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Anti-B-50 (GAP-43) antibodies decrease exocytosis of glutamate in permeated synaptosomes

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Abstract

The involvement of the protein kinase C substrate, B-50 (GAP-43), in the release of glutamate from small clear-cored vesicles in streptolysin-O-permeated synaptosomes was studied by using anti-B-50 antibodies. Glutamate release was induced from endogenous as well as ^{3}H-labelled pools in a [Ca^{2+}]-dependent manner. This Ca^{2+}-induced release was partially ATP dependent and blocked by the light-chain fragment of tetanus toxin, demonstrating its vesicular nature. Comparison of the effects of anti-B-50 antibodies on glutamate and noradrenaline release from permeated synaptosomes revealed two major differences. Firstly, Ca^{2+}-induced glutamate release was decreased only partially by anti-B-50 antibodies, whereas Ca^{2+}-induced noradrenaline release was inhibited almost completely. Secondly, anti-B-50 antibodies significantly reduced basal glutamate release, but did not affect basal noradrenaline release. In view of the differences in exocytotic mechanisms of small clear-cored vesicles and large dense-cored vesicles, these data indicate that B-50 is important in the regulation of exocytosis of both types of neurotransmitters, probably at stages of vesicle recycling and/or vesicle recruitment, rather than in the Ca^{2+}-induced fusion step.

Keywords: B-50/GAP-43; Glutamate; Ca^{2+}; Exocytosis; Synaptosome, permeated; ATP dependency

1. Introduction

Neurotransmission involves the recruitment, docking, priming, fusion and recycling of neurotransmitter vesicles. Many of the proteins involved in these Ca^{2+}-dependent stages of exocytosis have now been identified and are present in presynaptic nerve terminals, where they constitute the molecular apparatus for the storage and secretion of neurotransmitters (for reviews see Schweizer et al., 1995; Südhof, 1995). Two classes of secretory neurotransmitter vesicles can be distinguished in nerve terminals on an ultrastructural basis, namely small clear-cored vesicles and large dense-cored vesicles. Small clear-cored vesicles, which store neurotransmitters such as glutamate, γ-amino-butyric acid (GABA), glycine and acetylcholine, are released swiftly at the active zone and undergo multiple cycles of exo- and endocytosis within the nerve terminal. In contrast, large dense-cored vesicles, which contain catecholamines or a variety of neuropeptides, cannot refill after exocytosis and therefore need to be recycled via the trans Golgi network (for review see Kelly, 1993). B-50 (also known as GAP-43, neuromodulin and F1) is a nervous tissue-specific, protein kinase C substrate that so far has been shown to be important in the exocytosis of large dense-cored vesicles only. In neurons of the adult nervous

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system, most B-50 is attached to the presynaptic membrane, where it binds calmodulin at unstimulated [Ca^{2+}]. Its role in Ca^{2+}-induced exocytosis was demonstrated by introducing anti-B-50 antibodies (immunoglobulins (IgGs)) into streptolysin-O-permeated synaptosomes (Dekker et al., 1989, 1991; Hens et al., 1993a). In this system, the N-terminal-directed anti-B-50 IgG NM2, which inhibits phosphorylation of rat B-50 at Ser-41, dephosphorylation and B-50/calmodulin binding, inhibited the Ca^{2+}-induced release of noradrenaline, dopamine and cholecystokinin-8 (CCK-8), whereas the more C-terminal-directed anti-B-50 IgG NM6 was without effect on Ca^{2+}-induced release (Hens et al., 1993b, 1995, 1996b). An increasing number of studies, using molecular approaches (Ivins et al., 1993; Imaizumi et al., 1995; Gamby et al., 1996), and intracellular application of anti-B-50 IgGs (Norden et al., 1991) and exogenous B-50 in permeated cells (Vitale et al., 1994), have confirmed that B-50 has a role in neurotransmitter release in other experimental systems besides the nerve terminal.

Although high levels of B-50 mRNA expression have been reported in particular for catecholaminergic neurons (Kruger et al., 1993), no specific colocalization of B-50 with any type or group of neurotransmitters has been observed in adult rat brain (Benowitz et al., 1988). In mature, non-damaged nervous tissue, B-50 expression is highest in neurons found in the human associative brain areas (Neve et al., 1987, 1988; Ng et al., 1988; Benowitz et al., 1989) and rat hippocampal (Oestreicher et al., 1986; Oestreicher and Gispen, 1986; Benowitz et al., 1988; De La Monte et al., 1989) and olfactory areas (Verhaagen et al., 1989), in which glutamate is the major neurotransmitter. In these brain regions the B-50 protein is found mainly in synapses. In fact, we have shown, using ultrastructural immunocytochemistry that B-50 is abundantly present at the plasma membrane of all synaptosomes prepared from rat cerebral cortex (Van Lookeren Campagne et al., 1989). In the hippocampus, long-term enhancement of glutamatergic transmission (long-term potentiation) is closely correlated with an NMDA receptor-dependent increase in B-50 phosphorylation (Gianotti et al., 1992; Ramakers et al., 1995). These data and the observation that glutamate is the major excitatory neurotransmitter in mammalian brain prompted us to investigate the role of B-50 in the Ca^{2+}-induced release of glutamate stored in small clear-cored vesicles (Storm-Mathisen et al., 1983; Naito and Ueda, 1985; Burger et al., 1989; Shupliakov et al., 1992). So far, however, accurate interpretation of the results of studies on the molecular mechanism of glutamate release has been hampered to some extent by the high levels of endogenous glutamate in synaptosomes (for reviews see Nicholls, 1993; Verhage et al., 1994), and by the ability of intact nerve terminals to release glutamate not only in a Ca^{2+}-dependent manner, but also in a Ca^{2+}-independent, non-vesicular manner (for review see Adam-Vizi, 1992). Therefore, based on our previous permeation protocols (Hens et al., 1993a,b, 1995), we developed a permeated synaptosome system to study the role of B-50 in glutamate release from small clear-cored vesicles, using D-aspartate loading (McMahon and Nicholls, 1990; Terrian et al., 1991) to reduce endogenous glutamate levels in the cytosolic compartment and Ca^{2+}/EGTA buffers to trigger exocytosis. First, we characterized the Ca^{2+} sensitivity and ATP dependence of Ca^{2+}-induced glutamate release from permeated synaptosomes. Next, we introduced monoclonal, B-50-neutralizing IgGs and studied their effects on Ca^{2+}-induced glutamate release in comparison to those on Ca^{2+}-induced noradrenaline (NA) release. We show that both Ca^{2+}-induced noradrenaline and glutamate release were inhibited by B-50 IgGs, indicating that B-50 is not only involved in the release of large dense-cored vesicles but also in the release of small clear-cored vesicles. Our data revealed two major differences in the effects of the anti-B-50 IgGs on the exocytosis of these different types of neurotransmitters.

