JOURNAL OF NEUROCHEMISTRY | 2008 doi: 10.1111/j.1471-4159.2008.05264.x

PKC θ activity maintains normal quantal size in chromaffin cells

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Abstract

Protein kinase C (PKC) activity mediates multiple neurosecretory processes, but these are poorly understood due in part to the existence of at least 12 PKC isoforms. Using amperometry to record quantal catecholamine release from chromaffin cells, we found that both broad spectrum PKC antagonists and rottlerin, a selective inhibitor of the *novel* isoforms PKC θ and PKC δ, decreased quantal size and the number of secretory events recorded per stimulus. In contrast, drugs that selectively inhibit the *atypical* and *conventional* PKC isoforms had no effect on these parameters. While both PKC θ and δ were expressed in chromaffin cells, mice deficient for PKC θ, but not for PKC δ, exhibited lower quantal size than wild-type and were insensitive to rottlerin. Finally, an inhibitory PKC θ pseudosubstrate produced rottlerin-like responses in wild-type mice, indicating that the lack of rottlerin response in the PKC θ mutants was not the result of a form of compensation. These findings demonstrate neurosecretory regulation by a novel PKC isoform, PKC θ, and should contribute to defining mechanisms of activity-dependent regulation of neurosecretion.

Keywords: amperometry, catecholamine, chromaffin cell, exocytosis, protein kinase C, rottlerin.
J. Neurochem. (2008) 10.1111/j.1471-4159.2008.05264.x

Studies in mast cells, neuroendocrine cells, and neurons demonstrate that calcium-dependent protein kinase C (PKC) activity plays an important role in the regulation of exocytosis (Burgoyne *et al.* 2001). Protein kinase Cs encompass a family of at least 12 serine/threonine kinases involved in a multitude of cellular signaling cascades. They are divided into three groups based on their activation requirements. The c*onventional* PKC isoforms α, β_I, β_{II} and γ, are Ca^{2+}-dependent and activated by either diacylglycerol (DAG) or phosphatidylserine (PS). The n*ovel* PKC isoforms δ, ε, μ, η, and θ, are activated by DAG or PS but do not require Ca^{2+}. Finally, the *atypical* PKC isoforms, ζ, τ, and λ, are activated by PS but not DAG or Ca^{2+} (Dempsey *et al.* 2000; Way *et al.* 2000).

Protein kinase C isoforms phosphorylate multiple proteins implicated in secretory transmission, including SNAP25 (Nagy *et al.* 2002; Shoji-Kasai *et al.* 2002), Ca^{2+} channels (Yokoyama *et al.* 1997; Gerstin *et al.* 1998; Sena *et al.* 2001), Na^+ channels (Yanagita *et al.* 2000), synaptotagmin I (Hilfiker *et al.* 1999), annexin 7 (Caohuy and Pollard 2002), MARCKS (Fujise *et al.* 1994), Munc18 (Barclay *et al.* 2003), and protein phosphatase 2A (Zhang *et al.* 2007). It is thus not surprising that PKC activity has been implicated in the regulation of secretory vesicle trafficking, recruitment, level of transmitter accumulation, exocytic fusion, and recycling (Gillis *et al.* 1996; Scepek *et al.* 1998; Stevens and Sullivan 1998, Chen *et al.* 1999; Cousin and Robinson 2000; Nagy *et al.* 2002; Yang *et al.* 2002; Zhang *et al.* 2007). These roles are, however, complicated and controversial, in large part because of the multiplicity of isoforms and difficulties in analyzing drugs effects that affect PKC activity. For example, the classical activators of PKC activity, phorbol esters, also activate Munc13, an action that has been claimed to underlie phorbol ester augmentation of transmitter release (Rhee *et al.* 2002), although other studies report that effects of phorbol esters can be blocked by PKC inhibitors (Scepek *et al.* 1998), and recent findings suggest that both Munc13 and PKC may be required for phorbol ester response (Wierda *et al.* 2007).

Received December 6, 2007; revised manuscript received January 21, 2008; accepted January 21, 2008.

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Abbreviations used: DAG, diacylglycerol; HBSS, Hank's balanced salt solution; PBS, phosphate-buffered saline; PKC, protein kinase C; PS, phosphatidylserine.

