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PRESYNAPTIC PLASTICITY: THE REGULATION OF Ca^{2+} -DEPENDENT TRANSMITTER RELEASE

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1. INTRODUCTION

There is a growing consensus that the most crucial element necessary for the nervous system to learn and thus to acquire new knowledge, lies at the synaptic contacts between neurons. This is a rather old idea, since already Ramón y Cajal (1894) proposed that learning would be achieved by the growth of synaptic terminals. During the twentieth century many investigators have tried to obtain experimental evidence for this hypothesis. Although in some cases structural changes of synaptic contacts have been reported to be correlated with changes in synaptic efficiency (see for instance, Lee *et al.*, 1980; Desmond and Levy, 1983), these changes are rather elusive and difficult to quantify. It is more likely that changes at the molecular scale, i.e. in gene expression, in receptor properties and/or second messengers may be put in evidence as underlying synaptic plasticity. Such subtle changes probably involve processes that may be revealed using micro physiological, biochemical or molecular biological methods.

The relevance of synaptic changes for learning and memory received much attention from theorists, since Hebb (1949) introduced the idea that the efficacy of a synapse changes if, and only if, there is a conjunc-

tion between the activity of the synaptic terminal and the firing of the post-synaptic neuron. A direct experimental proof of this theory is extremely difficult to obtain in view of the fact that under normal conditions the synaptic changes underlying the formation of memory traces are distributed over a large number of neurons and may be extremely small. This difficulty can be circumvented by using certain experimental preparations that can serve as models for memory traces. Among these preparations the following are more commonly used, namely associative learning in gastropod molluscs: *Aplysia* as used by Hawkins *et al.* (1983) and *Hermisenda* as used by Alkon *et al.* (1982) and long-term potentiation (LTP) in the hippocampus (Bliss and Lomo, 1973).

The studies in the molluscan models has yielded a number of important results regarding the nature of the synaptic changes responsible for non-associative and associative plasticity. One of the mechanisms that was advanced in this respect is the modulation of the action potential duration due to a cAMP mediated decrease in a potassium current induced by the facilitatory transmitter serotonin in *Aplysia*, that leads to a prolonged increase in transmitter release and thus to synaptic facilitation (Hawkins *et al.*, 1983). In *Hermisenda* associative learning appears to be mediated by a change in the excitability of certain neurons due to a change in the degree of phosphorylation of several potassium channels (Alkon *et al.*, 1982). A common mechanism is that most of the plastic changes in synaptic efficiency encountered in

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these experimental models, involve changes in the amount of transmitter released. Both *increases* and *decreases* have been reported. For example the long-term behavioural habituation of the gill withdrawal reflex of *Aplysia* is accompanied by a decrease in transmitter release, whereas the synaptic facilitation that is responsible for short- and long-term sensitization of the same reflex results from an increase of transmitter release.

The mechanisms underlying LTP of the hippocampus have been intensively investigated using physiological and biochemical methods (for a recent review see Bliss and Collingridge, 1993). LTP can be induced in several ways. The most common way is to use a high frequency train of pulses (tetanus) as a trigger, although series of volleys given at the theta rhythm frequency are also effective (Rose and Dunwiddie, 1986). Currently, it is accepted that hippocampal LTP comprises several stages, i.e. the induction or early phase which starts within seconds and may last about 1 hr and the late phase that corresponds to the long-term maintenance of the synaptic enhancement. Furthermore LTP has been observed outside the hippocampal formation, namely in the neocortex (Bindman *et al.*, 1991; Crepel *et al.*, 1991; Artola *et al.*, 1990), the amygdala and septal area (Racine *et al.*, 1983) and the nucleus accumbens (Boeijinga *et al.*, 1992). Notwithstanding the fact that there is general agreement on the nature of the events responsible for the *initiation* phase of LTP, namely the increase in $[Ca^{2+}]$ in the postsynaptic cell through the activation, although not exclusively, of NMDA receptors (Collingridge *et al.*, 1983; Gustafsson *et al.*, 1987), there is little consensus about the expression of the *persistent* phase of LTP. Based on electrophysiology, some current views favour a postsynaptic mechanism (Kauer *et al.*, 1988; Muller *et al.*, 1988; Foster and McNaughton, 1991), whereas other favour, at least partly, a presynaptic mechanism (Malinow *et al.*, 1989; Malinow and Tsien, 1990; Bekkers and Stevens, 1991) or an initial presynaptic followed by a postsynaptic process (Davies *et al.*, 1989). Recently, Malgaroli and Tsien (1992) showed that glutamate application at synapses between hippocampal neurons in culture produces LTP of the frequency of spontaneous miniature synaptic currents and that this effect is due to a sustained enhancement of quantal transmitter release with no appreciable change in postsynaptic responsiveness. On biochemical grounds, there is indirect evidence indicating a change of the phosphorylation level of certain presynaptic proteins such as B-50 (GAP-43; Bär *et al.*, 1980, 1982; Tielen *et al.*, 1983). Furthermore the experiments of Bliss and co-workers indicate that LTP is maintained, at least in part, by a sustained increase in transmitter (e.g. glutamate) release (Bliss *et al.*, 1988, 1986; Errington *et al.*, 1987; Lynch *et al.*, 1989a). These findings were challenged by Ben-Ari and co-workers (Anikszteijn *et al.*, 1989, 1991), but confirmed by recent observations in our group (Ghijsen and Lopes da Silva, 1991; Ghijsen *et al.*, 1992). Evidence from studies on structural changes in LTP points either to presynaptic (Applegate *et al.*, 1987), or to postsynaptic events (Chang and Greenough, 1984) or to both (Lee *et al.*, 1980). It appears that the expression of LTP is mediated through AMPA postsynaptic recep-

tors and possibly changes in the dynamics of the corresponding receptor-ionophore complex.

In order to understand long-term changes in synaptic transmission it is necessary to know in detail the molecular mechanisms underlying synaptic transmission and its modulation. As discussed above, plastic changes in synaptic efficiency encountered in many experimental models, involve changes in the amount of transmitter released. The attention given to these presynaptic components in synaptic plasticity is relatively scarce as compared to the postsynaptic processes. This may be largely due to the small size and poor attainability of nerve terminals *in situ*. However, using biochemical and molecular biological techniques and isolated nerve terminals, synaptosomes and cell free systems, a number of basic mechanisms involved in transmitter release are now being unravelled. This review attempts to discuss these basic mechanisms in the light of their possible role in synaptic plasticity. Significant progress has recently been made on the understanding of secretion of transmitter granules from endo/exocrine and neurosecretory tissue with the use of novel electrophysiological techniques. These granules are different from CNS small synaptic vesicles and, at least in some respects, from CNS "large, dense-cored vesicles" and are therefore not included in this review. Several excellent reviews have recently appeared on this topic (Almers, 1990; Burgoyne, 1990; Gomperts, 1990). In *in vivo* experimental models of synaptic plasticity, it is difficult to study changes in synaptic transmission as occurring in isolated pre- and postsynaptic components. Nevertheless, an analysis of presynaptic processes in isolation is necessary to understand which components are responsible for overall synaptic changes in intact systems. A reductionistic approach is chosen here to evaluate the phenomena altering the presynaptic stimulus secretion coupling.

Nerve terminals employ a complex set of cytoplasmic regulatory mechanisms within a very small area to tune the process of neurotransmitter secretion upon arrival of action potentials. The presynaptic stimulus-secretion coupling is not static. The same impulse arriving at the presynaptic nerve terminal, may induce a variable response depending on the recent history of the synapse. Nerve terminals exhibit time- and activity-dependent properties, i.e. the previous experience may affect the stimulus-secretion coupling. Furthermore, the capacity of the presynaptic nerve terminal to transmit signals within a given time is limited, since the pool of transmitter molecules available is limited. Changes in response can sometimes be easily surveyed, like the fading of responses upon exhaustion of the available transmitter pool, but may also be very complex. The fundamental properties of these regulatory mechanisms are often incompletely understood and the precise impact of mutual interactions even less. However, recent advances based on new molecular and cellular approaches has yielded valuable new information that significantly added to our understanding of the presynaptic stimulus-secretion coupling and presynaptic plasticity.

In the first part of this review we will present evidence for several simple forms of presynaptic

plasticity. These processes may operate by changing the secreted amounts of a given neurotransmitter or by changing the timing of this secretion upon stimulation. These pathways include intracellular Ca^{2+} buffering, regulated phosphorylation and dephosphorylation of proteins, energy requirement (ATP-regulation), protein translocations, reshaping of electrical activity and structural changes.

In the second part of this review, we consider the possibility that the mixture of the released substances may change under the influence of the stimulation conditions and of previous events. In this way, the overall signal transduction in the synapse may undergo plastic changes. These possibilities include changes in the released cocktail of substances and differential regulation of production, packaging, trafficking and secretion of two or more co-existing signal molecules.

In the third part of this review, we suggest several ways in which the local environment of the nerve terminal may influence the presynaptic input-output relation. These influences are evoked by factors including classical transmitters, neuromodulators and other messengers that activate presynaptic receptors or directly modulate the intracellular cascade leading to secretion.

2. ACTIVITY DEPENDENT CHANGES IN THE EXTENT AND KINETICS OF TRANSMITTER RELEASE

Neuronal transmitter secretion or exocytosis is a highly specialized process, that differs in many respects from constitutive secretion, a general feature of all cells. It may also differ from the regulated secretion in non-neuronal cells, such as endo- and exocrine cells. A large number of factors that regulate neuronal secretion are neuron-specific and possibly even specific for the secretion of a particular transmitter only. Apparently, the specific demands of the swift and precise chemical communication between neurons require a unique (neuron specific) organization. This includes the highly specialized nerve terminals containing small synaptic vesicles that accumulate fast-acting neurotransmitters and the active zone of the presynaptic membrane where synaptic vesicles are clustered and recycled and that contains a high density of Ca^{2+} channels.

In addition, neuronal forms of secretion exist that resemble the non-neuronal secretion. The latter comprise larger vesicles, that originate from the Golgi-apparatus in the cell soma and contain transmitters that also occur outside the nervous system, such as neuropeptides and catecholamines.

In both cases, the timing and the extent of release depend on and may vary with the stimulation pattern of action potentials arriving through the axon. It is clear that the elementary coupling between an action potential and the extent and timing of release, the stimulus-secretion coupling, is not static. During repetitive stimulation, the stimulus-secretion coupling is known to vary depending on the characteristics of the stimulation pattern and may lead both to impairment as well as potentiation of secretion (see for a review, Zucker, 1989). These forms of plasticity

may, in some cases, be transient and easy to survey as in the case of fatigue where the supplies of transmitter(-vesicles) are temporarily exhausted. However, in other cases presynaptic activity may evoke more complex and longer lasting changes in components involved in the stimulus-secretion coupling as in the case of protein phosphorylation/dephosphorylation. It cannot be easily surveyed which molecular mechanisms are responsible and which processes are determining or rate-limiting in these changes. In all these cases, presynaptic plasticity is exclusively evoked by the stimulation pattern of its own axon, without involvement of external factors and it concerns the timing and extent of release of one particular transmitter only. In this first part we focus on this most basic form of presynaptic plasticity and discuss the possible mechanisms within the terminal that may be responsible for such variations in its stimulus-secretion coupling.

2.1. THE RELATIONSHIP BETWEEN Ca^{2+} -ENTRY, INTRATERMINAL Ca^{2+} -ELEVATION AND TRANSMITTER RELEASE

Whereas Ca^{2+} may only be one of the possible triggers for release processes in non-neuronal cells, it is now well established that in the mammalian CNS exocytosis is triggered when depolarization opens Ca^{2+} -channels and Ca^{2+} enters the presynaptic terminal. The relationship between Ca^{2+} -entry and elevation of intracellular free $[\text{Ca}^{2+}]$ ($[\text{Ca}]_i$) on the one hand and neurotransmitter secretion on the other, is certainly complex. Many different Ca^{2+} -transport mechanisms exist in the membrane and many intracellular sites contribute to the regulation of intracellular Ca^{2+} . Not surprisingly, the relationship between $[\text{Ca}]_i$ or $[\text{Ca}]_i$ -elevation and secretion appears to be far from linear (Zucker, 1989; Verhage *et al.*, 1991b). This has also contributed to the formation of several alternative or additional hypotheses to the "Ca²⁺ hypothesis" (Dudel *et al.*, 1983; Parnas and Segal, 1989; Hohnner *et al.*, 1989). However, new sensitive techniques, including (perforated) patch clamp, digital imaging of intracellular free Ca^{2+} and photolabile Ca^{2+} chelators have strengthened the Ca^{2+} hypothesis (Zucker and Haydon, 1988; Mulkey and Zucker, 1991; Thomas *et al.*, 1991). A number of studies have shown that Ca^{2+} -entry and $[\text{Ca}]_i$ -elevation are related to use-dependent changes and other long-term adaptations in a number of ways (see for a review, Zucker, 1989). In addition, Ca^{2+} appears to have secondary functions in the fine-tuning of exocytosis, like the translocation of proteins, the activation of kinases and phosphatases and its own clearance from the presynaptic cytosol.

Voltage gated Ca^{2+} channels in the presynaptic membrane are the principal mechanism to enable rapid Ca^{2+} entrance and exocytosis. These channels form the first possible target for the regulation of secretion. The duration of the "open" state of these channels is one of the important factors that determines the amount of transmitter released. In the case of fast-acting transmitters the coupling between Ca^{2+} channels and exocytosis may be very tight and this relationship between open-time and release may be

direct; in other cases there may only be an indirect relationship. The open-time depends on the duration of depolarization as the action potential reaches the terminal and on the activity-status of the channel protein, i.e. the channels may undergo inactivation, intrinsically or by chemical modifications, such as phosphorylation/dephosphorylation cycles.

Ca²⁺ channels have now been grouped into different classes on the basis of functional and pharmacological criteria (L-, N-, T- and P-type; Nowycky *et al.*, 1985; Miller, 1987; Tsien *et al.*, 1988; Bean, 1989; Llinas *et al.*, 1989). Although there may be overlapping criteria, several distinct characteristics have been formulated for each class such as activation threshold, inactivation kinetics, conductance, pharmacology and cellular distribution. Some types may be preferentially located in nerve terminals and different types of Ca²⁺ channels may be involved in the secretion of different transmitters. However, the identification of those Ca²⁺ channels present in nerve terminals is, for practical reasons, still far from complete. It is therefore still unclear whether the channels that were originally characterized in dorsal root ganglion and Purkinje cell bodies, are present in presynaptic nerve terminals and coupled to transmitter secretion. The pharmacological criteria to discriminate between the different types have nevertheless yielded indications with respect to this issue. However, a large number of studies have produced apparently conflicting data on the role of certain types of Ca²⁺ channels in exocytosis. Blockade of all four classes of channels has been found to inhibit exocytosis. In fact, the original classification may not hold for the Ca²⁺ channels coupled to exocytosis and recent evidence suggests that presynaptic N- and P-type channels may in fact represent an overlapping superfamily of channels with overlapping pharmacological profiles with respect to the sensitivity for different fractions of α -FTX- and ω -conotoxins (see Mintz *et al.*, 1991; Hillyard *et al.*, 1992; Pocock and Nicholls, 1992). In order to be able to measure transmitter release, stimuli are often chosen which are rather different from those encountered under physiological conditions, for instance a persistent depolarization by [K⁺]_o-elevations. Under these conditions, Ca²⁺ channels that are not coupled to exocytosis *in vivo* may be recruited. Artificially long depolarizations and Ca²⁺ channel open-states will produce longer Ca²⁺ influx and allow Ca²⁺ to diffuse over artificially long distances and reach the site of exocytosis from remote sites.

It has been suggested that L-type Ca²⁺ channels occur in nerve terminals, largely derived from studies on the isolated nerve terminal preparation (synaptosomes). Binding sites for specific blockers of L-type channels, 1,4-dihydropyridines (DHPs), exist in this preparation and inhibition of the ⁴⁵Ca uptake was observed (Turner and Goldin, 1985; Suszkiw *et al.*, 1986; Dunn, 1988). Furthermore, the specific agonist BAY-K was found to evoke Ca²⁺ uptake (Woodward *et al.*, 1988) and stimulate release in a number of preparations, such as brain slices. Still, the majority of studies have found no effect of DHPs (Nachshen and Blaustein, 1979; Daniell *et al.*, 1983; Miller and Friedman, 1984; Rampe *et al.*, 1984; Suszkiw *et al.*, 1986; Rivier *et al.*, 1987) and in those

cases where an inhibition was reported, this effect was only limited. It was suggested by Suszkiw *et al.* (1986) that DHP-mediated inhibition of Ca²⁺ fluxes is derived from preparations contaminated with "dendrosomes". Furthermore, DHP binding sites were found on brain microsomes (Cruz *et al.*, 1987) and mitochondria (Zernig and Glossmann, 1988), which contaminate synaptosomal preparations (see Verhage *et al.*, 1988 and references therein). Due to the lack of inactivation and the relatively high conductance of L-type channels, artificially long depolarizations as with K⁺, may produce coupling between this channel and exocytosis in some experimental models (see above), although this coupling is probably not relevant *in vivo*. All together, there is little direct evidence for an inhibition of [Ca]_o-elevation and exocytosis by DHPs in nerve terminals. However, some secretion, especially of neuropeptides may occur outside synapses and may be triggered through L-type Ca²⁺ channels.

