Journal of Neurochemistry, 2001, **76**, 1153–1164

IPF, a vesicular uptake inhibitory protein factor, can reduce the Ca^{2+}-dependent, evoked release of glutamate, GABA and serotonin

Yutaka Tamura,*[,1] Eric D. Özkan,*[,2] David G. Bole* and Tetsufumi Ueda*[,†,‡]

**Mental Health Research Institute and Departments of †Pharmacology and ‡Psychiatry, Medical School, The University of Michigan, Ann Arbor, Michigan, USA*

Abstract

Synaptic vesicles in the nerve terminal play a pivotal role in neurotransmission. Neurotransmitter accumulation into synaptic vesicles is catalyzed by distinct vesicular transporters, harnessing an electrochemical proton gradient generated by V-type proton-pump ATPase. However, little is known about regulation of the transmitter pool size, particularly in regard to amino acid neurotransmitters. We previously provided evidence for the existence of a potent endogenous inhibitory protein factor (IPF), which causes reduction of glutamate and GABA accumulation into isolated, purified synaptic vesicles. In this study we demonstrate that IPF is concentrated most in the synaptosomal cytosol fraction and that, when introduced into the synaptosome, it leads to a decrease in calcium-dependent exocytotic (but not calcium-independent) release of glutamate in a concentration-dependent manner. In contrast, α-fodrin (non-erythroid spectrin), which is structurally related to IPF and thought to serve as the precursor for IPF, is devoid of such inhibitory activity. Intrasynaptosomal IPF also caused reduction in exocytotic release of GABA and the monoamine neurotransmitter serotonin. Whether IPF affects vesicular storage of multiple neurotransmitters *in vivo* would depend upon the localization of IPF. These results raise the possibility that IPF may modulate synaptic transmission by acting as a quantal size regulator of one or more neurotransmitters.

Keywords: exocytosis, neurotransmitter, quantal size, regulation, vesicular storage.

J. Neurochem. (2001) **76**, 1153–1164.

It is now widely accepted that glutamate functions as the major excitatory neurotransmitter in the vertebrate central nervous system (Fonnum 1984; Collingridge and Bliss 1987; Cotman *et al*. 1987; Watkins *et al*. 1990). As such, proper glutamate transmission is required for learning and memory formation (Collingridge and Bliss 1987; Cotman *et al*. 1988; Zalutsky and Nicoll 1990; Malgaroli and Tsien 1992; Bliss and Collingridge 1993). Aberrant glutamate transmission has been implicated in a variety of neurological and psychiatric disorders, including epilepsy, ischemia-induced neuronal cell death, Huntington's chorea, Alzheimer's disease and schizophrenia (Choi and Rothman 1990; Meldrum 1991; Coyle and Puttfarcken 1993; Bradford 1995; Bunney *et al*. 1995; Chapman 1998; Moghaddam and Adams 1998). Glutamate transmission is postsynaptically mediated by various, distinct types of receptors, namely, AMPA, kainate and NMDA receptor/channels, and metabotropic receptors coupled to phosphatidyl inositol bisphosphate lipase C and adenylate cyclase (Sladeczek *et al*. 1988; Monaghan *et al*. 1989; Watkins *et al*. 1990; Nakanishi 1992; Hollmann and Heinemann 1994).

Presynaptically, despite the past controversy, evidence has now accumulated to strongly support the concept that synaptic vesicles play a pivotal role in glutamate synaptic transmission (Ueda 1986; Nicholls 1989; Maycox *et al*. 1990; Özkan and Ueda 1998). Both highly specific,

Received 28 June, 2000; revised manuscript received 17 October, 2000; accepted 19 October, 2000.

Address correspondence and reprint requests to T. Ueda, MHRI, University of Michigan, 205 Zina Pitcher Place, Ann Arbor, MI 48109–0720, USA. E-mail: tueda@umich.edu

[1]On leave from the Department of Pharmacology, Fukuyama University School of Pharmacy, 1 Gakuen-cho, Fukuyama, Hiroshima 729–02, Japan.

[2]Present address: Central Michigan University, Department of Psychology, Mt. Pleasant, MI 48859, USA

Abbreviations used: DMSO, dimethylsulfoxide, IPF, inhibitory protein factor.

