Vesicle transport and neurotransmitter release in central nerve terminals
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Chapter 2

A biochemical approach to study sub-second endogenous release of diverse neurotransmitters from central nerve terminals

Endogenous neurotransmitter release at the sub-second timescale

ABSTRACT

Exocytosis in central nerve terminals is rapidly triggered by the influx of calcium through high voltage sensitive Ca\(^{2+}\)-channels. Mainly due to their small size, studies in which neurotransmitter release from these terminals was determined at the sub-second time-scale are still rather limited. Here we describe the use of a pneumatic rapid mixing device, allowing application of short (≥ 50 ms) K\(^+-\)depolarizing pulses to purified nerve terminals, synaptosomes, to trigger endogenous release of different transmitter types. A consistent, Ca\(^{2+}\)-dependent exocytotic release of the amino acid transmitters glutamate and GABA was observed after 100 ms depolarization, from synaptosomes purified from rat and mouse brain. In contrast, no Ca\(^{2+}\)-dependent release of aspartate could be observed upon such a short depolarization. For determination of amino acid release after longer depolarizations (> 100 ms), transporter blockers had to be added to prevent clearance of the vesicularly released transmitters. Ca\(^{2+}\)-dependent release of the neuropeptide cholecystokinin did not occur until 250 ms depolarization. In addition, the time-courses of amino acid and cholecystokinin release were clearly different. The fast Ca\(^{2+}\)-dependent release of all transmitters was selectively and strongly inhibited by the P/Q-type Ca\(^{2+}\)-channel blocker \(\omega\)-Agatoxin IVA. In conclusion, this approach allows direct measurement of Ca\(^{2+}\)-dependent release of diverse endogenous neurotransmitters from central nerve terminals upon depolarization pulses in the physiologically relevant, sub-second, timescale.
INTRODUCTION

When studying neurotransmitter release from central nerve terminals, one can discriminate between fast acting transmitters such as glutamate, GABA and acetylcholine and the slower acting catecholamines and neuropeptides. The fast acting transmitters are released from SVs, and neuropeptides from LDCVs (De Camilli and Jahn, 1990; Kelly, 1993). Release of transmitter from both vesicle types is Ca$^{2+}$-dependent, though their regulation is different with respect to kinetics and calcium sensitivity (Zhu et al., 1986; Verhage et al., 1991b).

Electrophysiological studies showed indirectly that exocytosis of the amino acids from central nerve terminals has to be a very rapid (< ms) process (Borst and Sakmann, 1996). In contrast, the release of neuropeptides has been proposed to be much slower (Burgoyne and Morgan, 1995). Indeed, capacitance measurements of the plasma membrane during exocytosis directly showed the difference in kinetics of exocytosis and endocytosis of the SVs from goldfish retina bipolar neurons (Mennerick and Matthews, 1996) and LDCVs from neurohypophysial nerve terminals (Seward et al., 1995). However, similar studies on the dynamics of transmitter release from the different vesicle types in small central nerve terminals on a physiological relevant, sub-second, timescale are technically not feasible yet. Instead, assays used to study neurotransmitter release from brain slices or isolated nerve terminals, synaptosomes, are generally performed on a second to minute timescale. Turner and Goldin (1989) developed a rapid superfusion system allowing measurement of release of radioactively prelabeled amino acids from synaptosomes with a 60 ms time resolution. Interestingly, these and other investigators have discriminated two different components when studying amino acid release from synaptosomes; a fast one completed within 1-2 s depolarization, and a slower one lasting for minutes (Herrero et al., 1996; McMahon and Nicholls, 1991; Turner and Goldin, 1989). However, direct measurement of the fast endogenous release component at the millisecond timescale has not yet been reported.

