Vesicle transport and neurotransmitter release in central nerve terminals
Leenders, A.G.M.

Citation for published version (APA):
Chapter 3

Activity-dependent neurotransmitter release kinetics: correlation with changes in morphological distributions of small and large vesicles in central nerve terminals


ABSTRACT

In central nerve terminals transmitter release is tightly regulated, and thought to occur in a number of steps. These steps include vesicle mobilization and docking prior to neurotransmitter release. Intrasynaptic changes in vesicle distribution were determined by electron microscopical analysis and neurotransmitter release was monitored by biochemical measurements. We correlated K⁺-induced changes in distribution of SVs and LDCVS with the release of their transmitters. For SVs amino acid release as well as recruitment to and docking at the active zone were activated within a second depolarization. In contrast, the disappearance of LDCVs and the release of the neuropeptide CCK were much slower, and no docking was observed. Studies with diverse Ca²⁺-channel blockers indicated that mobilization and neurotransmitter release from both vesicle types were regulated by multiple Ca²⁺-channels, although in a different way. Neurotransmitter release from SVs was predominantly regulated by P-type Ca²⁺-channels, whereas primarily Q-type Ca²⁺-channels regulated neurotransmitter release from LDCVs. The different Ca²⁺-channels types directly regulated mobilization of and neurotransmitter release from SVs, whereas, by their cooperativity to raise the intracellular Ca²⁺ concentration above release threshold, they more indirectly regulated LDCV exocytosis.
INTRODUCTION

Neurotransmitter release from presynaptic nerve terminals is highly regulated in a temporal and spatial manner. One can discriminate between fast amino acid release from SVs in the active zone, and slow neuropeptide release from LDCVs at remote sites in the terminal. Differences in kinetics and calcium sensitivity suggest that exocytosis of the two vesicle types is differently regulated (Burgoyne and Morgan, 1995; Verhage et al., 1991b). It is well established that neurotransmitter release from SVs occurs within 1 ms after the Ca\textsuperscript{2+} trigger (Almers, 1990). The existence of a readily releasable pool of SVs at the active zone in the direct vicinity of Ca\textsuperscript{2+}-channels would allow such a fast release. In contrast to SVs, no readily releasable pool of LDCVs has been observed in central nerve terminals (Burgoyne and Morgan, 1995; Verhage et al., 1991b). Therefore, prior to fusion, LDCV mobilization and docking should occur after stimulation. Kinetics of LDCV exocytosis appear to vary greatly depending on cell type, size of the release site and identity of the transmitter investigated (Bruns and Jahn, 1995; Chow et al., 1992; Seward et al., 1995; Thomas et al., 1993; Ninomiya et al., 1997). However, very little is known about the kinetics of LDCV mobilization, docking and fusion in central nerve terminals (Bean et al., 1994). Exocytosis of both vesicle types is dependent on the entry of Ca\textsuperscript{2+} through high voltage activated Ca\textsuperscript{2+}-channels. Using pharmacological tools the P-/Q- and N-type Ca\textsuperscript{2+}-channels have been identified and shown to be involved in exocytosis of the SVs (Dunlap et al., 1995). Recent studies indicate that refilling, like fusion, is a Ca\textsuperscript{2+}-dependent process (Heinemann et al., 1993; Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998), but which Ca\textsuperscript{2+}-channel types are involved is unknown. Exocytosis of LDCVs in neuroendocrine cells, seems to be predominantly mediated by Ca\textsuperscript{2+}-influx through the L-type Ca\textsuperscript{2+}-channels (Dunlap et al., 1995), but very little is known about the identity of the Ca\textsuperscript{2+}-channel subtype(s) regulating the mobilization of LDCVs and release of their neurotransmitters in central nerve terminals.

Refilling of the readily releasable pool determines to a major extent the capacity of a terminal to respond to repetitive stimulation. Refilling of SVs from the reserve pool has been determined to occur within seconds (Neher, 1998; Ryan et al., 1993). Direct real-time imaging of secretory granules in endocrine cells indicate that refilling occurs in several steps, including recruitment, docking and priming of vesicles.
Mobilization and fusion of small and large vesicles

(Steyer et al., 1997). In order to investigate the molecular mechanisms involved, it would be desirable to determine directly these separate steps of the synaptic vesicle cycle. In the present study we used electron microscopical analysis to visualize some of these steps. We analysed changes in intraterminal distribution of both vesicle types upon stimulation in synaptosomes. As a model system, highly purified synaptosomes from rat cortex were used, allowing these ultrastructural studies in a synchronously stimulated population of central nerve terminals. To approach physiological relevance, depolarizations at the millisecond time-scale were applied by use of a rapid mixing device. The changes in vesicle distribution were correlated with neurotransmitter release from both vesicle types by biochemical measurement of the release of diverse transmitters from the synaptosomes upon the same millisecond depolarizations. Clear differences in kinetics and Ca$^{2+}$-channel regulation of mobility and neurotransmitter release between SVs and LDCVs were apparent.