The most common opinion appears to favour an association between presynaptic secretion and the α -FTX- and ω -conotoxin sensitive Ca²⁺ channels (Reynolds *et al.*, 1986; Miller, 1986; Hirning *et al.*, 1988; Herdon and Nahorski, 1989; Keith *et al.*, 1989; Maggi *et al.*, 1990; Woodward *et al.*, 1988; Lundy *et al.*, 1992; Pocock and Nicholls, 1992). The ω -conotoxins inhibit Ca²⁺ uptake into, and catecholamine release from, synaptosomes (Reynolds *et al.*, 1986; Woodward, 1988; Rivier, 1987; Pocock and Nicholls, 1992), acetylcholine release (Yeager *et al.*, 1987; Ahmad and Miljanich, 1988), serotonin release (Reynolds *et al.*, 1986), vasopressin release (Dayanithi *et al.*, 1988) and dynorphin release (Terrian *et al.*, 1989). However, this blocker only showed marginal effects on K⁺ evoked [Ca]_o-elevation in a mixed population of isolated nerve terminals and on the release of the major excitatory neurotransmitter in the brain, glutamate and showed also a poor effectivity on the major inhibitory transmitter, GABA (Keith *et al.*, 1989; Thate and Meyer, 1989; Turner and Goldin, 1989; Pin and Bockaert, 1990; Pocock and Nicholls, 1992). The agatoxin sensitive Ca²⁺ channels may consist of several subtypes (Bowers *et al.*, 1987; Llinas *et al.*, 1989; Bindokas and Adams, 1989; Adams *et al.*, 1989; see for a review, Jackson and Parks, 1989) and may be widely expressed in nerve terminals (Bowers *et al.*, 1987; Llinas *et al.*, 1989; Lundy *et al.*, 1992; Pocock *et al.*, 1992; Pocock and Nicholls, 1992). Recent evidence suggest that this toxin specifically blocks the secretion of fast-acting transmitters (Llinas *et al.*, 1989; Bindokas *et al.*, 1991; Pocock and Nicholls, 1992; Turner, 1992). From these data the picture emerges that ω -conotoxin sensitive Ca²⁺ channels (N-type) may be involved in secretion of monoamines, but not of the fast-acting amino acid transmitters. Conversely, agatoxin sensitive Ca²⁺ channels may be involved in secretion of the fast-acting amino acid transmitters, but not of the monoamines. A striking example of this suggestion is presented by Pocock and Nicholls (1992), showing that in a mixed population of mammalian nerve terminals agatoxins potently inhibit secretion of glutamate, but have no effect on nor-adrenaline secretion. In addition, the secretion of neuropeptides appears to be blocked predominantly

by the agatoxins, suggesting that one population of Ca^{2+} channels is involved in the secretion of both the classical neurotransmitters and of co-localized neuropeptides (Pocock and Verhage, manuscript in preparation).

More than one type of Ca^{2+} channel may be present in a single nerve terminal. This possibility gives the system extra opportunities to tune the Ca^{2+} influx and thus the timing and extent of secretion and the cocktail of transmitters released. The frequency of burst activity regulates the contribution of inactivating currents (mainly N- and P-like currents). In the mammalian neurohypophysis the burst activity of action potentials initially shows a 20 Hz frequency which subsequently diminishes to 5 Hz (Yeager *et al.*, 1987; Yoshikami *et al.*, 1989). It may be assumed that N-type currents present in the synapses of the neurohypophysis will contribute only to the initial high frequency, while the L-type currents will dominate during the later stages of the bursting activity (see Nowicky, 1991). In addition, if there is an unique association between certain transmitter types and Ca^{2+} channel types, stimulation patterns could differentially evoke the secretion of one of more co-existing transmitters (see Section 3 for further discussion).

Several mechanisms are described that regulate the activity of presynaptic ion channels. Long lasting changes in the currents generated by these channels have been implicated in mechanisms of synaptic plasticity. However, the main body of evidence regarding these mechanisms is derived from studies on "postsynaptic" cells. It is unclear whether similar processes are relevant to the plasticity of the presynaptic nerve terminal. Studies on isolated nerve terminals (Tibbs *et al.*, 1989; Zoltay and Cooper 1990; Barrie *et al.*, 1991) and the squid giant axon (Robitaille and Charlton, 1992) indicate that modulation of presynaptic K^+ channels may be an important factor in the modulation of the presynaptic input-output relation. Phosphorylation of presynaptic K^+ channels may prolong presynaptic depolarizations upon incoming action potentials, and may thus lengthen the periods of Ca^{2+} influx (presynaptic Ca^{2+} channels involved in fast-acting transmitter secretion appear not to inactivate readily, McMahon and Nicholls, 1991) and potentiate transmitter release. In fact, this mechanism may be a major pathway in presynaptic plasticity, because presynaptic PKC activation was shown to induce this increased depolarization, Ca^{2+} influx and glutamate release (Barrie *et al.*, 1991). *In vivo*, this PKC stimulation and the proposed lengthening of the depolarization at the nerve terminal may occur through repetitive (high frequency) stimulation, when Ca^{2+} levels gradually build up in the presynaptic cytosol or through presynaptic receptor occupation and phospholipase stimulation, possibly in concert with other second messengers (see Section 3 for further discussion). Similar mechanisms are known to operate on presynaptic ion-channels in *Aplysia* (see for a review, Siegelbaum and Tsien, 1983): phosphorylation by cAMP dependent kinase and protein kinase C alters the gating properties of presynaptic Ca^{2+} channels, K^+ channels and Cl^- channels and modulates transmitter secretion.

The elevation of intracellular free Ca^{2+} is not a

simple consequence of the extent of Ca^{2+} channel opening and Ca^{2+} influx. It must also be taken into account that the physiologically relevant elevation of $[\text{Ca}]_i$ represents a spatially restricted, transient, non-equilibrium situation in a complex system. A number of factors are possibly involved in this system:

- Different types of Ca^{2+} conductances may contribute to the intracellular elevation of Ca^{2+} (see above).
- Ca^{2+} channels may not be distributed randomly in the presynaptic membrane. Neighbouring Ca^{2+} channels may influence each other and the elevation of $[\text{Ca}]_i$ may provide a feedback signal that modulates the gating of the channels.
- The majority of Ca^{2+} ions that enter the nerve terminal may be absorbed by Ca^{2+} -buffering components in the cytosol of the nerve terminal.
- In the longer term, low capacity Ca^{2+} transporters may influence $[\text{Ca}]_i$, such as Ca^{2+} pumps and exchangers in the plasma membrane and Ca^{2+} mobilization from internal stores.

Ca^{2+} mobilization from internal stores within the nerve terminal may not be as prominent as it is in cell bodies and dendrites and in many non-neuronal cells. There is little direct evidence for such Ca^{2+} stores within the nerve terminal. Several observations evidence IP_3 production and IP_3 releasable Ca^{2+} pools within the isolated nerve terminal preparation (Ghandi and Ross, 1987; Audigier *et al.*, 1988), but these stores could be localized in other compartments than the presynaptic terminals. In electron microscopical analyses of nerve terminals, endoplasmatic reticulum-like structures have been found (McGraw *et al.*, 1980), but in a limited number of terminals. Often these structures were found in compartments devoid of synaptic vesicles (i.e. membrane sacs, isolated microsomes or "dendrosomes") and < 5% of the synaptosomes have identifiable endoplasmatic reticulum-like structures (Verhage and Ghijsen, unpublished observations). Moreover, indications for elevations of presynaptic free Ca^{2+} and/or transmitter release evoked by activators of phospholipase C are scarce in synaptosomes or intact presynaptic nerve terminals.

Presynaptic Ca^{2+} channels occur predominantly in the active zone. The kinetics of fast-acting transmitter exocytosis (0.2–2 msec) give Ca^{2+} ions little time to diffuse before triggering exocytosis. It is clear that high-capacity mechanisms, that allow a large Ca^{2+} influx in a very short time are necessary to obtain such fast kinetics. Therefore, a subcellular organization appears to exist in which Ca^{2+} channels are very close to the synaptic vesicles at the active zone (see Zucker and Stockbridge, 1983; Simon and Llinas, 1985; Zucker and Fogelson, 1986; Smith and Augustine, 1989). This is supported by ultrastructural observations (see Dreyer, 1973; Heuser *et al.*, 1979; Pumplun *et al.*, 1981) and fluorescent monitoring of Ca^{2+} (see Smith and Augustine, 1988; Swandulla *et al.*, 1991; Llinas *et al.*, 1992). Furthermore, the highest density of presynaptic Ca^{2+} channels is located directly opposite to the highest density of

postsynaptic receptors (Robitaille *et al.*, 1990). Converging Ca^{2+} influxes may occur as a result of the array of Ca^{2+} channel pore-mouths in the active zone. In this case, incoming Ca^{2+} fluxes from different Ca^{2+} channels will immediately start to overlap (Zucker and Fogelson, 1986).

These arguments suggest that an incoming action potential produces a rapid and local high elevation of free Ca^{2+} underneath the active zone membrane which is effective in triggering the exocytosis of fast-acting transmitters from the small synaptic vesicles that are lined-up at the active zone membrane. The high potency of cytosolic Ca^{2+} -buffering systems and the fact that Ca^{2+} channels rapidly close as a result of repolarization will further limit the spread and duration of $[\text{Ca}]$ -elevations in the terminal to this small area underneath the active zone. In such a model, the only obvious parameters that may modulate the extent and timing of transmitter release are the local factors that determine these local Ca^{2+} -elevations and release, i.e. the probability and duration of Ca^{2+} channel opening, the potency of local Ca^{2+} buffering components and the local availability of transmitter vesicles and Ca^{2+} sensitive triggers.

An important parameter in the regulation of Ca^{2+} channel opening and the duration of this opening may be the activity of K^+ channels to repolarize the terminal. Modulation of these channels may shorten or lengthen the local depolarization and may thus modulate the number of activated Ca^{2+} channels and their effectiveness in producing a local high Ca^{2+} -elevation and exocytosis (Delaney *et al.*, 1991; Jackson *et al.*, 1991; Robitaille and Charlton, 1992). Furthermore, elevations in intracellular free Ca^{2+} may modulate Ca^{2+} channel activity by changes in the phosphorylation state of the channel proteins. In postsynaptic cells there is good evidence for such mechanisms (see for instance, Armstrong, 1989), but it is presently unclear whether these act on the presynaptic Ca^{2+} channels involved in exocytosis. Probably, the kinetics of such phosphorylation-dephosphorylation reactions are too slow anyhow to play an important role in modulating the channel activity during the brief periods of opening, but in the longer term, during repetitive stimulation and prolonged elevations of intracellular free Ca^{2+} , such mechanisms may contribute to changes in the probability of opening and conductance of the Ca^{2+} channels involved in exocytosis. However, in isolated CNS nerve terminals two subsequent periods of chemical depolarizations produced identical Ca^{2+} responses (Verhage *et al.*, 1991c). Finally, basic thermodynamic constraints may play a role in the modulation of presynaptic Ca^{2+} influx. The channels exploit a combination of diffusional and electrophoretic motions of Ca^{2+} ions, whereas only diffusional motion is utilized in the internal and external compartment. As a result, during the non-equilibrium situation of Ca^{2+} channel opening, the local internal Ca^{2+} concentration rises steeply but also the local external Ca^{2+} concentration may decrease in the small volume of the synaptic cleft. In the local field around the Ca^{2+} channel both the chemical gradient over the membrane and the electrical field strength may decrease significantly. As these two parameters form the driving force for Ca^{2+} to enter the terminal,

their decrease will limit Ca^{2+} influx. This process may help to rapidly terminate Ca^{2+} influx during a single depolarization, but may also be prominent during repetitive stimulation when residual Ca^{2+} builds up in the internal compartment. In fact, such processes may, together with cytosolic Ca^{2+} -buffering components, help to maintain an unequal distribution of free Ca^{2+} inside the terminal for longer periods during repetitive and prolonged stimulation. Experimental evidence confirms that the extracellular Ca^{2+} concentration may indeed decrease significantly during such stimulation (Wadman *et al.*, 1985; Melchers *et al.*, 1988) and that prolonged, local elevations in intracellular free Ca^{2+} may exist (see below).

The above model of local Ca^{2+} -elevations has been elaborated over the last decade (see Zucker and Stockbridge, 1983; Simon and Llinas, 1985; Zucker and Fogelson, 1986; Smith and Augustine, 1988). However, it has been difficult to obtain unambiguous experimental evidence, mainly for practical reasons such as the poor accessibility of CNS nerve terminals to electrophysiological studies and Ca^{2+} imaging. Studies on an unusually large nerve terminal, that of the squid giant axon, have revealed the occurrence of Ca^{2+} "hot spots" associated with the tips of this terminal (Smith and Augustine, 1988; Swandulla *et al.*, 1991; Llinas *et al.*, 1992). We were able to obtain other, indirect evidence for the relevance of local elevations of $[\text{Ca}]$, in CNS nerve terminals with the use of the mobile-carrier Ca^{2+} -ionophore ionomycin (Verhage *et al.*, 1991b). As discussed above, a Ca^{2+} channel is opened by depolarization, allowing about 10^3 ions per msec to emerge from an individual channel. Consequently, the Ca^{2+} concentration in the immediate vicinity of the mouth of the channel, $[\text{Ca}^{2+}]_{\text{local}}$, will be substantially higher than that in the bulk cytoplasm, $[\text{Ca}^{2+}]_{\text{cyto}}$ and thus $[\text{Ca}]_{\text{local}} > [\text{Ca}]_{\text{ave}}$, i.e. the average concentration in the terminal. In contrast, the ionophore will induce a uniform and gradual elevation of Ca^{2+} within the terminal, such that $[\text{Ca}^{2+}]_{\text{local}} = [\text{Ca}^{2+}]_{\text{ave}} = [\text{Ca}^{2+}]_{\text{cyto}}$, since these ionophores insert randomly into the membrane and typically transport about one ion per msec (Pressman, 1976). It follows that when the same level of $[\text{Ca}^{2+}]_{\text{ave}}$ is evoked either by depolarization or by a low dose of ionomycin, a neurotransmitter regulated by $[\text{Ca}^{2+}]_{\text{local}}$ will be much more effectively released by depolarization than by ionomycin, whereas a neurotransmitter released by $[\text{Ca}^{2+}]_{\text{cyto}}$ will be released more effectively by ionomycin than by depolarization. Stimulation of isolated nerve terminals with both chemical depolarization and the ionophore clearly show that fast-acting (classical) transmitters are released by $[\text{Ca}^{2+}]_{\text{local}}$ in the micro-environment of voltage-activated Ca^{2+} channels while other transmitters, not clustered at the active zone such as the neuropeptides accumulated in large, dense-cored vesicles are released by bulk $[\text{Ca}^{2+}]_{\text{cyto}}$ (Verhage *et al.*, 1991c) (Fig. 1). This indicates that fast-acting transmitters indeed appear to be released by a closely coupled interaction with the Ca^{2+} -dependent effectors of secretion and the Ca^{2+} channel. It also indicates that other, slow transmitters, such as the neuropeptides, are only released when a significant rise in the bulk presynaptic $[\text{Ca}^{2+}]_{\text{cyto}}$ overrules the

Resting terminal

Chemical depolarization

Ionomycin

FIG. 1. Proposed mechanism for the differential release of classical transmitters and neuropeptides and the relevance of high local elevations of intracellular free Ca^{2+} underneath the active zone plasma membrane. Drawings represent nerve terminals containing two types of synaptic vesicles. Under resting conditions ($[\text{Ca}]_{\text{ave}} = 150 \text{ nM}$) no release occurs (upper scheme). When the terminal is depolarized by high K^+ (middle scheme), voltage-activated Ca^{2+} channels in the active zone open. The Ca^{2+} concentration in the immediate proximity of the inner aspect of the channel is high before Ca^{2+} is able to diffuse radially into the bulk cytoplasm and produce a $[\text{Ca}]_{\text{ave}} = 400 \text{ nM}$. The Ca^{2+} -sensitive trigger for classical transmitter exocytosis experiences the high Ca^{2+} concentration in the micro-environment of the mouth of the open channel which is necessary for classical transmitter release (see also Fig. 2). Stimulation with low ionomycin (bottom scheme) produces a similar $[\text{Ca}]_{\text{ave}}$ (400 nM), but through random penetration of nerve terminal membrane. As in the case of K^+ depolarization this elevation of $[\text{Ca}]_{\text{cyto}}$ is sufficient to activate the neuropeptide release mechanism, which is sensitive to relatively small Ca^{2+} -elevations (see also Fig. 2), but does not produce the high elevations in $[\text{Ca}]_{\text{local}}$ to evoke release of classical transmitters (modified from Verhage *et al.*, 1991b).

powerful homeostatic mechanisms for restoring $[\text{Ca}^{2+}]$. *In vivo*, this may be the case only when repetitive action potentials enter the terminal. Indeed, the ratio of neuropeptide to fast-acting neurotransmitter release from electrically stimulated brain slices was found to increase with the frequency of stimu-

FIG. 2. The differences in Ca^{2+} sensitivity of the release mechanisms for the different transmitter classes. The volume average intracellular free Ca^{2+} concentration may be titrated by different low doses of the calcium ionophore ionomycin in combination with adjustments of the extracellular Ca^{2+} concentration while the intracellular free Ca^{2+} concentration is measured with fura-2 (on the horizontal axis). Through these titrations (see Verhage *et al.*, 1991b), it appears that the release mechanisms for the different types of transmitter exhibit different Ca^{2+} sensitivity. Neuropeptides and catecholamines are released upon reasonably small increases of the free Ca^{2+} concentration in the presynaptic nerve terminals, whereas classical transmitters (amino acid transmitters) are only released upon large increases in free Ca^{2+} , above the detection limit of fluorescent probes for intracellular free Ca^{2+} (taken from Verhage *et al.*, 1989, 1991a,b).

lation (Bartfai *et al.*, 1988; see Section 3 for further discussion).

The mobile-carrier ionophore can also be used to titrate the intracellular free Ca^{2+} concentration to obtain information on the affinity of the release mechanism. Whereas slow-acting transmitters are released by small elevations of the cytosolic Ca^{2+} concentration, the Ca^{2+} concentration effective in triggering fast-acting transmitter release is very high, in fact beyond the detection limit of intracellular Ca^{2+} indicators, i.e. $> 10 \mu\text{M}$ (Fig. 2). These observations again confirm the model of local high Ca^{2+} elevations. Moreover, it suggests that the affinity for Ca^{2+} of the fast transmitter release mechanism is rather low. A relatively low affinity of the putative Ca^{2+} sensitive trigger allows a large off-rate (fast dissociation) of the interaction with this trigger, again a mechanism to maximize transmission velocity. On the other hand, exocytosis may also be terminated in a Ca^{2+} -independent manner, for instance by inactivation in analogy with Na^+ channel inactivation. In contrast, slow-acting transmitter exocytosis shows a high affinity for Ca^{2+} (Verhage *et al.*, 1991a,c, 1992b), in agreement with the slow kinetics of their release and the small Ca^{2+} -elevations that may reach remote sites in the terminal where the large, dense-core vesicles are found (a further discussion is given in Section 3).