In this study, we have examined the effects of chronic PKC activity on quantal neurosecretion using pharmacological PKC inhibitors and mouse mutant lines, thus avoiding reliance on phorbol ester activation. Our results demonstrate that an ongoing activity of a particular novel PKC isoform, PKC θ, contributes either directly or indirectly to the maintenance of normal quantal size, and may contribute to the regulation of the number of quantal events per stimulus. These results should thus contribute to elucidating how cellular activity modulates neurosecretion.

Materials and methods

Primary chromaffin cell cultures from mice and rats

Chromaffin cell cultures were prepared as described previously (Mosharov *et al.* 2006). Adrenal glands from 1-week-old Sprague–Dawley rats and approximately 3-week-old mice were dissected in ice-cold Hank's balanced salt solution (HBSS) (5 mM KCl, 440 μM KH_2PO_4, 4 mM $NaHCO_3$, 138 mM NaCl, 340 μM Na_2HPO_4, 5.6 mM D-glucose, Invitrogen, Carlsbad, CA, USA). The capsule surrounding the adrenal glands was removed and the remaining gland was sectioned into two pieces. After several washes with HBSS, the tissue was incubated with Ca^{2+} -free collagenase IA (250 units/mL, Worthington Biochemical Corp., Lakewood, NJ, USA) in HBSS for 30 min at 30°C while stirring. The tissue was rinsed three times and triturated gently in HBSS containing 1% heat-inactivated bovine serum albumin and 0.02% Dnase I. Dissociated cells were collected at 1000 *g* for 3 min and resuspended in culture medium (Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 2 mM glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin). Cells from one mouse or two rat pup adrenal glands were plated onto poly-D-lysine and laminin coated 1 cm^2 glass wells in 50 mm dishes. After 1–2 h, the dishes were flooded with the culture medium. Cells were maintained in a 7% CO_2 incubator at 37°C. All measurements were conducted 3–4 days post-plating.

Amperometric recordings

Recordings were performed as described previously (Staal *et al.* 2004). Briefly, a 5 μm diameter carbon fiber electrode held at +700 mV was positioned over the chromaffin cell (Newport Corp. micromanipulator MX300R, Irvine, CA, USA) and lowered until the tissue was slightly depressed (Pothos *et al.* 1998). The secretagogue solution contained in mM: 92 NaCl, 40 KCl, 10 HEPES, 1 Na_2HPO_4, 2 $MgCl_2$, 1.2 $CaCl_2$, ~300 mOsm and pH 7.4 At 700 mV dopamine is oxidized, resulting in the donation of two electrons to the electrode and the number of molecules reaching the electrode can be estimated from the resulting current (Sulzer and Pothos 2000). The current was filtered using a 4-pole 10 kHz Bessel filter built into an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA), sampled at 100 kHz (PCI-6052E, National Instruments, Austin, TX, USA) and digitally filtered using 1 kHz filter. The first derivative of the trace was further filtered at 300 Hz. The Igor (Igor Pro, Wave Metrics, Lake Oswego, OR, USA) routine used for analysis and filtering was written in house and is available for free download from our laboratory website (Mosharov and Sulzer 2005). Recordings compared within a single figure were conducted on the same day.

Western blotting of cultured chromaffin cells

Chromaffin cells were collected in media containing in mM: 10 HEPES, 10 KCl, 1.5 $MgCl_2$, 1 dithiothreitol and protease inhibitor cocktail, Complete Mini; Roche Diagnostics, Indianapolis, IN, USA and homogenized in a Dounce homogenizer. The homogenate was centrifuged for 10 min at 500 *g*, the supernatant was collected, and protein concentrations were measured using the bicinchoninic acid assay (Pierce, Rockford, IL, USA). Prior to loading onto the sodium dodecyl sulfate–polyacrylamide gel, proteins were denatured by mixing the supernatant with an equal volume of 2 × sample loading buffer (31 mM Tris–HCl pH 6.8, 10% glycerol, 1% sodium dodecyl sulfate, 2% β-mercaptoethanol, 0.1% bromophenol blue) and by boiling for 5 min. Overnight incubation with primary PKC antibodies was performed at 4°C. All primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and used at 1 : 100. Bands were quantified using the FluorChem 8800 imaging system after incubating the nitrocellulose membranes in chemiluminescent substrate (SuperSignal Ultra; Pierce).