MATERIALS and METHODS

Materials
Percoll was obtained from Pharmacia Biotech (Uppsala, Sweden). ω-Agatoxin IVA and ω-Conotoxin MVIIIC were obtained from Alomone labs (Jerusalem, Israel), ω-Conotoxin GVIA was obtained fromm Sigma. L-trans-pyrolidine-2,4,-dicarboxylate (L-trans-PDC) was purchased from Tocris Cookson (Bristol, UK). 1-(4,4-diphenyl-3-butenyl)-3-piperidine carboxylic acid hydrochloride (SK&F 89976-A) was kindly provided by dr. Skidmore (Smith, Kline and French, Welwyn, UK). Silicone oil (dow corning 550) was from Mavon B.V. (Alphen a/d Rijn, the Netherlands). All other chemicals were obtained from Sigma (Brunschwig Amsterdam, The Netherlands) or Janssen (Beerse, Belgium) and were of the highest purity available.

Synaptosomal preparation
Synaptosomes were prepared from male Wistar rat (180-220 gr) cortex, rapidly dissected on ice after decapitation. They were purified by Percoll density gradient centrifugation essentially as described by Dunkley et al. (1988). The synaptosomal fractions in the 10-15% and the 15-23% Percoll interfaces were pooled and washed
twice in artificial cerebrospinal fluid (aCSF) which contained (in mM): NaCl (132), KCl (3), MgSO₄ (2), NaH₂PO₄ (1.2), HEPES (10) and D-Glucose (10) + 2 mM CaCl₂. Synaptosomes were kept on ice in aCSF + 2 mM CaCl₂ at a protein concentration of 2 mg/ml until use in the release assay, which was within 4 hours after isolation. Protein concentration was determined according to Bradford (1976) with Bovine Serum Albumine as a standard.

Short depolarization assay
Synaptosomes (80 µg protein) were pelleted and resuspended in 40 µl aCSF supplemented with 50 µM ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), 1 mg/ml Bacitracine, 50 µM of the glutamate/aspartate uptake carrier blocker, L-trans-PDC (Bridges et al., 1991) and 10 µM of the the GABA uptake carrier blocker SK&F 89976-A (Yunger et al., 1984). If appropriate, the synaptosomes were preincubated at 37°C for 5 min with the Ca²⁺-channel toxins, ω-Agatoxin IVA, ω-Conotoxin MVIIC or ω-Conotoxin GVIA. Using a rapid mixing device the synaptosomes were given a depolarization varying from 0 to 1000 ms. This mixer consists of two syringes, each controlled by a pneumatic dispenser (type 1000-XLE, EFD Inc. USA). By a pressure pulse (0.35 bar) 200 µl of high K⁺ medium (depolarization medium, see below) is released from syringe 1, depolarizing the synaptosomes. After a preset time (0 - 1000 msec), controlled by a digital timer, syringe 2 released 1 ml ice-cold stop medium (release measurements) or fixative (electron microscopical analysis) (pressure pulse 1.8 bar) to stop the reaction. The compartment containing the synaptosomes and syringe 1 are thermostrated at 37°C and syringe 2 is kept at 0-4°C by ice. Depolarization media: aCSF containing 50 mM KCl in the presence of 2 mM Ca²⁺ (for total release) or 50 µM EGTA (for the Ca²⁺-independent release), NaCl was iso-osmotically replaced by KCl. Stop medium: aCSF containing 10 mM EGTA. Fixative: 2% paraformaldehyde and 2.5% glutaraldehyde.

Electron microscopical analysis
After fixation synaptosomes were embedded in epon, ultrathin sections (80 nm) were stained with uranyl acetate and lead citrate and examined in a Philips 201 electron microscope, essentially as described previously (Breukel et al., 1997a). For each...
Mobilization and fusion of small and large vesicles

Experimental condition 10 micrographs (39500×) showing 30 to 40 synaptosomal sections, were randomly taken and number of LDCVs in these sections was counted. For each experimental condition 25 synaptosomal sections with a clearly visible active zone were selected for analysis of SV distribution. SVs were counted in 50 nm frames starting from the active zone (Hess et al., 1993) and related to the total SV content per terminal. Only those SVs associated, i.e. in visible contact, with the active zone membrane were counted as docked SVs. Both the selecting of sections and the vesicle counting was performed blindly and independently by different persons.

Measurements of released neurotransmitters
For analysis of released transmitters, 700 µl of the synaptosomal suspension was centrifugated through 300 µl of 45:55% (vol.:vol.) mixture of silicone oil and dinonylphthalate for 2 min at 15000g. A 100 µl aliquot from the supernatant was pipetted onto 10 µl ice-cold Homoserine (1 µM)/trichloroacetic acid (TCA) (10%) solution for amino acid analysis, and 500 µl was brought onto 1 ml ice-cold methanol for CCK analysis. Both samples were stored at -20°C. Amino acid levels (glutamate and GABA) were determined by reversed phase HPLC as described previously (Verhage et al., 1989). Nonsulfated CCK-8 (CCK) release was quantified by radioimmunoassay (RIA), using the rabbit antiserum C221 (Breukel et al., 1998). In this RIA, sulfated CCK-8 and CCK-6 displayed 100% cross-reactivity, and sulfated CCK-7, CCK-4 and CCK-3 did not cross-react (< 0.07%).