Further evidence for the relevance of a local Ca^{2+}

elevation at the active zone is observed by the differential effects of different Ca^{2+} chelators depending on the Ca^{2+} -binding kinetics of these chelators (Adler *et al.*, 1991; Swandulla *et al.*, 1991). The slow binding kinetics of EGTA still allow exocytosis to occur in excess intracellular EGTA, while other Ca^{2+} chelators with faster binding kinetics give rise to a powerful inhibition of exocytosis when present in the nerve terminal cytosol of the squid giant axon (Adler *et al.*, 1991). This again indicates that exocytosis of fast-acting transmitters can occur through a kinetic lag between the local accumulation of high amounts of Ca^{2+} ions and their absorbance by the bulk cytosol and its Ca^{2+} -binding components.

Surprisingly, the coupling between directed Ca^{2+} -entry, local elevations and fast transmitter exocytosis appears to persist with the use of permanent chemical depolarizations (Verhage *et al.*, 1991b). In experiments with timed quenching of extracellular Ca^{2+} (McMahon and Nicholls, 1991) it appears that this coupling is an ongoing process and that fast transmitter exocytosis still occurs after 30 sec permanent depolarization. Firstly, this indicates that recruitment of transmitter vesicles is an ongoing process during prolonged stimulation, but secondly, these observations also suggest that the unequal distribution of Ca^{2+} may persist for many seconds. However, a nerve terminal with a diameter of approximately 1 μm seems too small to maintain a gradient of Ca^{2+} for more than a fraction of a second after Ca^{2+} -entry at the active zone and before Ca^{2+} diffuses into the bulk cytoplasm and equilibrates. This may indicate that physical barriers exist for Ca^{2+} ions to translocate between the submembrane area and the bulk cytoplasm of the terminal. In this case plasticity of fast transmitter release may be entirely dependent on factors within this putative " Ca^{2+} domain". Some evidence for such domains comes from the squid giant synapse (Llinas *et al.*, 1992) and our preliminary data using confocal scanning microscopy suggest that some mammalian CNS synapses may also contain such restricted areas. Alternatively, local Ca^{2+} -elevations may be obtained for longer periods without physical barriers if cytosolic Ca^{2+} buffering is potent enough to absorb all Ca^{2+} ions that diffuse from the submembrane area. It has been shown in oocyte cytosolic extracts, that soluble Ca^{2+} -buffering components indeed restrict an elevation of free Ca^{2+} to distances smaller than the diameter of a cell (Allbritton *et al.*, 1992). When an individual Ca^{2+} channel at the active zone is open, the Ca^{2+} concentration in the immediate proximity of the inner aspect of the channel will be high on purely geometric grounds, before it is able to diffuse radially into the bulk cytoplasm. It follows that, regardless of the overall dimensions of the terminal, if individual Ca^{2+} -sensitive triggers for fast-acting transmitter exocytosis were located with sufficiently close proximity to individual voltage-gated Ca^{2+} channels, then they would experience the high Ca^{2+} concentration in the micro-environment of the mouth of the open channel. Several time-consuming processes may allow Ca^{2+} to accumulate for a short instant within the micro-environment, long enough to trigger exocytosis. First, Ca^{2+} diffusion into the cytosol proceeds with limited velocity (diffusion co-efficient 10^{-5} – 10^{-6} m^2/sec ; Llinas, 1981a;

Neher, 1986; Allbritton *et al.*, 1993). Furthermore, the saturated Ca^{2+} buffer components must diffuse away into the bulk cytosol while free components must enter the submembrane area. This diffusion is probably delayed by interaction between Ca^{2+} and/or Ca^{2+} -buffer components with cytoskeletal components. Finally, the actual binding of Ca^{2+} by free Ca^{2+} buffers may require conformational changes (for instance, in the case of the so-called "EF-hand" Ca^{2+} -binding proteins, see Stoclet *et al.*, 1987).

In conclusion, the Ca^{2+} signal that results from short, single stimulations to the presynaptic nerve terminal and opening of Ca^{2+} channels may be restricted to a small submembrane area where it triggers classical transmitter release. The signal may be transmitted further as Ca^{2+} -calmodulin complexes and through the activation of Ca^{2+} sensitive kinases and phosphatases (Fig. 3).

Several estimations indicate that soluble Ca^{2+} buffering components alone may account for a potent chelation of Ca^{2+} (without uptake of Ca^{2+} into internal stores or Ca^{2+} efflux). The intracellular concentration of the Ca^{2+} -binding protein calmodulin was estimated to be in the high micromolar range (see Smith and Augustine, 1988; Kennedy, 1989) and a still increasing number of Ca^{2+} -binding proteins is reported to occur in high concentration in the soluble fraction. These Ca^{2+} -binding proteins may be of vital importance in the plasticity of the stimulus-secretion coupling. The central role of the Ca^{2+} -binding protein calmodulin and the calmodulin dependent kinase II has been emphasized already in many models as a molecular switch leading to prolonged changes in cellular responsiveness and memory storage (see Lisman, 1989; Kennedy, 1989). Homologous recombination in mice, deleting the α -isoform of the kinase, has provided experimental evidence that this kinase is indeed important in neuronal plasticity, i.e. spatial learning (Silva *et al.*, 1992a,b). Finally, it has been suggested that certain Ca^{2+} -binding proteins are expressed depending upon the specific needs of certain nerve cells. For instance, parvalbumin appears to be specifically expressed in the fast spiking GABAergic interneurons of the hippocampus (Kamphuis *et al.*, 1989) and it has been suggested that parvalbumin is responsible for the regulation of the rapid and short Ca^{2+} transients in these cells (Blaustein, 1988).

An illustration of this potent Ca^{2+} buffering in the cytosol can be obtained biochemically by studying the isolated nerve terminal preparation (synaptosomes). The volume of the synaptosome cytosol was estimated to be between 3.5 and 4.5 μl per mg protein (Nagy and Delgado-Escueta, 1984; Blaustein, 1985). Depolarizations of long duration using $[\text{K}^+]_o$ -elevations, yield only an increase in $[\text{Ca}]_i$ of 100–200% over basal levels, i.e. from a resting level of 100–200 nM to 400 nM (Ashley *et al.*, 1984; Verhage *et al.*, 1988, 1989; McMahon and Nicholls, 1991). Thus, roughly 1 pmol Ca^{2+} per mg protein is necessary to account for this elevation. At the same time, the $^{45}\text{Ca}^{2+}$ uptake in a split preparation (Verhage *et al.*, 1989) amounts to 2 nmol/sec/mg protein (after chelating superficially bound Ca^{2+} with excess EGTA). This indicates that in this case more than 99% of Ca^{2+} influx does not contribute to the intracellular

FIG. 3. Schematic representation of the proposed submembrane events that terminate the actual Ca^{2+} signal and restrict the elevation of intracellular free Ca^{2+} to a small submembrane area, but that, at the same time, translate the Ca^{2+} signal into other signalling pathways such as protein kinase C (PKC), calmodulin-dependent kinase (CaM-K II) and phosphatase (calcineurin). In addition, the release of classical transmitters from small synaptic vesicles is triggered by unknown Ca^{2+} -dependent factors (XY). Only some examples of substrates for the enzymes are mentioned; those for which the firmest evidence exists that they may indeed be substrates *in situ*. Especially in the case of phosphatase calcineurin, true substrates still need to be confirmed when better pharmacology as been used to test activity *in situ*. The kinases and phosphatases may also affect each other (such as the phosphorylation of calcineurin by PKC or CaM-kinase II, the dephosphorylation of CaM-K II by calcineurin) as well as themselves (such as the autophosphorylation of CaM-kinase II and PKC- α).

free Ca^{2+} -elevation. If endogenous Ca^{2+} -buffering systems would not exist, the intracellular Ca^{2+} concentration would rise to the millimolar range. Conversely, if all Ca^{2+} influx were to contribute to the $[\text{Ca}]$ -elevation, one single Ca^{2+} channel, with a common conductance of 20 pS, could account for the $[\text{Ca}]$ -elevation. Similar conclusions are reached through electrophysiological/fluorimetric studies of Ca^{2+} fluxes and $[\text{Ca}]$ -elevations. For instance, in melanotrophs Ca^{2+} currents carry an amount of Ca^{2+} sufficient to increase intracellular Ca^{2+} to 0.1 mM, but the free Ca^{2+} concentration only increases to approximately 1 μM (Thomas *et al.*, 1990).

It is difficult to evaluate the role of the potent cellular Ca^{2+} buffering in the light of the non-equilibrium situation that exists during exocytosis. The estimations on Ca^{2+} influx and $[\text{Ca}]$ -elevation as discussed above merely reflect the equilibrium situation *after* termination of the events. It is clear that detailed kinetic models are necessary to fully understand the relevant Ca^{2+} fluxes and (local) $[\text{Ca}]$ -elevations (see Allbritton *et al.*, 1992). In any case, the absorbance of free Ca^{2+} and the spatial limitation of Ca^{2+} -elevations are crucial to the plasticity of the input-output relation of the nerve terminal.

The entering pulse of Ca^{2+} may thus be largely absorbed by Ca^{2+} -buffering components. The availability of these components is probably both a *regulating* and a *regulated* parameter. Several proteins like B-50 (GAP-43) and MARCKS protein release at-

tached calmodulin upon Ca^{2+} -elevation (Masare *et al.*, 1986; Alexander *et al.*, 1987, 1988; Graff *et al.*, 1989; De Graan *et al.*, 1990; McIlroy *et al.*, 1991; Hartwig *et al.*, 1992). The capacity of these proteins to bind and release calmodulin is dependent on the phosphorylation state of the protein. The phosphorylation site overlaps with the calmodulin-binding site and only the non-phosphorylated form has affinity for calmodulin. Such a mutual exclusive mechanism could provide a potent mechanism for the plasticity of the nerve terminal. It is known that high frequency stimulation of the hippocampus, that is capable of establishing long term potentiation in these synapses, also induces an increased presynaptic PKC activity and phosphorylation of B-50 (Bär *et al.*, 1980, 1982; Tielen *et al.*, 1983; Lovinger *et al.*, 1985, 1986; Gianotti *et al.*, 1992) and thus a decreased capacity to bind calmodulin and to buffer incoming Ca^{2+} by releasing calmodulin. Other proteins may regulate Ca^{2+} -binding protein availability similarly, such as myosin I, which show similar cellular location, phosphorylation and Ca^{2+} -dependent calmodulin association/dissociation (Pollard *et al.*, 1991; Tan *et al.*, 1992). On the other hand, other factors may absorb calmodulin. For instance Ca^{2+} -calmodulin kinase II was shown to be able to trap several molecules of calmodulin for tens of seconds at moderate Ca^{2+} -elevations (Meyer *et al.*, 1992). In this way, the kinase may steadily recruit

FIG. 4. Schematic representation of several self-reinforcing mechanisms proposed to play a role in presynaptic plasticity. Several pathways involving calmodulin, calmodulin-dependent kinase, calmodulin-binding proteins and protein kinase C all co-operate to decrease the intracellular Ca^{2+} -binding capacity and thus to allow the spreading of Ca^{2+} gradients into the cytoplasm of the presynaptic nerve terminal during high activity. CaM: calmodulin, CaM-kinase: Ca^{2+} -calmodulin-dependent protein kinase II, PKC: protein kinase C.

calmodulin upon successive nerve impulses and thus lower cytosolic Ca^{2+} buffering capacity (see Fig. 4).

Absorbance of Ca^{2+} does not terminate the signalling cascade initiated by Ca^{2+} channel opening. Instead, Ca^{2+} activates other signalling molecules like Ca^{2+} -dependent kinase and phosphatase and travels as a Ca^{2+} -calmodulin complex into the cytosol (Fig. 3). In the case of binding of Ca^{2+} to calmodulin several pathways are known that contribute to the stimulus secretion coupling. Moreover, it can not be excluded that the actual exocytosis only occurs *after* binding of Ca^{2+} to calmodulin. Calmodulin is a major Ca^{2+} -binding component (see for a review, Kennedy, 1989). In the resting nerve terminal the free Ca^{2+} concentration is in the order of 10^{-7} M and calmodulin occurs predominantly in the free form. When, upon stimulation, the Ca^{2+} concentration rises above 10^{-6} M four Ca^{2+} -binding sites are occupied. Rather than free Ca^{2+} , the Ca^{2+} -calmodulin complex and probably other Ca^{2+} -protein complexes, act as second messengers through activation of Ca^{2+} -calmodulin-dependent kinase (CaM-kinase II) and Ca^{2+} -dependent phosphatase (calcineurin). Both calmodulin itself and the two enzymes, are highly concentrated in brain and in nerve terminals (Palfrey *et al.*, 1983; Ouimet *et al.*, 1984; Erondy and Kennedy, 1985). The activated kinase phosphorylates synapsin I, which may induce cytoskeletal rearrangements and changes in release parameters (see Greengard *et al.*, 1993; Rosahl *et al.*, 1993; see also below). At the same time, free Ca^{2+} elevation and a high level of the Ca^{2+} -calmodulin complex trigger several processes to prevent the terminal from over-stimulation. Firstly, Ca^{2+} -calmodulin initiates Ca^{2+} removal from the terminal

by stimulation of the plasma membrane Ca^{2+} -pump. Secondly, free Ca^{2+} -elevation may initiate opening of additional Cl^- and K^+ channels [$\text{K}_{(\text{Ca})}$ channels], reshaping depolarization extent and frequency (see Marty, 1989; Hille, 1984). Thirdly, the Ca^{2+} -calmodulin-dependent phosphatase calcineurin is activated, which may remove the autophosphorylation of CaM-kinase II. This phosphatase may therefore play a role in the limitation of postsynaptic potentiation and occurrence of posttetanic depression. The relatively slow kinetics of Ca^{2+} -dependent phosphatases are in line with this idea (see Schwartz and Greenberg, 1987; depression follows facilitation with some delay, see Zucker, 1989). However, even more complex mechanisms can be involved in these posttetanic changes in the stimulus-secretion coupling, since phosphatase activity is under control of regulated phosphatase inhibitors (see Kennedy, 1989), Ca^{2+} -independent phosphatases may also be of importance (Lisman, 1989) and both CaM-kinase and phosphatases have additional actions. In the light of this complex cross-talk of Ca^{2+} -activated mechanisms it is difficult to predict the overall consequence of large increases in the Ca^{2+} -calmodulin/free calmodulin ratio during repetitive stimulation. As judged by the affinities of various calmodulin dependent mechanisms for the Ca^{2+} -calmodulin complex, the first mechanisms to be activated are the (auto)phosphorylated form of CaM-kinase II ($K_d = 0.06$ nM) and cAMP phosphodiesterase ($K_d = 0.1$ nM). Interestingly, the calmodulin-dependent phosphatase calcineurin is activated with smaller Ca^{2+} -elevations than the unphosphorylated calmodulin-dependent kinase CaM-kinase II ($K_d = 6$ and 45 nM respectively, dissociation constants from Cohen

and Klee (1988) and Meyer *et al.* (1992)). Taking into consideration that outside of the submembrane area the Ca^{2+} concentration may only rise gradually and that any phosphorylation of CaM-kinase may be removed by calcineurin it is difficult to understand how the kinase may reach the autonomous autophosphorylated form, which has been implicated in long-term changes in cellular responsiveness in general and presynaptically in the recruitment of synaptic vesicles for exocytosis. To explain this, it may be assumed that calmodulin targets are differentially distributed, in analogy with the distribution of small synaptic vesicles and the localized entry of Ca^{2+} . Strategically localized targets with lower affinities may be more important modulators of the free calmodulin concentration than others. For instance, the presence of B-50 and MARCKS on the inner leaflet of the plasma membrane may be important in the regulation of local calmodulin availability (see Figs 3 and 4), although their affinities for calmodulin are relatively low ($K_d = 100$ nM and 3 nM respectively; Cohen and Klee, 1988).

During high activity of the nerve terminal, the local Ca^{2+} -buffering components will progressively be saturated. In addition to the Ca^{2+} -calmodulin complex, free Ca^{2+} -elevation may now spread into the bulk cytoplasm, inducing important additional effects (Fig. 4). Ca^{2+} can bind to the Ca^{2+} -sensitive elements involved in the mechanism of large, dense-cored vesicle release at ectopic sites in the terminal and thus stimulate neuropeptide exocytosis (and exocytosis of co-localizing catecholamines). The free Ca^{2+} -elevations in the cytoplasm also evoke effects indirectly involved in the stimulus-secretion coupling like stimulation of presynaptic metabolism both in general and specific metabolism of transmitters and activation of protein kinase C, which translocates to the plasma membrane manipulating the phosphorylation state of relevant proteins like B-50, MARCKS, tyrosine hydroxylase and presumably Ca^{2+} and K^+ channels. Furthermore, Ca^{2+} -elevations may stimulate adenylate cyclase activity (Andreasen *et al.*, 1983; Westcott *et al.*, 1979) and thus increase cytosolic cAMP-levels, which in turn stimulates cAMP kinase. However, breakdown of cAMP by cAMP phosphodiesterase may be stimulated to at least similar extent by a rise in cytosolic free Ca^{2+} and Ca^{2+} -calmodulin complexes (see Cohen and Klee, 1988).

In conclusion, during repetitive stimulation the regulation of $[\text{Ca}]$ may get into self-reinforcing cycles where Ca^{2+} -elevations in the bulk cytoplasm occur as a result of lengthening of depolarizations, saturation of soluble Ca^{2+} -buffering components and impaired calmodulin availability (Fig. 4).