Immunocytochemistry

Cultured chromaffin cells were fixed by slow addition of 4% paraformaldahyde in phosphate-buffered saline (PBS) for 5 min. Dishes were rinsed three times with PBS, then permeabilized with PBS-Triton (0.1%) for 1 h. Fixed cells were then incubated with respective primary antibodies (1 : 50) in PBS-Triton at 4°C overnight. All secondary antibodies (CY3, Alexafluor 484) for immunohistochemistry were obtained from Jackson ImmunoResearch (West Grove, PA, USA) and Invitrogen respectively and added to dishes at 1 : 500 for 1 h.

PKC θ,δ null mice

Protein kinase C θ and δ null mouse lines were kind gifts from Drs D. Littman (NYU) and R. Messing (UCSF), respectively.

Statistical analysis

Statistical analysis was by ANOVA with Newman–Keuls *post hoc* test.

Results

Pharmacological profile of PKC inhibitors implicates θ or δ PKC isoforms

Secretory vesicle exocytosis was stimulated by application of 40 mM potassium secretagogue solution (see Materials and methods) and quantal catecholamine release was measured by amperometry in rat chromaffin cell cultures. In preliminary experiments, pre-incubation with the phorbol ester PDBU (phorbol 12,13 dibutyrate, 2 μM, 30 min) had no significant effect on exocytosis (number of quantal events per stimulus, or size and shape of quantal events; data not shown).

Several PKC inhibitors, however, produced pronounced effects on evoked quantal secretion. When cells were pre-incubated for 30 min with the broad spectrum PKC inhibitors, chelerythrine (2 μM) or calphostine (100 nM), the number of quantal events per stimulus was reduced to 33%

and 17% of control levels, respectively (Figs 1a and 2), consistent with a role for PKC in maintaining the readily releasable pool (Gillis *et al.* 1996; Stevens and Sullivan 1998). Protein kinase C inhibition by these drugs also markedly reduced quantal size (Q), and resulted in a lower amplitude (I_{max}), an increased duration at half-height ($t_{1/2}$) and longer decay τ (Figs 1b, c and 2). No effect was observed on the size, duration, or frequency of 'foot' events that precede the full spike (data not shown).

More specific inhibitors were used to help identify the PKC isoforms responsible for decreasing Q; to enhance selectivity, all inhibitors were used at concentrations within an order of magnitude of the IC_{50}. Gö 6976 (20 nM), which blocks conventional PKC isoforms, had no significant effect on Q, peak height, number of events or decay τ (Figs 1a–c and 2). Rottlerin (10 μM), an inhibitor selective for the novel PKC isoforms θ and δ, significantly decreased the number of quantal events, reduced *Q*, and altered additional spike shape parameters to the same extent as the broad spectrum PKC inhibitors (Figs 1a–c and 2).

A myristoylated 13 amino acid PKC θ pseudosubstrate peptide (10 nM, 30 min) that specifically binds to the substrate binding site and inactivates the enzyme, mimicked the effects of rottlerin and the broad spectrum PKC inhibitors (Figs 1 and 2). As a control, we also tested the myristoylated 13 amino acid PKC ζ pseudo-substrate peptide (10 nM), which affected peak shape but had no effect on *Q* or the number of events per cell (Figs 1a–c and 2).

Chromaffin cells express both δ and θ PKC isoforms

Western blots of protein extracts from chromaffin cell cultures and Jurkat cell lysates run as a positive control indicated the presence of both PKC δ and θ, as well as PKC α, an isoform present in most cell types (Fig. 3a). Primary chromaffin cell cultures, however, contain fibroblasts and other cell types that could express these PKC isoforms, and so we performed immunocytochemistry to confirm the presence of δ and θ in chromaffin cells (Fig. 3b). Immunolabel for PKC α, ζ, and γ were also examined for comparison: PKC α is distributed throughout the cytosol and PKC ζ in the nuclei of nearly all mammalian cells, while PKC γ provided a negative control as it is only found in neurons (Saito and Shirai, 2002). The results confirmed that PKC δ and θ are both present in chromaffin cell cytoplasm (Fig. 3b).