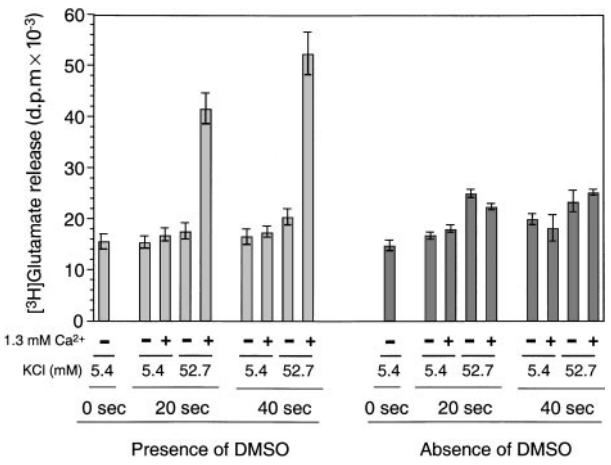


Fig. 1 Effect of DMSO presence, during the freeze/thaw period, on exocytotic release of glutamate. Synaptosomes were suspended in solution A (2 mg/mL) containing [^{3}H]glutamate, with or without 5% DMSO, and frozen for 3 min. Samples were then thawed and processed as described in Materials and methods. Glutamate release was allowed to proceed for 20 and 40 s. The amounts of [^{3}H] glutamate released at 0 s, 20 s (52.7 mM KCl, + Ca^{2+}), and 40 s (52.7 mM KCl, + Ca^{2+}) in the presence of DMSO represented 0.20 + 0.02%, 0.53 + 0.04%, and 0.66 + 0.06%, respectively, of the total [^{3}H] glutamate added to load synaptosomes. The data represent the mean ± SEM of 4 independent experiments, using 4 separate synaptosome preparations.

ATP-dependent, glutamate accumulation into isolated synaptic vesicles (Naito and Ueda 1983; Naito and Ueda 1985; Maycox *et al*. 1988; Cidon and Sihra 1989; Fykse *et al*. 1989; Tabb *et al*. 1992; Moriyama and Yamamoto 1995) and enrichment of glutamate in the synaptic vesicles (Storm-Mathisen *et al*. 1983; Burger *et al*. 1989) have been demonstrated. A variety of evidence indicates that glutamate is taken up into synaptic vesicles via a mechanism coupled to the electrochemical proton gradient $\Delta\mu_H{}^+$. This gradient is composed of the membrane potential ($\Delta\psi$) and the transmembrane pH difference (ΔpH), generated by a vacuolar type of proton-pump ATPase in the synaptic vesicle membrane. Although the membrane potential alone can drive glutamate uptake (Maycox *et al*. 1988; Cidon and Sihra 1989; Tabb *et al*. 1992), both elements of $\Delta\mu_H{}^+$, namely membrane potential and pH gradient, are important for maximal uptake activity seen under physiologically relevant Cl^- concentrations (Naito and Ueda 1985; Tabb *et al*. 1992; Wolosker *et al*. 1996; Bellocchio *et al*. 2000). It has recently been demonstrated that BNPI, a brain-specific Na^+-dependent inorganic phosphate transporter (Ni *et al*. 1994) functions as a vesicular glutamate transporter (Bellocchio *et al*. 2000; Takamori *et al*. 2000). However, it has a rather restricted distribution in the brain and hence is thought to represent an isoform of the vesicular glutamate transporter (Bellocchio *et al*. 2000). Nicholls and associates (Nicholls and Sihra 1986; McMahon and Nicholls 1991); Kish and Ueda (1991); Wilson and associates (Mehta *et al*. 1996) and Takamori *et al*. (2000) have provided evidence that glutamate is released from synaptic vesicles by an exocytotic mechanism.

Vesicular accumulation of glutamate thus plays a crucial role in directing the common biochemical glutamate toward the neurotransmitter pathway and diverting it away from the general metabolic pathway (Ueda 1986; Özkan and Ueda 1998). Since branch points in biochemical pathways are often sites of regulation, we postulated that vesicular glutamate uptake may be subject to such regulation. In light of these considerations, we obtained evidence for the existence of a potent inhibitory protein (Lobur *et al*. 1990; Özkan *et al*. 1997) that may serve as an endogenous modulator for this process. This protein, designated as inhibitory protein factor (IPF), inhibits ATP-dependent accumulation of not only glutamate but also GABA (γ-aminobutyric acid), whereas it exhibits no inhibition of Na^+-dependent glutamate uptake into synaptosomes (Özkan *et al*. 1997). IPF appears to be derived from the α subunit of the cytoskeletal protein fodrin (non-erythroid or brain spectrin), by at least two steps of cleavage, generating the distinct N-terminal sequence YHRFKELSTLRRAKLED-SYR, although the alternative possibility is not entirely ruled out that it is synthesized *de novo* according to the nucleotide sequence of a specific 'IPF mRNA'. In contrast to IPF, neither isolated fodrin nor its α subunit exhibits inhibitory activity. Although fodrin is known to occur in virtually all cells, contributing to the cytoskeletal structure (Goodman *et al*. 1995), the cellular and subcellular localization of IPF, as well as its physiological function, remains to be elucidated. In this communication, we report evidence suggesting that IPF is associated with synaptic vesicles. Moreover, in an effort to demonstrate that IPF functions within the nerve terminal as a potential regulator of exocytotic release of glutamate (and possibly of other neurotransmitters as well), we have introduced isolated IPF into synaptosomes and examined its effect on subsequent, depolarization-induced Ca^{2+}-dependent release of glutamate, GABA and serotonin. We present evidence here that isolated IPF, when permeated into synaptosomes, leads to a decrease in exocytotic release of these neurotransmitters. Part of this work was previously presented in abstract form (Tamura *et al*. 1998).