It cannot be excluded that the actual exocytosis only occurs *after* binding of Ca^{2+} to calmodulin. The original evidence for this last suggestion comes from the inhibitory effects of calmodulin antagonists on transmitter release (see for a review, DeLorenzo, 1981). However, these drugs may not be without side-effects and may, among other effects, obstruct mitochondrial metabolism and thus indirectly inhibit the ATP-dependent secretion. An intriguing challenge to the proposed role for calmodulin is provided by the actions of Ba^{2+} . Barium ions appear to be able to replace Ca^{2+} in the process of secretion in a

number of model systems (Heldman *et al.*, 1989; Tagliatela *et al.*, 1989; Weiss *et al.*, 1990), although Ba^{2+} does not bind to calmodulin (Chao *et al.*, 1984). The Ba^{2+} -induced secretion is both in extent and kinetics similar to the Ca^{2+} -dependent secretion of many transmitters. The two types of stimuli are not additive, suggesting that the two stimuli share the same pool of transmitter. Furthermore, calmodulin antagonists, like those used by DeLorenzo (1981) were at certain doses ineffective in blocking Ba^{2+} -induced release whereas Ca^{2+} -dependent release was well inhibited (Verhage *et al.*, in preparation). Therefore, it appears that calmodulin-independent secretion exists. However, there is still no direct proof for this suggestion, because all experiments were performed in systems that do contain endogenous calmodulin and/or Ba^{2+} may not replace Ca^{2+} but may rather replace the Ca^{2+} -calmodulin complex in the process of secretion. In addition, the Ba^{2+} -induced release may arise from rather low affinity processes while Ba^{2+} is effectively trapped in high concentrations inside nerve terminals since these lack Ba^{2+} efflux pathways (see also Augustine *et al.*, 1987).

2.2. Ca^{2+} -SENSITIVE TRIGGERS AND FUSION COMPLEXES

Upon the entrance of Ca^{2+} in the nerve terminal, exocytosis is triggered. Although research has made great progress during the last few years, the nature of the actual trigger mechanism (a Ca^{2+} -sensitive trigger or Ca^{2+} receptor) is still unclear. It can generally be predicted that a Ca^{2+} -sensitive trigger, at least for the fast-acting transmitters, is located in close proximity of the Ca^{2+} channels and synaptic vesicles docked at the plasma membrane ready for release. The latest concepts assume a complex of vesicle and plasma membrane proteins probably associated with the Ca^{2+} channels (see for instance, Bennett *et al.*, 1992a,b; Söllner *et al.*, 1993a,b; Bennett and Scheller, 1993; Südhof *et al.*, 1993; Hata *et al.*, 1993). The existence of such docking complexes implicates that many necessary steps on the way to exocytosis have been taken and the entrance of Ca^{2+} can instantaneously evoke conformational changes that produce secretion. In addition, to allow rapid succession of nerve impulses, a prompt termination and reset of the exocytosis machinery is of equal importance as the rapid onset of transmission. Therefore, rather low affinity Ca^{2+} -binding sites have been predicted to trigger exocytosis. Low affinity sites have by definition high off-rates, such that when an individual channel closes and the local Ca^{2+} gradient collapses, the ion will rapidly dissociate from the putative trigger without requiring slow processes such as Ca^{2+} -ATPase activity and other homeostatic mechanisms to completely restore intracellular free Ca^{2+} to basal levels. Alternatively, if the Ca^{2+} -sensitive factors were located exclusively on the synaptic vesicles, it is possible that each of these is used only once in many impulses and the interaction with Ca^{2+} may be more tight. Because the increase in secretion is highly non-linear, showing co-operativity, probably more than one binding site exists.

Confirmed members of synaptic docking-fusion complexes for fast-transmitter exocytosis are the ves-

icle protein synaptobrevin/VAMP (Trimble *et al.*, 1988; Baumert *et al.*, 1989), the plasma membrane protein syntaxin/HP-1 (Bennett *et al.*, 1992a; Inoue *et al.*, 1992) and the soluble, but probably membrane attached protein SNAP-25 (Hess *et al.*, 1992). These three proteins are proteolyzed each by different clostridial neurotoxins, botulinum and tetanus toxins, which are potent inhibitors of exocytosis (see for a review, Südhof *et al.*, 1993) and antibodies against syntaxin block exocytosis (Bennett *et al.*, 1993). Furthermore, these proteins interact with general (non-neuronal) vesicle fusion components, NSF and NSF-attachment proteins, to form so called 20S fusion particles (Söllner *et al.*, 1993a,b). Finally, homologues of at least synaptobrevin and syntaxin appear to be involved in evolutionary earlier forms of secretion (see Schekman, 1992; Bennett and Scheller 1993; McMahon *et al.*, 1993).

Although the role of the above proteins in docking–fusion complexes is now well established, these may not represent the actual Ca^{2+} -dependent trigger, because none of them appears to undergo Ca^{2+} -dependent changes. Two further constituents of small synaptic vesicles, synaptotagmin and synaptophysin, have been implicated in such Ca^{2+} -dependent changes. Interestingly, while the above constituents of fusion complexes appear to have homologues in conserved forms of (Ca^{2+} -independent) secretion like in yeast, no homologues for synaptotagmin are known to date in such systems that do not display regulated exocytosis. Synaptotagmin (p65), contains two repeats of a sequence on the cytoplasmic domain which are homologous to the Ca^{2+} - and phospholipid-binding domain of the protein kinase C family and phospholipase A_2 (Perin *et al.*, 1990, 1991; Brose *et al.*, 1992). Multimers of synaptotagmin form complexes with phospholipids and Ca^{2+} , when the Ca^{2+} concentration rises into micromolar range (Brose *et al.*, 1992; Davletov and Südhof, 1993). The Ca^{2+} dependence of this complex fits well with the suggested range of intracellular free Ca^{2+} underneath the active zone membrane (see above) and Ca^{2+} -association shows similar co-operativity as Ca^{2+} -dependence of secretion (see Zucker, 1989). Furthermore, synaptotagmin was found to co-precipitate with syntaxin (Bennett *et al.*, 1992a; Yoshida *et al.*, 1992), ω -conotoxin binding sites (N-type Ca^{2+} channels; Leveque *et al.*, 1992; Yoshida *et al.*, 1992) and the receptor for α -latrotoxin (Petrenko *et al.*, 1991), a toxin that produces massive synaptic vesicle exocytosis.

In a variety of systems, such as *C. elegans*, *Drosophila*, squids and mammals, synaptotagmin was found to play a role in vesicular exocytosis, generally as a negative regulatory element rather than an absolute requirement for fusion. PC12 cells lacking synaptotagmin are still able to release catecholamines and *C. elegans* and *Drosophila* mutants lacking synaptotagmin still show (limited) motor activity and respond to sensory stimuli (see for a review, Popov and Po, 1993). Recently, it was found that synaptotagmin competes with NSF-attachment proteins for a binding site on fusion–docking complexes (Söllner *et al.*, 1993b). On the basis of this finding it has been suggested that synaptotagmin represents a clamp to prevent fusion of the otherwise spontaneous process

involving ATP-hydrolysis induced by NSF and dependent on the presence of NSF-attachment proteins. Ca^{2+} -entry and submembrane $[\text{Ca}^{2+}]$ -elevation may trigger dissociation of synaptotagmin from this complex and allow fusion to precede. However, in the cell-free system used in these studies, Ca^{2+} dependence of synaptotagmin dissociation was not observed and a large excess of NSF-attachment proteins was necessary to displace synaptotagmin (Söllner *et al.*, 1993). Furthermore, the number of events taking place during the proposed fusion, dissociation of synaptotagmin, association of NSF-binding proteins, ATP-hydrolysis by NSF and lipid mixing/fusion, seem to be too complex and slow to match the extremely fast kinetics of neuronal fast-acting transmitter release. Probably the system is equipped with additional “clamps” to arrest the fusion process at later stages and can allow fusion to occur with simpler (faster) steps until final fusion.

These considerations suggest that additional factors may be involved in triggering evoked neuronal release. Potential additional components include other isoforms of synaptotagmin (Geppert *et al.*, 1991), synaptophysin/synaptoporin, members of the rab-family and rab-binding proteins and munc 18, a mammalian homologue of the unc-18 gene-product which is essential for transmitter release in *C. elegans* and which binds to syntaxin (Hata *et al.*, 1993). Synaptophysin and synaptoporin are two isoforms of a ubiquitous protein, present in all brain areas and co-localizing on synaptic vesicles (Fykse *et al.*, 1993). Synaptophysin was found to bind Ca^{2+} (Rehm *et al.*, 1986) and to form pores in lipid bilayers as a multimeric structure comparable to the formation of gap junctions by the related protein connexin (see Thomas *et al.*, 1988; Betz 1990). It has been suggested that synaptophysin is able to form the actual fusion-pore, probably in concert with a plasma membrane counterpart physophilin as a docking protein or receptor (Thomas and Betz, 1990). However, the role of Ca^{2+} in this pore formation is still unclear and the affinity of synaptophysin for Ca^{2+} and the pore-forming multimer was not confirmed by others (see Südhof and Jahn, 1991; Brose *et al.*, 1992). Synaptophysin may therefore not be the actual Ca^{2+} -sensor and rather represent additional components of the fusion-complex which come into play as soon as secretion is triggered.

Finally, the Ca^{2+} -calmodulin-dependent kinase II (CaM kinase II) is associated with synaptic vesicles probably through association to one of its substrates, synapsin I. Synapsin I is an abundant protein in nerve terminals, may bind to synaptic vesicles, microtubules, actin filaments, calmodulin and spectrin, is phosphorylated in a Ca^{2+} -dependent manner by CaM kinase II and has been implicated in the regulation of transmitter release through phosphorylation-dependent dissociation from the cytoskeleton (see for a review, Benfenati *et al.*, 1992; Valtorta *et al.*, 1992; Greengard, 1993). However, the kinetics of CaM kinase II may be too slow to be a likely candidate for a Ca^{2+} -sensitive trigger. Furthermore, transgenic mice lacking either the CaM kinase II gene or the synapsin I gene are well capable of releasing transmitter in a Ca^{2+} -dependent manner (Silva *et al.*, 1992a; Rosahl *et al.*, 1993), although several physio-

logical parameters were altered in these mice. This suggests that both CaM kinase II and synapsin I act as modulators rather than fundamental requirements of the release process, probably through dynamic interactions/rearrangements with components of the cytoskeleton.

From the above data, the picture emerges of preformed docking–fusion complexes containing many vesicular, plasma membrane and cytosolic proteins, which is withheld from fusion by certain factors until Ca^{2+} channel opening leads to release of this clamp and allows the otherwise spontaneous fusion to precede. The fundamental difference between this neuronal regulated exocytosis and constitutive exocytosis, a feature of all cells, lies in this regulated inhibition of fusion and the opening of Ca^{2+} channels. It is clear that the sites of Ca^{2+} -entry are located closely to the docking–fusion complexes. Ca^{2+} channels responsible for secretion are probably directly part of such complexes. The limited role for Ca^{2+} in components of docking–fusion complexes that have been resolved so far may also reflect a limited true Ca^{2+} dependency of the process. Because the events take place in a restricted area near the Ca^{2+} channel mouths, the true trigger for exocytosis may be a local large and rapid increase of divalent cations, rather than a specific (high affinity) binding of Ca^{2+} to a putative receptor. The selectivity of the process for Ca^{2+} may in this case arise from the selectivity of the Ca^{2+} channels, rather than from the selectivity of the fusion machinery. Support for this idea can be found in the limited correlation between bulk intracellular $[\text{Ca}^{2+}]$ -elevations and release (Verhage *et al.*, 1991b; Llinas *et al.*, 1992), the poor effectivity of Ca^{2+} released from photolabile chelators in the bulk cytosol (Höchner *et al.*, 1989), the poor inhibition of release by slow-kinetics Ca^{2+} -chelators (Adler *et al.*, 1991) and the fact that divalent cations that can pass through Ca^{2+} channels and accumulate inside nerve terminals, such as Ba^{2+} , are also able to replace Ca^{2+} in triggering transmitter release, but also with relatively low affinity ($\text{ED}_{50} \approx 0.5 \text{ mM}$).

Because many questions are still unanswered with respect to the molecular interactions in the micro-domain that regulates exocytosis of fast acting neurotransmitters, it is difficult to predict how these interactions are susceptible to plasticity. Probably, the docking–fusion configuration allows little variability, because the rapid kinetics of the process limit the number of steps and probably only allow simple conformational changes rather than enzymatic reactions and also because secretion is believed to be an all-or-nothing (quantal) process. On the shorter term, variation in the input–output relation of presynaptic terminals may simply depend on the number of Ca^{2+} ions in the direct environment of the release sites, on the number of available vesicle–plasma membrane–protein complexes, which all have equal probability to undergo fusion and on the local concentration of soluble co-factors such as ATP and probably others (such as NSF-binding proteins; Söllner *et al.*, 1993a,b). Furthermore, although several potential components of docking–fusion complexes, such as synaptophysin and synaptotagmin, are known to be phosphorylated, there are little indications for activity dependent changes in these pro-

tein modifications, as observed for other synaptic proteins, such as dephosphin, B-50/GAP-43, MARCKS and others (see Robinson, 1993; Dekker *et al.*, 1990). It is also unknown whether stoichiometric differences in these preformed fusion complexes exist, for instance in the stoichiometry between Ca^{2+} channels, docking–fusion proteins and Ca^{2+} sensors. Data so far suggest a predominantly invariable occurrence of the different components in docking–fusion complexes (Söllner *et al.*, 1993b; Hata *et al.*, 1993).

On the longer term, the presynaptic input–output relation may be complicated by the recruitment of new vesicles, removal and refilling of exposed vesicles, involving enzymatic reactions rather than simple conformational changes. The capacity of vesicular release depends on the recruitment and priming of the preformed complexes in concert with the spreading of Ca^{2+} gradients from the Ca^{2+} channel-mouths. In general, the number of Ca^{2+} -sensitive triggers, docking proteins and vesicles that are available for exocytosis to occur will be decisive for the capacity of exocytosis and will probably limit the secretion during high activity. This process has been correlated to short term depression or “fatigue” (Betz, 1970; Zucker, 1989). It has been suggested that this fatigue is primarily due to the limited availability of vesicles, rather than the availability of Ca^{2+} -sensitive triggers and docking–fusion complexes. Proteins like synaptotagmin and synaptophysin appear to be present in each vesicle and may thus not be limiting in this respect. However, the number of docking sites may be restrictive, also in view of the geometry of the presynaptic nerve terminal. It follows also, that the recovery of the secretion machinery from a secretion event is remarkably fast. Apparently, nerve terminals are capable of chemically transmitting impulses of more than 50 Hz. It strengthens the idea that Ca^{2+} only “tags” the secretion machinery, rather than a firm binding (with high affinity). Still, the apparent co-operativity of Ca^{2+} ions in secretion suggest multiple interactions of Ca^{2+} ions with Ca^{2+} sensors before exocytosis can take place. Finally, it can be expected that the removal and recycling of synaptic vesicles after release is determining in the longer term capacity of the nerve terminal to respond to high frequency stimulation.

More than one Ca^{2+} -sensitive trigger may exist and different signalling pathways may have different triggers. Given the complex relationship between Ca^{2+} -entry and transmitter output and the fact that release in other systems depends on several Ca^{2+} -dependent steps with each distinct kinetics and Ca^{2+} affinities (see Neher and Zucker, 1993; Thomas *et al.*, 1993), it seems likely that several Ca^{2+} -dependent processes contribute to neuronal regulated exocytosis. Furthermore, it can be expected that different molecular mechanisms exist for the secretion of different transmitter types. However, recent molecular biological approaches show a remarkable homology in secretion machinery between systems as far apart as yeast and mammals (see for reviews, Bennett and Scheller, 1993; Südhof *et al.*, 1993). However, with several exceptions like the binding of mammalian GDI and Mss4 to a yeast homologue of rab-proteins (sec4; Sasaki *et al.*, 1991; Burton *et al.*, 1993), many homologues are only effective in the

systems they naturally occur in and vesicular traffic and targeting is tightly regulated (see below). Different isoforms may be effective each in a particular species, cell type and vesicle type. In the case of regulated exocytosis, the selectivity of vesicle docking was proposed to be determined primarily by syntaxin and synaptobrevin, the receptors for the NSF/NSF-binding protein complex on the plasma and vesicle membrane respectively (Söllner *et al.*, 1993a,b). However, neuropeptide release from large, dense-cored vesicles appeared to be equally well inhibited by several clostridial neurotoxins as the classical transmitters (McMahon *et al.*, 1993a), although neuropeptide containing vesicles are clearly targeted differently from the small synaptic vesicles. As in the case of Ca^{2+} -dependent triggering of release, the selectivity of docking among different types of vesicles may also depend on other components in addition to the ones presently described. Members of the rab-family and rab-binding proteins are good candidates for such additional regulators (further discussion below).

2.3. THE AVAILABILITY OF TRANSMITTERS FOR RELEASE

As discussed above, the strict temporal demands and the spatially restricted events in neuronal regulated exocytosis may allow little variation in the direct Ca^{2+} -dependent triggering of release. Presynaptic plasticity may depend largely on factors that regulate and modulate the availability of synaptic vesicles for release. This is the basis for the capacity of the nerve terminal to respond properly to periods of high activity and to allow release of transmitters to occur at rates faster than the rate of synthesis or uptake into the vesicles. The precise regulation of this storage and recruitment determines the response of a nerve terminal upon stimulation and thus plays a central role in its plasticity.

The kinetics of vesicle migration and (re)filling are orders of magnitude slower than the actual vesicle fusion. Responses to prolonged or frequent impulses arriving at the nerve terminal must therefore be limited by the limited number of vesicles that are fusion-ready (docked at the active zone) and by the slow filling and migration of the vesicles. It can be predicted that as soon as the pool of vesicles already available at the active zone is exhausted, short term facilitation changes to (short term) depression or "fatigue" (see for a review, Zucker, 1989). The rate limiting step in this depression are thus the components responsible for vesicle (re)filling and migration. Interestingly, intense stimulation does not lead to depletion of synaptic vesicles. In general, even permanent depolarization or repeated electrical stimulation at amplitudes and frequencies higher than those encountered *in vivo*, only give rise to relatively subtle changes in the number of vesicles observed in synapses using electron microscopy and in the transmitter content of (isolated) nerve terminals in biochemical approaches. Only in clearly pathological conditions, for instance after application of α -latrotoxin, synapses can be found which are merely devoid of synaptic vesicles. This suggests that short term depression or fatigue applies to a subpopulation of fusion-ready vesicles, rather than the bulk of synaptic

vesicles found in the presynaptic terminal. This also emphasizes the relevance of processes that prime synaptic vesicles for release (the cellular mechanisms that yield fusion-ready vesicles) for presynaptic plasticity.