In the present study, we describe a novel method enabling direct measurement of fast exocytosis of diverse endogenous transmitters from isolated central nerve terminals, by use of a rapid mixing device (Fig. 1). In this device small aliquots of high K$^+$-medium are rapidly added from a syringe driven by a pneumatic dispenser to and
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mixed with synaptosomes, and after a controlled delay ranging between 0-1000 ms stopmedium containing low K⁺ and without Ca²⁺ is added from a separate syringe to terminate the depolarization. By combining the use of this rapid mixing device with sensitive HPLC amino acid analysis, a time resolution of 50 ms was achieved to measure K⁺-induced, Ca²⁺-dependent release of glutamate and GABA. In addition, sub-second release of the neuropeptide CCK could be measured. The sub-second vesicular release of all these transmitters was effectively regulated by P/Q-type Ca²⁺-channels, since release was strongly inhibited by their blocker α-Agatoxin IVA.

MATERIALS and METHODS

Synaptosomal preparation
Synaptosomes were prepared from male Wistar rat (180-220 gr) cerebrocortex or from whole forebrain of C57BL6 mice (4 to 5 months old), rapidly dissected on ice after decapitation, and highly purified by Percoll (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation as described by Dunkley et al. (1988). This procedure was approved by the Animal Experiments Committee, faculty of Biology, University of Amsterdam (1996).

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 (2mg/ml) were kept on ice in aCSF + 2 mM CaCl₂ 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 when prepared from rat cortex, 40 μg protein when prepared from mouse forebrain) were pelleted and resuspended in 40 μl (rat) or 20 μl (mouse) aCSF supplemented with 50 μM EGTA and 1 mg/ml Bacitracine, and were preincubated in a tube positioned in a holder at 37°C for 5 min. When release kinetics and Ca²⁺-channel involvement were determined, the transporter inhibitors L-
trans-PD C (50 μM) (Tocris, Bristol, UK) and SK&F89976-A (10 μM), kindly provided by dr. Skidmore (Smith, Kline and French, Welwyn, UK), or 200 nM ω-Agatoxin IVA (Alomone labs, Jerusalem, Israel), were added during this preincubation period. Subsequently synaptosomes were shortly (0-1000 ms) depolarized by use of a rapid mixing device. This mixer device (Fig. 1) consists of two syringes with a volume of 5 (syringe 1) and 40 ml (syringe 2), equipped with outlets of stainless steel (inner diameter 1 mm (syringe 1); 1.5 mm (syringe 2)), and each controlled by a pneumatic dispenser (type 1000-XLE, EFD Inc., East Providence RI, USA) which are connected to an air pressure outlet. The compartments for the synaptosomes and syringe 1 are kept at 37°C by thermocouples (Watlow, Winona MN, USA). On top of both syringes, storage reservoirs are present in order to supply media to the syringes. During depolarization of the synaptosomes and addition of stop solution, the fluid in these storage reservoirs is separated from both syringes by closure of valves. By a slight negative pressure in both syringes of 0.08 bar, spontaneous release of their contents was avoided. By a pressure pulse (0.35 bar) 200 μl of high K⁺ medium.

Fig. 1. Schematic drawing of the rapid mixing device.

The mixer device consists of two syringes (1, 2), each controlled by a pneumatic dispenser (3). By a pressure pulse (0.35 bar) depolarization medium is released from syringe 1, depolarizing the synaptosomes. And after a preset time (0 - 1000 ms), controlled by a digital timer (4), ice-cold stop medium is released from syringe 2, which terminates the depolarization. The compartment (5) containing the synaptosomes and syringe 1 are thermo-stated at 37°C and syringe 2 is kept at 4°C by ice.
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Endogenous neurotransmitter release at the sub-second timescale (depolarization medium, see below) is rapidly released from syringe 1 to stimulate the synaptosomes. After a preset delay ranging from 0 - 1000 ms, controlled by a digital timer (home made), syringe 2 released (pressure pulse 1.8 bar) 1 ml ice-cold stop medium to terminate the depolarization. For that purpose, the compartment of syringe 2 is kept at 0-4°C by ice. In a test experiment, the delay between the injections from syringe 1 and 2 was controlled by a laser beam positioned just above the tube with synaptosomes. The laser was coupled to an oscilloscope (LeCroy Type 9410) to visualize the delay. During addition of the depolarization and stop solutions synaptosomes were gently shaken by a vortex-like device (home made). Immediately after addition of the stop solution, the synaptosomes were placed on ice and separated from the extracellular medium by centrifugation as described below.

