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## Symposium 9: Exocytosis and neurosecretion

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Chair: David Adams

## Differential regulation of two modes of exocytosis by protein phosphatases

*A.T.R. Sim, School of Biomedical Sciences, The University of Newcastle, and Hunter Medical Research Institute, Callaghan, NSW 2308, Australia. (Introduced by D. Adams)*

Phosphorylation and dephosphorylation of nerve terminal proteins are involved in the regulation of neurotransmitter release. However, it is now clear that there are multiple modes of exocytosis in neurons and the specific roles for different kinases and phosphatases remain unknown. The most well characterised mode of exocytosis involves docking and fusion of a synaptic vesicle with the plasma membrane followed by full incorporation of the vesicle into the plasma membrane, so called full fusion exocytosis. Vesicles are subsequently recovered by Clathrin-mediated endocytosis. However, an alternative mode, termed kiss-and-run, has now been demonstrated, where neurotransmitter is released without complete fusion of the vesicle with the plasma membrane, and the vesicle is rapidly retrieved and refilled with neurotransmitter. Kiss-and-run therefore accelerates the turnover of the limited pool of synaptic vesicles in neurons and may have both beneficial and pathological outcomes. In studies to identify the molecular events underlying these different modes, we have shown that serine/threonine and potentially tyrosine phosphatases have specific regulatory roles.

We used selective pharmacological inhibitors of different protein phosphatases to investigate their roles in the different modes of exocytosis in neurons and mast cells. Two chemical depolarising agents were used (KCl and 4-aminopyridine) that can selectively induce full fusion and kiss-and-run exocytosis, and exocytosis was measured by two complementary assays that can distinguish between these 2 modes of exocytosis. Measurement of endogenous, soluble, glutamate release detects both full fusion and kiss-and-run modes of exocytosis. In contrast, measuring the release of the lipophilic styryl dye FM 2-10 , reflects the time dependent dissociation of the dye from vesicle membranes, and is therefore much less capable of detecting the rapid, transient exocytosis that occurs during kiss-and-run. Our results suggest that protein phosphatase 2A positively regulates the full fusion mode of exocytosis, whilst protein phosphatase 2B, in addition to its recognised role in regulating endocytosis, negatively regulates the kiss-and-run mechanism of exocytosis.

We have also studied the role of the Src family of tyrosine kinases in regulating these two modes of exocytosis. Inhibition of the Src family kinases, using the specific inhibitor, PP1 (10μM), significantly increased kiss-and-run release, but had no effect on full fusion release. The inactive analog, PP3, had no effect on either mode. Measurement of depolarisation induced changes in synaptosomal protein tyrosine phosphorylation did not show any association between Src kinase activity and kiss-and-run exocytosis. This indicates that the effect of Src kinase inhibition is either to remove a constitutive phosphorylation dependent restraint on exocytosis, perhaps mimicking an endogenous tyrosine dephosphorylation event that promotes the kiss-and-run mode, or to inhibit a depolarisation dependent activation of a member of the Src kinase family that is not Src.

Since the fundamental molecular machinery involved in full fusion exocytosis is highly conserved, we have also used a mast cell model to further investigate the molecular events underlying the control of full fusion exocytosis by PP2A. These studies indicate that translocation of PP2A from cytosolic to membrane-associated locations within the cell and the formation of transient complexes with myosin are critical for the regulation of exocytosis.

## Coupling G protein-coupled receptors to exocytosis

*P.D. Marley, Department of Pharmacology, University of Melbourne, Vic. 3010, Australia. (Introduced by D.J. Adams)*

The primary driving force for regulated exocytosis is the elevation of cytosolic $Ca^{2+}$. In excitable cells, this is normally achieved by extracellular $Ca^{2+}$ entering the cell through $Ca^{2+}$-permeable channels in the plasma membrane. Ionotropic receptors evoke exocytosis either by being themselves permeable to $Ca^{2+}$ or by being non-selective cation channels that depolarise the cell, so activating voltage-sensitive $Ca^{2+}$ channels (VOCCs). In contrast, the mechanisms by which G protein-coupled receptors (GPCRs) cause extracellular $Ca^{2+}$ entry are much less clear. Possible mechanisms have been exploring using the secretion of catecholamines evoked by histamine H1 receptors from adrenal chromaffin cells (Marley, 2003).

