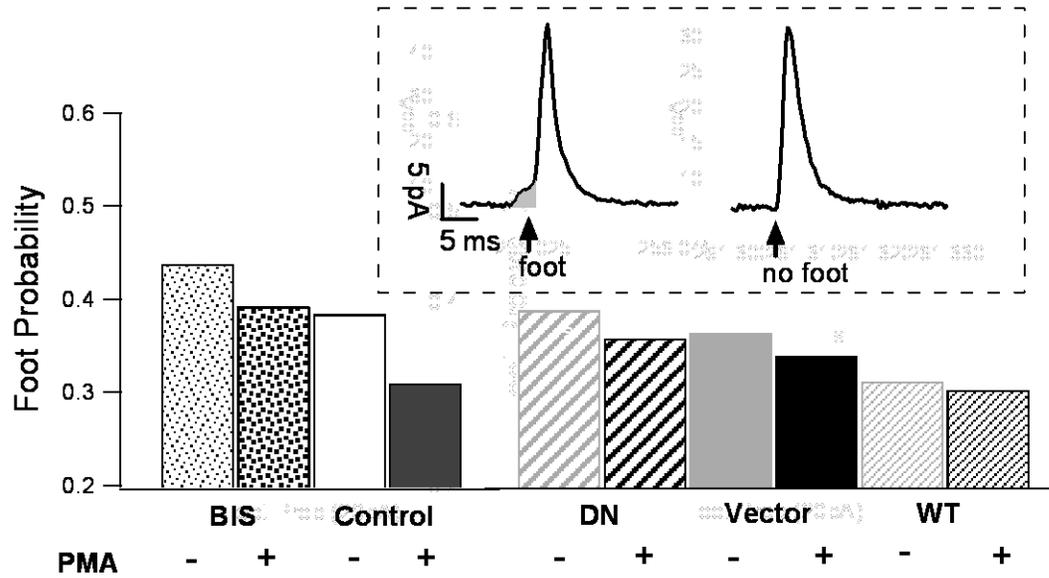
Fig. 4. The effects of PMA, BIS and PKCα expression on foot probability.

Representative amperometric spikes with and without a preceding foot are illustrated in

the inset. Foot probability is defined as the percentage of footed signals among all the

events that were obtained from the same experiments presented in Figs. 1 and 2, and have

amplitude > 20 pA (43 – 212 events).