Depolarization media: aCSF containing 50 mM KCl in the presence of 2 mM Ca$^{2+}$ (for total release) or 50 μM EGTA (for the Ca$^{2+}$-independent release), NaCl was iso-osmotically replaced by KCl. Stop medium: aCSF containing 10 mM EGTA.

Transmitter analysis
Quickly after terminating release, 700 μl of the ice-cold synaptosomal suspension was centrifuged through 300 μl of 45:55% (vol.:vol.) mixture of silicone oil and dinonylphthalate for 2 min in a BHG table centrifuge at 15000g in order to analyse released transmitters in the extracellular medium. From the supernatant a 100 μl aliquot was pipetted onto 10 μl ice-cold Homoserine (1μM)/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 analysis: Extracellular amino acid levels (glutamate, aspartate and GABA) were determined by reversed phase HPLC after precolumn derivatization with o-phtaldehyde in mercapto-ethanol, using Homoserine as an internal standard (Verhage et al., 1989). The detection limits for glutamate and GABA were 10 and 20 fmol, respectively.

CCK analysis: Nonsulfated CCK-8 (CCK) release was quantified by radioimmunoassay as described (Breukel et al., 1998) using the rabbit antiserum C221. The detection limit for CCK-8 was 1.75 fmol. In this radioimmunoassay, sulfated CCK-8 and CCK-6 display a 100% cross-reactivity, and sulfated CCK-7, CCK-4 and CCK-3 do not cross-react (< 0.07%). In short, freeze-dried pellets are reconstituted in buffer (20 mM sodium barbitone, 20 mM sodium azide and 2.5 mg/ml
BSA; pH 8.2) and incubated overnight with antiserum (1:6000 dilution) at 4°C. Nonsulfated CCK-8 is used as standard, \(^{125}\)I CCK-8 as tracer.

**Statistical analysis**

The data were statistically analyzed by the paired Student's \(t\)-test. The rejection of the null hypothesis was accepted as significant if \(P<0.05\).

**RESULTS**

*The rapid mixing device*

In Fig. 1 a schematic drawing of the rapid mixing device is shown. Application of a positive pressure pulse allowed instantaneous addition of aliquots of depolarizing high \(K^+\)-medium (200 \(\mu\)l) from syringe 1 to a small amount (20-40 \(\mu\)l) of synaptosomes. After a preset delay time, adjustable in steps of 10 ms, syringe 2 was pulsed to add excess (1 ml) stop-solution to the synaptosomes in order to terminate the depolarization reaction. In a test experiment, the delay between the injections from syringe 1 and 2 was controlled by means of a laser, and visualized on an oscilloscope. When adjusting a delay of 100 msec, injection of the stopsolution started between 104 and 110 msec after the start of injection from syringe 1 (n=4), indicating variations in reaction time of maximally 10%. Thermostratation of the depolarization at 37°C allowed determination of transmitter release at physiologic relevant temperature, whereas termination with ice-cold stop-solution and immediate placement on ice inhibited biological activity strongly after the depolarization.

*Fast \(K^+\)-induced amino acid release from rat and mice synaptosomes*

100 ms depolarizations were applied to rat cortex synaptosomes. Depolarizations were induced by 40 mM \(K^+\), either without or with 2 mM \(Ca^{2+}\) to measure \(Ca^{2+}\)-independent and total release, respectively. The \(Ca^{2+}\)-dependent, vesicular, release is then calculated by substracting the \(Ca^{2+}\)-independent from the total release. After 5 min preincubation at 37°C and subsequent 100 ms depolarization, the accumulative \(Ca^{2+}\)-independent release was 3.6 ± 0.4 nmol/mg protein for glutamate and 1.2 ± 0.1 nmol/mg protein for GABA (Fig. 2 A). On top of this \(Ca^{2+}\)-independent release a
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A. rat cortex synaptosomes
a. Glutamate
b. GABA
c. Ca-dependent release

B. mice forebrain synaptosomes
a. Glutamate
b. GABA
c. Ca-dependent release

Fig. 2. 100 ms K⁺-induced release of glutamate and GABA from rat and mice synaptosomes.