PKC θ maintains normal quantal size

To confirm independently if ongoing PKC θ activity was responsible for regulating quantal neurosecretion, we exam-

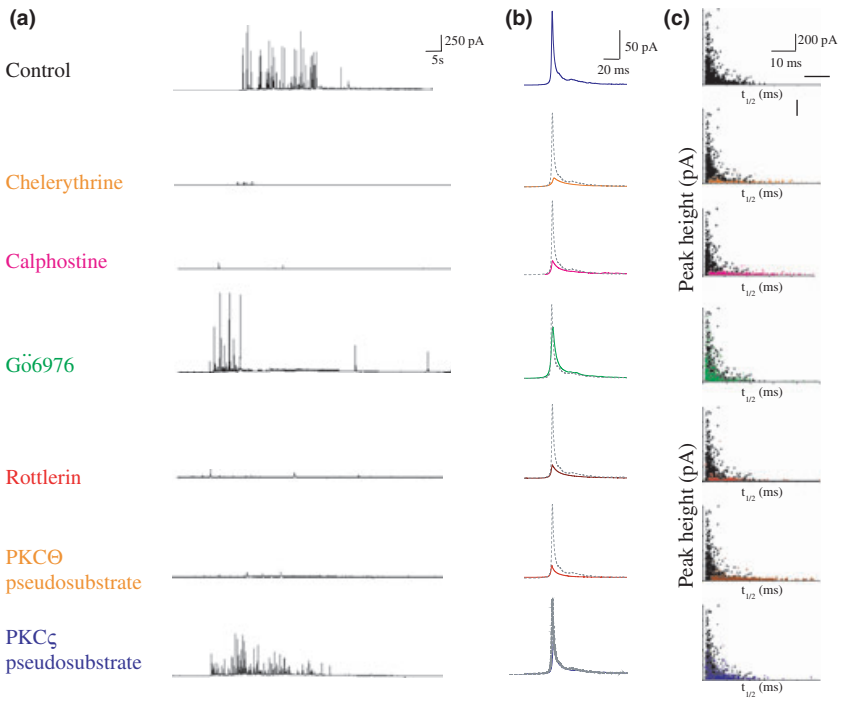

Fig. 1 Inhibition of PKCs alters the shape of amperometrically recorded evoked quanta. Chromaffin cell cultures were pre-treated with PKC inhibitors or vehicle for 30 min prior to recordings at the indicated concentrations. Cells were stimulated with a 3 s application of high K^+ medium, applied 10 seconds after the beginning of the recording. (a) Representative traces of amperometric measurements from chromaffin cells. (b) Averaged peak shape for all events for a given treatment. (c) The distribution of individual events based on their peak height (*y*-axis) and peak width ($T_{1/2}$; *x*-axis).

