Differing mechanisms of exocytosis for large dense core vesicles in chromaffin cells and small synaptic vesicles in dopamine neurons.

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Cell Biology of the Chromaffin Cell
R. Borges & L. Gandía Eds.
Instituto Teófilo Hernando, Spain, 2004

Synaptic neurotransmission depends on the exocytosis of neurotransmitter (NT) during the fusion of vesicles with the plasma membrane. A key goal for understanding the mechanism of exocytosis is to determine whether vesicles undergo full fusion or kiss and run exocytosis and how this process may be regulated. Studies using carbon fiber amperometry and capacitance measurements have demonstrated that the predominant mechanism of exocytosis for large dense core vesicles (LDCVs) in chromaffin cells is full fusion, although kiss and run exocytosis can also occur[1-4]. Several studies have demonstrated that phorbol esters and kinase inhibitors can modulate the mode of exocytosis in chromaffin cells[5-9]. The authors generally conclude that the target of these drugs is protein kinase C (PKC) although many of the drugs used are not selective for this kinase. To our knowledge, no studies to date have identified which of the 12 known PKC isoforms is responsible for the modulation of exocytosis. We used a variety of small inhibitory molecules and peptides to further investigate the role of PKC in chromaffin cell exocytosis and to identify the PKC isoform(s) responsible. Calphostine (100 nM), chelerythrine (2 □M), rottlerin (10 □M) and the myristoylated PKC□-pseudosubstrate peptide (10nM), but not Gö6976 (20 nM) or the myristoylated PKC□-pseudosubstrate peptide, 10 nM) markedly slowed the rising and falling phases of quantal events, increased the $t_{1/2}$ while decreasing the maximal peak height (Table 1, all drug concentrations are 2-3 fold their IC_{50} of PKC inhibition). These data suggest that PKC can regulate the formation and size of the fusion pore as well as shifting the mode of exocytosis from full fusion to kiss and run exocytosis. Rottlerin's (but not Gö6976) ability to alter the kinetics of neurotransmitter release suggest that PKC□ and/or PKC_ (but not PKC□ and PKC□) are the isozymes responsible. The presence of PKC□ and □ have been confirmed by western blotting of proteins from chromaffin cell cultures (which also contain fibroblasts) as well as immunohistochemistry (data not shown).

Our data in chromaffin cells suggest that PKC□ and/or PKC□ can switch the mechanism of exocytosis in chromaffin cells from the full fusion to kiss-and-run exocytosis. This is consistent with many previous reports implicating PKC in regulating the mechanism of exocytosis[5-9]. Interestingly, PKC□ and PKC□ are both Ca^{2+} independent

isoforms of PKC. Utilizing Ca^{2+}-independent isoforms of PKC may be an important attribute for a pathway regulating exocytosis, a process that is triggered by Ca^{2+}.

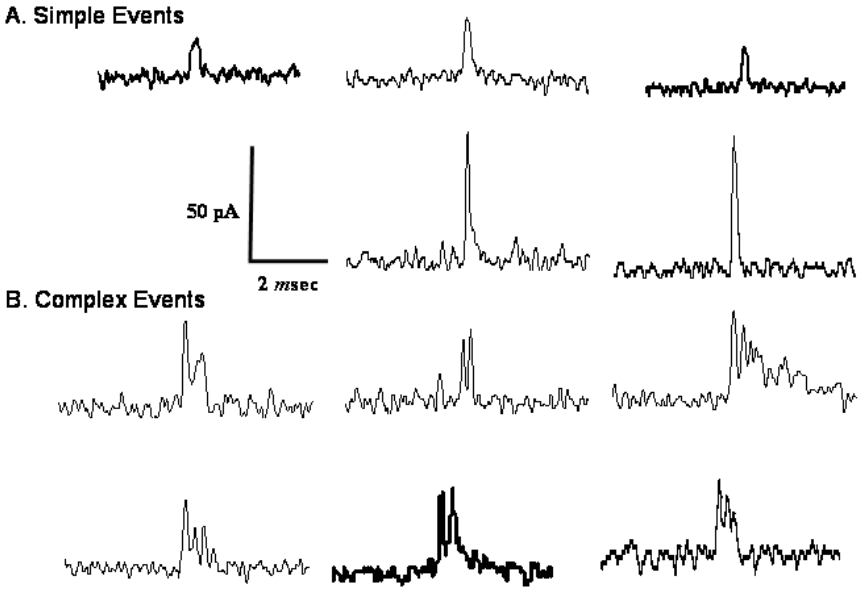

Figure 1. Representative traces quantal dopamine release from rat ventral midbrain dopamine neurons stimulated with K^+. To be considered an *event*, the derivative dI/dt must cross a 4.5 x RMS threshold. The event begins and ends when dI/dt = 0. **A)** Events with derivatives that cross the 3.0 x RMS threshold once in a rising trajectory are *simple*. **B)** Events that cross the 3.0 x RMS threshold multiple times are *complex*.

