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Minireview

Neurotransmitter release

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Axon terminals release more than one physiologically active substance. Synaptic messengers may be stored in two different types of vesicles. Small electron-lucent vesicles mainly store classical low molecular weight transmitter substances and the larger electron-dense granules store and release proteins and peptides. Release of the two types of substances underlies different physiological control. Release of messenger molecules from axon terminals is triggered by influx of Ca^{2+} through voltage sensitive Ca^{2+} channels and a rise in cytosolic Ca^{2+} concentrations. Neither the immediate Ca^{2+} target(s) nor the molecular species involved in synaptic vesicle docking, fusion and retrieval are known. It is, however, likely that steps involved in the molecular cascade of transmitter release include liberation of vesicles from their association with the cytonet and phosphorylation by protein kinase C of proteins which have the ability to alter between membrane bound and cytoplasmic forms and thus facilitate or initiate the molecular interaction between synaptic vesicles and the plasma membrane.

Exocytosis; Neurotransmitter; Protein kinase C; Release; Synapse; Vesicle

1. SITE AND CELLULAR SOURCE OF NEUROTRANSMITTER RELEASE

1.1. *Types of release and types of released messengers*

Upon arrival of an action potential the phasic release of a quantity of neurotransmitter is evoked from the terminal axon segment. This is the general physicochemical mechanism underlying the fast processing of information between nerve cells and also the transfer of signals from nerve to muscles and glands.

It is likely that all neurons release at their axon terminals more than one physiologically active substance. In addition to the classical low molecular weight messenger molecules like acetylcholine, noradrenaline and various amino acids also proteins and peptides are secreted [1,2]. This observation has abolished the former one-neuron-one-transmitter doctrine. Whether the substances coreleased with the classical transmitter substances serve as cotransmitters sustaining signal transfer or rather as modulators of the synaptic signal or even act at non-synaptic sites has to be discussed together with the classical transmitter regarding both the cellular preconditions and the molecular cascade of neurotransmitter release.

As has been shown for a variety of neurotransmitter substances, classical neurotransmitters are released in the form of small quantal packages and this is thought to be the result of the exocytotic secretion of the contents of individual synaptic vesicles. In addition to this phasic release mechanism there is a small but continuous release of neurotransmitter from nerve terminals which has also been referred to as transmitter leakage [3–6].

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1.2. *Consensus for exocytosis*

Exocytosis of neurotransmitter involves the release of soluble synaptic vesicle contents after controlled fusion with the presynaptic plasma membrane. Support for this view comes from ultrastructural analyses of nerve terminals on induced transmitter release, uptake of extracellular markers into synaptic vesicles, immunocytochemical analyses, the comparison of synaptic vesicle contents and products released, biochemical studies of synaptic vesicle life cycle and synaptic vesicle heterogeneity, and the quantal nature of the postsynaptic signal [6–13].

Using rapid freezing techniques it could be demonstrated at the frog neuromuscular junction that the exocytotic fusion of cholinergic synaptic vesicles occurs about 0.5 ms before the onset of the postsynaptic signal. When the synapse is intensely activated and synaptic vesicle recycling is impaired, the loss in synaptic vesicle numbers can be related directly to the counts of postsynaptically recorded transmitter quanta. If synaptic vesicle recycling is completely blocked, the incorporation of the synaptic vesicle membrane compartment into the presynaptic plasma membrane can be visualized by immunocytochemical methods using an-

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tibodies against synaptic vesicle membrane proteins [12]. Whereas it had been assumed for a long time that a single synaptic vesicle gives rise to a postsynaptic quantum, the observation at the neuromuscular junction that the classical quantal event may in fact consist of subquanta has come as a challenge to the original vesicle hypothesis [14,15]. Does one quantum correspond to the release of the contents of a single or of several synaptic vesicles? Are synaptic vesicles functionally heterogeneous with different release probabilities for different vesicle pools [3,7]?

For the neurotransmitter acetylcholine it has also been suggested [16] that it is not released by exocytosis of synaptic vesicles but through pores in the presynaptic plasma membrane which are formed by the association of membrane proteins referred to as mediatophore. The Ca^{2+}-activated opening of a mediatophore complex would give rise to the release of a quantum of acetylcholine. Similarly, the evoked release of amino acid transmitters from cytoplasmic rather than from synaptic vesicle stores has long been inferred. The protein subunit of the mediatophore complex has now been identified as a component of the synaptic vesicle proton pumping ATPase [17]. From studies on the vesicular uptake and storage of amino acids [18] and on the Ca^{2+} dependency of amino acid release from brain synaptosomes [5] strong evidence could be derived for exocytotic release of amino acid transmitters.