100 ms K⁺-induced glutamate (a) and GABA (b) release was determined from rat cortex (A) and mice forebrain (B) synaptosomes. K⁺ (40mM) stimulations were applied either in the presence of 50 μM EGTA (white bars) for Ca²⁺-independent release or in the presence of 2 mM Ca²⁺ (black bars, a,b) for total release. Ca²⁺-dependent release (c) was calculated by subtracting Ca²⁺-independent release from total release. Data are means ± SEM of 9 independent experiments.

Consistent Ca²⁺-dependent release was evoked by depolarization of 1.06 ± 0.30 nmol/mg protein for glutamate and 0.37 ± 0.10 nmol/mg protein for GABA (n= 9, p < 0.05). In synaptosomes, purified from mice forebrain similar values of Ca²⁺-dependent release of glutamate and GABA were observed upon 100 ms depolarization as found with rat synaptosomes (Fig. 2 B). The Ca²⁺-independent glutamate release in mice synaptosomes was somewhat lower than in rat synaptosomes. These results indicate that the rapid mixing device was appropriate to study sub-second amino acid release from synaptosomes purified from both rat and mouse brain. All other experiments described in this study were performed with rat cortex synaptosomes.
Kinetics of amino acid release

In order to study kinetics of amino acid release, synaptosomes were exposed to high K⁺-medium for increasing periods. However, when depolarization time was extended from 100 ms to 500 ms, this did not result in an increase of the Ca²⁺-dependent release of both glutamate (Fig. 3 A) and GABA (Fig. 3 B). Since prolonged exposure of synaptosomes to their released amino acids could lead to progressive reuptake by their respective transporters, we measured the effects of the specific transporter blockers L-trans-PDC (50 µM) for the glutamate/aspartate transporter (Bridges et al., 1991) and SK&F89976-A (10 µM) for the GABA transporter (Yunger et al., 1984) on amino acid release upon 100 and 500 ms depolarization. At these concentrations uptake of the amino acids was completely blocked in rat brain synaptosomes (Breukel et al., 1997b). In the presence of the blockers, the Ca²⁺-dependent release component at 100 ms for both glutamate and GABA was the same as that measured without blockers (Fig. 3). However, after 500 ms depolarization Ca²⁺-dependent glutamate release was almost doubled to 1.92 ± 0.30 nmol/mg protein (Fig. 3 A, p < 0.05), and Ca²⁺-dependent GABA release was increased to 0.95 ± 0.20 nmol/mg protein (Fig. 3 B, p < 0.05). Simultaneously, the blockers raised the Ca²⁺-independent release of both transmitters, glutamate from 3.6 to 6.3 nmol/mg protein and GABA.

Fig. 3. Sub-second release of glutamate and GABA: effect of transporter blockers.

Synaptosomes were preincubated in absence or presence of 50 µM L-trans-PDC and 10 µM SK&F89976-A for 5 min at 37°C. K⁺-evoked glutamate (A) and GABA (B) release was determined after 100 and 500 ms, either in the presence of 50 µM EGTA (white) for Ca²⁺-independent release or in the presence of 2 mM Ca²⁺ (total (white + black) bar) for total release. Ca²⁺-dependent release was calculated by subtracting Ca²⁺-independent release from total release and is indicated by the black part of the bar. Data are means ± SEM of 9 independent experiments. * p < 0.05 by paired student’s t-test.
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A. Aspartate

![Graph A](image)

B. Ca-dependent aspartate release

![Graph B](image)

