Calcium and transmitter release at nerve terminals
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Introduction
The study of transmitter release has been advanced by the use of two recently developed techniques: (i) the measurement of intracellular free Ca concentration ([Ca\(^{2+}\)]\(_i\)) in nerve terminals during synaptic transmission with presynaptically injected indicator dyes; and (ii) the control of presynaptic [Ca\(^{2+}\)], by use of injected photolabile Ca chelators. This review will summarize recent progress in elucidating the physiological mechanisms involved in transmitter release by application of these techniques to a number of synaptic preparations, as well as to hormonal secretion by endocrine cells.

Abbreviations used: BAPTA, bis-(O-aminophenoxo)ethane-N,N,N',N'-tetraacetic acid; EPSP, excitatory postsynaptic potential; GABA, \(\gamma\)-aminobutyric acid; LHRH, luteinizing hormone-releasing hormone; MEPSI, mini-excitatory postsynaptic potential; PTP, post-tetanic potentiation.

Transmitter release at ‘calcium domains’ in fast synapses
Fura-2 can be injected into crustacean motor axons near their terminals, filling synaptic boutons on the surface of the muscle [1]. Changes in [Ca\(^{2+}\)], can then be measured in the boutons while stimulating the motor neuron and recording postsynaptic potentials. Single spikes have barely detectable effects on [Ca\(^{2+}\)], raising it by perhaps 10 nM; nevertheless, each spike can evoke substantial transmitter release during a brief interval following the spike. Conversely, tetanic stimulation causes the measurable [Ca\(^{2+}\)], to rise gradually in the boutons during the tetanus, while transmitter release is still phasic and occurs only for a few milliseconds after each spike. Clearly, the rise in [Ca\(^{2+}\)] measured with fura-2 is poorly correlated temporally with phasic transmitter release.

At rapidly transmitting synapses, such as the glutamatergic neuromuscular junction of the cray-
fish claw opener muscle or the squid giant synapse, transmission begins in a fraction of a millisecond after the peak of the presynaptic spike [2], and in even less time after the presynaptic Ca current starts to flow [3]. In this short time, Ca has had time to diffuse only a few tens of nanometers from Ca channel mouths. Solution of the diffusion equation from arrays of Ca channels clustered into active zones provides an estimate of the temporal and spatial \([Ca^{2+}]+\), profiles caused by action potentials. Our most recent calculations consider diffusion with rapid but non-instantaneous binding to a slowly diffusible buffer whose parameters match those measured experimentally [4]. The number of Ca channels per active zone opened by a spike, the single channel current, the disposition of active zones, and removal of cytoplasmic Ca by active transport were all based on experimental measurements [5]. Since vesicular fusion occurs on average about 50 nm from Ca channels [6] segregated into active zones [7, 8], it is most interesting to investigate this distance from Ca channels that open in an active zone during an action potential. Our simulations show \([Ca^{2+}]+\), at this critical locus rising to a peak level of about 100 \(\mu\)M, and dropping rapidly (1/2 ms half width) to a residual \([Ca^{2+}]+\), of less than 10 \(\mu\)M in 5 ms. After tens of milliseconds, \([Ca^{2+}]+\), has diffusively equilibrated and is of the order of 10 nM above its initial level.

These calculations suggest that transmitter release is triggered by very brief, localized and high Ca concentrations in active zones. Present optical methods are unable to resolve such \([Ca^{2+}]+\), transients. However, several experimental results suggest they do exist. First, the weak effects of injected exogenous fast buffers on transmitter release are consistent only with such high \([Ca^{2+}]+\), levels acting on release sites [9]. Second, estimates of localized \([Ca^{2+}]+\), based on the magnitude of Ca-dependent potassium current triggered by spikes in hair cells are also very high [10]. Finally, cumulative measurements of repeated synaptic stimulation using the relatively insensitive Ca indicator \(\pi\)-aequorin-\(\beta\) reveal a spatial pattern of local \([Ca^{2+}]+\), exceeding 100 \(\mu\)M, resembling the disposition of active zones [11].