1.3. *Non-neuronal model systems*

Thus, the basic mechanism of evoked neurotransmitter release is analogous or perhaps identical to controlled secretory release from other secretory systems including endocrine and exocrine gland cells and possibly also mast cells, neutrophils or even oocytes or Paramaecium. There has been an increasing interest in non-neuronal model systems for studying the molecular mechanism underlying exocytosis in general but also to understand more fully neuronal secretion mechanisms [19]. Immediate access to the exocytotic release mechanism of these cells could be obtained by electropermeabilization [20], by permeabilization with digitonin and more recently with bacterial exotoxins [21–23] or with the patch clamp technique [24,25].

2. MORE THAN ONE TYPE OF VESICLE AND MORE THAN ONE TYPE OF EXOCYTOTIC CONTROL?

Before discussing some of the key mechanisms involved in exocytosis, a number of basic features of the neuronal secretory machinery have to be evaluated. This is important if it comes to a comparison with other secretory systems and the deduction of consensus mechanisms.

Neurons possess in their axons two principal structural types of secretory vesicles: the larger electron-dense or dense-cored vesicles (also referred to as granules) which store a variety of proteins and peptides and possibly also smaller molecular weight neurotransmitters. The function of these granules is thought to be mainly in the release of neuropeptides. The smaller electron-lucent vesicles do not store proteins and their function is in the rapid release of low molecular weight messengers. Both types release their contents by controlled exocytosis. This means, that axon terminals possess a differential organelle outfit for the release of high and low molecular weight substances. The two types of cell organelles differ not only with regard to their contents. At present there is some debate as to what extent they carry common membrane proteins. Whereas there are common features like the presence of a proton pumping ATPase [26], the presence of other common components is interpreted differently whether biochemical or ultrastructural evidence is used as the main argument [10,27–29]. A possible reason for some of the discrepancy may come from difficulties in comparing the adrenergic vesicle types 'large-dense cored', 'small dense-cored' and in addition 'electron-lucent' to the two principal vesicle types of other neurons.

It is, however, clear that the release characteristics of low and high molecular weight messengers differ with regard to both the activation pattern of the axon and the Ca^{2+} dependency [6,30]: at low action potential frequencies release of low molecular weight messengers (classical transmitters) predominates whereas release of peptides depends on higher frequencies. Furthermore, differential Ca^{2+} channels appear to be involved in triggering exocytosis from the two organellar sources [31]. This is paralleled by the observation that the small electron-lucent vesicles release their contents at morphologically specified release sites of the presynaptic membrane whereas the larger peptide-loaded granules release their contents distal to the synaptic cleft [32]. Furthermore, the association with surface proteins for the linkage to the cytonet is different between granules and smaller vesicles. Synapsin I which is thought to connect electron-lucent synaptic vesicles to actin and tubulin filaments [34] and which is involved in the control of neurotransmitter release [35] is not associated with the membrane of protein-storing secretory granules [36]. All these observations suggest that there are differences in the cellular and molecular preconditions for the release of low and high molecular weight messengers and thus for granules and small electron-lucent vesicles. Whether there are also differences in the final pathway of the exocytotic fusion event needs to be elucidated.

It has also been suggested that neurons contain only one principal type of vesicle, the dense-cored granule. After release of its peptide and protein contents and local membrane recycling this would turn into a smaller electron-lucent vesicle to be used in further cycles of

release and reloading of small messengers [37]. This would, however, need to involve also alterations in the membrane properties of the organelle. It is of interest that the small electron-lucent vesicle type has now been identified as a separate organelle also in neurosecretory axon terminals using vesicle-specific antibodies [36] and in non-neuronal cells in tissue culture transfected with a gene coding for a synaptic vesicle-specific protein [38,39]. Thus, large dense-cored granules and small electron-lucent vesicles may represent different vehicles for exocytotic release in a variety of cellular systems.

3. THE MOLECULAR CASCADE UNDERLYING EXOCYTOSIS

3.1. *Molecular events involved*

Due to the inaccessibility of the terminal axon segment and the small size of isolated synaptosomes, information concerning the molecular mechanism of transmitter release has also been gained from studies of model systems like the adrenal medulla or neutrophils and mast cells. It has to be seen to what extent both the mechanisms regulating exocytosis and the molecular cascade of vesicle fusion and fission are identical in all of these secretory systems and even between the two principal synaptic vesicle types.