Materials and methods

Purification of IPF and fodrin

IPF was purified by the method of Özkan *et al*. (1997). Two Mono Q-purified fractions rich in IPFα and IPF$\beta\gamma$, respectively, were used in the experiments described here. The latter preparation contained a mixture of IPFβ and IPFγ. Fodrin and its α subunit were purified from bovine brain by the procedures described by Bennett *et al*. (1986) and Calvert *et al*. (1980), respectively.

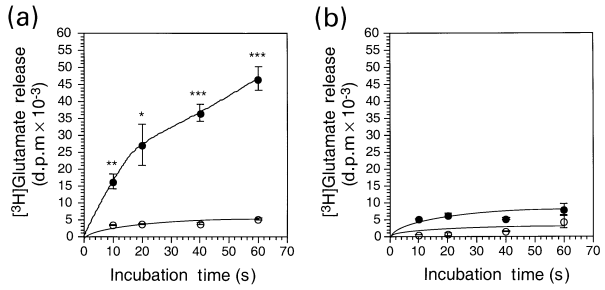


Fig. 2 Time course of glutamate release from permeabilized and resealed synaptosomes. Aliquots (150 μL) of [^{3}H]glutamate-loaded, permeabilized/resealed synaptosomes (2 mg/mL) were mixed with 150 μL each of solution RS 3 or RS 4 (a) and solution RS1 or RS2 (b), as described in Materials and methods, and incubated at 30°C for 0, 10, 20, 40 and 60 s. Samples were processed as described in Materials and methods. The value at 0-s incubation has been subtracted from all data. The values subtracted were 18 967 d.p.m. (Ca^{2+} present) and 20 337 d.p.m. (Ca^{2+} absent) for (a), and 16 348 d.p.m. (Ca^{2+} present) and 17 997 d.p.m. (Ca^{2+} absent) for (b), respectively. The amounts of [^{3}H] glutamate released during 60 s in the presence and absence of Ca^{2+} represented 0.67 + 0.034% and 0.04 + 0.002% for (a), and 0.05 + 0.02% and 0.03 + 0.015% for (b), respectively, of the total amount of [^{3}H] glutamate added to load synaptosomes. The data represent the mean ± SEM of 3 independent experiments, using 3 separate synaptosome preparations. (a) [^{3}H]glutamate release in the presence (●) or absence (○) of Ca^{2+} (1.3 mM) under depolarization conditions (i.e. in the presence of 52.7 mM KCl). (b) [^{3}H]glutamate release in the presence (●) or absence (○) of Ca^{2+} (1.3 mM) under non-depolarizing conditions (i.e. in the presence of 5.4 mM KCl). Unpaired Student's *t*-test *p*-values (open circle vs. closed circle): $^*p = 0.02$; $^{**}p = 0.005$; $^{***}p < 0.0001$.

Preparation of synaptosomes

Crude synaptosomes were prepared at 4°C by a modification of the method of Krueger *et al*. (1977). Male Sprague-Dawley rats (190–200 g) were killed by decapitation. The brain was removed within 1 min after decapitation and hemisected. Cerebellum, brainstem and white matter were removed from each hemisphere. One and a half grams of cerebrum were homogenized in 10 mL of ice-cold 0.32 M sucrose in 4 mM Tris–HCl, pH 7.4 (sucrose solution), with 12 up-and-down strokes in a glass-Teflon homogenizer. The homogenate was diluted with 5 mL of sucrose solution and centrifuged at 1000 g_{max} for 10 min. The supernatant was removed with a pipette and the pellet rehomogenized in 10 mL of sucrose solution with 4 up-and-down strokes, and centrifuged at 1000 g_{max} for 10 min. The supernatants were combined and centrifuged at 1000 g_{max} for 10 min, and the pellet discarded. The supernatant was then centrifuged at 12 500 g_{max} for 15 min; the pellet was washed by resuspending in 15 mL of sucrose solution and centrifuging at 12 500 g_{max} for 15 min. The resulting pellet (P2) was used as the crude synaptosome preparation in all experiments described here. Protein was quantified with the Coomassie Protein Assay Kit from Pierce (Rockford, IL, USA), with bovine serum albumin as standard.