With prolonged, instead of repetitive, stimulation, facilitation and depression are not distinguished, but appear as a large initial release, which subsequently decays. In the extreme case of permanent depolarization, the process of secretion sustains for a surprisingly long period. In isolated nerve terminals stimulated with permanent chemical depolarization, exocytosis of many different transmitters, from amino acids to neuropeptides, precedes until 3–5 min (see for instance, McMahon and Nicholls, 1991; Verhage *et al.*, 1991a,c, 1992b). On the basis of the large total release after the initial seconds, it may be excluded that all release occurs from vesicles that were already docked before the terminals were stimulated. It has been concluded that vesicles are recruited for secretion during this period and that the recruitment (preparing fusion-ready vesicles) is an ongoing process during nerve stimulation with slower kinetics than the actual secretion events. The phenomenon of fatigue, seen in electrophysiological experiments with high frequent stimulation, may be taken as an illustration of this kinetic difference: as soon as the initial reservoir of vesicles has been depleted as a result of high activity, new vesicles can not be recruited with the same frequency at which the terminal initially responded to incoming impulses.

Hence, persistently depolarized nerve terminals do show a sustained release, but do not release all their vesicles. Persistent depolarization yields a prolonged elevation of intracellular free Ca^{2+} , without a significant decline in cellular ATP during a few minutes (see Verhage *et al.*, 1991a). Furthermore, it appears that Ca^{2+} channels coupled to exocytosis of fast acting transmitters do not inactivate over a period of minutes (McMahon and Nicholls, 1991). The terminals are thus in a situation that obeys the fundamental requirements for exocytosis. Still, the terminals do not release all their vesicles. Only upon repolarization and renewed stimulation, the terminals release new vesicles (Verhage *et al.*, 1991c, 1992b,c). These experiments are performed in isolated terminals, which rules out the possibility of supply of new transmitter from the cell soma, but some transmitters may be synthesized and/or taken up into the vesicles locally. These observations again suggest that at least two pools of transmitter vesicles exist, one containing docked vesicles and those that can be recruited during activity and a second (reserve) pool of vesicles that can only be recruited during periods of rest. Similar conclusions were reached in studies of nerve terminals of the electric organ of the electric ray and rat ileum (Zimmermann and Denston, 1977; Suszkiw *et al.*, 1978; Agoston *et al.*, 1985; Whittaker, 1990). The distinct pools of transmitter appear to be a general phenomenon, shared by amino acid transmitters, catecholamines and neuropeptides (Verhage *et al.*, 1991a,b, 1992a–c). It suggests that certain factors in the process of secretion needs to be readjusted during periods of rest (availability of vesicles or reactivation of docking proteins/ Ca^{2+} -sensors).

Nerve terminals contain the necessary protein ma-

FIG. 5. Levels of different transmitters in nerve terminals isolated from the axons that supply new proteins and peptides. In the case of classical transmitters, as exemplified by the amino acid glutamate (left panel), the total amount of transmitter during a period of stimulation added to retained content of the nerve terminals amounts to a significantly higher value than the initial content, indicating the net production of transmitter in the isolated organelle to balance transmitter loss during high activity (ratio 1.19, data taken from Verhage *et al.*, 1991c). In the case of neuropeptide transmitters, as exemplified by cholecystokinin-8 (right panel), there is no net production of peptide. Instead, total release + retained content add up to a smaller value than the initial content (ratio 0.89), possibly due to breakdown of peptides during the experiment (data taken from Verhage *et al.*, 1991a). This indicates that in the case of neuropeptides, the nerve terminal depends entirely on the supply from the cell soma to balance loss of transmitters due to release.

chinery to produce most neurotransmitters from their precursors independently of the cell soma. In some cases this machinery is present within the vesicles, as in the case of noradrenaline and the neuropeptides. In other cases, such as the amino acids and acetylcholine, the synaptic vesicles can locally take up their transmitters (see for a review Maycox *et al.*, 1990). It is therefore believed that the availability of fast-acting transmitters for exocytosis, at least on the short term, is entirely dependent on the local conditions in the nerve terminal. Conversely, neuropeptide production is entirely dependent on the synthesis in the cell soma.

Both transmitter production and the packaging of transmitter in their vesicles may be an important factor in the response of the nerve terminal. It is not clear whether in physiological situations, the availability of transmitter molecules is rate-limiting to this response. It appears that a large reservoir of synaptic vesicles is present, although probably not directly releasable (a "reserve" pool, see above). In addition, several of the major transmitters, such as glutamate and GABA, are present in the presynaptic cytosol in high concentrations and can be taken up into the vesicles.

In general, mechanisms are available to balance possible discrepancies between transmitter demand and supply. The local production of amino acid transmitters can also be observed after isolation of the terminals. By perfusion of immobilized isolated nerve terminals, they can be stimulated repetitively for long periods in order to maximally burden the exocytosis capacity. Under these conditions, the rate of amino acid transmitter production is correlated to the number or the duration of stimulation periods

and to the amount of transmitter released (Fig. 5). For neuropeptides, which are produced and packaged in the cell soma region, no net production is found and the amount of neuropeptides retained in nerve terminals after stimulation simply decreases with the amount of neuropeptides released (Fig. 5). On the level of the (isolated) nerve terminal, there may thus be a simple decline of transmitter content with the number of effective stimuli because neuropeptides are not synthesized locally. On the whole-cell level, it is clear that a correlation between stimulation and neuropeptide production does exist. In general, stimulation of neurons results in increased synthesis rates of neuropeptides through the stimulatory effect of second messengers in the cell soma on transcription/translation of neuropeptide precursors (see for a review, Goodman, 1990). However, a discrepancy may occur between the stimulation intensity at the cell soma level and locally at the nerve terminal. Presynaptic nerve terminals receive modulatory inputs from other nerve terminals. For instance, presynaptic inhibition may induce a selective, local inhibition, while the soma area is stimulated. This situation will thus increase synthesis while release is inhibited, leading to an increase in the number of vesicles present in the terminal. Retrograde signals from the nerve terminal to the cell soma would be necessary to completely adjust neuropeptide synthesis. Retrograde transport from the terminals certainly occurs, but it is unclear whether this involves signals that help to match central production and local demands of neuropeptides. Alternatively, if no such retrograde adjustment exists the release capacity of the nerve terminal to release neuropeptides is

directed by the stimulation pattern of the remote cell body (further discussion in Section 3).

As indicated in Fig. 5, the availability of transmitter molecules may, at least for the fast-acting transmitters, not be rate-limiting in physiological situations, due to the large reservoir of transmitter in the presynaptic cytosol and of a pool of reserve vesicles and compensatory mechanisms. However, during long periods of high activity the reserve pool must be replenished by recycling of released vesicles. In many cases, neuronal secretion of fast-acting transmitters was found to be of quantal nature, although there is still considerable debate on the generality of this phenomenon in the CNS (see Edwards, 1991). The uniform size of these quanta suggests that the content of small synaptic vesicles is invariable and that vesicles are only subjected to secretion after they have been completely filled with transmitter. Possibly, there is such a regulation mechanism that allows only completely filled vesicles to enter the releasable pool, although it is difficult to understand how such a mechanism would sense the correct content of a vesicle. Furthermore, quantal analysis is typically performed during low activity. It may thus be that secretion involves quantal secretion with variable quantal size (see Larkman *et al.*, 1992), especially during burst activity.

The major classical transmitters all depend for their storage in vesicles on the proton gradient over the vesicle membrane generated by a H^+ -ATPase, but differentially depend on either the difference in membrane potential or specifically on ΔpH (see Maycox *et al.*, 1990). Probably as little as four transport proteins account for accumulation of transmitter in synaptic vesicles: the acetylcholine transporter (Koenigsberger and Parsons, 1980), a GABA/glycine transporter (Fyske and Fonnum, 1988; Hell *et al.*, 1988; Kish *et al.*, 1989), a glutamate carrier (Disbrow *et al.*, 1982; Naito and Ueda, 1983; Maycox *et al.*, 1988) and a non-specific monoamine carrier (see Njus *et al.*, 1986; Erickson *et al.*, 1992; Liu *et al.*, 1992). Large, dense-cored vesicles may only contain the monoamine carrier and contain peptides that they have received while budding from the Golgi apparatus. The ATP-dependence of the proton- and electrical gradients that drive vesicle loading is possibly one of the main ways, together with ATP-dependent establishment of docking–fusion complexes (Söllner *et al.*, 1993a,b), in which exocytosis is ATP-dependent. During periods of high activity and/or pathological conditions, the (local) availability of ATP may modulate the number of vesicles available for release and thus the extent of transmitter release. In addition, other aspects of vesicular exocytosis are dependent on ATP, such as phosphorylation of proteins involved in targeting, docking and fusion of vesicles.

In the long term, all transmitter systems depend on the cell soma to provide proteins involved in the production and regulation of transmitter exocytosis. It is not clear whether this central protein synthesis operates under feedback regulation generated by the nerve terminal fields. Depending on the activity status of the terminals there may be a variable demand of protein synthesis. Moreover, changes in protein synthesis, alternative splicing of messengers and post-translational protein modification may dictate

changes in the stimulus–secretion coupling capacity or efficacy. There are no clear indications for specific intracellular feedback signals from the terminals. Local recurrent interneurons may also contribute to such feedback regulation.

Specific targeting factors must be involved in vesicle migration to the nerve terminal, because they are specifically targeted to nerve terminals, not to dendrites. The first stages of this selective transport of vesicles and their content must be at the levels of the cell soma where proteins are directed into one of three pathways, the lysosomal pathway and the pathways for constitutive and regulated exocytosis. Being sorted into the latter pathway vesicles need to be transported selectively to the axon, not to dendrites. Although especially large, dense-cored vesicles may be found in dendrites and cell somata as well, it is generally believed that vesicles in the regulated exocytosis pathway, also in the case of large, dense-cored vesicles, are enriched in nerve terminals and are thus targeted selectively, or at least preferentially, to the axon. One important additional layer of selectivity arises from local processes in the nerve terminal provided by the selective interaction of vesicle constituents (synaptobrevins) and active zone proteins (syntaxins) in docking–fusion complexes (see Söllner *et al.*, 1993a,b). As discussed above, a large pool of vesicles is available within the presynaptic nerve terminal, but the majority of these vesicles are not readily releasable. Hence, local regulation of the docking process is an important factor that helps to establish and potentially adjust the release capacity of the nerve terminal.

In a wide variety of systems, from yeast to the mammalian nervous system, the importance of a group of small GTP-binding proteins of the p21^{ras} superfamily is emphasized in targeting to specific organelles (see for reviews, Bourne, 1988; McCormick, 1989; Balch, 1990; Zerial and Stenmark, 1993). It has been shown that different members of the sec4/ypt1/rab protein family are present on vesicles directed to different targets. Interchanging C-terminal fragments among different members of the rab-family in chimaeric proteins in mammalian cells changes their destination (Chavrier *et al.*, 1991), suggesting that these rab proteins contain a targeting signal in their C-terminal fragment. Since different members of the rab-family are found to be associated with different vesicles, it has been suggested that these proteins contribute to the targeting of these vesicles. However, similar experiments in yeast indicate that the presence of such chimaeras does not necessarily lead to mis-sorting of vesicles, suggesting that the sec4/ypt1/rab family of proteins are at least not the only factors that specify the target of vesicles (Brennwald and Novick, 1993). These data emphasize a different aspect of rab protein function, i.e. a molecular switch or timing device to control and facilitate establishment of the correct docking complexes that contain additional factors determining the specificity of the docking process (see also Bourne, 1988).

At least one member of the rab-family, rab3A, is specifically present on synaptic vesicles and appears to undergo cycles in membrane-bound and soluble forms (Fischer von Mollard *et al.*, 1990). As judged by its absence in Golgi area of the cell body (Matteoli

et al., 1991), rab3A probably associates with vesicles only at the level of the nerve terminal. For these reasons rab3A could be a local targeting/controlling factor to ensure correct targeting of small synaptic vesicles to the active zone of the nerve terminal. Because large, dense-cored vesicles are targeted differentially (not to the active zone; Zhu *et al.*, 1986; Thureson-Klein and Klein, 1990; Verhage, 1991b) and rab3A may be absent from large, dense-cored vesicles (which represents pinched off Golgi sacs), these vesicles may exploit different factors, probably other members of the rab-family.

The function of rab proteins depends on the GTP- or GDP-bound form. It has been suggested (Bourne, 1988) that the GTP-bound form represents the "on"-state, capable of targeting vesicles. The high GTP/GDP ratio in the cell soma ensures the maintenance of this on-state and the transport of the vesicles in the soma area. The acceptor membrane may contain factors to stimulate hydrolysis of GTP (GTP-ase activating proteins, GAPs; see for a review, McCormick, 1989), which terminate its targeting function. These GAPs may thus represent a specific target location and account for unidirectional transport. In addition, factors are described that inhibit GDP-dissociation from rab proteins (GDP-dissociation inhibitor, GDI; see Araki *et al.*, 1990; Sasaki *et al.*, 1990). GDI and probably other rab-binding proteins could further establish and confirm the correct targeting of vesicles. At this stage other proteins come into play to establish preformed fusion complexes and Ca^{2+} -dependent triggering (see above). Furthermore, binding of GDI may trigger the dissociation of the rab proteins from the transported vesicle and allow the proteins to return to their donor membranes or associate to endosomes involved in locally recycling vesicles.

Targeting of small synaptic vesicles is not a simple unidirectional transport from the cell body to the active zone. The vesicles undergo local recycling as part of endosomal organelles and individual vesicles. Both the total number of vesicles in the terminal and their capacity to locally recycle determine the capacity of the nerve terminal to properly transmit repetitive incoming stimuli and is therefore important for presynaptic plasticity. In general, cellular levels of small G proteins were found to correlate to neuronal plasticity. For instance, cellular levels of ras-like proteins were found to decrease upon classical conditioning paradigms (Nelson *et al.*, 1991). More specifically, the capacity to target recycling vesicles again to the active zone may be an important factor during high activity of the terminal. The association of rab3A to small synaptic vesicles depends on hydrophobic modifications of the protein (Musha *et al.*, 1992; Farnsworth *et al.*, 1991; Johnston *et al.*, 1991), which may function as a membrane anchor for the protein. Following exocytosis, the amount of rab3A associated with vesicles is decreased (Fischer von Mollard *et al.*, 1990). It is suggested that the hydrophobic moiety of rab3A is modified during these transitions (Johnston *et al.*, 1991). Hydrophobic modification may thus be a regulated parameter, similar to protein phosphorylation, but the hydrophobic residues of rab3A may also be shielded upon association of GDI, making the protein more soluble

(the hydrophobic modifications of rab3A are necessary for association of these GDI; Musha *et al.*, 1992; Kikuchi *et al.*, 1992). Little is known about the local traffic of members of the rab family. It is clear that the association/dissociation cycles may be relevant for ensuring and controlling vesicle recycling and docking, and for the capacity of the terminal to respond to repetitive signals, but it is, at this point, still difficult to point out which factors may be rate-limiting and which are primarily involved in establishing adjustments in the presynaptic input-output relation.

The availability of transmitter vesicles for release is also modulated through its interactions with the presynaptic cytoskeleton and through cytoskeletal rearrangements that influence presynaptic architecture and that of the active zone in particular. The synapsin family is a group of well studied proteins known to bind to a number of cytoskeletal components, such as microtubules, actin filaments and spectrin, as well as to synaptic vesicles (see for a review, DeCamilli and Greengard, 1986; Benfenati *et al.*, 1992; Valtorta *et al.*, 1992; Greengard 1993). Synapsins occur in high concentrations in the nerve terminal and are phosphorylated by cAMP-dependent and Ca^{2+} -calmodulin-dependent kinases, which reduces its affinity for vesicles and cytoskeletal elements. The activity of these kinases, especially the Ca^{2+} -dependent kinase, may thus be a mechanism to modulate transmitter release. Indeed, a number of studies have shown that presynaptic stimulation, Ca^{2+} -calmodulin kinase activity and synapsin phosphorylation correlate with the transmitter output (Nestler and Greengard, 1984; Llinas *et al.*, 1985; Nichols *et al.*, 1990). Both synapsin and the kinase may not be primary requirements for regulated exocytosis to function properly since mice lacking synapsin I or Ca^{2+} -calmodulin kinase II both exhibit no overt phenotype and show normal synaptic transmission, but the absence of these proteins has clear effects on certain aspects of synaptic plasticity (Silva *et al.*, 1992; Rosahl *et al.*, 1993). Mice lacking synapsin I exhibit no changes in K^{+} -evoked noradrenaline release and several electrophysiological parameters, but show a selective increase in paired pulse facilitation. Synapsin I may thus be a negative regulator of this type of presynaptic plasticity (see Rosahl *et al.*, 1993). This role of synapsin I may, also given the great abundance of this protein in the nerve terminal, relate to cytoskeletal rearrangements that may be induced by synapsin I. Changes in synapsin I phosphorylation alters its affinity for cytoskeletal components and affects for instance the bundling of actin filaments *in vitro* (Bähler and Greengard, 1987). This may be a factor in structural plasticity of the terminal. Apparently, different forms of the synapsin protein with different actin-bundling capacities can be obtained by alternative splicing (see DeCamilli and Navone, 1987; Südhof *et al.*, 1989; DeCamilli and Jahn, 1990).