2. Materials and methods

2.1. Materials

Brains of male Wistar rats weighing 100–120 g were used (dissected on ice after decapitation). [3,4-^{3}H]L-glutamic acid (35 Ci/mmol) was purchased from ICN Pharmaceuticals (Irvine, CA, USA). ATP and dithiothreitol were purchased from Boehringer (Mannheim, Germany), protein kinase C-(19–36) from Calbiochem (La Jolla, USA), and creatine phosphate, creatine phosphokinase, D-aspartate, EGTA, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), homoserine, oligomycin, pargyline and polymyxin B (7800 IU/mg of protein) from Sigma (St. Louis, MO, USA). Bovine serum albumin (bovine serum albumin; fraction V) and all other chemicals were obtained from Merck (Darmstadt, Germany). The Ca^{2+}/EGTA buffers were calculated and prepared as described by Föhr et al. (1993).

2.2. Purified proteins

Monoclonal anti-B-50 IgGs NM2 and NM6 (IgG1 kappa; Mercken et al., 1992) were purified by affinity chromatography on a protein G column (Pharmacia, Uppsala, Sweden), dialysed extensively against 1000 volumes of buffer A (124 mM NaCl, 5 mM PIPES; pH 6.8) and stored at −80°C as described earlier (Hens et al., 1995). Monoclonal, isotype control IgGs were obtained from the mouse hybridoma cell line F3-114-77-1 (gift from Dr. Gerhard Zenke, Sandoz, Switzerland). Tetanus toxin was separated into its heavy and light chain fragments by isoelectric focusing on a sucrose gradient with ampholyte under reducing conditions in 2 M urea as described earlier

(Weller et al., 1989). Before use, heavy and light chain fragments were dialysed extensively against 1000 volumes of buffer A containing 10 μM $ZnCl_2$ and stored at −80°C. Streptolysin-O was purified from culture supernatants of group A Streptococci (Richards strain) as described (Bhakdi et al., 1984) with slight modifications. In short, the (60%)-ammonium sulfate precipitate of culture supernatant was purified by subsequent chromatography on a Thiopropyl–Sepharose 6B column, an Alkyl–Superose 10/10 column and a Fractogel DEAE column (Pharmacia, Uppsala, Sweden). Streptolysin-O (0.5 mg of protein/ml; stored at −80°C in the presence of 1 mM dithiothreitol and 1 mg/ml of bovine serum albumin) was tested for its hemolytic activity using 1.25% (v/v) rabbit erythrocytes (Ahnert-Hilger et al., 1989) and caused 60% hemolysis at a dilution of 1:3000. Protein was measured by the technique of Bradford, using bovine serum albumin as the standard (Bradford, 1976).

2.3. Neurotransmitter release from streptolysin-O-permeated synaptosomes

Synaptosomes were isolated from rat cerebral cortex on Percoll–sucrose gradients (15–23% interface) as described earlier (Dunkley et al., 1988). Before permeation, synaptosomes were partially depleted of their cytosolic glutamate pool by 100 μM D-aspartate loading (30 min at 34°C) in permeation buffer [122.85 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1.15 mM NaH_2PO_4, 20 mM PIPES and 5.6 mM D(+)-glucose; oxygenated; pH 6.8]. After loading, exogenous D-aspartate was removed by three washes with permeation buffer (5 min, 15,000 × *g*, 4°C). This D-aspartate loading protocol has been extensively characterized in synaptosomes. D-aspartate partially depletes the endogenous glutamate pool without affecting the Ca^{2+}-dependent (vesicular) pool of glutamate (McMahon and Nicholls, 1990; Terrian et al., 1991). In our hands D-aspartate, loading reduced the synaptosomal glutamate content by 20% (data not shown). To measure radiolabelled glutamate release, synaptosomes were loaded with ^{3}H-glutamic acid (5 μCi/mg of protein) in permeation buffer supplemented with 2 mM $CaCl_2$ (5 min after a 5-min pre-incubation at 34°C) as described by Chittajallu et al. (1996). Excess radiolabel was removed by two washes with permeation buffer (5 min, 15,000 × *g*, 4°C) before D-aspartate loading as described above.

Synaptosomes (167 μg protein/ml) made permeable with streptolysin-O (2.1 μg protein/ml) in permeation buffer supplemented with 2.0 mM ATP, 5 mM CP, 5 IU/ml of creatine phosphokinase and 1 mg/ml of bovine serum albumin for 5 min at 25°C as described previously (Hens et al., 1995), unless stated otherwise. In the experiments in which endogenous noradrenaline was measured, the permeation buffer was supplemented with 10 μM of the monoamine oxidase inhibitor pargyline. The indicated free [Ca^{2+}] (generated by Ca^{2+}/EGTA buffers containing a final concentration of 10 mM EGTA) was present throughout the experiment. However, in some experiments, the results of which were presented in Figs. 3, 4 and 6, the Ca^{2+} trigger was added 2.5 min after the start of permeation in 10^{-8} M Ca^{2+} (buffered with 1 mM EGTA). IgGs, toxins or inhibitors were present throughout the permeation experiment. Incubations were stopped by centrifugation for 25 s at 10,000 × *g*. Neurotransmitter release was measured in the supernatant samples. The total neurotransmitter content was measured in supernatant samples of synaptosomes solubilized with 0.05% (v/v) Triton X-100 (5 min, 25°C).

In an alternative permeation protocol (used in Fig. 2 only), the synaptosomes (167 μg protein/ml) were incubated in permeation buffer with 3.0 IU/ml streptolysin-O (Murex, Utrecht, the Netherlands) at 0°C for 20 min. Excessive, unbound streptolysin-O was removed by a single centrifugation step (3 min, 7000 × *g*, 4°C) and synaptosomes were resuspended in permeation buffer. Streptolysin-O-pre-treated synaptosomes were permeated in permeation buffer supplemented with 2.0 mM ATP, 5 mM CP, 5 IU/ml of CPK, 1 mg/ml of bovine serum albumin and the indicated free [Ca^{2+}] (in 10 mM EGTA) for 5 min at 30°C as above.

2.4. Glutamate and noradrenaline analysis

Glutamate release in supernatant samples (stored in 5% (v/v) trichloroacetic acid at −80°C) was measured by liquid scintillation counting or by reversed-phase high performance liquid chromatography (HPLC) with fluorometric detection after *o*-phthaldialdehyde pre-column derivatization, using a methanol–phosphate buffer (35% methanol, 0.05 M sodium phosphate with 2% (v/v) tetrahydrofuran [adjusted to pH 7.0 with phosphoric acid]) as described earlier (Verhage et al., 1989). Homoserine was used as the internal standard for HPLC analysis. Glutamate release is expressed as a percentage of the total endogenous or ^{3}H-labelled synaptosomal glutamate content or as nmol glutamate/mg of synaptosomal protein.