Statistical analysis
The data were statistically analysed by the paired Student's t-test. The rejection of the null hypothesis was accepted as significant if p < 0.05. Difference in distribution of the SVs was tested with the χ2-test (p < 0.01).
Purified nerve terminals, i.e. synaptosomes, from rat cortex were taken as a model system for the central presynapse (Breukel et al., 1997a). Making use of a rapid mixing device, we were able to stimulate synaptosomes by short (50 millisecond) elevations of the extracellular K\(^+\) concentration. This ensured vesicle movement mainly involved in exocytosis and not in endocytosis, a process which probably takes sec-min (Neher, 1998). Although in neuroendocrine cells sub-second endocytosis of granules was found (Thomas et al., 1994), such a fast step has not been reported for SVs in central terminals. In addition, this technique limited regulated exocytosis of SVs to the active zone of the nerve terminals, in contrast with more prolonged K\(^+\)-stimulation that has been shown to induce fusion of these vesicles outside the active zone as well (Ceccarelli et al., 1988).

To study the mobility of vesicles in synaptosomes we performed an ultrastructural analysis by electron microscopy. The synaptosomal sections had an average diameter of 550 nm, containing about 55 SVs, 4 of which were docked at the active zone, indicating that in purified nerve terminals the functional vesicle distribution is preserved (Schikorski and Stevens, 1997), with a large reserve pool and some docked SVs, which may include the readily releasable pool. In 9% of these synaptosomal sections one or more LDCV were observed. We determined the changes in the distribution of both vesicle types within the nerve terminal upon stimulation by applying short K\(^+\)-induced depolarizations, lasting either 100 or 1000 ms, after which the nerve terminals were immediately fixed. Since SVs release their transmitter in the active zone, we randomly selected for each experimental condition 25 synaptosomal sections where the active zone was clearly visible (Fig. 1). Total number of SVs and active zone length in each section and the diameter of the section varied due to differences in plane of section through the terminals. This variety was, however, seen in every experimental condition and the frequency distributions within the total range for each parameter were not different between all separate experiments (Kolmogorov-Smirnov test, \(p > 0.05\)). The means of these parameters were therefore not different between controls and stimulated conditions (Table 1).
Mobilization and fusion of small and large vesicles

Fig. 1. Synaptosome sections.
Sections of synaptosomes with clearly visible active zone, defined by a thicker presynaptic plasma membrane accompanied by a still attached (part of the) post synaptic density. SVs docked at the active zone are indicated by arrow heads. Fig. 1A, section which clearly shows clustering of the SVs at the active zone. Fig. 1B, section with a random distribution of SVs throughout the terminal. Scale bar: 0.1 μm.

Table 1. Synaptosomal section parameters

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Synaptosomal section diameter (nm)</th>
<th>Active zone length (μm)</th>
<th>Total number of SVs/synaptosomal section</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 7)</td>
<td>555.5 ± 14.5</td>
<td>0.395 ± 0.02</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>100 ms / 2 mM Ca²⁺ (n = 3)</td>
<td>520.3 ± 45.3</td>
<td>0.343 ± 0.05</td>
<td>52 ± 6</td>
</tr>
<tr>
<td>1 s / 2 mM Ca²⁺ (n = 7)</td>
<td>536.3 ± 11.5</td>
<td>0.349 ± 0.01</td>
<td>54 ± 4</td>
</tr>
<tr>
<td>1 s / 50 μM EGTA (n = 6)</td>
<td>549.4 ± 12.6</td>
<td>0.368 ± 0.02</td>
<td>54 ± 3</td>
</tr>
</tbody>
</table>

For each stimulus condition average ± SEM of section diameter (nm), active zone length (μm) and total amount of SVs is given, these parameters show no significant differences between the different conditions tested.

Distribution of SVs within the terminals was quantified by determining the amount of SVs at successive distances from the active zone in steps of 50 nm (≈ vesicle diameter) (Hess et al., 1993) (Fig. 2A). The intrasynaptosomal distribution of SVs after depolarization was significantly different from that observed in control, non depolarized, synaptosomes (χ²-test, p < 0.01, n=3-6). In the control synaptosomal
Fig. 2. Depolarization induced redistribution of SVs at the active zone.