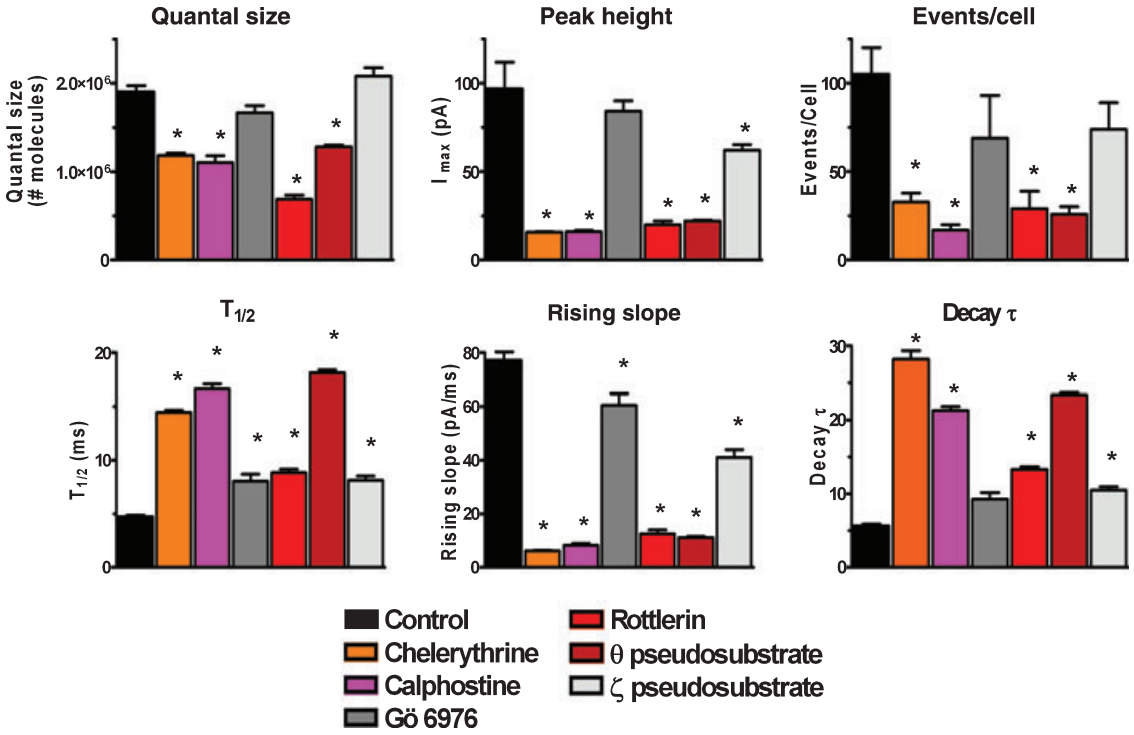

Fig. 2 Effect of inhibitors on quantal parameters. Cells were pre-treated with inhibitors for 30 min at concentrations indicated in Fig. 1. The data are the average of the median ± SEM from 13–24 cells. *, different than untreated controls, $p < 0.05$ by ANOVA, Newman–Keuls *post hoc* test.

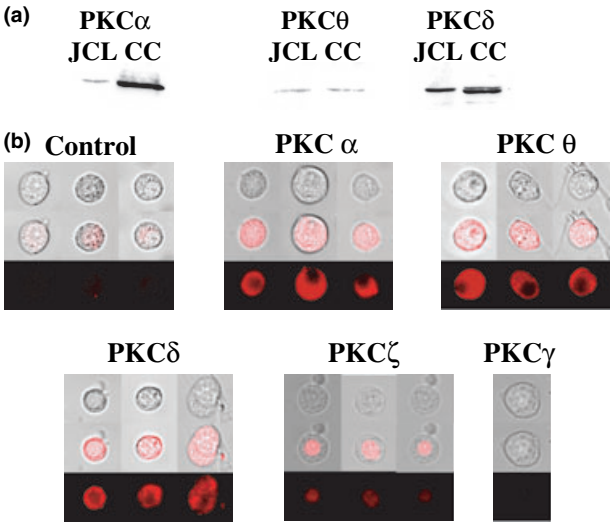

Fig. 3 Western blots and immunohistochemistry confirm the presence of PKC δ and θ. (a) Western blots of jurkat cell lysates (JCL) or chromaffin cell (CC) lysates confirm the expression of PKC δ and θ. PKC α was used as a positive control. (b) Chromaffin cells were stained using primary antibodies Ab against the PKC isoforms indicated (control: no primary antibody). PKC ζ has been previously shown to localize to the nucleus and PKC γ is a neuronal isoform that is not found in peripheral cell types including chromaffin cells.

ined knockout mouse lines deficient in the subtypes inhibited by rottlerin, PKC δ and θ (Gerstin *et al.* 1998; Kim *et al.* 2004). While recent data indicates that PKC δ activity inhibits tyrosine hydroxylase via effects on protein phosphatase-2A (Zhang *et al.* 2007), and its absence might thus be expected to increase *Q*, we observed no significant alteration of quantal parameters in PKC δ null mice (Fig. 4). In contrast, chromaffin cells from PKC θ null mice demonstrated smaller Q, slower rising and falling slopes and longer $t_{1/2}$ (Fig. 5), essentially resembling the effects of rottlerin and the PKC θ pseudosubstrate inhibitor. There was, however, no significant reduction in the number of quantal events per stimulus in PKC θ null cells ($p = 0.4$ vs. wild-type, *t*-test).

If PKC inhibitors exerted effects on quantal neurosecretion by PKC θ inhibition, the response to the inhibitors should be occluded in PKC θ null cells. We indeed found that quanta from PKC θ null cells had no rottlerin response, with no decrease in quantal size and no significant change in the number of evoked quanta per stimulus.