Noradrenaline in supernatant samples (stored in 100 μM glutathione at −80°C) was analysed by HPLC after liquid–liquid extraction and pre-column derivatization with the fluorogenic agent 1,2-diphenylethylenediamine as described earlier (Van der Hoorn et al., 1989). Noradrenaline release is expressed as a percentage of the total noradrenaline content or as pmol noradrenaline/mg of synaptosomal protein. Ca^{2+}-induced release was calculated by subtracting noradrenaline release in the presence of 10^{-8} M Ca^{2+} from noradrenaline release in the presence of elevated [Ca^{2+}], e.g., 10^{-4} M Ca^{2+}.

2.5. Statistics

Data are presented as the means ± S.E.M. Statistical analysis was performed with an analysis of variance

(ANOVA) followed by a two-tailed Student's *t*-test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Glutamate release from streptolysin-O-permeated synaptosomes

To study the effects of anti-B-50 IgGs on the exocytosis of transmitter glutamate, we developed a glutamate release assay with streptolysin-O-permeated synaptosomes. Because the batches of streptolysin-O, which had been used earlier to study Ca^{2+}-induced release of noradrenaline and CCK-8 in this preparation (Hens et al., 1993a,b, 1995), contained unidentified contaminants which interfered with the fluorometric detection of glutamate by HPLC, we used highly purified streptolysin-O (see Section 2) to permeate synaptosomes. At a basal $[Ca^{2+}]$ of 10^{-8} M, streptolysin-O increased the efflux of endogenous glutamate in a dose-dependent manner (Fig. 1): from $14.1 \pm 0.6\%$ of the total synaptosomal glutamate content in the absence of streptolysin-O to $56.6 \pm 1.5\%$ with 5.2 μg/ml streptolysin-O, the highest [streptolysin-O] tested. When this experiment was performed in the presence of a stimulating $[Ca^{2+}]$ of 10^{-4} M, the efflux of glutamate increased from $13.6 \pm 0.6\%$ of the total synaptosomal glutamate content in the absence of streptolysin-O to $64.8 \pm 2.1\%$ with 5.2 μg/ml streptolysin-O (Fig. 1). Thus, in the presence of 2.1 μg/ml streptolysin-O, an elevation of the $[Ca^{2+}]$ from 10^{-8} to 10^{-4} M resulted in a significant ($P < 0.005$) increase in the release of glutamate, referred to as Ca^{2+}-induced glutamate release, amounting to 1.5 ± 0.4 nmol glutamate/mg ($6.9 \pm 1.7\%$ of the total glutamate content). Significant Ca^{2+}-induced glutamate release was absent at [streptolysin-O] < 2.1 μg/ml. In the presence of 5.2 μg/ml streptolysin-O, Ca^{2+}-induced glutamate release was 1.8 ± 0.6 nmol/mg ($8.2 \pm 2.6\%$ of the total glutamate content). In all further experiments a [streptolysin-O] of 2.1 μg/ml was used. At this [streptolysin-O] Ca^{2+}-induced glutamate release from ^{3}H-labelled pools was $5.1 \pm 1.0\%$ of the total synaptosomal ^{3}H-glutamate incorporation ($P < 0.001$). ^{3}H-Glutamate release was $55.0 \pm 1.0\%$ at 10^{-8} M Ca^{2+} and $60.1 \pm 0.8\%$ at 10^{-4} M Ca^{2+} ($n = 4$; data not shown), thus resembling endogenous glutamate release at this [streptolysin-O].

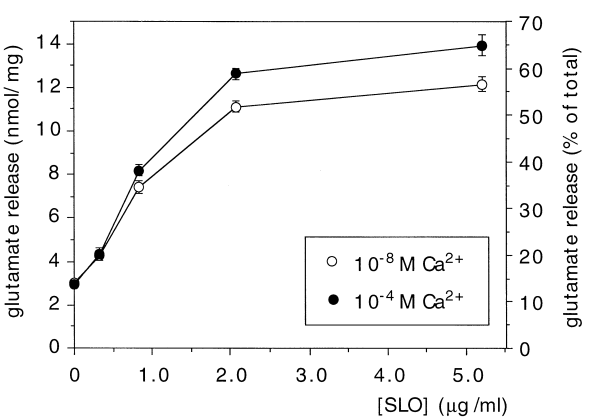

Fig. 1. Effect of streptolysin-O on the efflux of endogenous glutamate from synaptosomes. Synaptosomes were permeated with the indicated concentrations of streptolysin-O in the presence of either 10^{-8} M (open circles) or 10^{-4} M (filled circles) of free Ca^{2+} for 5 min at 25°C. Glutamate release is expressed in nmol/mg of synaptosomal protein and as a percentage of total glutamate content (21.5 ± 2.8 nmol/mg). Data are the means ± S.E.M. (bars) from 13–29 observations obtained in six independent experiments (ANOVA: $P < 0.001$).

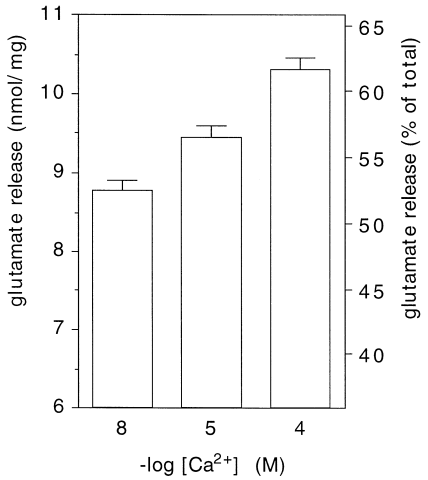

Fig. 2. Ca^{2+}-dependent efflux of endogenous glutamate from streptolysin-O-pre-treated synaptosomes. With this alternative permeation procedure, the synaptosomes were first pre-incubated with streptolysin-O (20 min on ice) and washed once to remove unbound streptolysin-O, before being permeated at the indicated free $[Ca^{2+}]$ for 5 min at 30°C as described in Section 2. Glutamate release is expressed in nmol/mg of synaptosomal protein and as a percentage of total glutamate content (16.7 ± 1.0 nmol/mg). Data are the means ± S.E.M. (bars) from 18–39 observations obtained from five independent experiments (ANOVA: $P < 0.001$).

To test whether the glutamate efflux of more than 50% of the total synaptosomal content, observed in the presence of [streptolysin-O] ≥ 2.1 μg/ml at basal $[Ca^{2+}]$ (Fig. 1), was due to permeational damage of intracellular membrane structures, an alternative permeation protocol was used to limit the effect of streptolysin-O to the synaptosomal plasma membrane (Fig. 2). The alternative permeation procedure was based on the observation that at 0°C, streptolysin-O only binds to the outer membrane and does not form pores. Pore formation by streptolysin-O requires physiological incubation temperatures (Hugo et al., 1986; Ahnert-Hilger et al., 1989). Therefore, synaptosomes were incubated for 20 min on ice with a 10-fold higher [streptolysin-O] than used in previous permeation studies with synaptosomes (Hens et al., 1993a,b, 1995) and were washed once to remove unbound streptolysin-O before permeation

at 30°C (Fig. 2). The basal efflux of glutamate increased significantly from 52.4 ± 0.7% (at 10^{-8} M Ca^{2+}) to 56.4 ± 0.9% when the free [Ca^{2+}] was increased to 10^{-5} M ($P < 0.005$), or to 61.6 ± 0.8% in the presence of 10^{-4} M Ca^{2+} ($P < 0.001$). Ca^{2+}-induced glutamate release was 4.0 ± 1.1% in the presence of 10^{-5} M Ca^{2+} and 9.2 ± 1.1% in the presence of 10^{-4} M Ca^{2+}. Thus, basal glutamate release was not reduced by the alternative streptolysin-O-permeation protocol, demonstrating that streptolysin-O does not permeate intrasynaptosomal, glutamate-containing compartments. Taken together these data demonstrate that streptolysin-O-permeated synaptosomes retain their structural integrity and can be used for functional interference studies under the present experimental conditions.