**Synaptic facilitation**

A simple model of transmitter release involves Ca ions binding rapidly to a low-affinity secretory trigger molecule, which in turn triggers a slower exocytotic process when fully occupied with five Ca ions. Ca binds co-operatively to reflect the highly non-linear dependence of release on extracellular \([Ca^{2+}]+\) [12-14]. Binding to the secretory trigger must be fast, for it is barely affected by slow buffers like EGTA and only modestly affected by rapid buffers like \(\text{bis-(O-aminophenoxy)-ethane-N,N',N''-tetraacetic acid (BAPTA)}\) [9, 15]. Furthermore, the Ca affinity of the binding sites must be low because transmission is still not saturated at normal levels of Ca influx (normal levels of external \([Ca^{2+}]+\)), even for multiple action potentials at high frequency. In such a process, the small residual \([Ca^{2+}]+\), present for tens to hundreds of milliseconds following activity before diffusional equilibration is complete would correspond to some trigger sites still being occupied with Ca ions. A subsequent spike would result in a higher proportion of Ca-binding site occupancy on the trigger molecules, and hence a facilitated release of transmitter. However, simulations of such a process significantly underpredict facilitation [4], suggesting that it does not arise simply from the co-operativity of Ca ions binding to identical sites on a trigger molecule and the residual Ca still bound to such sites. A model with Ca binding to a second site, with higher affinity and slower kinetics, was able to simulate observed levels of facilitation (several hundred percent within a few milliseconds of a single action potential). At present, this remains our working hypothesis of how short term synaptic facilitation works.

**Consequences of calcium domains**

Recognizing that local transient high \([Ca^{2+}]+\), triggers secretion accounts for numerous otherwise anomalous results. When \([Ca^{2+}]+\) in the medium is increased, a highly non-linear relationship (at least fourth power) between Ca influx per spike and transmitter release per spike is seen, when Ca influx is assessed from the changes in \([Ca^{2+}]+\), during a train of action potentials in small motor nerve terminals [16] or from measures of Ca current at the squid giant synapse [14]. This would be expected, since the increased influx is caused by an increase in single Ca channel currents, so that the \([Ca^{2+}]+\), in the neighbourhood of Ca channels in active zones increases approximately proportionately to increased macroscopic Ca influx. On the contrary, increasing Ca influx by prolonging action potentials or using larger depolarizations has a less dramatic effect on transmitter release. Release per spike increases with something like a first to third power dependence on measured or inferred Ca influx [3, 14, 16, 17]. These manoeuvres do not increase the Ca influx per channel; rather they increase the number of channels that open. If few channels open, this would result in recruitment of
release from vesicles within reach of the additional open Ca channel domains, and release would increase nearly linearly with macroscopic Ca influx. If many channels open, the \([\text{Ca}^{2+}]\), from neighbouring domains may overlap at a releasable vesicle, local \([\text{Ca}^{2+}]\), will summate from these multiple channel openings, and some of the co-operativity of Ca action in exocytosis will be expressed, leading to a more than linear but less than fully non-linear relationship between release and macroscopic Ca current. A similar explanation accounts for the observation [3, 18] that a given Ca current at large potentials, with many open channels causing overlapping channel \([\text{Ca}^{2+}]\), domains at vesicles, causes more release than the same Ca current at low potentials, with few channels opening far from each other, albeit with increased single channel current [19, 20].

**Calcium and PTP**

The above considerations make clear that measurements of \([\text{Ca}^{2+}]\), should not bear a simple relationship to the level of transmitter release evoked by spikes, since it is not possible to measure the relevant presynaptic \([\text{Ca}^{2+}]\), in space and time. However, indicator dyes do provide accurate measures of \([\text{Ca}^{2+}]\), at release sites following electrical activity, when the sharp \([\text{Ca}^{2+}]\), gradients near Ca channels have subsided. Measurements of post-tetanic \([\text{Ca}^{2+}]\), correlate well with post-tetanic potentiation (PTP) of transmitter release [1], suggesting that this residual \([\text{Ca}^{2+}]\), directly causes PTP, or is in equilibrium with Ca bound to some site which causes PTP. This is supported by the finding that chelation of residual \([\text{Ca}^{2+}]\), with a slow buffer (EGTA) that has no effect on transmitter release, and increasing single spikes blocks both the measured accumulation of residual Ca in a tetanus and potentiation caused by that tetanus [15]. However, like synaptic facilitation, PTP cannot be explained simply by supposing that residual free \([\text{Ca}^{2+}]\), sums with peak local \([\text{Ca}^{2+}]\), in active zones during action potentials to generate higher local \([\text{Ca}^{2+}]\), peaks and potentiate release. Such a model requires a much lower peak-to-residual \([\text{Ca}^{2+}]\), ratio in active zones than is expected from the considerations of the previous section, and predicts a faster decay of PTP of transmitter release than of \([\text{Ca}^{2+}]\), contrary to observation [1]. Rather, it appears that residual Ca acts presynaptically at a separate site to linearly potentiate release, perhaps by a calmodulin-dependent phosphorylation of synapsin I to liberate vesicles from cytoskeletal-binding sites and permit them to dock at release sites [21].