Discussion

Of the various second messenger systems that mediate neurosecretion, PKC has received the most attention, as its regulation by Ca^{2+}, DAG, and lipids makes it a good candidate for activity-dependent regulation of pre-synaptic

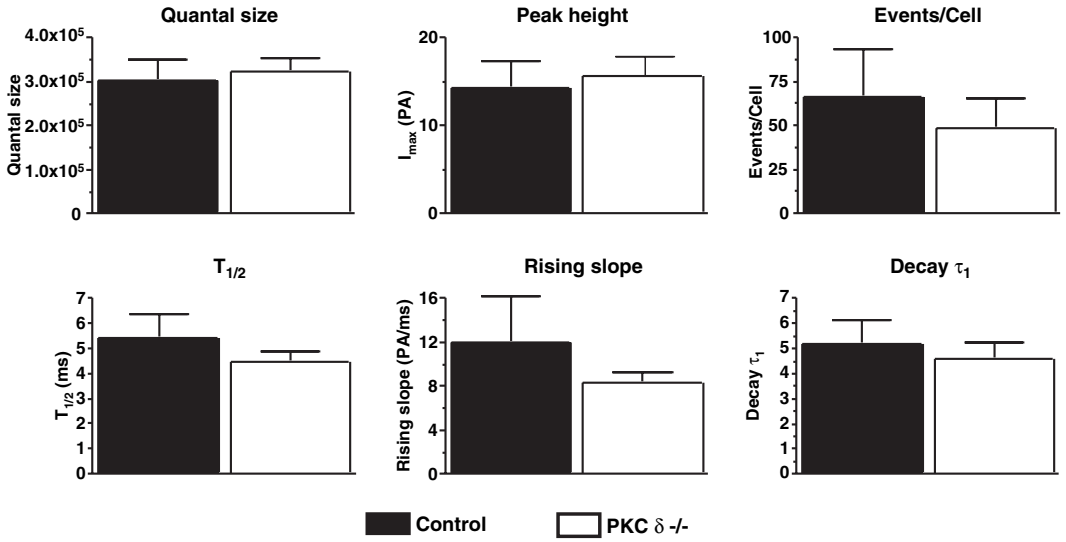

Fig. 4 Effect of PKC δ ablation on quantal catecholamine release. Data are the average of the median ± SEM from 10–15 cells. No parameters in the mutant cells were different than wild-type, $p > 0.05$ by ANOVA, Newman–Keuls *post hoc* test.

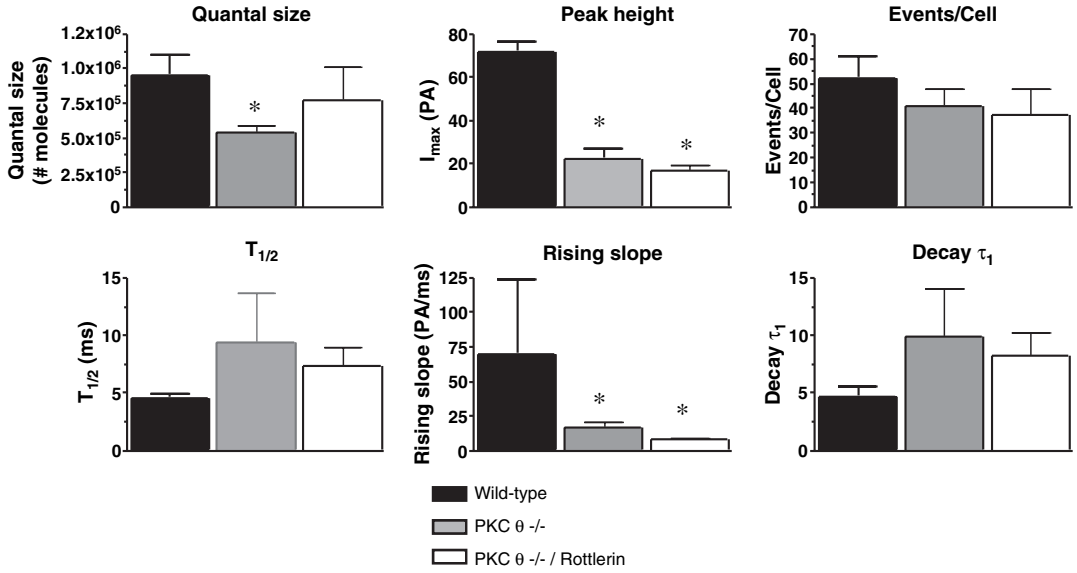

Fig. 5 Effect of PKC θ ablation on quantal catecholamine release. Chromaffin cells were exposed to rottlerin for 30 min or normal medium only prior to stimulation. The data are the average of the median ± SEM from 12–15 cells. *, different from wild-type $p < 0.05$ by ANOVA, Newman–Keuls *post hoc* test.