Fig. 2A. Distribution of SVs. SVs were counted in 50 nm frames starting from the active zone and expressed as percentage of total amount of vesicles per synaptosomal section. The χ²-test showed a significant difference in distribution after depolarization compared to non-depolarized controls (p < 0.01). Subsequent analysis using paired student’s t-test revealed differences between 0-150 nm and 200-500 nm (p < 0.05). Fig. 2B. SVs associated with the active zone membrane were counted as docked vesicles and presented as docked vesicles per active zone per synaptosomal section (*p < 0.05). For each experimental condition, distribution and docking of SVs in 25 synaptosomal sections were determined. Data represent means ± SEM of 3-6 independent experiments. Fig. 2C. Kinetics of Ca²⁺-dependent release of endogenous glutamate, GABA and CCK from rat cortex synaptosomes. Both total (40 mM K⁺ + 2 mM Ca²⁺) and Ca²⁺-independent (40 mM K⁺ + 50 μM EGTA) release was measured after indicated depolarization times and after 5 min preincubation with L-trans-PDC (50 μM) and SK&F 89976-A (10 μM). Ca²⁺-dependent release was calculated by subtracting Ca²⁺-independent release from the total release. The Ca²⁺-independent levels were 5.50 ± 0.50 nmol glutamate/mg protein, 1.20 ± 0.09 nmol GABA/mg protein and 0.180 ± 0.01 pmol CCK/mg protein, and did not change between 50 ms to 1 s depolarizations. Note the different axes shown for the three transmitters. Data represent means ± SEM of 6 independent experiments.
sections 41.9% of all the SVs were located within 150 nm distance from the active zone. This amount was significantly increased when synaptosomes were shortly depolarized (58% after 100 ms, 54.9% after 1 s, p < 0.05). In contrast, at a distance of 200-500 nm away from the active zone a decrease of the number of SVs was observed after depolarization, from 45.2% in control sections to 33.5% after depolarization (both at 100 ms and 1 s, p < 0.05). The depolarization-induced shifts in intraterminal SV distribution were not accompanied by changes in their total amount (see Table1). This redistribution of the SVs that takes place towards the active zone, clearly indicates the recruitment of SVs to their release site. This depolarization-induced recruitment of SVs was critically Ca\(^{2+}\)-dependent, since no changes were observed when Ca\(^{2+}\) was replaced by 50 μM EGTA (Fig. 2A).

To study whether such a fast recruitment of SVs from the reserve pool leads to replenishment of the releasable pool, we examined depolarization induced changes in the amount of vesicles that are docked at the active zone. Docked SVs are defined as SVs in direct contact with the active zone membrane. In control sections 3.84 \pm 0.4 docked SVs/active zone per synaptosomal section were counted, which increased to 4.89 \pm 0.2 (p < 0.05) after 100 ms and to 5.02 \pm 0.2 (p < 0.05) after 1 s depolarization (Fig. 2B). This depolarization induced increase in docking of the SVs at the active zone did not occur in the absence of Ca\(^{2+}\) (Fig. 2B).

We correlated these ultrastructural findings on fast SV mobilization with neurotransmitter release from the SVs. K\(^+\)-induced depolarizations of different durations (50 ms -1 s) showed that the kinetics of the Ca\(^{2+}\)-dependent release of the SV transmitters glutamate and GABA (Fig. 2C) are in close agreement with the fast mobility and increased docking of SVs to the active zone in these terminals. Within 50 ms substantial amounts of both amino acids were released, reaching a plateau within 1 sec. Apparently, upon sub-second stimulation not only release is activated but there is also a very rapid replenishment by recruitment and docking of SVs from the reserve pool.

For quantification of the number of LDCVs in synaptic terminals, 10 electron micrographs containing 30 to 40 sections of synaptosomes were randomly selected for each experimental condition (Fig. 3A). The number of synaptosomal sections with LDCVs were counted. Per terminal, regularly only one LDCV was detected, but occasionally two or three were seen, which were randomly distributed in the cytosol
Fig. 3A. Representative electron micrograph, showing about 35 synaptosomal sections used for LDCV analysis. Some synaptosomal sections contain an active zone (arrow heads). Arrows indicate LDCVs. Scale bar: 0.2 μm. Fig. 3B. Fraction of synaptosomal sections that did contain LDCVs before (0 ms) and after (100 or 1000 ms) K⁺-depolarization in the presence of 2 mM Ca²⁺, and after 1000 ms K⁺-depolarization in the presence of 50 μM EGTA. For each condition the number of LDCVs on 10 electron micrographs, i.e. in 350 synaptosomal sections, were counted. Data represent means ± SEM of 4-7 independent experiments. * p < 0.05 (tested with paired student's t-test).
Mobilization and fusion of small and large vesicles

and most of them in sections where no active zone was visible (Verhage et al., 1991b). In contrast to SVs, we never observed a LDCV docked at the plasma membrane, neither in controls nor after depolarization. The fraction of synaptosomal sections containing LDCVs (9%) did not differ from control when synaptosomes were depolarized for 100 ms (Fig. 3B). However, after 1 s depolarization the fraction of synaptosomal sections containing LDCVs was reduced to 4.5% (Fig. 3B, p < 0.001). This reduction in LDCV content was observed in the presence of 2 mM Ca\textsuperscript{2+}, and did not occur when 50 μM EGTA was present.