The Ca^{2+} -calmodulin kinase II is a multimeric protein that binds many calmodulin molecules and can be autophosphorylated. This kinase has affinity for microtubules (Vallano *et al.*, 1985) and specifically binds to synaptic vesicles (Huttner *et al.*, 1983; Schiebler *et al.* 1986; Benfenati *et al.*, 1992). In the

autophosphorylated form the kinase may be active over prolonged periods, while the counteraction of Ca^{2+} -calmodulin-dependent phosphatase (calcineurin) is restricted to periods of elevated $[\text{Ca}^{2+}]$ and Ca^{2+} -calmodulin complexes. This autonomous state has therefore been considered as a molecular switch in changing the synaptic input-output relation (see for instance, Lisman, 1989; Kennedy, 1989). CaM-kinase II is only fully autophosphorylated, when several Ca^{2+} -calmodulin complexes bind to the kinase. It is unlikely that a large percentage of cellular calmodulin (an rough estimate amounts to 10–100 μM ; Kennedy, 1989) occurs in the Ca^{2+} -bound form upon single nerve impulses (see Kennedy, 1989). Furthermore, according to the affinities of the kinase and the phosphatase (see Cohen and Klee, 1988; Meyer *et al.*, 1992), the latter is activated with smaller increases in free Ca^{2+} . Hence, upon repetitive stimulation, during the gradual rise of $[\text{Ca}]$, and of the concentration of Ca^{2+} -calmodulin complexes, the phosphatase will be activated earlier than the kinase. Therefore, it is difficult to understand how the activity of CaM-kinase II can ever become autonomous. Possibly, the balance between kinase and phosphatase activity is influenced *in vivo* by kinetic differences and differences in spatial organization, like the close association of CaM-kinase II with synaptic vesicles and the active zone. In mice lacking the kinase, an impaired performance in spatial learning and a reduced incidence of long term potentiation was found (Silva *et al.*, 1992a,b). These data clearly establish a role for the kinase as a modulator of synaptic transmission. However, it can not be excluded that these modulatory actions originate from postsynaptic processes. The fact that deletion of CaM-kinase II and its major presynaptic substrate synapsin I have more or less opposite effects on synaptic plasticity (the former selectively *increases* paired pulse potentiation, the latter appears to selectively *decrease* long term potentiation; Silva *et al.*, 1992; Rosahl *et al.*, 1993), argues against a co-localization of the two phenomena in presynaptic terminals.

In contrast to detailed descriptions of small synaptic vesicle proteins, little is known about large, dense-cored vesicle proteins. Presumably these proteins are not found in all vesicles but associated with subpopulations (see Südhof, 1989; Walch-Solimena *et al.*, 1993). Either the Ca^{2+} -calmodulin complex or the activated CaM-kinase II may contribute to the migration of large, dense-cored vesicles, which, however, lack synapsin I (Navone *et al.*, 1984). In addition, other members of the rab family may be involved in the targeting of large, dense-cored vesicles.

3. DIFFERENTIAL TRANSMITTER RELEASE: CO-TRANSMISSION AND CHANGES IN THE RELEASED COCKTAIL OF TRANSMITTERS

Many neurotransmitters have been identified in the brain. The characteristics of their release from nerve terminals show differences in a number of properties, such as subcellular distribution, number of molecules released per impulse and release kinetics. This

suggests that different mechanisms of stimulus-secretion coupling exist. The majority of nerve terminals appear to be arranged in such a way that signals are transmitted with maximal velocity. These arrangements include the close approximation of the presynaptic terminal to the target cell, the accumulation of large amounts of transmitters at this site in small synaptic vesicles and the secretion of transmitters that induce very swift responses onto the target cell. In addition, other transmitter systems exist which serve a transmitting role with much slower dynamics, comparable to the dynamics of non-neuronal secretion. These systems use different transmitters, inducing relatively slow effects on the target cell and possibly on neighbouring cells that may develop in a gradual way. There is now ample evidence suggesting that many nerve terminals in fact have both kinds of transmitter at their disposal, co-existing within one synapse. Key contributions by Lundberg and Hökfelt (1983), DeCamilli and Navone (1987) and Smith and Augustine (1988) have led to the concept of multiple signalling within the synapse, suggesting that different stimulation patterns may induce the secretion of a different mixture of these co-localized transmitters. This aspect has potentially important implications for synaptic plasticity and the “weight” of synaptic coupling. The availability of more than one signal molecule and the proposed ability to selectively modulate each of these signals allows the system to display a variety of responses depending on the stimulation conditions. In addition, co-transmitters are usually slow-acting transmitters with slow release kinetics and long extracellular half-lives. This allows the synaptic coupling to undergo relatively long-lasting changes in addition to the brief “classical” transmission performed by fast-acting transmitters. In this second part, we attempt to review cellular mechanisms that may account for the capacity of nerve terminals to release more than one transmitter and the ability to use these release mechanisms differentially.

Transmitters are now divided into three categories (see also Iversen and Goodman, 1986; McGeer *et al.*, 1987), on the basis of their postsynaptic effects and their extracellular half-life. The amino acid transmitters, glutamate, GABA, aspartate and glycine together with acetylcholine can all induce very swift responses on the postsynaptic cell through the opening of postsynaptic ion-channels. These transmitters are rapidly taken up from the synaptic cleft or broken down by ectoenzymes. This group of transmitters is named “ionotrope” or fast-acting transmitters. It is generally believed that these transmitters are locally produced and packaged exclusively in small vesicles that can locally be recycled. These vesicles are clustered at the active zone that can produce a massive fast release, in the case of isolated nerve terminals in the order of nmoles per mg synaptosomal protein (Table 1).

The monoamines noradrenaline, dopamine and serotonin, also called “metabotrope” or intermediate transmitters, induce slower effects mediated by second messenger cascades and are taken up or broken down less effectively. The monoamines are among the first substances recognized as neurotransmitters. Still, their exact role in signal transduction has remained

TABLE 1. SUMMARY OF DIFFERENCES BETWEEN DIFFERENT CLASSES OF NEUROTRANSMITTERS

	Amino acids	Catecholamines	Neuropeptides
Postsynaptic action	< 5 msec	Seconds/minutes	Seconds/minutes
Vesicle supply	Local recycling	Mixture (?)	From soma
Vesicle type	Small clear cored	Both small and large Both clear and dense-cored	Large, dense-cored
Implicated in vesicle transport/regulation	rab 3A synapsin I	?	? (No rab3A and synapsin I)
Release site	Active zone	Both (?)	Ectopically/non-synaptic
Released amounts	nmol/mg protein	pmol/mg protein	fmol/mg protein
Reuptake/breakdown	Very effective	Moderately effective	Slow
Release kinetics	Biphasic	Seconds	Minutes
Apparent Ca^{2+} -sensitivity	> 2 μM	300–400 nM	< 200 nM
ATP-requirement	Absolute	(?)	Modulatory role (?)

For details see text.

relatively indistinct, as compared to the clear-cut role of the fast-acting transmitters. The release of catecholamines can exhibit, depending on the system, characteristics of both the rapid signalling pathway of fast-acting neurotransmitters present in small synaptic vesicles and a slower signalling pathway as “neuromodulators” contained in large, dense-cored vesicles. Catecholamines are believed to be stored in both types of vesicles or in separate, morphologically distinct categories (for a review see Thureson-Klein and Klein, 1990). It appears that monoamines can be locally produced and taken up into vesicles, but it has been observed that synthesizing enzymes, such as dopamine β -hydroxylase and probably other proteins are secreted together with the transmitter (see below). Hence, a constant supply of protein from the soma would be required in this case. The extent of release in the case of isolated nerve terminals is in the order of pmoles per mg synaptosomal protein (Table 1), except in some specific brain areas, such as the striatum and peripheral postganglionic terminals, where the released amounts may be higher.

Finally, the group of neuropeptides induces even slower effects. They are believed to be released distant from the active zone and at non-synaptic sites (see Zhu *et al.*, 1986) with slow kinetics (Verhage *et al.*, 1991a, 1992b) and in minute amounts. In the case of isolated nerve terminals their release is in the order of fmoles per mg synaptosomal protein. Neuropeptides are broken down extracellularly by slow enzymatic processes and persist for minutes in the extracellular space. No reuptake mechanisms of the active substances have been found and are therefore called “slow” transmitters or “neuromodulators” (Table 1).

A few substances with possible transmitter or neuromodulatory role fall outside the above categories (such as ATP, adenosine, nitric oxide). Furthermore, several exceptions have now been described for the classification of transmitters in ionotrope and metabotrope type: glutamate, GABA and acetylcholine have all found to interact with classes of specific receptors that are not linked to an ion channel but rather to the generation of second messengers and as such belong to metabotropic signalling pathways

3.1. TRANSMITTER PRODUCTION, STORAGE AND TRANSPORT

All fast-acting transmitters are produced locally in the nerve terminal. The production of these transmitters is often not exclusively for the benefit of transmitter purposes. Amino acid transmitters are also metabolic intermediates and are, except GABA, also incorporated into proteins. Choline is not only the precursor of the transmitter acetylcholine, but also a constituent of membrane phospholipids. An equilibrium exists between the metabolic pools and pools for transmitter purposes, dependent on their relative size and the synthesis/break-down capacity. These fast-acting transmitters and also several monoamine transmitters are locally accumulated in their active form in synaptic vesicles by specific, energy dependent uptake mechanisms present in the vesicle membrane. Accumulation depends on ATP-driven proton and chloride gradients across the vesicle membrane, which allows accumulation of these transmitters against their concentration gradient (see Maycox *et al.*, 1990). Finally, selective carriers are present in the nerve terminal membrane to retrieve these transmitters or their metabolites. These circumstances imply that for these transmitters, the nerve terminal behaves as an independent unit, at least on the short term. Determining factors in the release capacity, i.e. the availability of transmitter molecules, is exclusively dependent on local variables. Upon high activity, the terminal is able to accelerate transmitter synthesis (see Fig. 5) and presumably to accelerate accumulation into vesicles. On the other hand, a large reserve pool of transmitter appears to be present in nerve terminals to counteract acute shortage during high activity (Suszkiw *et al.*, 1978; Agoston *et al.*, 1985; Verhage *et al.*, 1991c). Production and accumulation of transmitter may not be a highly regulated process in physiological situations and have rather slow kinetics. Under pathological conditions, when ATP-levels are persistently low and electrochemical gradients over plasma- and vesicle membrane decrease, the terminal may lose its capacity to prepare for exocytosis and terminals may become deficient. In many systems, both the CNS and periphery, ATP appears to be stored together in single synaptic vesicles with different transmitters like acetylcholine (Dowdall

et al., 1974) and norepinephrine (Winkler *et al.*, 1981).

In the case of neuropeptide release, the terminal is clearly not an independent unit. For the production and supply the terminal is entirely dependent on the cell soma. The regulated transcription and translation of neuropeptide genes may be crucial in presynaptic plasticity as far as neuropeptide release is concerned. In addition, the neuropeptide containing vesicles may locally accumulate monoamine transmitters. Studies using immunogold double labelling have shown that these vesicles can contain more than one transmitter, either two peptides or a peptide with a monoamine transmitter (for a review see Lundberg and Hökfelt, 1983; Bartfai *et al.*, 1988; Hökfelt, 1991). Large, dense-cored vesicles contain factors to process large precursor peptides to the active neuropeptides (see Boarder, 1989; Eipper *et al.*, 1992). Neuropeptides are synthesized as large precursor peptides and packaged in the endoplasmic reticulum and Golgi complex in the cell soma. Post-translational processing, probably exclusively inside the vesicle (see Boarder, 1989), yields the active peptides. These circumstances imply that in addition to local variables involved in the regulation of transmitter release, such as recruitment and the actual release, additional components of targeting the vesicles towards the terminal and intravesicular production of the active transmitters are involved in the regulation of neuropeptide release (Fig. 6). Furthermore, the central production of the precursor peptides must be regulated in accordance with the local demands in the terminal.

The catecholamines and serotonin also depend on supply from the cell soma, in addition to the local production and packaging. The anabolic enzyme for noradrenaline, dopamine β -hydroxylase, is found to be released with the transmitter (Axelrod, 1972). Furthermore, a family of proteins is believed to be present in monoaminergic synaptic vesicles to obviate

hyperosmolarity of the vesicles and released with the transmitter upon exocytosis (see Gershon and Tamir, 1984). Constant supply of these proteins is thus necessary, for which the only likely source is the protein synthesis in the cell soma. Furthermore, pharmacological manipulations (summarized in McGeer *et al.*, 1987) suggests that vesicles are packaged with monoamines in the cell soma and then migrate to the terminals by axonal transport, although it is clear that small synaptic vesicles can locally synthesize and accumulate monoamine transmitters (Smith, 1972; Njus *et al.*, 1986; Maycox *et al.*, 1990).

It has been assumed that the presynaptic neuron may exhibit plastic properties both in targeting of neuropeptide containing vesicles and the intracellular cleavage of precursor peptides. In invertebrates different terminals from one identified neuron may contain a different set of peptides derived from the same precursor peptide (Fisher *et al.*, 1988). Mechanisms that regulate this apparent differential targeting are still obscure. It is also uncertain whether accelerated activity at the synapse may lead to "premature" release of vesicles. Clearly, such a process would modify the synaptic transmission. In the case of classical transmitters it would lead to release of variable quanta derived from not completely equilibrated (filled) vesicles, in the case of neuropeptides it would lead to release of precursor peptides rather than the active peptide. There is evidence to suggest that precursor peptides are released (CCK-33 instead of CCK-8, somatostatin-28 instead of -14 etc.), but it is uncertain whether this specifically occurs during high activity. In general, precursor peptides may undergo their final stages of cleavage in the extracellular space. Furthermore, postsynaptic receptors may exist for more than one form of certain neuropeptides (for instance somatostatin-14 and -28, see Bell and Reisine, 1993; different CGRP-like peptides, see Goltzman and Mitchell, 1985). These suggestions again indicate that if high activity produces more release of not completely processed vesicles, this would have great impact on synaptic transmission. Similar considerations may apply to the intracellular conversion of dopamine to noradrenaline. Accelerated release may induce a shift from predominantly noradrenaline secretion to predominantly dopamine secretion. Again, these two transmitters may induce quite different modulations to synaptic transmission.

3.2. DIFFERENTIAL RELEASE OF DIFFERENT TRANSMITTERS

Different transmitter systems have many distinct characteristics with respect to production, accumulation in different vesicles, reuptake after release and postsynaptic effects (Table 1). In addition, the actual release process exhibits unique characteristics for different transmitters, especially between the group of transmitters accumulated in small synaptic vesicles and those in large, dense-cored vesicles. Small synaptic vesicles containing classical transmitters and large, dense-cored vesicles containing neuropeptides and catecholamines are differentially distributed in the nerve terminal (see Zhu *et al.*, 1986; Verhage *et al.*, 1991b). Small clear cored vesicles occur in clusters,

FIG. 6. Schematic representation of the different aspects discriminated for the regulation of neuronal neuropeptide secretion.

FIG. 7. Kinetic differences in the release of different classes of neurotransmitters upon permanent depolarization (at $t = 0$). The net Ca^{2+} -dependent, exocytotic components of release are plotted (data taken from Verhage *et al.*, 1991a,c, 1992b,c). The release of all transmitters is terminated after 3–5 min although at this moment still a large majority of the transmitters is present within the terminals. This “reserve” pool can be released during consecutive stimulation periods, but only after periods of repolarization and restoration of the intracellular free Ca^{2+} concentration to basal levels.

lined up at the active zone. In contrast, large, dense-cored vesicles are distributed and released more ectopically (see also DeCamilli and Navone, 1987). The two types of vesicles represent two different, differentially regulated exocytotic pathways. These differences include the effective stimuli, the activation and inactivation kinetics of the release process, the ATP-dependence, the apparent Ca^{2+} sensitivity and the factors present on the different vesicles. Since the two different exocytotic pathways may often be present in individual nerve terminals, these different means of generating signals add extra dimensions to the plastic properties of the nerve terminal.

All classical transmitters, the amino acids, acetylcholine and the catecholamines, show an initial burst of release upon depolarization (Fig. 7, see also, for instance, Drapeau and Blaustein, 1983; Adam-Vizi and Ashley, 1987; Nicholls, 1989; Turner and Goldin, 1989). This fact is likely to be correlated to their accumulation in small synaptic vesicles, which are docked at the active zone, ready for release. In addition, a slow release component is detected, more apparent for amino acids than for catecholamines, which is probably related to vesicle recruitment and recycling of small synaptic vesicles. The release of neuropeptides shows a gradual and rather slow build-up (Fig. 7). This does not necessarily indicate that the intrinsic properties of neuropeptide secretion are different from those of classical transmitters. The slow release of peptides may also be explained by the fact that the large, dense-cored vesicles in which peptides are accumulated, occur at a more ectopic site and both these vesicles themselves and the Ca^{2+} ions that trigger the secretion have to travel longer distances to reach the plasma membrane and to release their content (see also Peng and Zucker, 1993). In the case of Ca^{2+} ions these also have to overcome the powerful cytosolic Ca^{2+} buffering of the presynaptic cytosol. This idea is strengthened by the finding that neuropeptide release, in contrast to catecholamine release, is predominantly dependent on the same population of Ca^{2+} channels as the amino acid

transmitters (Pocock *et al.*, in preparation). Since these channels may be present predominantly in the active zone and neuropeptide containing vesicles are localized predominantly at ectopic sites within the terminals, the homeostatic mechanisms to buffer intracellular Ca^{2+} represent a significant factor in the delay of neuropeptide release and help to explain the higher effective stimuli necessary for neuropeptide release as compared to release of classical transmitters (see below). In any case, nerve terminals that contain both types of vesicles will very swiftly release a classical transmitter with a short extracellular half-life and very rapid ionotropic effects on a postsynaptic cell and, at the same time, slowly secrete neuropeptides and catecholamines with longer extracellular half-lives that evoke slower effects within a larger area of target cells. One of these signals may dominate depending on the pattern of stimulation, the previous experience of the terminal and the cell soma that synthesizes the neuropeptides.