3.2. Protein kinase C inhibitors and Ca^{2+}-induced glutamate release

The role of protein kinase C in the release of glutamate after a single Ca^{2+} trigger was studied by introducing protein kinase C inhibitors into streptolysin-O-permeated synaptosomes at concentrations that almost completely inhibited protein kinase C-mediated B-50 phosphorylation in this preparation (results not shown; Hens et al., 1993a). Neither the autoinhibitory protein kinase C peptide protein kinase C-(19–36) at 3×10^{-5} M nor the kinase inhibitor H-7 at 10^{-4} M significantly affected basal or Ca^{2+}-induced glutamate release from permeated synaptosomes (Fig. 3). The mixed kinase/calmodulin antagonist polymyxin B (Hens et al., 1996a, and references therein), which has been shown to inhibit Ca^{2+}-induced noradrenaline and CCK-8 release (Hens et al., 1993a,b), also completely inhibited Ca^{2+}-induced glutamate release in the same assay.

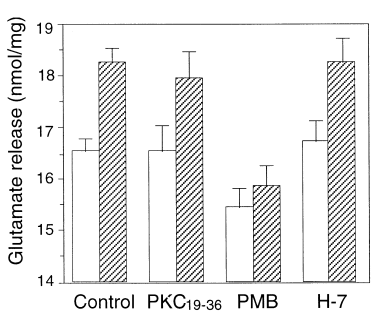

Fig. 3. Lack of effect of protein kinase C inhibitors on Ca^{2+}-induced glutamate release. Effects of protein kinase inhibitors protein kinase C-(19–36) (3×10^{-5} M) or H-7 (10^{-4} M) on Ca^{2+}-induced glutamate release. Polymyxin B (PMB; 200 IU/ml) was used as a positive control (see also: Hens et al., 1993a,b, 1996a,b). Synaptosomes were permeated for 2.5 min at 25°C before either 10^{-8} M (open columns) or 10^{-4} M (filled columns) free Ca^{2+} was added for 2.5 min. Inhibitors were present throughout the experiment. Glutamate release is expressed in nmol/mg of protein. Data are the means ± S.E.M. (bars) of 15–30 observations obtained from four independent experiments (ANOVA: $P < 0.001$). Total glutamate content was 29.3 ± 2.5 nmol/mg.

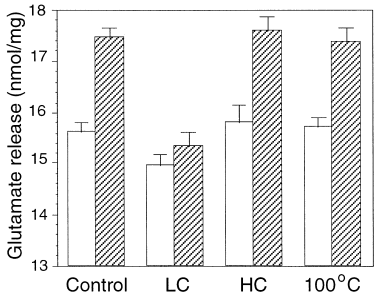

Fig. 4. Tetanus toxin LC inhibits Ca^{2+}-induced glutamate release. Effects of tetanus toxin light chain (LC), heavy chain (HC) and heat-inactivated LC ('100°C', 5 min) on Ca^{2+}-induced glutamate release from synaptosomes that were permeated at a basal [Ca^{2+}] of 10^{-8} M for 2.5 min at 25°C before either 10^{-8} M (open columns) or 10^{-4} M (filled columns) free Ca^{2+} was added for 2.5 min. Tetanus toxin HC, LC or heat-inactivated LC were present throughout the experiment at a concentration of 300 nM. Glutamate release is expressed in nmol/mg of synaptosomal protein. Data are the means ± S.E.M. (bars) of 16–32 observations obtained from four independent experiments (ANOVA: $P < 0.001$). Total glutamate content was 30.9 ± 0.9 nmol/mg.

3.3. Characterization of Ca^{2+}-induced glutamate release

To establish the exocytotic nature of Ca^{2+}-induced glutamate release from streptolysin-O-permeated synaptosomes, the effects of the tetanus toxin low and heavy chain fragments (Schiavo et al., 1992) were tested on Ca^{2+}-induced glutamate release (Fig. 4). Introduction of the light chain fragment of tetanus toxin at 300 nM completely inhibited Ca^{2+}-induced glutamate release and slightly reduced basal glutamate release (0.7 ± 0.3 nmol/mg; $P < 0.05$). Identical amounts of the heavy chain fragment of tetanus toxin or heat-inactivated light chain fragment were without effect on Ca^{2+}-induced glutamate release (Fig. 4), demonstrating that the inhibition of Ca^{2+}-induced glutamate release is mediated by the heat-labile light chain fragment protein. The light chain fragment also inhibited Ca^{2+}-induced ^{3}H-glutamate release almost completely in a dose-dependent manner: from 5.7 ± 1.2% (controls without light chain fragment) to 3.1 ± 1.2% in the presence of 300 nM light chain fragment ($P < 0.05$) or 1.3 ± 1.0% in the presence of 1000 nM light chain fragment ($P < 0.001$), corresponding to an inhibition of 45 and 76%, respectively. Basal [^{3}H]-glutamate release was not affected by any of the treatments (results not shown). Control experiments in which Western blot analysis was used showed that B-50 is not a substrate for the proteolytic activity of tetanus toxin (data not shown).

Next, the Ca^{2+} sensitivity of glutamate release from both endogenous and [^{3}H]-labelled pools was studied in streptolysin-O-permeated synaptosomes (Fig. 5). An increase in the free [Ca^{2+}] from 10^{-8} M Ca^{2+} increased glutamate release in a [Ca^{2+}]-dependent manner. This Ca^{2+}-induced glutamate release was significant at 3×10^{-6} M Ca^{2+} ($P < 0.05$) and peaked at 3×10^{-5} M Ca^{2+} glutamate release amounted to 8.1 ± 1.8% of the total