plasticity. The precise roles played by PKC, however, are unclear due in large part to the variety of PKC subtypes and broad range of possible substrates. We have used immunocytochemistry, PKC inhibitors, and mutant mouse lines in conjunction with amperometric detection of evoked quantal neurosecretion from adrenal chromaffin cells to identify the quantal neurosecretory parameters regulated by PKC and to identify which PKC subtype is involved.

The most striking response to broad spectrum PKC inhibition was a marked decrease in quantal size. This was surprising, as PKC δ inhibits tyrosine hydroxylase activity via affects on protein phosphatase-2A (Zhang *et al.* 2007), and PKC inhibition might have been expected to increase rather than decrease quantal size. Our results indicate that quantal size is regulated by the ongoing activity of the novel isoform, PKC θ, as (i) PKC θ was present in chromaffin cell cytoplasm, (ii) rottlerin, a selective PKC δ/θ inhibitor,

decreased quantal size, (iii) a myristoylated pseudosubstrate peptide inhibitor highly specific for PKC θ similarly decreased quantal size, (iv) while PKC δ null chromaffin cells possessed normal quantal sizes, PKC θ null chromaffin cells had small quantal sizes, (v) quantal secretion from PKC θ null chromaffin cells was unaffected by rottlerin.

There are multiple means by which PKC activity might regulate quantal size, including effects on the vesicular pH or ion gradients, vesicle or membrane catecholamine transporters, regulation of vesicle size, the manner of fusion, and regulation of cytosolic transmitter levels via effects on transmitter synthesis and breakdown (Sulzer and Pothos 2000). We found that decreased quantal size by PKC inhibition was accompanied by decreases in the rising and falling slopes of quantal events, as expected from simulated transmitter diffusion during exocytic release (Mosharov and Sulzer 2005). In contrast, simulations indicate that the increased duration at half-height is independent of altered quantal size, suggesting that PKC inhibition might slow degranulation of catecholamine from the vesicle dense core matrix. It is unlikely that the change in Q is due to premature closing of the fusion pore prior to full fusion, as this would be expected to produce a higher proportion of stand alone 'feet.' Analysis of the data showed no such difference, nor any differences in the number of molecules per foot, or the duration or amplitude of feet.

Both broad spectrum and PKC θ inhibition decreased the number of quanta evoked per stimulus. In contrast to the effects on quantal size, however, PKC θ null chromaffin cells did not produce fewer evoked quanta per stimulus than wild-type cells. There was, nevertheless, an occlusion of rottlerin's effects on the number of quantal events in the null cells. The results suggest a compensatory adjustment by the mutant cells to maintain a normal releasable pool of vesicles and that additional mechanisms are involved; many molecular processes determine which vesicles will be released upon a given stimulus, including those involved in vesicle transport, docking, priming, and fusion (Becherer and Rettig 2006).

It is interesting to speculate on the significance of PKC regulation of quantal size, since these isoforms are activated by lipids rather than rapid changes in Ca^{2+} levels. This may provide a low-pass temporal filter, so that transmitter release is controlled by an integrated history of previous cellular activity. For example, modulatory neuronal inputs that provide sustained changes in novel PKC activity via sustained changes in Ca^{2+} and lipids could transynaptically regulate quantal size in post-synaptic neurons.

Acknowledgments

We thank Dr D. Littman (NY University) for the PKC θ null mice, Dr R Messing (UCSF) for the PKC δ null mice and Dr R Messing (UCSF) and M. Lindau (Cornell) for discussion of the results. This work was supported by NINDS, NIDA, the National Alliance for Research in Schizophrenia and Depression (NARSAD) and the Picower and Parkinson's Foundations.

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