**Sodium and PTP**

Besides Ca, Na accumulation in nerve terminals has also been implicated in PTP [22, 23], and recent optical measurements of \([\text{Na}^{+}]\), show order-of-magnitude rises of 10 mM in nerve terminals during tetani causing PTP (K. Delaney and D. Tank, personal communication). It has been suggested that elevated \([\text{Na}^{+}]\), operates by releasing Ca from internal stores, but a tetanus in Ca-free medium still loads terminals with Na but does not result in any rise in \([\text{Ca}^{2+}]\), [24]. A reversal of Na/Ca exchange leading to a leak of Ca into Na-loaded terminals has also been proposed, but no rise in \([\text{Ca}^{2+}]\), could be detected in terminals following a Na load when Ca was washed back into the medium [24]. Instead, three lines of evidence indicate that Na accumulation reduces the rate of active extrusion of Ca during and after a tetanus, slowing the removal of Ca that has entered through Ca channels during action potentials. First, increasing the Na load with ouabain to block Na extrusion enhances and prolongs PTP in proportion to an increase in the accumulation of Ca in a tetanus and slowing of its post-tetanic decay. Second, reducing the Na load (by depolarizing in a Na-free medium) results in a more rapid extrusion of a Ca load. Third, blocking Na/Ca exchange with lithium slows post-tetanic Ca removal and increases its tetanic accumulation. Apparently Na/Ca exchange participates in Ca removal from terminals, and diminishing this process by reducing the transmembrane Na gradient enhances and prolongs the elevation in \([\text{Ca}^{2+}]\), that causes PTP.

**Modulation of transmission**

We have studied two processes that alter synaptic transmission at the crayfish neuromuscular junction: presynaptic inhibition and serotonergic modulation. \(\gamma\)-Aminobutyric acid (GABA)ergic synapses form between inhibitory motor nerve terminals and excitatory terminals, as well as onto muscle cells. By filling both the inhibitor and exciter with fura-2, the terminals of each can be identified by stimulating either alone and observing the selective \([\text{Ca}^{2+}]\), rise. Stimulating both simultaneously results in a reduction of Ca accumulation in excitatory terminals [16], as GABA opens presynaptic chloride channels [25] and shunts action potentials [26], reducing the number of Ca channels opened by a spike in exciter terminals.

Serotonin enhances both spontaneous and evoked glutamate release from exciter terminals by a cyclic AMP- and phospholipase C-dependent process [27]. Since neither resting \([\text{Ca}^{2+}]\), nor Ca
influx during action potentials is enhanced [28]. serotonin must work ‘downstream’ of Ca in the secretory process, either by sensitizing the receptor to \([\text{Ca}^{2+}]\), or by mobilizing vesicles to release sites and increasing the store of releasable transmitter.

**Effect of the voltage change during a spike on transmission**

A number of properties of synaptic transmission have suggested that the time course of transmitter release is determined primarily by the time course of the presynaptic membrane potential acting directly on the exocytotic machinery, in concert with a rise in \([\text{Ca}^{2+}]\). Examples of such properties are the difference in time course of transmitter release and presynaptic \([\text{Ca}^{2+}]\), measured after an action potential, and the fact that large depolarizations evoke greater release than small depolarizations leading to the same macroscopic Ca influx. As indicated above, these properties could also be consequences of the release of transmitter by localized \([\text{Ca}^{2+}]\), not measurable with present indicators and the different spatial distributions of submembrane \([\text{Ca}^{2+}]\), at different membrane potentials. A number of other experiments on neuromuscular junctions were originally interpreted as evidence that presynaptic membrane potential directly triggers transmitter release [29], but these were all shown to arise from procedural artefacts [30]. Recent simulation studies [31] also purport to show that the collapse of Ca domains in active zones could not explain the time course of transmitter release. In particular, its relative constancy at different levels of release (when changing extracellular \([\text{Ca}^{2+}]\) or following prior stimulation) and its temperature sensitivity were thought to be inconsistent with theoretical predictions. However, when the parameters of simulations were adjusted to better fit experimental procedures, and exocytosis was represented as a slow temperature-sensitive process following binding of Ca to trigger molecules [4], the simulations matched experimental results.