**Fig. 4. Sub-second aspartate release.**

A. K⁺-evoked Ca²⁺-independent (white bars) and total (black bars) aspartate release was determined after 100 ms and 500 ms either in the presence or absence of 50µM L-trans-PDC. B. Ca²⁺-dependent aspartate release, calculated by subtracting Ca²⁺-independent release from total release, determined after 100 ms, 500 ms and 15 s either in the presence (white bars) or absence (black bars) of 50 µM L-trans-PDC. Data are means ± SEM of 9 (100 ms and 500 ms) or 5 (15 s) independent experiments. * p < 0.05 by paired student's t-test.

from 1.1 to 1.65 nmol/mg protein. Since these results indicated partial clearance of vesicular glutamate and GABA release by activation of their transporters, kinetic studies were performed in the presence of both blockers.

With respect to the putative transmitter aspartate, no Ca²⁺-dependent release could be observed after 100 ms and 500 ms depolarizations of the synaptosomes (Fig. 4). Preincubation with L-trans-PDC (50 µM) increased the Ca²⁺-independent release of aspartate from 3.19 ± 0.30 to 5.75 ± 0.60 nmol/mg protein, but still no Ca²⁺-dependent release was seen (Fig. 4 A). Ca²⁺-dependent aspartate release (0.76 ± 0.30 nmol/mg protein, p < 0.05) could not be detected until a depolarization lasting 15 s was applied. This Ca²⁺-dependent component was however completely blocked by 50 µM L-trans-PDC (Fig. 4 B).

To measure the kinetics of Ca²⁺-dependent amino acid release, K⁺-induced depolarizations of different durations (50 ms-3 min) were applied in the presence of both 50 µM L-trans-PDC and 10 µM SK&F89976-A. Though Ca²⁺-independent release of glutamate was slightly increased upon sub-second depolarization, already at the lower threshold of the mixing device, i.e. 50 ms depolarization, a significantly higher total release of glutamate was observed, resulting in a Ca²⁺-dependent release of glutamate of 0.7 ± 0.3 nmol/mg protein (p < 0.05, Fig. 5 B). Similarly, a
Fig. 5. Time-course of K⁺-induced release of the amino acids glutamate and GABA.

Fig. 6. Time-course of K⁺-induced release of the neuropeptide CCK.
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Fig. 5. Time-course of K⁺-induced release of the amino acids glutamate and GABA.
40 mM K⁺-induced release was determined after 5 min preincubation with 50 μM L-trans-PDC and 10 μM SK&F89976-A. Total release (2 mM Ca²⁺ (•)) and Ca²⁺-independent release (50 μM EGTA, (O)) of glutamate (A), GABA (C), is plotted against the time (s) of depolarization, on a logarithmic scale. Ca²⁺-dependent release was calculated by subtracting Ca²⁺-independent release from total release and is shown in B (glutamate) and D (GABA). Data are means ± SEM of 6 independent experiments.

Fig. 6. Time-course of K⁺-induced release of the neuropeptide CCK.
40 mM K⁺-induced release was determined after 5 min preincubation. Total release (2 mM Ca²⁺ (•)) and Ca²⁺-independent release (50 μM EGTA, (O)) of CCK (A), is plotted against the time (s) of depolarization, on a logarithmic scale. B. Ca²⁺-dependent release was calculated by subtracting Ca²⁺-independent release from total release. Data are means ± SEM of 6 independent experiments.

Ca²⁺-dependent release of GABA being 0.28 ± 0.05 nmol/mg protein, p < 0.05, Fig. 5 D) was estimated after 50 ms depolarization. For both glutamate and GABA at least two components could be discriminated in the time-course of Ca²⁺-dependent release. A rapid component reaching a plateau of about 1.3 nmol/mg protein after 250 ms depolarization for glutamate (Fig 5B), and of about 0.3 nmol/mg protein between 50 and 250 ms depolarization for GABA (Fig. 5D). After prolonged (> 1 s) depolarization a second phase of Ca²⁺-dependent glutamate and GABA release were observed increasing to 4.3 ± 0.9 and 2.5 ± 0.3 nmol/mg protein, respectively, after 3 min depolarization.