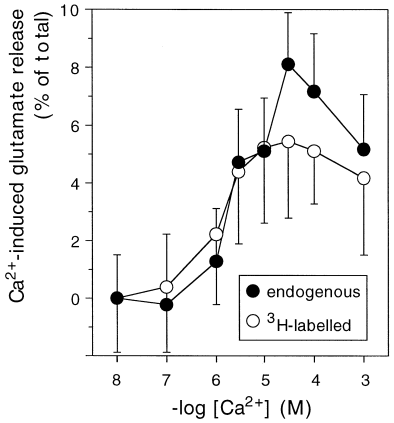

Fig. 5. Ca^{2+}-induced glutamate release from endogenous and ^{3}H-labelled pools in streptolysin-O-permeated synaptosomes. Synaptosomes were permeated in the presence of the indicated free [Ca^{2+}] for 5 min at 25°C. Ca^{2+}-induced glutamate release from endogenous (filled circles) and ^{3}H-labelled (open circles) pools was calculated by subtracting basal glutamate release (at 10^{-8} M Ca^{2+}) from the glutamate release at the indicated [Ca^{2+}]. Ca^{2+}-induced release is expressed as a percentage of the total endogenous or ^{3}H-labelled glutamate content. Basal glutamate release was $59.1 \pm 1.3\%$ for ^{3}H-glutamate and $56.0 \pm 1.0\%$ for endogenous glutamate (total content: 21.7 ± 2.6 nmol/mg). Data are the means $\pm$S.E.M. (bars) of 20–40 observations obtained from five independent experiments for endogenous glutamate release (upward error bars) and 13–28 observations obtained from four independent experiments for ^{3}H-labelled glutamate release (downward error bars). Ca^{2+}-induced glutamate release is significant at [Ca^{2+}] $\geq 3 \times 10^{-6}$ M (ANOVA: $P < 0.001$ for endogenous as well as ^{3}H-labelled glutamate).

endogenous glutamate content, corresponding to 1.8 ± 0.4 nmol/mg protein and to $5.4 \pm 2.6\%$ of the total ^{3}H-labelled glutamate content. This Ca^{2+}-induced glutamate release from permeated synaptosomes was strikingly similar to the magnitude of 30-mM K^+-evoked, Ca^{2+}-dependent glutamate release from intact rat cerebrocortical synaptosomes, which amounted to $9.0 \pm 2.3\%$ in our hands ($24.4 \pm 1.7\%$ in the presence of 100 μM EGTA; $33.4 \pm 1.5\%$ in the presence of 2 mM Ca^{2+}; 5-min incubation at 25°C; $n = 3$). At 10^{-3} M Ca^{2+}, the highest [Ca^{2+}] tested, Ca^{2+}-induced glutamate release was reduced to 64 (endogenous) or 77% (^{3}H-labelled) of the maximal Ca^{2+}-induced glutamate release observed. No significant difference was observed between the Ca^{2+} dependence of glutamate released from ^{3}H-labelled pools and endogenous pools (Fig. 5).

To study the ATP requirement of Ca^{2+}-induced glutamate release, synaptosomes were permeated in the absence or presence of 2 mM exogenous ATP. To allow full equilibration of ATP in the synaptosomes, they were permeated with streptolysin-O 2.5 min prior to the Ca^{2+} trigger (Fig. 6). We found that Ca^{2+}-induced glutamate release was only partially ATP-dependent: in the absence of ATP Ca^{2+}-induced glutamate release was 0.8 ± 0.4 nmol/mg ($P < 0.05$), corresponding to 57% of the maximal Ca^{2+}-induced glutamate release observed in the presence of 2 mM ATP and an ATP regenerating system (Helms et al., 1990), i.e., creatine phosphate and creatine phosphokinase (1.3 ± 0.4 nmol/mg; $P < 0.01$). Ca^{2+}-induced glutamate release in the presence of 2 mM ATP, but without an ATP regenerating system, was 1.2 ± 0.4 nmol/mg (94% of the maximal Ca^{2+}-induced glutamate release with an ATP regenerating system). Neither basal glutamate release nor glutamate release in the presence of 10^{-4} M Ca^{2+} was significantly different between the treatments (Fig. 6).

3.4. Effects of anti-B-50 IgGs on glutamate release

The involvement of B-50 in Ca^{2+}-induced glutamate release was studied in comparison to its established involvement in noradrenaline release (Hens et al., 1995) by introducing two different monoclonal anti-B-50 IgGs (NM2 and NM6) into streptolysin-O-permeated synaptosomes (Fig. 7). The epitope characterization of NM2 and NM6 has been described in detail elsewhere (Oestreicher et al., 1994). In short, NM6 recognizes an epitope located in the C-terminal domain (amino acids 132–213) of rat B-50, whereas NM2 recognizes the N-terminal domain (amino acids 39–43) of rat B-50 containing the unique protein kinase C phosphorylation site at Ser-41. We have previously shown that anti-B-50 IgG NM2 inhibited B-50 phosphorylation, dephosphorylation and B-50/calmodulin binding, whereas anti-B-50 IgGs NM6 is without effect on these parameters (Hens et al., 1995). Anti-B-50 IgGs NM2 and NM6 were tested at a single concentration of 50 μg/ml IgG on Ca^{2+}-induced release of endogenous glutamate (Fig. 7A) and noradrenaline (Fig. 7B). Ca^{2+}-induced glutamate release (control: $6.3 \pm 2.1\%$; $P < 0.05$) was sig-

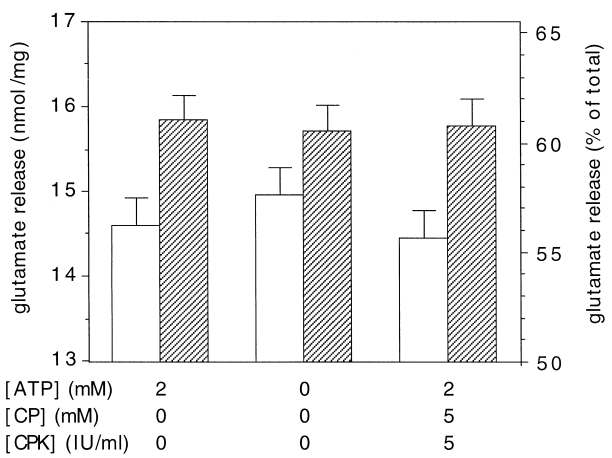

Fig. 6. ATP dependence of Ca^{2+}-induced glutamate release from permeated synaptosomes. Synaptosomes were permeated for 2.5 min at 25°C before either 10^{-8} M (open columns) or 10^{-4} M (filled columns) free Ca^{2+} was added for 2.5 min. ATP and creatine phosphate were present at the indicated concentration in mM and creatine phosphokinase in IU/ml. Glutamate release is expressed in nmol/mg of synaptosomal protein and as a percentage of total glutamate content (25.9 ± 4.9 nmol/mg). Data are the means $\pm$S.E.M. (bars) of 21 observations obtained from five independent experiments (ANOVA: $P < 0.005$).