Since the exocytic machinery is believed to be similar in chromaffin cells and neurons, we examined the kinetics of NT release from ventral tegmental area dopamine neurons in culture. Using carbon fiber amperometry to measure NT release, we found that ~20% of quantal events recorded from neurons did not possess the "simple" (Fig 1a) amperometric spike shape usually seen with single vesicle exocytosis in other cell types. Rather, we observed a "complex" spike shape with multiple peaks (Fig 1b), usually with sequentially decreasing amplitudes, consistent with the release of NT via the high frequency (~4 kHz) flickering of a small diameter (~2 nm) fusion

pore. Complex events had significantly larger quantal sizes than simple events, suggesting that the latter arose from the partial release of transmitter via a single flicker of the fusion pore. The incidence of complex amperometric spikes was enhanced by staurosporine and reduced by phorbol-12,13-dibutyrate (PDBU), demonstrating that exocytosis in neurons can also be regulated by second messenger systems, possibly PKC, although other systems cannot be ruled out at this point.

TABLE 1. Effect of PKC Inhibition on Kinetics of Exocytosis.

Cells were stimulated with 40 mM K^+ administered by puff pipette (2 μm diameter, 10 psi, 3 sec). Catecholamine release was detected amperometrically from at least three cells under control conditions before inhibitors were added to the media.

Treatment	# of Cells	$t_{1/2}$ (ms) (n)	Peak Height (pA)	Quantal Size ‡ (molecules)	Rising Slope ($^{pA}/_{ms}$)	Decay
Control	13	4.9±0.1 (1359)	105± 8.1	2,197,000 ± 90,000	83 ± 3	6 ± 0
Calphostine (10 nM)	22	15.5±0.9* (226)	16.3±1.5*	1,139,00±150,000*	9 ± 1*	20 ± 1*
Chelerythrine (2 μM)	13	14.7±0.4* (718)	16.1±0.7*	1,237,000 ± 68,000*	6 ± 0*	25 ± 2*
Gö6976 (20 nM) Inhibits α and β	11	6.5±0.3 (693)	95.5±5.8	1,954,000 ± 127,000	67 ± 4	8 ± 1
Rottlerin (10 μM) Inhibits δ and ε	10	10.5±0.5* (315)	22±2.7*	847,000± 80,000*	14 ± 2*	15 ± 1*
PKC ζ Pseudo-substrate	24	17.1±0.6* (635)	24.6±1.5*	1,437,00± 70,000*	13 ± 1*	23 ± 1*
PKC ι Pseudo-substrate	8	7.9±0.2 (594)	65.7±3.4	2,295,00± 164,000	43 ± 3	10 ± 0*

‡ Data are the average ± SEM from all cells.

* $P < 0.05$, compared to control using ANOVA.

While the dimensions of the initial fusion pore of LDCVs and SSVs appear to be similar, exocytosis of NT by SSVs appears to be

accomplished by the rapid flickering (opening and closing) of the fusion pore, whereas exocytosis of LDCVs is primarily accomplished by full fusion. Although the fusion pore of LDCVs can flicker, this most likely reflects the failure of the fusion pore to radially expand into full fusion[1-2] which is in contrast to SSVs where the fusion pore opens and closes (or rhythmically fluctuates in size) once or several times without undergoing full fusion. Furthermore, the flickers in SSVs were ~1000 fold shorter than those observed in LDCV foot events and the SSV flickering frequency was ~25 fold faster. Our results suggest that although full fusion is the dominant form of exocytosis for LDCVs, dopaminergic SSVs release NT through a rapidly flickering fusion pore, a novel mechanism that is distinct from kiss and run exocytosis as well as full fusion.

ACKNOWLEDGEMENTS:

Supported by the Parkinson's Disease Foundation and the National Institute on Drug Abuse.

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