Kinetics of CCK release
In order to determine whether release of LDCV neuropeptide transmitters from central nerve terminals did occur at the sub-second time scale, the kinetics of CCK release was also measured. Release of this abundant brain neuropeptide was measured simultaneously with that of the amino acids, i.e. from the same sample of synaptosomes. Although a Ca²⁺-independent CCK release of ± 0.25 pmol/mg protein from synaptosomes was detectable, which did not increase in time by K⁺-depolarization, Ca²⁺-dependent release of CCK was detected not until 250 ms depolarization, being 0.08 ± 0.02 pmol/mg protein (Fig. 6, p < 0.05). Ca²⁺-dependent CCK release increased thereafter monophasically to 0.89 ± 0.32 pmol/mg protein at 3 min.
Ca\textsuperscript{2+}-channel regulation of sub-second transmitter release

Ca\textsuperscript{2+}-dependent release is thought to represent vesicular release triggered by Ca\textsuperscript{2+}-influx through high voltage activated Ca\textsuperscript{2+}-channels. Since presynaptic P/Q-type Ca\textsuperscript{2+}-channels have been prominently associated with transmitter release, we used ω-Agatoxin IVA, a blocker of these Ca\textsuperscript{2+}-channels, to evaluate their regulation of fast Ca\textsuperscript{2+}-dependent transmitter release. After 5 min of preincubation with 200 nM ω-Agatoxin IVA, the Ca\textsuperscript{2+}-dependent release of both glutamate and GABA induced by 500 ms depolarization was completely blocked. In addition, also CCK release was strongly inhibited, by 80 ± 5 % (Fig. 7). No effect of 200 nM ω-Agatoxin IVA on the K\textsuperscript{+}-induced Ca\textsuperscript{2+}-independent release of the amino acids and CCK was observed (not shown).

DISCUSSION

In this study we presented a rapid mixing device, enabling simultaneous measurement of endogenous release of amino acid and neuropeptide transmitters from presynaptic nerve terminals upon depolarization at the physiologically relevant sub-second timescale.

Fig. 7. Inhibition of sub-second Ca\textsuperscript{2+}-dependent release by ω-Agatoxin IVA.
Synaptosomes were preincubated for 5 min at 37°C in the presence or absence of 200 nM ω-Agatoxin IVA. Release was measured after a 500 ms K\textsuperscript{+}-induced depolarization. Ca\textsuperscript{2+}-dependent release of glutamate, GABA and CCK is shown for controls (black bars) and ω-Agatoxin IVA preincubated (white bars) synaptosomes. Data are means ± SEM, of 8 (glutamate + GABA) and 6 (CCK) independent experiments. * p < 0.05 by paired student's t-test.
By adapting pneumatic dispensers equipped for fast reproducible sampling of small droplets, we designed a technique to add instantaneously μl volume amounts of depolarizing solution to a suspension of purified nerve terminals. An adjustable time-delay between two interconnected dispensers coupled to syringes filled with stimulation and stop medium, respectively, allowed measurement of transmitter release upon depolarization as fast as 50 ms. The sub-second depolarization times applied in our experiments probably reflect real release times. The finding of Turner and Dunlap (1995) that amino acid release is prolonged after termination of rapid depolarization is not applicable under our conditions since depolarization was terminated by addition of ice-cold EGTA-medium, making release by either transporter reversal or exocytosis rather unlikely.

The difference in initial time-course between Ca\textsuperscript{2+}-dependent amino acid and CCK release indicate the differential regulation of exocytosis of the transmitter types. The applicability of this fast release assay for mouse synaptosomes as well creates the interesting possibility to study the consequence of genetically deletion of certain presynaptic proteins on initial burst of transmitter release.