Adrenal chromaffin cells express one of the highest densities of H1 receptor of any tissue and these are of critical importance in protecting against anaphylactic shock. The chromaffin cell H1 receptors are coupled through *Pertussis* toxin-resistant G proteins to the hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate and the generation of inositol 1,4,5-trisphophate (IP3), which mobilises $Ca^{2+}$ from intracellular stores. Of the GPCRs expressed by chromaffin cells, histamine H1 receptors are particularly effective at evoking exocytosis, having almost half the efficacy of powerful nicotinic receptor agonists. The great majority of this secretory response is inhibited by antagonists to L, N and P/Q-type VOCCs, indicating histamine recruits VOCCs, however histamine has complex effects on the membrane potential of these cells (Wallace *et al.*, 2002). Initially there is a transient hyperpolarisation that is abolished if intracellular $Ca^{2+}$ stores are depleted and which is due to activation of small conductance $Ca^{2+}$-activated $K^+$ (SK) channels by store $Ca^{2+}$ released by IP3. The hyperpolarisation is followed after 10-20 s by a slow depolarisation and an increase in frequency of spontaneous action potentials. The latter two effects persist after store depletion and after block of SK channels, and are accompanied by an increase in membrane resistance and by a small inward current. The latter are in part the result of the closure of a $K^+$ channel responsible for a novel M current that helps set the resting membrane potential. How the H1 receptors cause the closure of these channels is presently unknown, however the secretory response to histamine is not prevented by inhibitors of IP3 receptors or ryanodine receptors, by depletion of intracellular $Ca^{2+}$ stores, or by protein kinase C inhibitors (Donald *et al.*, 2002). The identity of the cause of the rest of the depolarisation also remains unknown. H1 receptors regulate the activity of at least five classes of $K^+$ channels in chromaffin cells, however the secretory response is not prevented by blocking SK channels, intermediate- or large-conductance $Ca^{2+}$-activated $K^+$ (IK or BK) channels, $K_{ATP}$ channels, delayed rectifier channels or A type channels, and inward rectifier $K^+$ channels are not expressed in these cells.

The results from such studies raise a number of important questions, including (i) what is the molecular mechanism by which GPCRs inhibit $K^+$ channels, (ii) through what other channels can GPCRs depolarise cells, and (iii) why do some GPCRs evoke large secretory responses, while others have very low efficacy, while apparently activating similar signaling pathways in the same cells?

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Wallace D.J., Chen C. & Marley P.D. (2002) *Journal of Physiology,* **540**, 921-929.

## PI-3 kinase type II C2α is essential for ATP-dependent priming of neurosecretory granules prior to exocytosis

*F.A. Meunier[1], G. Hammond[1], P.J. Parker[1], G. Schiavo[1], F.T. Cooke[2] and J. Domin[3], [1]Cancer Research UK, London Research Institute, Lincoln's Inn Fields Laboratories, 44 Lincoln's Inn Fields, London WC2A 3PX, UK,[2]Biochemistry and Molecular Biology, University of College, London, Darwin Building, Gower Street, W1E 6BT, London, UK and [3]Renal section, Faculty of Medicine, Imperial College School of Medicine, London W12 0NN, UK. (Introduced by D.J. Adams)*

Phosphatidylinositol 3-kinases (PI3K) are implicated in a variety of synaptic functions including axonal guidance and long-term depression and potentiation (reviewed in Osborne *et al*., 2001). However, a direct involvement of this class of enzymes and their lipid products in neuroexocytosis has been questioned (Chasserot-Golaz *et al.*, 1998), based on the low sensitivity of exocytosis to PI3K inhibitors wortmannin and LY294002 (Martin *et al.*, 1997; Wiedemann *et al.*, 1996).

Neurotransmitter release from synaptosomes and hormonal secretion from chromaffin cells are only sensitive to high concentrations of the PI3K inhibitors wortmannin and LY294002, pointing to a possible role for the less sensitive PI3K-C2α. In support of this, PI3K-C2α was detected on a subpopulation of mature secretory granules abutting the plasma membrane in neurosecretory cells. Furthermore, both PI3K inhibitors and sequestration of PI3K-C2α with specific antibodies selectively prevented ATP-dependent priming in permeabilised chromaffin cells.

Transient over-expression of PI3K-C2α in PC12 cells potentiated evoked secretion, whereas its dominant negative mutant abolished exocytosis, suggesting PtdIns3*P*, the main catalytic product of this enzyme plays a role in neuroexocytosis. Consistent with this, treatment of PC12 cells transiently expressing PtdIns3*P*-sequestering FYVE domain with low concentrations of wortmannin selectively abolished early endosomal staining and revealed a full co-localisation of the FYVE domain with PI3K-C2α on PC12 granules. Finally sequestration of PtdIns3*P* by the FYVE domain also abolished secretion from PC12 cells demonstrating that PtdIns3*P* production is needed in the process of acquisition of fusion competence secretory vesicles undergo, during or following docking to the plasma membrane.