LDCV neurotransmitter release was studied by measuring release of the neuropeptide cholecystokinin (CCK), abundantly present in rat cortex in GABAergic interneurons (Hendry et al., 1984; Raiteri et al., 1993). As shown in Fig. 2C, no Ca\textsuperscript{2+}-dependent release of endogenous CCK could be detected until 250 ms depolarization, at which time it reached 0.08 ± 0.02 pmol/mg protein (p < 0.05) and gradually increased upon longer depolarizations. Both ultrastructural and biochemical studies indicate that the mobility and release kinetics from LDCVs are much slower than that of the SVs.

Both SV and LDCV mobilization was critically dependent on Ca\textsuperscript{2+}-influx (Figs. 2A, 3B). Since neurotransmitter release is regulated by Ca\textsuperscript{2+} entry through multiple high-voltage activated Ca\textsuperscript{2+}-channels (Wheeler et al., 1994; Dunlap et al., 1995; Randall and Tsien, 1995), we determined whether neurotransmitter release and mobilization of SVs and LDCVs were regulated by different Ca\textsuperscript{2+}-channel type(s), by using ω-Agatoxin IVA and ω-Conotoxin MVIIIC, to block the P- and Q-type Ca\textsuperscript{2+}-channels, and ω-Conotoxin GVIA, to block the N-type Ca\textsuperscript{2+}-channel. In order to activate all possible Ca\textsuperscript{2+}-channels involved, K\textsuperscript{+}-induced depolarization lasting 500 ms was applied, after preincubation for 5 min at 37°C with the different toxins. ω-Agatoxin IVA (Fig. 4A) inhibited release of all three transmitters, although amino acid release was inhibited with a tenfold higher affinity (IC\textsubscript{50} ± 10 nM) than CCK release (IC\textsubscript{50} ± 100 nM). This high affinity and complete inhibition of amino acid release by ω-Agatoxin IVA suggest regulation of neurotransmitter release from SVs by primarily P-type Ca\textsuperscript{2+}-channels, whereas the incomplete inhibition of CCK release with a lower affinity could indicate regulation of neurotransmitter release from LDCVs by mainly Q-type Ca\textsuperscript{2+}-channels (Sathe et al., 1993; Wheeler et al., 1994). Indeed, ω-Conotoxin MVIIIC which has been reported not to discriminate between P- and Q-type Ca\textsuperscript{2+}-channels (Wheeler et
Fig. 4. Regulation of exocytotic amino acid and CCK release by Ca$^{2+}$-channels.

Dose dependency of $\omega$-Agatoxin IVA (A), $\omega$-Conotoxin MVIIC (B) and $\omega$-Conotoxin GVIA (C) on the exocytotic release induced by a 500 msec depolarization. Control release was: glutamate: 1.3 ± 0.1 nmol/mg protein; GABA: 0.64 ± 0.05 nmol/mg protein and CCK: 219.6 ± 26 fmol/mg protein. The Ca$^{2+}$-dependent release in the presence of the blockers is given as a percentage of these control levels. Data represent means ± SEM of 4-8 independent experiments.

al., 1994), inhibited release of all transmitters with equal affinity (IC$_{50}$ ± 500 nM, Fig. 4B) causing maximally 80% inhibition of amino acid release and 100% inhibition of CCK release at 10 µM. In contrast, the N-type Ca$^{2+}$-channel blocker $\omega$-Conotoxin GVIA apparently affected only the neurotransmitter release from SVs (maximally 80% inhibition of glutamate release and 30% for GABA) and not the neurotransmitter release from LDCVs (Fig. 4C). Involvement of Ca$^{2+}$-channels in mobilization of the vesicles was determined by application of a 1 s depolarization, after preincubation for 5 min at 37°C with the different toxins at saturating concentrations. When the P- and Q- type Ca$^{2+}$-channels were blocked by $\omega$-Agatoxin IVA or $\omega$-Conotoxin MVIIC, both the redistribution of the SVs (Fig. 5A) and the decrease in LDCVs (Fig. 5B) after depolarization were prevented. Similar to the effects on neurotransmitter release, $\omega$-Conotoxin GVIA only blocked the redistribution of the SVs, but did not affect the decrease in synaptosomal LDCV content (Fig. 5A+B). All three toxins prevented the increase in the amount of docked SVs at the active zone as well, which was 5.02 ± 0.2 after a 1s depolarization with 2 mM Ca$^{2+}$ (Fig. 2B) and 3.1 ± 0.4 in the presence of one of the toxins (n = 4). The L-type Ca$^{2+}$-channel blockers nimodipine and nifedipine (100 nM) did neither affect mobility of SVs and LDCVs, nor affected release of any of the transmitters investigated (data not shown).
Mobilization and fusion of small and large vesicles

Fig. 5. Depolarization induced redistribution of SVs and decrease in LDCV amount is Ca\(^{2+}\)-channel dependent.