Recently, experiments have been described using the caged Ca chelator nitr-5, presynaptically injected into crayfish motor nerve terminals, to probe the separate roles of \([\text{Ca}^{2+}]\) and membrane potential in evoking transmitter release [32]. When nitr-5 partially loaded with Ca is exposed to ultraviolet illumination, it is converted to a Ca buffer with somewhat lower Ca affinity, resulting in a higher equilibrium level of free \([\text{Ca}^{2+}]\) [33]. Only a modest elevation of \([\text{Ca}^{2+}]\), is possible, from a resting level of about 150 nM to about 1 μM. This results in a modest increase (quadrupling) of the frequency of miniature excitatory postsynaptic potentials (MEPSPs), representing the spontaneous release of transmitter quanta. Pairs of action potentials repeatedly delivered in a low \([\text{Ca}^{2+}]\) medium (0.5 mM) with 2 mM of the Ca channel-blocker Mn2+, or in a solution containing no added Ca2+ and somewhat elevated Mg2+, failed to evoke detectable EPSPs before photolysis of nitr-5, but after photolysis led to a brief increase in the probability of appearance of an MEPSP immediately following a spike. Since spikes evoked no EPSPs before u.v. light exposure, it was assumed that \([\text{Ca}^{2+}]\), influx had been effectively blocked in the solutions used, and that membrane potential was therefore acting directly on the release mechanism that was ‘sensitized’ to voltage by the elevation in \([\text{Ca}^{2+}]\).

When we tested the efficacy of these solutions in blocking Ca influx in terminals filled with fura-2, we found that the same stimulus pattern used in the earlier study was accompanied by a readily detectable rise in presynaptic \([\text{Ca}^{2+}]\) [34]. Higher frequencies (100 Hz) also evoked large EPSPs, but these were not detectable at the lower frequencies used in the previous study. Apparently, the Ca influx was insufficient to evoke detectable transmitter release at these frequencies in low \([\text{Ca}^{2+}]\) medium, which is not surprising given the high degree of Ca co-operativity in triggering exocytosis and the large facilitation that these synapses display. We found that Ca influx could only be prevented effectively by adding a Ca chelator like EGTA to the extracellular solution in sufficient concentration to overcome the native buffering of Ca in the extracellular space and especially in the synaptic cleft [35].

We used this solution to block Ca influx during spikes, and asked what effect they had on transmitter release triggered by an elevation in presynaptic \([\text{Ca}^{2+}]\). With nitr-5, we again found only a small elevation in MEPSP frequency upon photolysis, but now this was unaffected by action potentials. We also tried a different caged Ca chelator, DM-nitrophen [36]. This compound is photolysed to products that hardly bind Ca2+ at all, permitting a higher elevation of \([\text{Ca}^{2+}]\), probably to about 5 μM and perhaps even higher. Photolysis of nitrophen elevated transmitter release to high rates (up to about 10,000 quanta/s), nearly approaching the rate of release achieved by an action potential in normal medium [37]. However, this activation of transmission was also unaffected by spikes when Ca influx was blocked with external EGTA [34]. Similar experiments in the squid giant synapse [38]
also failed to show any effect of the potential change during an action potential on transmitter release evoked by release of Ca from presynaptically injected nitrophen, when Ca influx was blocked with external EGTA.