In addition to the activation of exocytosis, different transmitters exhibit differences in the way this process is terminated. Under physiological circumstances, exocytosis may merely be limited by the repolarization, the inactivation of Na^{+} or Ca^{2+} currents and absorbance of intracellular free Ca^{2+} by Ca^{2+} pumps, exchangers and other Ca^{2+} -binding proteins. However, experimental conditions have been designed to maximally burden the exocytotic capacity in an attempt to characterize the recruitment of transmitter during tetanic activity. Such studies have shown that the release of different transmitters is terminated/inactivated in different ways. In nerve terminals, transmitter release is not as massive as in certain non-neuronal cells, where $> 50\%$ of the available signal molecules and vesicles is released upon a single period of stimulation. In the case of permanent chemical depolarization using high $[\text{K}]_o$, release of all major transmitters is limited to a fraction of the total content, despite the fact the intracellular Ca^{2+} is elevated and ATP levels are found not to drop dramatically under these conditions (Verhage *et al.*, 1991c). The retained fraction of transmitter is nevertheless available for release as soon as the terminal has been repolarized and stimulated again (Verhage *et al.*, 1991c).

Different transmitters appear to be differentially dependent on ATP. Almost complete inhibition of the catecholamine release was observed upon prior ATP depletion of synaptosomes (Verhage *et al.*, 1992b). For amino acid transmitters this was also observed (Kauppinen *et al.*, 1988) and confirmed in our laboratory (Verhage *et al.*, 1991c). In contrast, the release of the two most abundant neuropeptides, CCK-8 and Met-enkephalin, was also inhibited but a significant release component was ATP-independent (Verhage *et al.*, 1991a, 1992c). It was suggested that ATP is not an absolute requirement for the neuropeptide release mechanism and the neuropeptide release may depend on fundamentally different mechanisms from those operating in classical transmitter release. Alternatively, it is possible that all transmitter release mechanisms have comparable ATP-dependence. Although overall ATP/ADP ratios do not drop significantly during K^{+} -depolarization, spatial differences in ATP concentration (or in ATP-binding

competition) may occur in the terminal and underlie the present results, since neuropeptide release, in contrast to classical transmitter release, was found to involve events at remote sites of the nerve terminal (ectopic distribution of neuropeptide containing vesicles and stimulation of release by delocalized Ca^{2+} entry).

As elaborated in Section 2, the release of amino acids may selectively be stimulated with local Ca^{2+} entry through the active zone Ca^{2+} channels, yielding local high elevations of free Ca^{2+} . In contrast, the release of two of the most widely distributed neuropeptides, CCK-8 and Met-enkephalin, is preferentially evoked by delocalized Ca^{2+} -entry, in line with the ectopic distribution of neuropeptide containing large, dense-cored vesicles (Verhage *et al.*, 1991b). The fact that prolonged localized Ca^{2+} -entry is more effective for fast-acting transmitter release than delocalized Ca^{2+} -entry, suggests that Ca^{2+} gradients may exist over the length of the terminal. However, models of Ca^{2+} diffusion (Simon and Llinas, 1985; Zucker and Fogelson, 1986; Neher, 1986) predict a rapid, equilibration of free Ca^{2+} over the terminal (roughly 1 μm) and thus do not account for the existence of standing Ca^{2+} gradients. Two explanations were put forward in Section 2 to account for the fact that neuropeptides and amino acids are nevertheless differentially released. It was proposed that nerve terminals contain submembrane compartments, " Ca^{2+} domains", that provide a Ca^{2+} diffusion barrier. Alternatively, a kinetic difference may exist between the triggering of exocytosis on the one hand and the dilution of free Ca^{2+} into the bulk cytoplasm and the cytoplasmic Ca^{2+} buffering on the other hand. The Ca^{2+} entering the terminal triggers exocytosis of classical transmitters before the ions dilute into the cytoplasm and/or bind to Ca^{2+} -buffering components. This explanation requires the presence of the factors involved in exocytosis to be very close to the Ca^{2+} channel, or even associated with it. In this case the Ca^{2+} gradients would be transient and spatially limited, allowing full exocytosis of classical transmitters, but limited exocytosis of peptides. Transmitters from large, dense-cored vesicles, i.e. neuropeptides and co-localizing catecholamines, may thus only be fully released upon persistent high activity of the terminal, when the spatially limited Ca^{2+} gradients originating from active zone Ca^{2+} channels begin to saturate cytosolic-buffering components and to produce increases in the free Ca^{2+} concentration within the whole presynaptic cytosol. This implies that during low activity incoming action potentials are coupled to secretion of classical transmitters exclusively, whereas high activity yields additional release of neuropeptides while supplies of classical transmitter probably get progressively exhausted.

Titration of the cytoplasmic $[\text{Ca}]_i$ of nerve terminals using ionomycin, revealed indications for different Ca^{2+} regulation of the release mechanism of the three major classes of transmitters, as summarized in Fig. 2. The release of neuropeptides obeys a more or less linear relationship with the volume average (bulk) $[\text{Ca}]_i$ (Verhage *et al.*, 1991a,b), while this relationship is clearly non-linear for the classical transmitters (Verhage *et al.*, 1989, 1991b). This observation is complementary to the idea that the ectopic exocytosis of large, dense-cored vesicles is restricted

by cytosolic Ca^{2+} buffering. It must be emphasized that the $[\text{Ca}]_i$, as indicated in Fig. 2, may differ between the peptidergic, catecholaminergic and amino acid terminals and titration with ionomycin is an indirect method. However, peptides do not regularly occur in distinct peptidergic terminals, but co-localize with classical transmitters (in serial sections of isolated nerve terminals, very few terminals were found to only contain large, dense-cored vesicles and no small vesicles, Verhage and Ghijsen, unpublished results).

The hypothesis of selective stimulation of large, dense-cored vesicle exocytosis upon high activity is strengthened by several experimental observations. Differences in effective stimuli and stimulus frequencies have been observed in a few cases where electrically evoked release of co-localizing transmitters can be studied (cat submandibular gland, Lundberg *et al.*, 1982, 1984; splenic nerve of the pig, Lundberg *et al.*, 1986; substantia nigra, Lindefors *et al.*, 1985a,b; raphe nucleus in the spinal cord, Iverfeldt *et al.*, 1986). Nerve terminal storage of small molecular transmitters is often 50–1000 times higher than that of peptide transmitters (see Bartfai *et al.*, 1988). Thus, the nerve terminal is equipped for more frequent use of the small molecular transmitters than of the peptide transmitters. Generally, it is believed that peptide transmitters are released at higher frequencies than small molecular transmitters. In the systems where the two groups can be studied in parallel, facilitation of release of small molecular transmitters occurs in the frequency range 1–10 Hz (Burnstock *et al.*, 1964) while peptide release shows facilitation between 5–40 Hz (Diez-Guerra *et al.*, 1987; see Bartfai *et al.*, 1988 for a review) up to 80 Hz for the secretion of oxytocin from neurohypophysis nerve terminals (see Nowycky, 1991). A particular difference is observed in splenic nerve (Lundberg *et al.*, 1986), where release of neuropeptide-Y is observed only by bursting pattern stimulation, while this pattern is not effective in releasing the principle (classical) transmitter noradrenaline. These findings indicate that indeed neuropeptide release may occur specifically during high activity of the terminal. This mechanism poses a powerful tool for the plastic properties of nerve terminals. The released cocktail of transmitters shifts depending on the stimulation pattern and the recent history of the nerve terminal. A single stimulus to the nerve terminal may thus only produce small synaptic vesicles exocytosis at the active zone and replacement with new vesicles. $[\text{Ca}]_i$ -elevations are restricted to the micro-environment of the active zone. Hence, this type of presynaptic stimulation yields a swift and brief communication between an individual presynaptic terminal and an individual postsynaptic cell. High activity, in addition, may induce saturation of Ca^{2+} -buffering components, spread of Ca^{2+} gradients and the actual Ca^{2+} -dependent triggering of co-transmitter release. This type of presynaptic stimulation yields repetitive swift and brief communication in a confined synapse and additionally produces more global and slower signals that persist for longer periods.

The molecular mechanisms underlying differential release are still largely obscure. The spatial organization of the nerve terminal is certainly an important factor in differential release. The cellular factors that

help to establish this organization are therefore of primary interest, i.e. the docking of small synaptic vesicles at the active zone membrane, the assemblance of the active zone with its specific components and potentially arrangements specific for the ectopic secretion of neuropeptides. Fundamental differences in the molecular factors present on small synaptic vesicle release and on large dense cored vesicles exist. The lack of a very pure preparation of neuropeptide vesicles from the CNS still hampers a good comparison, but a large number of indirect indications suggest that proteins implicated in the uptake and intravesicular metabolism of classical transmitter and also in small synaptic vesicle transport, recycling, targeting and release are generally not present in large, dense-cored vesicles or in other related neural crest-derived, neuropeptide-containing vesicles (see DeCamilli and Jahn, 1990; Südhof and Jahn, 1991; Walch-Solimena *et al.*, 1993). For instance, synapsins are highly abundant on the surface of small synaptic vesicles (Huttner *et al.*, 1983). However, synapsins appear to be absent on large, dense-cored vesicles (Navone *et al.*, 1984). Synapsins are therefore probably an important component in the architecture and the local specified area with several specific features to produce exocytosis at the active zone. Rab3a may also be specific for small synaptic vesicles (see above) as well as synaptophysin (see discussion in Walch-Solimena *et al.*, 1993). However, the sensitivity of neuropeptide release for clostridial neurotoxins (McMahon *et al.*, 1992) suggests that some proteins, i.e. the synaptobrevins and syntaxins, are involved in the function of both types of vesicles, although each type of vesicle may exploit unique isoforms of these proteins. Synaptotagmins may also be a factor involved in the function of both vesicle types (Walch-Solimena *et al.*, 1993). These three proteins implicated in the function of both vesicle types may therefore represent core factors in neuronal regulated exocytosis. Factors specific for large, dense-cored vesicle regulation are still obscure. It is clear that these vesicles to some extent use different mechanisms, since their release exhibits unique characteristics, such as the release kinetics and the site of release. However, these distinct characteristics may largely arise from a negative selection, i.e. the absence of factors that direct small vesicles to the active zone and help to build up a large reservoir of releasable transmitter that can be released exceptionally fast. On the other hand, neuropeptide release, similar to that of classical transmitters, shows release of subpopulations of vesicles, while others are withheld from fusion and in invertebrates several different neuropeptides from a single precursor polypeptide may be transported to different areas of the cytoplasm (see for instance, Fisher *et al.*, 1988). Therefore, large, dense-cored vesicle trafficking and release may not be just a more simple (evolutionary older) version of small vesicle trafficking and release, but may in fact be more complex and heterogeneous.

4. LOCAL MODULATION OF TRANSMITTER RELEASE BY EXTERNAL FACTORS

Neuronal signals are integrated on the level of the cell soma and its dendrites. In addition, local modulation

on the level of the nerve terminals may occur. The presynaptic nerve terminal is equipped with a variety of presynaptic receptors. These receptors are a possible gateway for the local environment to influence the nerve terminal function. Furthermore, signalling substances may diffuse directly from outside the terminal into the presynaptic cytosol and play a modulatory role in its intracellular stimulus-secretion coupling. Hence, in addition to the principle integrative aspects of the cell body and its dendrites, there may be regulation of the presynaptic secretion by local factors in the nerve terminal area. Clearly, these local events may provide an important extra dimension in presynaptic regulation and contribute to the plasticity of the nerve terminal.

4.1. THE ORIGIN OF EXTERNAL SIGNALS ONTO PRESYNAPTIC NERVE TERMINALS

Presynaptic receptors have been implicated in the self-regulation of secretion by a feedback mechanism of released transmitter on its own receptors ("autoreceptors"). This suggestion is difficult, if not impossible, to test because it is presently impossible to follow individual transmitter molecules and discriminate between those originating from neighbouring or remote nerve terminals and those secreted from the nerve terminal itself. A theoretical argument against such mechanisms is provided by the fact that the interior of the nerve terminal appears to be already equipped with a number of mechanisms for rapid termination of the secretion process before the relatively slow mechanisms of receptor occupation, second messenger generation and second messenger effects can come into action. The exact localization of presynaptic receptors may be determining regarding which signals they receive. If presynaptic receptors are largely present in the active zone, it seems unlikely that signals from other nerve terminals have a large contribution to the events in the highly impenetrable synaptic cleft. In this case it remains uncertain what the impact of the generation of second messenger will be relative to the massive Ca^{2+} influx and (local) elevation of $[\text{Ca}]_i$. On the other hand, if presynaptic receptors are located on more remote sites of the terminal, they may receive signals from neighbouring or remote terminals and may thus be a target for modulation of the presynaptic stimulus-secretion coupling by external factors. Localization studies on an ultrastructural level may provide new information in this issue. It is also still largely unknown whether presynaptic receptors on a given population of nerve terminals are predominantly receptive for the transmitter(s) that these terminals secrete and whether receptors sensitive to foreign transmitters are also present. One example of the latter case is presynaptic inhibition by adenosine (Silinsky, 1986; Prestwich *et al.*, 1987). Adenosine may not be a major transmitter itself, but may largely be a breakdown product of ATP.

In addition to the nerve terminals themselves, postsynaptic cells and probably astrocytes may also release signalling substances that influence the presynaptic stimulus-secretion coupling. In the case of postsynaptic cells there is accumulating evidence that

these can send signals back to the opposing nerve terminal ("retrograde messengers", see below).

In addition to these three possible origins of signals onto the presynaptic terminal, from the own terminal, from neighbouring terminals or from the opposing postsynaptic neuron, presynaptic agonists may originate from more remote sites. Several agonists, especially neuropeptides and hormones, may occur in the extracellular compartment over long periods and may thus diffuse over large areas. As a result of this long extracellular half-life and also the high affinity of their receptors, these substances can be predicted to induce more global and uniform effects on a population of nerve terminals than the classical transmitters which are known to be cleared rapidly from the extracellular compartment by specific uptake carriers. This uniformity of the response of course depends on the set of receptors present on a nerve terminal population.

4.2. THE IMPACT OF PRESYNAPTIC RECEPTOR ACTIVATION ON THE STIMULUS-SECRETION COUPLING

Whatever the origin of the agonists for the presynaptic receptors may be, their modulatory role may certainly contribute to the plasticity of the stimulus-secretion coupling. This contribution depends on the signalling cascade that follows binding to presynaptic receptors and the timing of these events relative to the stimuli arriving through the axon (action potentials).

A wealth of information is available on receptors and their coupling to intracellular second messenger systems in cell bodies and dendrites. Many have also been implicated in the presynaptic modulation, largely on the basis of pharmacological manipulations. Both excitatory and inhibitory effects of the presynaptic receptors have been described, depending

on the second messenger they exploit. However, the evidence for many presynaptic receptor pathways is still not as unambiguous as in the case of "postsynaptic" receptors. For instance, there is little direct proof, largely for practical reasons, for the presynaptic localization of the receptor proteins (see above). Many indications are derived from complex model systems like nerve terminal-enriched preparations (which do contain microsomes, dendrosomes etc.) and brain slices. In the latter case the role of glial cells may have been underestimated in some cases. Furthermore, it is uncertain whether the set of presynaptic G proteins is similar to those of postsynaptic cells and it is not clear how potent presynaptic internal Ca^{2+} stores are.

Still, the existence of several presynaptic receptors is beyond doubt. Generally, these may affect the nerve terminal function and its plasticity in two ways. Firstly, their activation may directly evoke secretion. Secondly, their activation may modulate the stimulus-secretion coupling and may thus only be relevant in combination with the arrival of action potentials through the axon. Especially the latter effect is potentially an important aspect of presynaptic plasticity. The timing of presynaptic receptor activation relative to the arrival of action potentials may yield Hebbian mechanisms (see Fig. 8). In both cases, the secretion of different (co-localized) transmitter can be modulated differentially. As suggested in the first two parts of this review, the secretion of "classical" transmitters on the one hand and co-localizing peptides and catecholamines on the other hand is already differentially regulated. The two types of secretion arise from different parts of the terminal, from different vesicles and presumably depend on different effective stimuli. If this suggestion holds, the secretion from large, dense-cored vesicles may be more susceptible to a modulating input by presynaptic receptors. The

FIG. 8. Schematic representation of the proposed additivity of presynaptic receptor stimulation and electrical stimulation through the arrival of action potentials through the axon. If the two events are timed in phase, this may yield transmitter release and provide a mechanism for presynaptic plasticity.

release of classical transmitters from the clustered small synaptic vesicles is a highly localized event with a low/moderate affinity for Ca^{2+} and massive, local Ca^{2+} accumulations produced by high capacity Ca^{2+} channels. In contrast, co-localizing transmitters may be released with higher affinity mechanisms and smaller, more gradual Ca^{2+} -elevations at ectopic sites. Presynaptic receptors normally produce second messengers (with relatively slow kinetics and low capacity as compared to ion channels) and may also be located at ectopic sites in the terminal membrane. Therefore, modulation of the stimulus-secretion coupling may affect the secretion of neuropeptides and catecholamines (from large, dense-cored vesicles) to a greater extent than the secretion of classical transmitters. However, presently there is little information on the modulation of large, dense-cored vesicle secretion by presynaptic receptors.