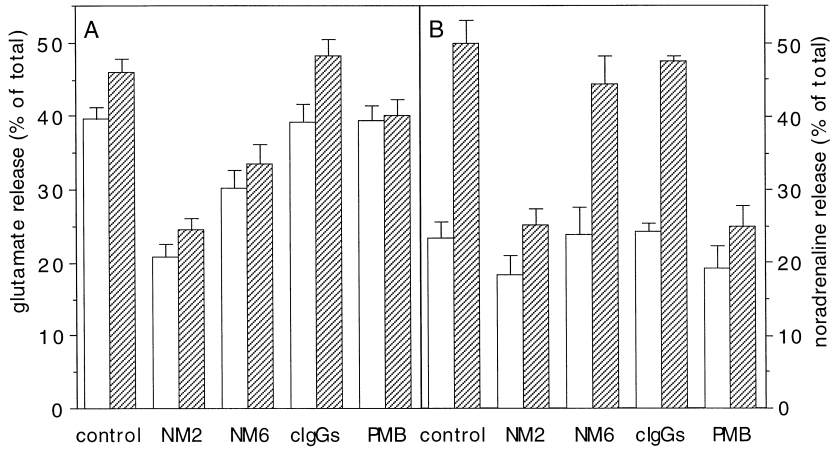

Fig. 7. Effect of anti-B-50 IgGs NM2 and NM6 on the release of endogenous glutamate and noradrenaline noradrenaline from permeated synaptosomes. Synaptosomes were permeated with 1.25 μg/ml streptolysin-O in the presence of 10^{-8} M (open columns) or 10^{-4} M (filled columns) free Ca^{2+} for 5 min at 25°C. Anti-B-50 IgGs and idiotypic control IgGs (cIgGs) were present throughout the experiment at 50 μg of IgG/ml. As positive control polymyxin B (PMB) was present at 200 IU/ml. The release of endogenous glutamate (A) and noradrenaline (B) was determined in the very same sample of permeated synaptosomes and is expressed as a percentage of the total glutamate content (37.7 ± 6.5 nmol/mg) and total noradrenaline content (9.7 ± 1.2 pmol/mg). Data are the means ± S.E.M. (bars) of 17–39 observations obtained from five independent experiments (ANOVA: $P < 0.001$ for glutamate as well as NA).

nificantly decreased in the presence of anti-B-50 IgGs NM2 ($3.7 \pm 2.4\%$) and NM6 ($3.2 \pm 3.7\%$), corresponding to an inhibition of 42 and 49%, respectively (Fig. 7A). The mixed kinase/calmodulin inhibitor polymyxin B (Hens et al., 1996a), used as a positive control in these experiments, inhibited Ca^{2+}-induced glutamate release completely (90% inhibition). Interestingly, anti-B-50 IgGs NM2 and NM6 also decreased basal glutamate release significantly from 15.0 ± 0.5 nmol/mg (control) to 7.8 ± 0.7 nmol/mg in the presence of NM2 ($P < 0.001$) or 11.4 ± 0.9 nmol/mg in the presence of NM6 ($P < 0.005$), corresponding to an inhibition of 48 and 24%, respectively. Thus, NM2 decreased basal glutamate two-fold more potently than NM6 ($P < 0.005$), whereas polymyxin B was without any effect (Fig. 7A). Idiotypic control IgGs (50 μg/ml) were without effect on Ca^{2+}-induced or basal glutamate release (Fig. 7A).

The effects of anti-B-50 IgGs on Ca^{2+}-induced noradrenaline release (Fig. 7B), which have been published earlier (Hens et al., 1995), were measured here in the same supernatant samples to enable direct comparison with Ca^{2+}-induced glutamate release from a distinct pool of vesicles. In fact, we found that the effects of the anti-B-50 IgGs on Ca^{2+}-induced glutamate and noradrenaline release differed. In accordance with our previous data, Ca^{2+}-induced noradrenaline release (control: $26.4 \pm 3.8\%$; $P < 0.001$) was almost completely blocked (74% inhibition) in the presence of the anti-B-50 IgG NM2 ($6.8 \pm 3.5\%$) and hardly affected (22% inhibition) in the presence of the anti-B-50 IgG NM6 ($20.5 \pm 5.4\%$; $P < 0.005$). As expected, the positive control polymyxin B blocked Ca^{2+}-induced noradrenaline release almost completely (78% inhibition). Moreover, basal noradrenaline release was not significantly affected by any of the treatments. Thus, in contrast to the effects on noradrenaline release, the anti-B-50 IgGs NM2 and NM6 reduced Ca^{2+}-induced glutamate release only partially and caused a large reduction (NM2 ≫ NM6) in basal glutamate release.

To investigate to what extent the effects of anti-B-50 IgGs NM2 and NM6 on basal glutamate release may be due to an inhibition of vesicle recycling and/or recruitment, we also tested the anti-B-50 IgGs on glutamate release from ^{3}H-labelled pools (Fig. 8). A major advantage

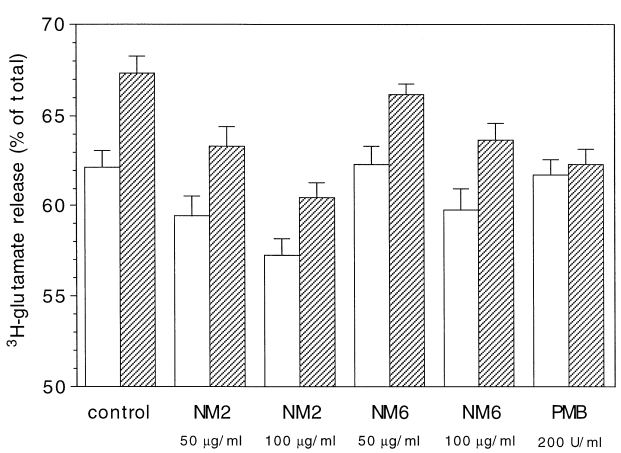

Fig. 8. Effect of anti-B-50 IgGs NM2 and NM6 on [^{3}H]-glutamate release from permeated synaptosomes. Synaptosomes were permeated in the presence of 10^{-8} M (open columns) or 10^{-4} M (filled columns) free Ca^{2+} for 5 min at 25°C. Anti-B-50 IgGs were present throughout the experiment at 50 or 100 μg of IgG/ml. As positive control polymyxin B (PMB) was present at 200 IU/ml. Glutamate release is expressed as a percentage of the total ^{3}H-glutamate incorporation. Data are the means ± S.E.M. (bars) of 8–12 observations obtained from three independent experiments (ANOVA: $P < 0.001$). Ca^{2+}-induced ^{3}H-glutamate is significant at any treatment with anti-B-50 IgGs ($P < 0.05$).

of using [^{3}H]-glutamate under our assay conditions is that it is largely taken up into the releasable pool of vesicular glutamate. Once released from the vesicle, the [^{3}H]-glutamate is strongly diluted in the permeation buffer with micromolar concentrations of endogenous glutamate, coming from the cytosol, the mitochondria and the vesicles. Thus, the re-uptake of [^{3}H]-glutamate into recycling vesicles is expected to be small. We observed only a small reduction in basal [^{3}H]-glutamate release ($4.8 \pm 1.3\%$; $P < 0.005$) in the presence of 100 μg/ml anti-B-50 IgG NM2, representing an inhibition of 7.7%, thus, about 10-fold less than the decrease observed for endogenous glutamate release. NM6 IgG and lower concentrations of NM2 IgG were without a significant effect on basal [^{3}H]-glutamate release. Ca^{2+}-induced [^{3}H]-glutamate release (control: $5.2 \pm 1.3\%$; $P < 0.005$) was partially inhibited by the anti-B-50 IgGs NM2 (25.3% inhibition at 50 μg/ml and 38.9% at 100 μg/ml IgGs) and NM6 (26.0% inhibition at 50 μg/ml and 26.8% at 100 μg/ml IgGs), whereas Ca^{2+}-induced [^{3}H]-glutamate release was almost completely (88.8%) inhibited by polymyxin B at 200 IU/ml,used at positive control. Thus, the anti-B-50 IgGs only partially (maximal 40%) inhibited Ca^{2+}-induced [^{3}H]-glutamate release and hardly, if at all, reduced basal ^{3}H-glutamate release from streptolysin-O-permeated synaptosomes (Fig. 8).