A 1 s depolarization was applied after 5 min preincubation of the synaptosomes with the distinct toxins at saturating concentrations (200 nM \(\omega\)-Agatoxin IVA and 1 \(\mu\)M for both \(\omega\)-Conotoxins). **Fig. 5A.** Distribution of SVs in the synaptic terminal as function of distance to the active zone. The redistribution of the SVs is blocked by all three toxins and by 50 \(\mu\)M EGTA (p < 0.05). **Fig. 5B.** Fraction of sections with LDCVs after various treatments. Depolarization in the presence of 2 mM Ca\(^{2+}\) decreased the number of LDCVs. This decrease in LDCVs is abolished when Ca\(^{2+}\) is replaced by 50 \(\mu\)M EGTA or when \(\omega\)-Agatoxin IVA or \(\omega\)-Conotoxin MVII C are present (p < 0.05), but not with \(\omega\)-Conotoxin GVIA. Data represent means \(\pm\) SEM of 3-6 independent experiments.

The effects of the separate Ca\(^{2+}\)-channel toxins on vesicle distribution and neurotransmitter release exceed 100\%, which may be explained by cooperative regulation of these processes by multiple Ca\(^{2+}\)-channels (Dunlap et al., 1995; Mintz et al., 1995). We tested the interaction between Ca\(^{2+}\)-channels in regulation of release of amino acids and CCK by investigating additivity of the toxin effects. If the Ca\(^{2+}\)-channel subtypes drive release of separate vesicle pools, the combined application of toxins should result in an additive inhibition of the release. If, however, these subtypes interact to regulate release of vesicles from a common pool, combined application of two toxins should result in a non-additive inhibition. For these studies maximal inhibitory concentrations of the toxins were applied for both \(\omega\)-Conotoxins (10 nM GVIA and 1 \(\mu\)M MVII C, Fig.4). We applied a concentration of 10 nM (=IC\(_{50}\)) of \(\omega\)-Agatoxin IVA, since the maximal inhibitory concentration exerted a complete block of amino acid release (Fig. 4A) and to block primarily the P-type and hardly the Q-type Ca\(^{2+}\)-channels (Wheeler et al., 1994). Combined application of \(\omega\)-Conotoxin GVIA with either \(\omega\)-Agatoxin IVA or \(\omega\)-Conotoxin MVII C did not inhibit
additively the release of the amino acids (Figs. 6A+B). Only the combination of ω-Conotoxin MVIIIC with ω-Agatoxin IVA blocked additively the release of both amino acid transmitters (Fig. 6C), probably reflecting the overlapping blockade of both P- and Q-type Ca\(^{2+}\)-channels by both toxins. The combined toxin effects on CCK release were completely different from those observed on amino acid release. In contrast to the absence of a separate effect of ω-Conotoxin GVIA on CCK release (Fig. 4C), an additional inhibition of 30-40% was obtained when this toxin was applied in combination with either ω-Agatoxin IVA or ω-Conotoxin MVIIIC (Figs. 6A+B). The combined application of ω-Agatoxin IVA and ω-Conotoxin MVIIIC also caused a synergistic effect resulting in a complete block of CCK release (Fig. 6C). The synergistic effects of the toxins on CCK release indicate a more indirect regulation of LDCV release by different Ca\(^{2+}\)-channel types. The synergy probably reflects the cooperativity of different Ca\(^{2+}\)-channels in raising the global intracellular Ca\(^{2+}\) concentration above the threshold necessary for LDCV release at distant sites in the terminals.

![Fig. 6](image_url)  

**Fig. 6. Regulation of exocytotic amino acid and CCK release by multiple Ca\(^{2+}\)-channels.**

**Fig. 6A.** Combination of ω-Agatoxin IVA (10 nM) and ω-Conotoxin GVIA (10 nM).  
**Fig. 6B.** ω-Conotoxin MVIIIC (1 μM) and ω-Conotoxin GVIA (10 nM).  
**Fig. 6C.** ω-Agatoxin IVA (10 nM) and ω-Conotoxin MVIIIC (1 μM). The Ca\(^{2+}\)-dependent release in the presence of the blockers is given as a percentage of control levels (see fig. 3). Data represent means ± SEM of 4-8 independent experiments. Additivity (*) or synergism (**) (p < 0.05) was tested by paired student's t-test.
DISCUSSION

This study unravels the exocytotic part of the synaptic vesicle cycle into distinct steps. Clear differences in mobility and neurotransmitter release between SVs and LDCVs upon depolarization of central nerve terminals became apparent. Furthermore, exocytosis of both vesicle types was differentially regulated by multiple Ca\(^{2+}\)-channels. By combining ultrastructural and biochemical approaches we showed that depolarization not only induced neurotransmitter release from SVs but also their recruitment and docking. This is the first direct determination of activity-dependent recruitment and docking of SVs in central nerve terminals. So far, only a Ca\(^{2+}\)-dependent translocation of vesicles has been described in the *Drosophila* neuromuscular junction, although under resting conditions (Koenig et al., 1993).