There are few clear examples of direct induction of transmitter release by presynaptic receptors. This may be evoked by opening of presynaptic Ca^{2+} channels or Ca^{2+} mobilization from internal stores upon activation of such receptors. Presynaptic glutamate receptors of the NMDA-type are in principle able to produce sufficient elevations in intracellular free Ca^{2+} to overrule the powerful Ca^{2+} -buffering systems in the presynaptic cytosol and induce transmitter release. There is some evidence for such mechanisms in the striatum (Wang, 1991; Wang *et al.*, 1992; Desce *et al.*, 1992). Part of this effect may be due to interactions of NMDA with the high-affinity glutamate uptake-carrier and reduced glutamate uptake. It remains to be determined whether such mechanisms are physiologically relevant (see Erecinska, 1987; Bernath, 1992). Furthermore, receptor-activated Ca^{2+} mobilization from internal stores may in principle induce transmitter release directly. Several presynaptic receptors couple to phospholipase C activation, such as acetylcholine receptors of the muscarinic type (see for instance, Raiteri, 1982; Diamant *et al.*, 1988), 5-HT (Kaczmarek and Levitan, 1987; Delaney *et al.*, 1991), several neuropeptide receptors such as CGRP (Oku *et al.*, 1988), galanin (Martire *et al.*, 1991), vasoactive intestinal polypeptide (see for a review, Chesselet, 1984) and a recently characterized member of glutamate receptors, the metabotropic, tACPD sensitive receptor (Baskys and Malenka, 1991; Calabresi *et al.*, 1992; Rainnie and Shinnick-Gallanger, 1992). As discussed in Section 2, there is already relatively little information as to the potency of presynaptic internal Ca^{2+} stores. The indications for such a mechanism being coupled to transmitter release in the mammalian CNS are even more scarce and indirect (see for instance, Diamant *et al.*, 1988; Weiss *et al.*, 1990). This lack of evidence strengthens the suggestion that in most cases, presynaptic secretion may not directly be manipulated by presynaptic receptor activation and intracellular Ca^{2+} -elevation, but that the activation may in general rather *modulate* the secretion evoked by depolarization. Rather than a straight forward stimulation of release, a synergistic effect of low frequency axonal stimulation and receptor occupation may evoke a plastic behaviour of the nerve terminal (Fig. 8). Intracellular Ca^{2+} mobilization could "prime" the terminal through small elevations of free Ca^{2+} and/or

saturation of the Ca^{2+} -buffering capacity; that in itself does not produce transmitter release, but potentiates transmitter release when action potentials trigger release. In this case, the timing of presynaptic receptor occupation is thus decisive for its impact. Simultaneous occupation of presynaptic receptors and arrival of action potentials through the axon will produce an enhanced release, whereas non-simultaneous occurrence of the two events is without effect. Such timed actions of presynaptic receptors may lower the effective frequency of stimulation for catecholamine and neuropeptide release (Fig. 8). An example of such plastic mechanisms is the metabotropic glutamate receptor. Its activation by tACPD was found to have an inhibitory effect on presynaptic activity (Baskys and Malenka, 1991; Calabresi *et al.*, 1992; Rainnie and Shinnick-Gallanger, 1992), but may in concert with high-frequency stimulation lead to potentiation (see Anwyl, 1991), which has been attributed to a "priming" effect of inositol triphosphates by a subthreshold elevation of free Ca^{2+} . Alternatively, the other branch of phospholipase C activation, through diacylglycerol and its activation of protein kinase C is even more important in nerve terminals. Recent data from the laboratory of Sanchez-Prieto (Herrero *et al.*, 1992) suggest that the potentiating effect of metabotropic glutamate receptors during high frequency stimulation may arise from a synergy between diacylglycerol-induced activation of protein kinase C and arachidonic acid, which may be released from postsynaptic cells during high-frequency stimulation (see below). This is in line with an older observation that presynaptic G proteins are involved in LTP (Goh and Pennefather, 1989).

Apart from direct induction of transmitter release, modulation of evoked presynaptic transmitter release by presynaptic receptor activation can occur at any step in the signalling cascade leading to transmitter exocytosis, such as the modification of presynaptic ion channels, modulation of docking-fusion complexes, cellular Ca^{2+} -buffering, (transmitter) metabolism, the availability of synaptic vesicles etc., and may affect all transmitter systems. Relatively few presynaptic receptors are known to potentiate release. Generally, modulating presynaptic input was found to inhibit presynaptic secretion.

One pathway that potentiates presynaptic secretion acts through protein kinase C (PKC, see Tanaka, 1984; Nichols *et al.*, 1987; Shu and Selmanoff, 1988; Shuntoh *et al.*, 1988; see also Fig. 3). The activation of PKC attenuates the presynaptic input-output relation by manipulating the repolarization through phosphorylation of K^{+} channels and hereby prolonging Ca^{2+} channel opening and Ca^{2+} entrance (see Tibbs *et al.*, 1989; Barrie *et al.*, 1991; Herrero *et al.*, 1992), by regulating the availability of Ca^{2+} buffering components through phosphorylation of B-50 and MARCKS-protein (Alexander *et al.*, 1987, 1988; De Graan *et al.*, 1990; Graff *et al.*, 1989; McIlroy *et al.*, 1991; Hartwig *et al.*, 1992), by modulating transmitter production through phosphorylation of tyrosine hydroxylase (Woodrow *et al.*, 1992) and probably by modulating recruitment, docking or release of small synaptic vesicles through phosphorylation of P96 (Robinson 1991, Sim *et al.*, 1991). These mechanisms may also be involved in the maintenance

of long term potentiation, since presynaptic PKC-activity was found to correlate with the induction of LTP and increased transmitter release has been implicated in the mechanisms underlying LTP (Bär *et al.*, 1980, 1982; Tielen *et al.*, 1983; Lovinger *et al.*, 1985, 1986; Gianotti *et al.*, 1992). Generally, PKC may only be fully activated during high activity (as in the case of LTP-induction) and increases in free Ca^{2+} in the bulk presynaptic cytoplasm. The activated kinase has a longer lifetime than the Ca^{2+} -elevations and may therefore continue to modulate factors involved in secretion when the axonal stimulation is stopped and hereby potentiate the presynaptic stimulus-secretion coupling during new stimulations.

Inhibition of the presynaptic stimulus-secretion coupling by presynaptic receptor activation may be more general than potentiation (see Chesselet, 1984). A well known mechanism of presynaptic inhibition is the activation of presynaptic GABA-receptors and their modulation of ionic balance by ligand-gated ion channels. Both presynaptic GABA_A-receptors coupled to Cl^- channel and GABA_B-receptors, coupled to K^+ channel opening inhibit secretion by hyperpolarization (see for instance, Peet and McLennan, 1986; Ong *et al.*, 1990; Heidelberger and Matthews, 1991). By hyperpolarizing the terminal, depolarizing stimuli fail to pass the threshold for the activation of Ca^{2+} channels involved in exocytosis, or the probability of opening is decreased. Effectively, the voltage dependence of the Ca^{2+} channels and thus of the triggering of transmitter release, is shifted to more depolarized potentials. Many other presynaptic receptors have been described to mediate inhibition of the presynaptic stimulus-secretion coupling (see Chesselet, 1984), such as α_2 -adrenergic receptors (see for instance, Starke, 1977; Maura *et al.*, 1982), adenosine A₁-receptors (Prince and Stevens, 1992; Barrie and Nicholls, 1993), opioid receptors (see for instance, Hagan and Hughes, 1984; McWilliam and Campbell, 1987). For these receptors the most likely targets are presynaptic K^+ channels and Ca^{2+} channels, either via G proteins or through intracellular messengers.

In the case of receptors coupled to stimulation or inhibition of cAMP-production by adenylate cyclase activation of these receptors may have priming or additive effects. Possibly Ca^{2+} -entry and intraterminal elevation of free Ca^{2+} stimulates adenylate cyclase activity to produce cAMP and activate cAMP-dependent protein kinase II to phosphorylate synapsins. Additional modulation of cAMP production by presynaptic receptors may thus either potentiate or reduce this effect, dependent on the type of G protein (G_s or G_i) involved in the cascade. Furthermore, cAMP production and activation of cAMP-dependent protein kinase II is known to phosphorylate other relevant substrates, for instance ion channels (see Siegelbaum and Tsien, 1983; Huganir and Greengard, 1983) and cytoskeletal components (see Nairn *et al.*, 1985). In the case of cAMP coupled receptors and probably also cGMP coupled receptors (Branisteanu *et al.*, 1988) activation of these receptors may not only lead to potentiation, but generally to inhibition of transmitter release (see above). In *Aplysia*, cAMP-dependent phosphorylation of K^+ channels induce depolarization (impaired repolariza-

tion; Siegelbaum and Tsien, 1983) and consequently potentiation of transmitter release.

The availability of the G proteins that couple receptors to their effector systems may be an integrative aspect of the presynaptic input-output relation. In general, stimulation of G protein coupled receptors induce the dissociation of the heterotrimeric G proteins into their free subunits. Because G proteins appear to share the same pool of regulatory β subunits, the stimulation of one type of receptors induces the release of free β subunits. These may bind free catalytic α subunits of all types of G proteins, suppress all G-protein mediated signal transduction pathways and thus invoke negative feedback in the cross talk between different signalling cascades.

In conclusion, a variety of mechanisms exist in the presynaptic terminal to modulate synaptic transmission and to integrate different chemical and electrical signals onto the terminal. K^+ channels play a pivotal role. PKC-induced phosphorylation of certain K^+ channels leads to a decrease in K^+ permeability and potentiate transmission, whereas cAMP and probably also cGMP induced phosphorylation of certain K^+ channels may increase K^+ permeability and inhibit transmission. In addition, presynaptic receptors may increase K^+ permeability through G protein interactions.

4.3. OTHER MESSENGERS

In general, agonists for presynaptic receptors are regular transmitters, which are themselves released from presynaptic terminals. In addition to these classical signals, new classes of intercellular messengers are being identified. These messengers are believed to be released from postsynaptic cells and have been implicated in retrograde signalling to the presynaptic terminals or other cells. These postsynaptic cells and dendrites do not contain the regular exocytosis machinery and signals arise merely from evoked synthesis and free diffusion. Two such diffusible messengers have been characterized in detail: arachidonic acid and nitric oxide.

Arachidonic acid and its metabolites were the first substances to be proposed as neuronal messengers that could pass freely across cell membranes in *Aplysia* (Piomelli *et al.*, 1987) and implicated in retrograde signalling in LTP (see Collingridge, 1987; Stevens, 1989). In *Aplysia*, the oxidation of arachidonic acid is the main activation step leading to presynaptic modulation of K^+ currents and elevating serotonin induced presynaptic inhibition of the same channels (Belardetti *et al.*, 1987). In mammals, arachidonic acid itself may also be an important signalling substance (see for a review Axelrod *et al.*, 1988). Arachidonic acid (Lynch *et al.*, 1989b; Williams, 1989; Freeman *et al.*, 1990) and its metabolites (Freeman *et al.*, 1991) were found to modulate transmitter release by unknown mechanisms. The implication of retrograde signalling by arachidonic acid (or its metabolites) was supported by some experimental evidence in brain slices and the intact animal (Williams *et al.*, 1989; Lynch *et al.*, 1989b). The mechanism of these proposed retrograde signals may lie in the differential inhibition of synaptic transmitter-uptake mechanisms, i.e. a potent

inhibition of excitatory transmitter reuptake (Yu *et al.*, 1987; Barbour *et al.*, 1989; Volterra *et al.*, 1992), but a small inhibition of inhibitory transmitter reuptake (Yu *et al.*, 1987). In this sense arachidonic acid may thus not be a true retrograde signal towards the presynaptic terminal. It may rather potentiate the effect of a constant amount of presynaptically released neurotransmitter by increasing its extracellular half-life. Alternatively, arachidonic acid was also found to produce a true presynaptic modulation by a synergistic effect with presynaptic glutamate receptor activation on PKC activity, proposed to potentiate presynaptic stimulus-secretion coupling through phosphorylation of K^+ channels (Herrero *et al.*, 1992). Still several questions remain unresolved. In many studies, especially in LTP, the effective concentrations of arachidonic acid appear to be quite high and arachidonic acid appears to be produced in high concentrations also by nerve terminals themselves (see Bradford *et al.*, 1983). Finally, the effects of arachidonic acid on synaptic transmission are slow relative to the expression of LTP.

Nitric oxide is a gas produced in neurons through NO-synthase. The neuronal form of this enzyme appears to be highly regulated. Its activity can potentially be stimulated by selective stimuli such as calcium elevations (Bredt *et al.*, 1991). Especially the link between NMDA-receptor stimulation and NO production is now well established, particularly in the cerebellum (see for a review Snyder, 1992). In this case, NO may thus be largely a signal arising from postsynaptic cells. In other cases NO-synthase may be present in nerve terminals, for instance of the myenteric plexus (Llewellyn-Smith *et al.*, 1992). In this case NO may serve a more "classic" anterograde signalling role. Nitric oxide is a free radical and may act on many targets. One well-documented target is cGMP formation. Since it is also a free diffusible substance this signal can easily translocate from its site of production to adjacent compartments, such as the presynaptic terminal. Evidence from a trans-synaptic signalling role for NO comes from studies using haemoglobin, which binds extracellular NO. In this way evidence is produced for a trans-synaptic role of NO under toxic conditions (see for instance, Nowicki *et al.*, 1991; Dawson *et al.*, 1992). In synaptic plasticity, comparable results are obtained for long-term depression in the cerebellum and long-term potentiation in the hippocampus (see Snyder, 1992). However, NO-synthase was not detected in the postsynaptic cells involved in LTP. Furthermore, NO is a free radical with a half-life of only a few seconds. The evoked production of NO is limited by the kinetics of the synthesizing enzyme. Detailed kinetic and diffusional models are necessary to confirm whether a significant amount of NO may reach the presynaptic nerve terminal and can serve as a true retrograde messenger.

New retrograde messengers may be characterized in the future. Other free radicals may function in this way, like carbon monoxide (see Verma *et al.*, 1993). Another potential new group is the polyamines which can be released from postsynaptic cells in a Ca^{2+} dependent manner and can modulate presynaptic

transmitter release (Bondy and Walker, 1986; Fage *et al.*, 1992).

5. CONCLUSIONS

Presynaptic nerve terminals have several means to tune their response to axonal stimulation and to exhibit integrative properties. This presynaptic plasticity is expressed in the variation of the timing and the extent of transmitter release with previous experience of the terminal. In addition, the nerve terminal may vary the mixture of several signalling substances that are being secreted.

On the shortest term (msec), the variation in the input-output relation of presynaptic terminals may lay predominantly in the available number of preformed fusion complexes between vesicles, proteins and the plasma membrane and in the number of Ca^{2+} ions that reach the direct environment of these release sites. On the longer term, the presynaptic input-output relation is influenced by processes like the recruitment of new vesicles, the removal and refilling of exposed vesicles, involving enzymatic reactions rather than simple conformational changes. Furthermore, different transmitters from different vesicles may be differentially recruited and released. Finally, on the longest term presynaptic plasticity may involve structural changes and changes in presynaptic (transmitter) metabolism and other changes in the stimulus-secretion coupling capacity or efficacy. These changes may no longer be taken as specific to terminals and involve protein synthesis at the level of the cell body, other neurons and the extracellular matrix.

The capacity of the nerve terminal to transmit incoming action potentials depends on the recruitment and priming of the preformed complexes in concert with the spreading of Ca^{2+} gradients from the Ca^{2+} channel-mouths. In general, the number of Ca^{2+} -sensitive triggers, docking-fusion proteins and vesicles that are available for exocytosis to occur will be decisive for this capacity and will probably limit the secretion during high activity. It is likely that migration of new vesicles towards the active zone and clearance of elevated Ca^{2+} at the site of exocytosis take longer than exocytosis and repolarization. Upon repetitive stimulation, the former process will thus create a progressive shortage of vesicles at the active zone, a phenomenon known as short-term depression or fatigue (DeCastello and Katz, 1954; see also Zucker, 1989); the latter will create an accumulation of residual Ca^{2+} at the active zone. This phenomenon is known as short-term facilitation or augmentation (Katz and Miledi, 1968; see also Attwood and Wojtowicz, 1986).

To fully understand many aspects of presynaptic plasticity, it is important to understand the fate of Ca^{2+} ions from their entry at the interface between plasma membrane Ca^{2+} channels and the presynaptic cytosol. This is an extremely complex issue for several reasons. The compartment formed by docked vesicles, proteins involved in exocytosis and the Ca^{2+} channel is very small. The event of exocytosis is very brief and represents a non-equilibrium situation. Many components within this small compartment bind Ca^{2+} . Furthermore, more than 99% of the Ca^{2+}

uptake does not contribute to the elevation of cytosolic free Ca^{2+} -concentration, as it is measured with fluorescent probes. This observation stresses the importance of Ca^{2+} -buffering components within the terminal. Slight changes in this capacity will have profound effects on the free Ca^{2+} concentration and therefore on the stimulus-secretion coupling. Due to the powerful cytosolic Ca^{2+} buffering, Ca^{2+} gradients may persist within the terminal despite the already small overall dimensions of the nerve terminal. As a result, local submembrane events, such as the actual release process, may be regulated by different concentrations of Ca^{2+} , rather than the events in the presynaptic cytosol, such as vesicle recruitment and docking and probably also the release of neuropeptides and catecholamines from large, dense-cored vesicles. In fact, this submembrane compartment may be as small as the Ca^{2+} channel-mouth and it is probably incorrect to speak of Ca^{2+} concentrations, because of the local absence of water molecules. Possibly, the actual release process does not involve processes that are highly selective for Ca^{2+} . Instead, initiation of classical transmitter release may be a process involving a brief exposure to high amounts of divalent cations, with certain preference for Ca^{2+} . True Ca^{2+} dependence may arise from the Ca^{2+} selectivity of the ion channels in the presynaptic membrane. Two arguments have been put forward here for such a mechanism. First, reactions with a high off-rate require low affinity interactions and, second, several other divalent cations appear to be able to replace Ca^{2+} in the process of triggering transmitter release, especially those that can not be taken up or extruded by the terminal, as in the case of Ba^{2+} . Further Ca^{2+} dependence of transmitter release may arise from the Ca^{2+} -dependent enzymatic processes that account for vesicle recruitment and docking and cytoskeletal rearrangements that facilitate exocytosis, such as the activity of Ca^{2+} -dependent kinases and phosphatases.

Each of the events, local and more remote, will respond differently upon repeated stimulation and Ca^{2+} entrance. Finally, both types of events are again dependent on the regulation of Ca^{2+} channel gating, the regulation of membrane repolarization and the stimulation pattern that reaches the terminal. Different types of Ca^{2+} channels may be involved in the release of different transmitters.

In addition, a variety of mechanisms exist in which local chemical signals modulate the presynaptic stimulus-secretion coupling and integrate with the electrical signals arriving through the axon. K^{+} channels may play a pivotal role as a target for these local signals by their ability to modulate transmitter release in both a positive or a negative direction.

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