The number of molecular events which are implicated in the mechanism of synaptic vesicle exocytosis is expanding rapidly. They include an increase in the cytosolic Ca^{2+} concentration into the micromolar range [40,41] or even higher [31], Mg-ATP and the activation of protein kinases, in particular of protein kinase C [40,42–44], synaptic vesicle-associated proteins like synapsins I and II or a chromaffin granule-binding protein [45–47], the phosphoproteins B-50 (GAP-43) [48] and P92 (possibly the MARCKS protein) [8], cytoskeletal proteins like actin and fodrin [23,50–54], GTP-binding proteins [55–59], arachidonic acid, and even others [24,60,61]. It is, however, not clear how these various molecular components interact and how their interaction is regulated.

3.2. *The central role of Ca^{2+}*

In action potential evoked synaptic transmission the elevation of the intracellular Ca^{2+} level following Ca^{2+} influx through voltage-gated Ca^{2+} channels is presumably the very first step of the cascade [41]. In this respect the nerve terminal differs from non-neuronal secretory systems where exocytosis is initiated by the action of receptors at the cell membrane [62]. However, it could be demonstrated that in a number of experimental conditions neurotransmitter can also be released in the absence of extracellular Ca^{2+} [63,64]. There is even evidence that neurotransmitter released in the absence of Ca^{2+} may be derived from a different intracellular pool than that in the presence of Ca^{2+} [65]. Together this documents the potential of intracellular Ca^{2+} stores in modulating or – under certain experimental conditions – in sustaining axonal transmitter release. The concentration of Ca^{2+} in the nerve terminal is controlled by a complex system of soluble buffers, organellar stores and membrane transport systems with varying Ca^{2+} transport affinities and capacities and every distortion will result in a modulation of transmitter release [66,67]. Ca^{2+} is sequestered inside the nerve terminal in the smooth endoplasmic reticulum, synaptic vesicles, mitochondria, and possibly also calcisomes. Furthermore, there exist intracellular buffering systems like Ca^{2+}-binding proteins [68,69]. Inositol polyphosphates have been assigned a key role in Ca^{2+} intracellular homeostasis. Inositol 1,4,5-trisphosphate is formed on receptor-mediated and G-protein-controlled turnover of phosphatidylinositol 4,5-bisphosphate together with diacylglycerol, an activator of protein kinase C. The inositol trisphosphate functions via specific receptors in the release of Ca^{2+} from intracellular stores, presumably the endoplasmatic reticulum and/or the calcisome [70]. In axon terminals receptor-mediated turnover of synaptic phosphoinositides for mobilizing Ca^{2+} from internal stores may serve mainly as a secondary and modulatory mechanism. It is a major disadvantage that we still do not know the immediate Ca^{2+} target(s).

3.3. *Molecular cascade downstream of the Ca^{2+} influx*

An early step in the initiation of vesicle exocytosis is presumably the mobilization of synaptic vesicles from their association with the cytonet [8,34] and possibly the removal of a cytoskeletal undercoating of the presynaptic release site [23]. This may explain the involvement of synapsin I and various cytoskeletal proteins in the release process. Ca^{2+} and protein phosphorylation play a major role in the control of these processes [23,45,71]. Furthermore, protein kinase C-dependent protein phosphorylation is likely to play a key role in the initiation of the exocytotic event. There is strong evidence that activation of protein kinase C potentiates transmitter release [72–75]. This enzyme is unique in that its soluble form is capable of being translocated to a membrane-bound form [76,77]. The endogenous activators of the enzyme are thought to be diacylglycerol and Ca^{2+} [78]. Two of its presynaptic targets which are by now well defined are the B-50 protein [79,80] and the MARCKS protein [81]. We still know, however, very little of how the substrate proteins for protein kinase C interpose between synaptic vesicles and the presynaptic plasma membrane. A protein of M_r 92 000 (P92, presumably identical with the MARCKS protein) associated with synaptic plasma membranes has been found to bind to synaptic vesicles after protein kinase C-dependent phosphorylation [8,82]. It may thus act as a mediator for docking the synaptic vesicle to its release site. Other evidence demonstrates that antibodies against the B-50 protein introduced into brain

synaptosomes inhibit transmitter release implicating the involvement of this protein in exocytosis [48]. Similarly exocytosis is inhibited by intracellular application of antibodies against the chromaffin-granule binding protein [46].