Notwithstanding the heterogenous nature with regard to their transmitter content, the rather pure synaptosome preparation used enabled us to study mobilization of the more homogeneous populations of the two morphological distinct vesicle types in central nerve terminals upon synchronous depolarization. The synaptosomal section parameters (Table 1) and the vesicle distribution in a large reserve pool and a small docked pool at the active zone strongly indicate that the synaptosomes retained their functional vesicle distribution (Schikorski and Stevens, 1997). The rapid application of the fixative to the synaptosomes in suspension immediately after their stimulation should allow fast fixation of the terminals, as was shown with cultured hippocampal neurons where rapid perfusion with fixative abolished synaptic transmission after about one second (Rosenmund and Stevens, 1997). Such a delayed fixation could explain the similar effects of 100 ms and 1 s depolarization on SV mobilization observed in our study. We cannot exclude effects of the fixative on transmitter release as reported by Smith and Reese (Smith and Reese, 1980) or on certain steps in the vesicle cycle which may interfere with the vesicle distribution. However, the K\(^+\)-induced and Ca\(^{2+}\)-channel dependent changes in vesicle distribution suggest functional relevance of the ultrastructural observations. Gross depletion of vesicles by the fixative is rather unlikely since the number of vesicles counted in the synaptosomal sections is in proportion with the amount found in hippocampal boutons by Schikorski and Stevens (1997). Our observation, that besides neurotransmitter release also the recruitment and docking steps of the vesicle cycle
were activated by second depolarizations, implies that at the active zone the fused SVs are rapidly replaced. This would be a very efficient mechanism of these central nerve terminals to get ready for subsequent stimulations. The recruitment and docking are much faster than the 5-12 seconds required for refilling of the readily releasable pool as measured in a number of different preparations (Neher, 1998). Recruitment and docking are only two steps that contribute to refilling. After docking a priming step has been postulated to make the docked SVs competent for fusion (Südhof, 1995). The time required for this last step could explain the difference in time required for recruitment and docking that we observed and refilling measured by others. But another explanation may be that this faster refilling rate is induced by the strong depolarization, as was recently shown in the giant calyx of Held synapse where high frequency stimulation enhanced the refilling rate from seconds to milliseconds (Wang and Kaczmarek, 1998).

The biochemically measured neurotransmitter release could involve full fusion of SVs in the synaptosomes. Morphologically we observed, however, only occasionally fusion by omega-like structures in the active zone, but this could be an underestimation because fixation might be too slow to capture more of these structures. The fact that these short depolarizations did not change the total amount of SVs, whereas SV docking increased, indicates that per active zone only a limited amount of SVs release their transmitter content upon sub-second K⁺-depolarization.

To get more insight whether release from such a small amount of vesicles in each synaptosome would be consistent with our data, we compared the morphological properties of the synaptosomes with the biochemically measured release. Pellets containing 300 µg synaptosomal protein were fixed for electron microscopy, and the resulting blocks contained 1.5 mm³ of tissue. Taking an average synaptosomal diameter of 550 nm (Table I) and considering synaptosomes as spherical structures, we calculated a total amount of about 2.10¹⁰ synaptosomes in such a block. Assuming that about 2/3 of the population of synaptosomes in the cortex is glutamatergic containing one active zone, and a content of 5000 molecules of glutamate in one vesicle (Rusakov and Kullmann, 1998), we estimated a release of about 0.4 nmol glutamate/mg protein if only one vesicle would fuse upon depolarization. As shown in Fig. 2C we actually measured a release of 1.25 nmol glutamate/mg protein, which would be consistent with involvement of 3 vesicles. Although based on a few assumptions, our calculation does not support the
hypothesis postulated by Korn et al. (1982) that maximally one vesicle would fuse upon each stimulation. Instead, our data would be more consistent with the multi-vesicular release hypothesis suggested by Tong and Jahr (1994). Interestingly, our assumption indicates that only a fraction of the docked vesicles would be actually released during sub-second depolarization. Indeed, prolonged K\(^+\)-depolarization up to 3 min is required to deplete the total vesicular releasable pool of 5 nmol glutamate/mg protein (Verhage et al., 1991b), which would be in accordance with depletion of the total docked pool. Preliminary observations in our laboratory confirm such a decrease in the docked vesicular pool upon K\(^+\)-depolarization lasting tens of sec (unpublished observation). Alternatively, our observation that the total amount of vesicles in the synaptosomes did not change upon short depolarization could be explained by a fast "kiss and run" release mechanism where vesicles fuse only partially after which rapid endocytosis occurs (Fesce et al., 1994).

The results indicate that not only SV neurotransmitter release, but also their recruitment and docking is regulated by Ca\(^{2+}\)-channels, although these processes may be interrelated with each other. This finding is in agreement with recent studies showing that the refilling of SVs is strongly facilitated by Ca\(^{2+}\)-influx (Stevens and Wesseling, 1998; Wang and Kaczmarek, 1998). The Ca\(^{2+}\)-dependence of SV recruitment could reflect involvement of proteins activated by elevated [Ca\(^{2+}\)]\(_i\), such as synapsin I, which can be phosphorylated by CaM-kinase II (Pieribone et al., 1995; Rosahl et al., 1995) or C\(_2\)-domain containing Ca\(^{2+}\)-binding proteins such as rabphilin, Doc2 and synaptotagmin (Goda and Südhof, 1997).