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## Pre- and postsynaptic factors controlling synaptic efficacy at central synapses

*M.C. Bellingham and M.L. Kerr, School of Biomedical Sciences, University of Queensland, St. Lucia, Queensland 4072, Australia. (Introduced by D.J. Adams)*

Synaptic efficacy is a measure of the strength of postsynaptic electrical signals arising from synaptic release of chemical neurotransmitters. Both pre- and postsynaptic factors can alter synaptic efficacy. Most synapses are located on dendrites, whose passive and active electrical properties can distort recorded signals arising from remote synapses. These distortions are avoided at certain central synapses, such as that between the endbulbs of Held and bushy cells in the cochlear nucleus, where the glutamate-releasing presynaptic terminals of cochlear nerve fibres direct contact the bushy cell soma. Whole cell patch clamp recordings of eEPSCs were made from bushy cells (n=113) in cochlear nucleus slices obtained from postnatal day (P) 4-21 rats anaesthetised with sodium pentobarbitone (20 mg/kg i.p.), in order to investigate pre- and postsynaptic factors contributing to developmental changes in synaptic efficacy.

**Postsynaptic changes**: At endbulb-bushy cell synapses, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated single fibre evoked EPSCs (eEPSCs) increase in amplitude with age while N-methyl-D-aspartate receptor (NMDAR)-mediated eEPSCs decrease in amplitude and decay time constant. The functional characteristics of AMPARs and NMDARs depend on subunit composition. NMDARs with NR2B subunits have high $Ca^{2+}$ permeability and long decay time constant, are typically more common in neonatal brains and may play an important role in synapse development. We investigated developmental changes in subunit composition of postsynaptic receptors using subunit-specific antagonists. Ifenprodil (10μM), an NR2B subunit-selective NMDAR antagonist, reduced NMDAR eEPSC amplitude to 24±3% (mean±SEM, n=13) of control in P4-8 rats, significantly greater than NMDAR EPSC reduction in P10-17 rats (40±4% of control, n=13) suggesting that NR2B subunits are exchanged during development to probably NR2A subunits. Pentobarbitone (100 μM), which selectively inhibits AMPARs containing GluR2 subunits, reduced AMPAR EPSC amplitude in P4-6 rats to 51±2% of control (n=4), to 73±5% (n=3) at P8-11 and to 40±14% (n=3) in P12-15 rats. The intracellular polyamine spermine blocks $Ca^{2+}$-permeable AMPARs lacking GluR2 subunits at positive voltages. After inclusion of spermine (100μM) in the electrode solution, the mean rectification index (RI) of AMPAR EPSC I-Vs increased with age (P4-6, RI=1.2±0.5 (n=5), P7-11, RI= 4.5±0.5 (n=8), P12-15, RI= 5.6±0.8 (n=10). suggesting that AMPARs in older animals are likely to lack GluR2 subunits and be more $Ca^{2+}$ permeable.

**Presynaptic changes**: Paired stimuli at 5-140 ms caused marked facilitation of $2^{nd}$ eEPSC amplitude at P4-7 (mean ratio±SEM at 10 ms = 1.7 ± 0.1, n=17), marked depression at >P11 (0.6±0.05, n=17), and a mixture of facilitation and depression at P8-10 (1.0±0.15, n=18). Depletion of the synaptic vesicle pool by 10 stimuli at 100 Hz caused eEPSC amplitude depression at all ages (P4-7, 0.03±0.04, n=7; P8-10, 0.02±0.03, n=6; >P11, 0.13±0.08, n = 13). Recovery from depletion was similar at short delays but slower at >P11 for longer delays. Varying external $Ca^{2+}$ caused larger changes in eEPSC amplitude and paired pulse ratio at ages <P11, indicating that sensitivity of synaptic release to external $Ca^{2+}$ altered with development.

**Conclusions**: These data suggest that presynaptic factors regulating $Ca^{2+}$-sensitive synaptic release and short term plasticity, and that subunit composition of postsynaptic AMPARs and NMDARs can be rapidly modified during synaptic development. It is proposed that $Ca^{2+}$ influx through NMDA receptors may contribute to these developmental changes, so to increase synaptic efficacy with large and rapid AMPA responses at mature synapses.