4. Discussion

4.1. The Ca^{2+} sensitivity of glutamate release

Here, we show with streptolysin-O-permeated synaptosomes that Ca^{2+} induces the release of glutamate from endogenous as well as ^{3}H-labelled pools in a concentration-dependent fashion (Fig. 5). The vesicular nature of this Ca^{2+}-induced glutamate release was demonstrated by using the isolated LC fragment of tetanus toxin, which cleaves the synaptic vesicle-associated protein synaptobrevin-2/VAMP-2 (Schiavo et al., 1992) and blocks Ca^{2+}-induced glutamate release in permeated synaptosomes (Fig. 4) at a step following vesicle docking (Hunt et al., 1994). We found that the Ca^{2+}-induced glutamate release from permeated synaptosomes ($8.1 \pm 1.8\%$) was highly reproducible and strikingly similar in magnitude to the K^+-evoked, Ca^{2+}-dependent glutamate release from intact rat cerebrocortical synaptosomes ($9.0 \pm 2.3\%$). Because exocytosis can be considered as a cascade of Ca^{2+}-regulated steps preceding (e.g., vesicle recruitment, docking and priming) and following (e.g., endocytosis) Ca^{2+}-triggered vesicle fusion (for reviews see Schweizer et al., 1995; Südhof, 1995), the Ca^{2+} sensitivity of glutamate release represents a combination of these distinct Ca^{2+}-sensitive steps along the exocytotic process in permeated synaptosomes. Consequently, the submaximal Ca^{2+}-induced glutamate release at 10^{-3} M Ca^{2+} (Fig. 5) may be due to an inhibition of endocytosis at this [Ca^{2+}] (Von Gersdorff and Matthews, 1994), because a block of endocytosis is known to impede exocytosis (Koenig et al., 1989).

The Ca^{2+} sensitivity of glutamate release resembled that seen for noradrenaline, dopamine and CCK-8 release in permeated synaptosomes (Hens et al., 1993a,b, 1996b), reaching a maximum between 3×10^{-5} and 10^{-4} M Ca^{2+}. These relatively high intracellular Ca^{2+} concentrations indeed occur underneath the presynaptic plasma membrane at sites of vesicle fusion (Adler et al., 1991; Verhage et al., 1991; Llinás et al., 1992) and are in accordance with the assumption that vesicular neurotransmitter release is a low Ca^{2+} affinity process (for reviews see Nicholls, 1993; Verhage et al., 1994). It is generally accepted that glutamatergic vesicles are released by a rapid, highly localized and large rise in $[Ca^{2+}]_i$ at the Ca^{2+} channel-rich active zones, whereas neuropeptides are released by fusion of large dense-cored vesicles at ectopic sides outside these active zones (for review see Kelly, 1993) and require a smaller and more diffuse rise in $[Ca^{2+}]_i$ to trigger fusion (for review see Verhage et al., 1994). Although we cannot exclude that a shift in the Ca^{2+} sensitivity of the release machinery occurred as a result of leakage of Ca^{2+}-dependent proteins from the synaptosomal interior after permeation, we feel that this possibility is less likely because we obtained identical results for the Ca^{2+} sensitivity in experiments in which the synaptosomes were permeated before application of the Ca^{2+} trigger. Therefore, our data suggest that this difference in the Ca^{2+} sensitivity of exocytosis of amino acid and peptidergic neurotransmitters may be due to a difference in the efficacy (for instance density and location of the Ca^{2+} channels) to reach the required [Ca^{2+}] at the sites of vesicle fusion. Glutamate is released from pre-docked vesicles which fuse in the close vicinity of Ca^{2+} channels at the active zone (Bennett et al., 1992), whereas neuropeptides are released at remote sites which are less accessible to Ca^{2+} influx in intact nerve terminals. In permeated synaptosomes, however, Ca^{2+} influx is not restricted to the active zone, and this may explain why the Ca^{2+} sensitivity for the different types of neurotransmitter vesicles was not essentially different in this preparation.

4.2. ATP and the exocytosis of glutamate

Our data for permeated synaptosomes show that Ca^{2+}-induced glutamate release is at least partially (40%) ATP dependent (Fig. 6). At least three ATP-dependent processes can be distinguished that may affect the magnitude of Ca^{2+}-induced glutamate release from permeated synaptosomes. Firstly, the storage of glutamate in vesicles requires a vesicular H^+/ATPase to generate an electrochemical proton gradient (Maycox et al., 1988; Burger et al., 1989; Carlson and Ueda, 1990). Secondly, ATP is required for the formation of a stable 20 S fusion complex

containing the *N*-ethylmaleimide-sensitive ATPase NEM-sensitive fusion protein (NSF) (Söllner et al., 1993), an important event preceding Ca^{2+}-induced vesicle fusion (for review see Schweizer et al., 1995). In fact, *N*-ethylmaleimide was found to inhibit Ca^{2+}-induced noradrenaline release in permeated release systems (Frye and Holz, 1985; Hens and De Graan, unpublised data) and in vitro fusion of [^{3}H]-glutamate-loaded vesicles (Kish and Ueda, 1991), but this reagent could not be tested on Ca^{2+}-induced glutamate release from permeated synaptosomes because it seriously interfered with streptolysin-O-permeation (Hens and De Graan, unpublished data). Thus, the ATP-independent release (Fig. 6) could reflect the exocytosis of glutamatergic vesicles, which were primed already at a step downstream of the ATP-dependent formation of the 20S fusion complex. Thirdly, ATP is hydrolysed by Ca^{2+}-activated protein- and lipid kinases involved in exocytosis. In permeated PC12 cells activation of phosphatidylinositol-4-phosphate 5-kinase appears to be essential in priming vesicles for fusion (Hay et al., 1995). Although compelling evidence demonstrates that activation of protein kinase C or Ca^{2+}/calmodulin-dependent protein kinase II facilitates glutamate release in synaptosomes (for reviews see Greengard et al., 1993; Sánchez-Prieto et al., 1996), we observed that the Ca^{2+}-induced glutamate release from permeated synaptosomes was not affected by the autoinhibitory protein kinase C peptide protein kinase C-(19–36) or kinase inhibitor H-7 at concentrations that almost completely inhibited protein kinase C-mediated B-50/GAP-43 phosphorylation in this preparation (Fig. 3). Similar findings were obtained for Ca^{2+}-induced noradrenaline and CCK-8 release (Hens et al., 1993a,b). These data therefore suggest that the phosphorylation state of proteins implicated in exocytosis, rather than the protein kinase C-mediated protein phosphorylation per se, is important in the regulation of glutamate release after the influx of Ca^{2+}. Our observations are in accordance with other studies demonstrating that a reduction in the ATP/ADP ratio of synaptosomes reduced Ca^{2+}-dependent glutamate release (Sánchez-Prieto et al., 1987; Verhage et al., 1989).