The disappearance of co-localized LDCVs is much slower (sec), while they were distributed in central terminals distant from the active zone. This slow mobility and the delay in occurrence of CCK release indicate that LDCVs need at least 250 ms to translocate to the plasma membrane before release of their contents can occur, which would be in line with LDCV exocytosis from neurohypophysis terminals (Seward et al., 1995). However, melanotrophic and chromafinn cells showed exocytosis of LDCVs within a second (Chow et al., 1992; Thomas et al., 1993). This faster exocytosis most likely reflects the fusion of docked LDCVs in these cells (Parsons et al., 1995). In the central nerve terminals, we could not observe such a docked, readily releasable pool of LDCVs.
Both SV and LDCV mobility and neurotransmitter release depend on depolarization-induced Ca\(^{2+}\)-channel activation, but clear differences in this regulation by the different Ca\(^{2+}\)-channels between both vesicles types became apparent in this study. The SV exocytosis is regulated mainly by the P-type Ca\(^{2+}\)-channels, whereas LDCV exocytosis is primarily regulated by the Q-type Ca\(^{2+}\)-channels. The N-type Ca\(^{2+}\)-channels directly influence amino acid release only, with a larger inhibition of glutamate than of GABA release. Our data do not provide any indication for involvement of L-type Ca\(^{2+}\)-channels in LDCV release, unlike that found for LDCVs from most neuroendocrine cells (Dunlap et al., 1995). Although we used a heterogenous population of terminals with regard to transmitter content, the results of the experiments with combined application of the Ca\(^{2+}\)-channel toxins strongly suggest that on most terminals, if not all, diverse Ca\(^{2+}\)-channel subtypes are localized. The terminals used are, however, homogenous with regard to the small synaptic vesicles. The effects of the Ca\(^{2+}\)-channel toxins on recruitment and docking of these vesicles imply that multiple Ca\(^{2+}\)-channels are present on each terminal. A non-uniform distribution of the Ca\(^{2+}\)-channel subtypes on the central terminals (Reid et al., 1997; Reuter, 1995) could account for the differences in contribution of each Ca\(^{2+}\)-channel subtype to release of the different transmitters. Alternatively, there could be an uneven distribution of the different Ca\(^{2+}\)-channel subtypes on the nerve terminal, with the Q-type Ca\(^{2+}\)-channels preferentially localized outside the active zone, which may explain their large contribution to LDCV exocytosis, in contrast with P- and N-type Ca\(^{2+}\)-channels which are thought to be localized in the active zone (Reuter, 1996). In the active zone the joint control of SV neurotransmitter release by P- and to a lesser extent N- and Q-type Ca\(^{2+}\)-channels occurs probably by direct interactions with the synaptic core complex proteins (el Far et al., 1995; Sheng et al., 1994). Regulation of LDCV exocytosis by these Ca\(^{2+}\)-channels is clearly different and probably involves a more indirect mechanism. The effects of separate toxins imply that activation of the Q- and (to a lesser extent) P-type Ca\(^{2+}\)-channels is sufficient to raise Ca\(^{2+}\) above threshold for LDCV exocytosis, whereas N-type Ca\(^{2+}\)-channels alone can not provide enough Ca\(^{2+}\) to trigger their release. The finding that blockade of N-type Ca\(^{2+}\)-channels alone did not affect CCK release, whereas it did in the presence of P- or Q-type blockers, could be explained by cooperativity between the different Ca\(^{2+}\)-channels in elevation of global intracellular Ca\(^{2+}\) needed to trigger LDCV exocytosis. The low affinity of \(\omega\)-Conotoxin MVIIIC for N-type Ca\(^{2+}\)-channels
Mobilization and fusion of small and large vesicles

(Hillyard et al., 1992; Wheeler et al., 1994) and our finding of the cooperative regulation of CCK release by Ca\textsuperscript{2+}-channels sensitive to this toxin and ω-Conotoxin GVIA could explain the complete inhibition of CCK release by a high concentration of ω-Conotoxin MVIIC. The inability of ω-Conotoxin MVIIC to inhibit glutamate and GABA release completely can be explained by the observed non-additive regulation of SV neurotransmitter release by these Ca\textsuperscript{2+}-channel types. Thus, whereas SV neurotransmitter release is tightly coupled to colocalized P-/Q- and N-type Ca\textsuperscript{2+}-channels, LDCV neurotransmitter release depends on Ca\textsuperscript{2+}-influx irrespective of the Ca\textsuperscript{2+}-channel type(s) involved. This differential regulation of mobilization and neurotransmitter release of both vesicle types would be in accordance with the selective local recruitment of SVs at the active zone upon a single or a few action potentials, whereas LDCV release at distant sites in the terminal requires high intensity, repetitive, stimulation, as proposed previously (Bartfai et al., 1988; Verhage et al., 1991b).

ACKNOWLEDGMENTS

We like to thank Ank Frankhuijzen of the Rudolf Magnus Institute, Utrecht University for excellent technical assistance in the CCK analysis. A.G.M. L. is supported by grant 903-42-016 of the Netherlands Organization of Scientific Research.