Phosphorylation of the neuron-specific B-50 protein has been correlated to transmitter release, long-term potentiation and also neurite outgrowth [44,83,84]. The B-50 protein has been reported to bind to the inside of the plasma membrane [85] to the actin rich neuronal membrane skeleton [86] and also to calmodulin [87]. Similarly the MARCKS protein, which is enriched in brain but can also be detected in a number of other tissues, becomes phosphorylated on induced transmitter release from synaptosomes and on activation of chromaffin cells [74,88–90]. Both the B-50 and the MARCKS protein are fatty-acylated [80,91], and in the fatty-acylated form they could be membrane anchored. Fatty-acylation and thus membrane anchoring may be regulated by protein kinase C-dependent phosphorylation [92,93]. However, additional properties of the protein may be essential for its targeting to specific domains of the nerve cell membrane [94]. Regarding the strong evidence for protein phosphorylation in neurotransmitter exocytosis, the importance of dephosphorylation events in this process cannot be overestimated [95,96].

When docking of synaptic vesicles is achieved, this step is followed by an as yet undefined mechanism of membrane fusion and fission. It is thought that the interaction of the two membrane compartments is initiated by a channel-like structure possibly involving corresponding protein channels in the synaptic vesicle membrane and the presynaptic plasma membrane [8,97,98]. Eventually soluble messengers may be released through a short-lived 'fusion pore' [24,99] and the synaptic vesicle membrane may be recycled for re-use [100]. From electrophysiological experiments it has been concluded that only about 100–200 μs elapse between the influx of Ca^{2+} and transmitter release [101]. Thus, the process leading to the fusion of small electron-lucent synaptic vesicles must be very rapid. This may be another aspect where exocytosis of larger granules and small synaptic vesicles differ.

4. MODULATION OF TRANSMITTER RELEASE

Synaptic transmission modulates and is subject to modulation. This is of great importance e.g. for synaptic information processing and modulation processes involved in behavioural plasticity phenomena. Releasing nerve terminals possess receptors for other transmitter substances, and practically all neurotransmitter substances also act back to the terminals from which they were released via presynaptic receptors (autoreceptors). The modulation may involve receptor-mediated modifications of e.g. K^+ or Ca^{2+} channels and thus control the availability of extracellular Ca^{2+} for transmitter release. External messengers may, however, also act via receptor-activated G-proteins controlling phospholipase C activity and thus the molecular cascade involved in vesicle exocytosis. This would result in changes in the intraterminal Ca^{2+} level, the concentration of diacylglycerol for activation of protein kinase C, and possibly other messenger systems [51,102]. In order to fully understand the molecular cascade underlying exocytosis at the axon terminal it may thus be necessary to clearly differentiate between two events: the cascade following the arrival of the action potential and Ca^{2+} influx leading to exocytotic release on the one hand, and additional mechanisms which are involved in modulating this event (e.g. by presynaptic receptor mediated mechanisms) and causing plasticity phenomena like potentiation or possibly also decrement on the other. Since secretion from model systems like mast cells or neutrophils is activated under physiological conditions by receptor-mediated mechanisms only (and not by action potentials), the sensitivity and preponderance of the molecular mechanisms involved in release may differ [102–104].

5. RESTING RELEASE

At least for low molecular weight transmitter substances there is in addition to quantal also a non-quantal transmitter release [4,5]. Non-quantal release is preponderant under resting conditions, independent of external Ca^{2+}, and results in a steady molecular leakage of transmitter [105]. Whereas quantal release is potentiated on depolarization of the axon terminal this is not the case for non-quantal release. Neither the mechanism of this Ca^{2+}-independent leakage nor its physiological implications are clear. At the neuromuscular junction it has been suggested to result from the transient incorporation of the vesicular acetylcholine transporter into the presynaptic plasma membrane on vesicle exocytosis [106]. This would allow the transmitter to leave the nerve terminal by a carrier-mediated mechanism and, accordingly, the released neurotransmitter would be derived from a non-vesicle bound, cytoplasmic pool.

6. SYNOPSIS

Whilst by now many of the principal building-stones making up the molecular control mechanisms involved in neurotransmitter release are known, they still have to be placed into the right order. The molecular cascade of the exocytotic event at the nerve terminal remains an enigma. In particular, the Ca^{2+} target(s) and the molecular (protein and/or lipid) components directly involved in synaptic vesicle docking, fusion and retrieval need to be identified.

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