4.3. B-50 and regulated exocytosis of glutamate and noradrenaline

Several studies have demonstrated a role for B-50 in the release of catecholamines and neuropeptides stored in large dense-cored vesicles, but not yet for B-50 in the release of glutamate, the major excitatory neurotransmitter stored in small clear-cored vesicles. Here, we demonstrated the important role of B-50 in the exocytosis of glutamate. This was done by introducing site-specific anti-B-50 IgGs into streptolysin-O-permeated synaptosomes. The permeation conditions with streptolysin-O were carefully established for glutamate release from synaptosomes (Figs. 1 and 2), and confirmed the results of previous studies showing that the streptolysin-O-permeated synaptosome system is a suitable system to apply IgGs into the synaptosomal interior and to study their effects on Ca^{2+}-induced exocytosis of noradrenaline, dopamine and CCK-8 (Dekker et al., 1989; Hens et al., 1993a,b, 1996b). Here, we used the permeated synaptosome system to study the effects of anti-B-50 IgGs on Ca^{2+}-induced release of glutamate, the dominant neurotransmitter released by this preparation.

Functional interference studies revealed two major differences between the effects of anti-B-50 IgGs on glutamate and noradrenaline release (Figs. 7 and 8). Firstly, Ca^{2+}-induced glutamate release was inhibited less potently than Ca^{2+}-induced noradrenaline release. Apparently, the anti-B-50 IgGs were not able to block Ca^{2+}-induced glutamate release completely. The anti-B-50 IgG NM2 at a concentration higher than 50 μg/ml IgG did not further increase the inhibition of Ca^{2+}-induced glutamate release (Fig. 8), whereas Ca^{2+}-induced noradrenaline release was almost completely (75%) inhibited in this preparation (Fig. 7B). This lower, inhibitory potency was not due to an inability of the IgG to reach the site of glutamatergic vesicle fusion at the active zone, because Ca^{2+}-induced glutamate release from permeated synaptosomes was inhibited by monoclonal anti-calcineurin IgGs in a concentration-dependent manner, with almost complete inhibition occurring at 20 μg/ml IgG (Hens et al., 1998), and by the light chain fragment of tetanus toxin (Fig. 4). Because the relative number of pre-docked, fusion-ready vesicles in isolated nerve terminals is larger for small clear-cored (glutamatergic) vesicles than for large dense-cored (NA-containing) vesicles (Verhage et al., 1991), the most likely interpretation of these data is that the anti-B-50 IgGs do not interfere with the ultimate, Ca^{2+}-induced fusion step of pre-docked vesicles.

The second major difference is that anti-B-50 IgGs significantly reduced the basal release of glutamate but not of noradrenaline. The N-terminal-directed IgG NM2 was two-fold more potent than the C-terminal-directed IgG NM6 (Fig. 7A), although the latter IgG has a three- to 10-fold higher affinity for B-50 (Hens et al., 1995). In the same batches of permeated synaptosomes, basal noradrenaline release was not affected (Fig. 7B), indicating the selectivity of the anti-B-50 IgGs to affect basal glutamate release. To study whether this differential effect on basal release may be due to interference with vesicle recycling and/or recruitment, we determined the effect of anti-B-50 IgGs [^{3}H]-glutamate release. The major advantage of [^{3}H]-glutamate is that it hardly contributes to refilling of the recycled and recruited glutamatergic vesicles under our experimental conditions in permeated synaptosomes. The [^{3}H]-glutamate, which is preferentially taken up into the releasable pool of vesicular glutamate during pre-loading, is strongly diluted upon release into the permeation buffer, which contains micromolar concentrations of endogenous glutamate. Therefore, [^{3}H]-glutamate re-uptake during the release assay is negligible. Indeed, we observed that basal glutamate release from ^{3}H-labelled pools was much less

affected by the anti-B-50 IgGs (Fig. 8). Basal noradrenaline release was not affected by the anti-B-50 IgGs, probably because noradrenaline-containing, large dense-cored vesicles, in contrast to glutamatergic vesicles, do not recycle after Ca^{2+}-induced vesicle fusion. The vesicle recycling dye FM1-43 was used earlier to demonstrate Ca^{2+}-dependent, stimulus-induced endocytotic activity in intact synaptosomes (Meffert et al., 1994). Permeated synaptosomes, however, cannot be used with FM1-43 and thus the effects of anti-B-50 IgGs on endocytosis could not be tested (Hens and De Graan, unpublished data). The most likely interpretation of our data is that the anti-B-50 IgGs disturb the exocytotic cascade of glutamate release at the stage of vesicle recycling and/or recruitment, and although the anti-B-50 IgGs seem to act differently in the exocytotic cascades of noradrenaline and glutamate release, B-50 may fulfil a common role in these processes. The anti-B-50 IgGs partially decreased Ca^{2+}-induced glutamate release, suggesting that the majority of the small clear-cored vesicles attached at the active zone, which are ready for immediate fusion upon Ca^{2+} influx, were not affected. The noradrenaline-containing vesicles are not ready for immediate fusion and are thus more susceptible for inhibition by anti-B-50 IgGs. Both types of vesicles are retrieved after fusion, but only the glutamatergic vesicles are assumed to immediately enter a new cycle of exocytosis. Consequently, we observed a decreased basal release of glutamate but not of noradrenaline in the presence of anti-B-50 IgGs.

In conclusion, comparison of the effects of anti-B-50 IgGs on glutamate and noradrenaline release indicates that B-50 is more likely to be involved in vesicle recruitment and/or recycling than in the Ca^{2+}-induced vesicle fusion event per se. In fact, it is known that B-50 interacts with the actin filaments of the cytoskeleton, which are important in vesicle recycling and recruitment (Meiri and Gordon-Weeks, 1990; Strittmatter et al., 1992; Hens et al., 1993c). In accordance with our previous reports (Hens et al., 1993b, 1995, 1996b), the N-terminal domain of the B-50 protein, recognized by the NM2 IgG, and comprising the unique protein kinase C site and calmodulin-binding domain, seems to be particularly important for B-50 functioning in exocytosis. Moreover, our data indicate that the permeated synaptosome system is a suitable model system to study the relevance of proteins involved in the recycling and recruitment of glutamatergic, small clear-cored vesicles, as well as the proteins involved in the Ca^{2+}-induced fusion event per se.

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