**Flash photolysis of caged Ca and phasic transmitter release**

In many of these experiments, nitrophen was photolysed by exposure to a bright (150 W) collimated xenon light source. However, rapid photolysis of nitrophen can be achieved using a focused xenon flash lamp. Rapid partial photolysis of nitrophen releases Ca which rebinds relatively slowly to unphotolysed nitrophen – a derivative of the slow Ca buffer EDTA. This is in contrast to observations made using nit-5 – a derivative of the fast Ca buffer BAPTA. Therefore, partial flash photolysis of presynaptically injected nitrophen generates a brief spike of [Ca\(^{2+}\)], up to about 100 μM in amplitude but lasting only about a millisecond before rebinding to unphotolysed chelator and establishing a new modestly higher equilibrium [Ca\(^{2+}\)], of about 1 μM. These consequences of nitrophen photolysis have been measured directly with the Ca indicator fluo-3 [39, 40]. Rapid photolysis of nitrophen evokes an intense phase of transmitter release resembling a normal EPSP [38] (H. Kamiya and R. Zucker, unpublished work), while flash photolysis of nit-5 barely increases MEPSP frequency. These results also suggest that phasic transmitter release requires high [Ca\(^{2+}\)], at release sites.

**Multiple sites of Ca action in secretion from chromaffin cells**

The simulations of [Ca\(^{2+}\)], changes in active zones during and following nerve activity suggest that short-term synaptic facilitation does not arise simply from residual [Ca\(^{2+}\)], summing with peak [Ca\(^{2+}\)], transients during action potentials and acting at a single class of binding site. The similar post-tetanic decays of [Ca\(^{2+}\)], and EPSP amplitude also argue against a simple residual Ca summation model, and suggest a separate site of Ca action. Recent experiments on chromaffin cells provide additional evidence for such a separate site of Ca acting to influence secretion. We have triggered secretion in chromaffin cells by photolysis of Ca-loaded nitrophen perfused into single cells, with [Ca\(^{2+}\)], measured with the indicator furaptra, and secretion due to exocytotic fusion of secretory granules monitored as an increase in cell membrane area measured as a membrane capacitance change [41]. Using bright photolytic flashes, we found that a large step rise in [Ca\(^{2+}\)], triggered a brief, intense phase of secretion (about 1000 fF/s decaying in 2 s or less) followed by a much slower persistent phase of about 40 fF/s. Although Ca clearly triggered the rapid phase of secretion, it was unclear whether the subsequent slow phase was caused by a Ca-dependent or Ca-independent mobilization of granules up to release sites, or by an independent parallel Ca-dependent pathway for secretion. One way to distinguish these possibilities was to manipulate a phenomenon we call the 'loading transient'. When a whole-cell configuration is established by breaking the membrane under the patch pipette, dilute nitrophen enters a cell containing millimolar [Mg\(^{2+}\)], and initially this excess Mg competes with Ca for cationic-binding sites on the nitrophen, displacing the bound Ca and elevating [Ca\(^{2+}\)], to about 10 μM for about 30 s, until the nitrophen concentration in the cell exceeds the [Mg\(^{2+}\)]. This loading transient can be minimized by filling the tip of the perfusion pipette with a dilute EGTA solution, and backfilling with the Ca/nitrophen/furaptra mixture. This procedure almost eliminated both the transient modest rise in [Ca\(^{2+}\)], and secretion during filling. We wondered whether cells subjected to a loading transient were 'primed' in some way, mobilizing granules to 'docking' points at the membrane from which they could be more readily secreted. In fact, we found that a given [Ca\(^{2+}\)], step evoked more intense transient secretion if it was preceded by this modest [Ca\(^{2+}\)], elevation for 30 s, suggesting the existence of a Ca-dependent mobilization of secretory granules to release sites.

In some cells, we observed that, following massive secretion, an additional flash leading to another large [Ca\(^{2+}\)], step often resulted in sudden reduction of membrane capacitance, which we think represents a Ca-dependent endocytosis or recovery of vesicular membrane from the cell surface. Thus, there may be three sites of Ca action in secretory cells: triggering fast exocytosis, mobilizing granules to release sites, and activating a vesicular recovery process.

**Ca-dependence of secretion at slow synapses**

Some synapses transmit effectively only to repeated stimulation, and the responses rise very slowly, reaching a peak seconds after stimulation ceases and decaying for tens of seconds afterwards. Peptidergic secretion often behaves this way, and we have recently studied such secretion at preganglionic terminals onto bullfrog sympathetic ganglion cells. Luteinizing hormone-releasing hormone
(LHRH) is released from dense core vesicles in these terminals with typical 'slow' characteristics [42]. These vesicles do not cluster in active zones under the membrane, but are dispersed throughout cytoplasm and do not appear to be docked at or even near release sites [43]. Therefore, secretion of these vesicles cannot be triggered rapidly by local high [Ca\(^{2+}\)] domains, since they must first be moved up to the membrane before exocytosis can occur. This probably accounts for the sluggish nature of release of LHRH from these terminals and the 'late, slow EPSP that it evokes postsynaptically.