Two components of amino acid release

Although actual triggering of presynaptic amino acid release occurs within one ms, a time resolution of 50 ms reasonably approaches isolated measurement of the synchronous, readily releasable transmitter pool as defined by Goda and Stevens (1994). According to this definition, the asynchronous phase of release would be consistent with the additional, slow release component of amino acids upon depolarization for s - min. Interestingly, two release components have been discriminated in biochemical studies before, although a detailed analysis of endogenous release within the first s of depolarization was not performed (Herrero et al., 1996; McMahon and Nicholls, 1991; Turner and Goldin, 1989). A rapid perfusion system for studying amino acid release with a similar time-resolution as obtained in our study has been applied, but concerned measurement of a radioactively prelabelled transmitter pool (Turner et al., 1993; Turner and Goldin, 1989). However, after short preincubation with radioactive amino acids the vesicular pool is only partially labelled, giving rise to rather undefined release components (Wilkinson and Nicholls, 1989). By estimating the sub-second endogenous Ca\textsuperscript{2+}-dependent amino acid release, probably the complete readily releasable vesicular pool is released.
an ultrastructural study, this vesicle pool was suggested to be equivalent with the vesicles docked at their release site in nerve terminals, the active zone (Schikorski and Stevens, 1997). Indeed, the amount of docked vesicles at the active zone in synaptosomes was changed upon sub-second depolarization, though a small increase instead of a decrease was observed (Leenders et al., 1999). Evidently, besides depolarization-induced fusion of vesicles a simultaneous fast recruitment of new vesicles from a reserve pool did occur to replenish the readily releasable pool, since this increase was accompanied by a similar decrease in vesicle amount distantly from the active zone (Leenders et al., 1999).

After the initial bursts of glutamate and GABA release, which reached a plateau between 50 ms and 250 ms, a second progressively increasing release component was observed up to 3 min depolarization (see Fig. 5). This slower component probably represented additional fusion of vesicles steadily recruited from the reserve pool, and the cumulative amount of endogenous amino acid release during this period closely resembled values reported previously by our and other laboratories estimated by other procedures (Herrero et al., 1996; McMahon and Nicholls, 1991; Verhage et al., 1991b). This slow release component accounted for 80% or more of the totally released amount of amino acids, thereby quantitatively masking the initial burst. The rapid mixing device used in the present study allowed selective measurement of the initial burst of release from a population of vesicles at or very near to their release site, enabling functional studies of certain exocytotic steps of the vesicle cycle.