...cytoplasm and do not appear to be docked at or these vesicles cannot be triggered rapidly by local even near release sites [43]. Therefore, secretion of would respond only to Ca that reaches vesicles... movement of vesicles up to the membrane. This process as fura-2, so it seemed interesting to relate release from such synapses to measurements of presynaptic [Ca\(^{2+}\)], during stimulation.

The results on slow synaptic transmission and hor-...s are partly due to the long diffusion path-
way of LHRH from release sites to postsynaptic targets [42]. Thus, no good measure of the rate of LHRH release is available, but the integral of the postsynaptic current provides a measure of total release. This was found to be linearly related to the integral of presynaptic changes in [Ca\(^{2+}\)], above a threshold level of at least 200 nM [44]. LHRH release was also found to be a roughly linear function of external [Ca\(^{2+}\)] [45]. Thus, the rate-limiting step at slow synapses may be a Ca-dependent mobilization of vesicles similar to that inferred for chromaffin cells, operating with little co-operativity and at much lower [Ca\(^{2+}\)], levels than the Ca-sensitive trigger for exocytosis at fast synapses.

**Future directions**

The results on slow synaptic transmission and hormonal secretion suggest multiple sites of Ca action in the secretory process. To what extent are these processes also present at fast synapses? Are the 'priming' step in chromaffin cells and the mobilization of dense core vesicles in peptide secretion related to processes such as facilitation or potentiation at fast synapses? If mobilization of vesicles is the rate-limiting step in LHRH secretion, must this still be followed by local high [Ca\(^{2+}\)], levels near Ca channels to trigger exocytosis as in fast synapses? These are the sorts of questions we are pursuing now.

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Identification of cytosolic protein regulators of exocytosis
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Introduction
In eukaryotic cells, proteins destined for the plasma membrane or the extracellular space are delivered along the secretory pathway. This comprises a series of sequential, vesicle-mediated transport steps, each of which requires the specific targeting of transport vesicles to the appropriate acceptor membrane and the subsequent fusion of vesicle and acceptor membranes. In this way, proteins destined to be secreted from the cell travel from the endoplasmic reticulum through the Golgi complex, and are sorted into secretory vesicles in the trans-Golgi network. These vesicles then fuse with the plasma membrane. This final membrane fusion event is known as exocytosis and results in the discharge of vesicle contents into the extracellular space as well as the incorporation of vesicle membrane protein into the plasma membrane. Exocytosis can be divided into two classes: constitutive and regulated. In constitutive exocytosis, secretory vesicles fuse with the plasma membrane immediately after formation, whereas in regulated exocytosis, secretory vesicles accumulate in the cytoplasm and only undergo fusion upon receipt of an appropriate signal (usually, but not always, an increase in the cytosolic free Ca²⁺ concentration). All cells exhibit constitutive exocytosis, but only "professional" secretory cells such as neurons, endocrine and exocrine cells undergo regulated exocytosis.

Over the past decade, our understanding of the proteins involved in vesicle budding and fusion in the early stages of the secretory pathway has increased dramatically, mainly as a result of in vitro reconstitution approaches (for review, see [1]). Such reconstitution typically exhibits an absolute requirement for ATP and cytosolic proteins, and several of the essential cytosolic proteins have been purified and characterized, including N-ethylmaleimidesensitive fusion protein (NSF), α, β- and γ-soluble NSF attachment proteins (SNAPs), ADP-ribosylation factor and 'coatamer' components. However, in vitro reconstitution of exocytotic membrane fusion has proved extremely difficult and so most information on the intracellular requirements for exocytosis has come from the use of cells whose plasma membranes have been porated to allow manipulation of the intracellular environment. Recently, such permeabilized cells have been exploited to identify cytosolic proteins involved in regulated exocytosis.

Abbreviations used: PKC, protein kinase C; PLA₂, phospholipase A₂; PMA, phorbol 12-myristate 13-acetate.
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