Release and transport of amino acids

A potential draw-back in our rapid mixing device is the fact that during depolarization the synaptosomes were exposed to increased amounts of released amino acids, allowing progressive reuptake by their respective transporters which would lead to underestimation of the actual vesicular release. In addition, preincubation and K⁺-induced reversal of amino acid transporters contributed to a relatively high background level of extracellularly accumulated amino acids (Nicholls and Attwell, 1990). We cannot fully exclude pressure effects on Ca²⁺-independent (unspecific) transmitter release, though control experiments did not show effects of the rapid high pressure application on leakage of the cytosolic enzyme lactate dehydrogenase out of the synaptosomes (results not shown). The Ca²⁺-independent component
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amounted to about 70% of the total release after 100 ms depolarization, and increased even further in the presence of transporter blockers. Apparently, during 100 ms K\(^+\)-pulses hardly any clearance of released amino acids by their transporters did occur, since Ca\(^{2+}\)-dependent release of glutamate and GABA was not affected by the blockers. However, after 500 ms depolarization the transporters evidently recycle about half of the vesicularly released amino acids. Therefore, the presence of blockers was required during extended depolarization times. 100 ms bursts of transmitter release could be appropriately measured in the absence of the blockers, thereby avoiding excessive increases in background levels as mentioned above. However, the small but significant Ca\(^{2+}\)-dependent component of sub-second amino acid transmitter release in the presence of the blockers is probably from vesicular origin, as suggested by the following observations: 1) Ca\(^{2+}\)-dependent release was significant during K\(^+\)-depolarization only. 2) No K\(^+\)-induced Ca\(^{2+}\)-dependent release of the non-vesicular aspartate could be observed within the first second. 3) The Ca\(^{2+}\)-dependent release of glutamate and GABA was inhibited by the P-, Q-type Ca\(^{2+}\)-channel blocker ω-Agatoxin IVA, whereas the Ca\(^{2+}\)-independent release was unaffected. Similarly, the Ca\(^{2+}\)-dependent CCK release was strongly inhibited by this blocker, in full agreement with the prominent role ascribed to these channels in regulating transmitter release in central nerve terminals (Dunlap et al., 1995). In addition, N-type Ca\(^{2+}\)-channels have been described to regulate transmitter release (Dunlap et al., 1995). Recently, we have reported cooperative regulation of sub-second transmitter release by multiple Ca\(^{2+}\)-channels as well (Leenders et al., 1999). Although in many studies, either with slices or synaptosomes, Ca\(^{2+}\)-dependent aspartate release has been observed; its vesicular origin is still questioned (Ghijsen et al., 1999; Pende et al., 1993; Zhou et al., 1995). Nicholls and Attwell (1990) postulated the hypothesis that in cerebral cortex aspartate is released by reversed operation of the plasmamembrane glutamate/aspartate transporter and not by exocytosis. Depolarization with extracellular Ca\(^{2+}\) present could even give more release via this route, since the exocytotically released glutamate may cause additional aspartate efflux via heteroexchange through the transporter. Indeed, in contradiction to glutamate, no K\(^+\)-induced Ca\(^{2+}\)-dependent aspartate release was observed in hippocampus CA1 region in the presence of the transporter blocker L-trans-PDC studied by in vivo microdialysis (Zuiderwijk et al., 1996). There are also studies, however, which challenge this hypothesis. In these studies aspartate release
has been reported to be regulated independently from glutamate release (Martin et al., 1991; Zhou et al., 1995) and aspartate was identified by immunogold electronmicroscopy in vesicle-enriched areas in hippocampal terminals (Gundersen et al., 1998). However, until now, only a glutamate specific vesicular transporter has been identified (Bellochio et al., 2000) and no accumulation of aspartate in SVs could be found (Naito and Ueda, 1985; Gundersen et al., 1995; Bellochio et al., 2000). In the present study, cerebral cortex synaptosomes did not show Ca\(^{2+}\)-dependent aspartate release upon sub-second depolarizations. Only after a depolarization of 15 s we were able to detect a Ca\(^{2+}\)-dependent aspartate release of 0.76 nmol/mg protein, which was completely blocked after preincubation with the glutamate/aspartate transporter blocker L-trans-PDC, in agreement with release of this amino acid via reversal of the transporters by exocytotically released glutamate, as proposed above.

**Sub-second CCK release**

Neuropeptides are thought to be stored in and released from LDCVs. The release of neuropeptides from these vesicles is even more poorly understood than that of the SVs (Bean et al., 1994). To study the kinetics of neuropeptide release from isolated cortex nerve terminals we determined the time-course of CCK release, one of the most abundant neuropeptides in the brain (Rehfeld, 1985; Raiter et al., 1993). Ca\(^{2+}\)-dependent CCK release from the synaptosomes did not occur until 250 ms depolarization. Since some, though Ca\(^{2+}\)-independent release or leakage of CCK from synaptosomes could already be detected after 50 ms, the delay in appearance of Ca\(^{2+}\)-dependent release of CCK could not be explained by limited sensitivity of the RIA assay used in our study. Thus, CCK release was much slower in onset than amino acid release. Seward et al. (1995) showed that a Ca\(^{2+}\)-dependent 'preparatory' step of about 600 ms preceded the Ca\(^{2+}\)-dependent release of LDCVs in neurohypophysial terminals. For CCK containing LDCVs in cortical nerve terminals a preparatory step of about 250 ms appeared to be necessary. Another difference with the amino acids is that there is almost no Ca\(^{2+}\)-independent CCK release, whereas the amino acids showed a clear increasing Ca\(^{2+}\)-independent release component. This indicates that there is only a vesicular pool of CCK released in a Ca\(^{2+}\)-dependent way, which is in agreement with previous studies (Verhage et al., 1991a).
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