Introduction of isolated IPF and fodrin into synaptosomes

Freshly prepared synaptosomes were frozen/thawed by a modification of the method of Nichols *et al*. (1989). The synaptosome preparation was resuspended in 200 μL of oxygenated solution A (140 mM K^+-gluconate; 4 mM KCl; 4 mM $MgSO_4$; 2 mM Tris–ATP; 20 mM HEPES; 50 μM glutamate, pH 7.4) at 3.75 mg/mL, except for experiments in Figs 1 and 2, in which the synaptosome protein concentration was 4 times higher. Isolated IPF was added to the synaptosome suspension. In control experiments, 50 μL of solution A were added instead of IPF. In vehicle experiments, 50 μL of the medium (300 mM NaCl in 20 mM Tris–HCl buffer, pH 7.4), in which IPF had been dissolved, were added, instead of IPF. To each mixture, 117.5 μL of solution A were added, followed by addition of 7.5 μL of L-[G-^{3}H]glutamate (50 Ci/mmol, Amersham Pharmacia Biotech, Piscataway, NJ, USA) (7.5 μCi). The total volume of the mixture was 375 μL, to which 375 μL of 10% dimethylsulfoxide (DMSO) (Fisher Scientific, Pittsburgh, PA, USA) in solution A were added, rendering the final concentration of DMSO 5%. The entire mixture (750 μL) was frozen for 3 min in an isopropyl alcohol/dry ice bath. The frozen samples were thawed at 1–4°C with periodic agitation over 10 min. Permeabilization and resealing of synaptosomes were carried out in the presence of Mg-ATP and the low concentration of chloride (4 mM) as described above, allowing selective glutamate uptake into synaptic vesicles within the synaptosome during the subsequent incubation at 30°C for 10 min. These conditions have been shown to give rise to maximal uptake of glutamate into isolated synaptic vesicles (Naito and Ueda 1985; Carlson *et al*. 1989; Fykse *et al*. 1989; Tabb *et al*. 1992; Hartinger and Jahn 1993; Wolosker *et al*. 1996; Bellocchio *et al*. 2000). This procedure permitted us to selectively monitor exocytotic release with ease. At the end of the thawing period, the samples were rapidly diluted with 10 mL of oxygenated balanced salt solution (BSS); 116.4 mM NaCl; 5.4 mM KCl; 1.3 mM $MgSO_4$; 0.92 mM Na_2HPO_4; 26.2 mM $NaHCO_3$; 11 mM glucose; 0.1 mM EGTA, pH 7.4) and centrifuged at 10 000 g_{max} for 10 min. The pellets were suspended in 1.5 mL of BSS and incubated for 10 min at 30°C. At the end of the incubation period, the extrasynaptosomal medium was removed by centrifugation at 10 000 g_{max} for 10 min. The pelleted synaptosomes were washed twice by resuspending in 10 mL of BSS and centrifuging at 10 000 g_{max} for 10 min. The washed synaptosome preparation was resuspended in 1.5 mL of oxygenated BSS. Isolated fodrin or α-fodrin was introduced into synaptosomes by the same method described above.

Assay for glutamate release

[^{3}H]Glutamate-loaded, resealed and washed synaptosomes (in 1.5 mL of BSS) were preincubated at 30°C for 1.5 min. Aliquots (150 μL) were mixed with 150 μL of the following preoxygenated release solutions at 30°C (RS 1–4) and incubated at 30°C for various periods of time as follows:

RS 1: 116.4 mM NaCl, 5.4 mM KCl, 1.3 mM $MgSO_4$, 0.92 mM Na_2HPO_4, 26.2 mM $NaHCO_3$, 11 mM glucose, 0.1 mM EGTA, and 4 mM DL-*threo*-β-hydroxy aspartate (pH 7.4)

RS 2: RS 1 plus 2.6 mM $CaCl_2$

RS 3: RS 1 plus 94.6 mM KCl minus 94.6 mM NaCl

RS 4: RS 3 plus 2.6 mM $CaCl_2$

Release was stopped by placing the samples on ice, followed by centrifugation at 10 000 g_{max} for 10 min at 4°C. Radioactivity in

the 250-μL aliquot of the supernatant was determined in a Beckman LS 6500 scintillation spectrophotometer.

Assay for GABA and serotonin release

The assay conditions for GABA and serotonin release were the same described for the glutamate release assay, except that (a) [^{3}H]glutamate was replaced by 7.5 μCi of 4-amino-*n*-[2,3–^{3}H] butyric acid (50 Ci/mmol) or 5-hydroxy[G-^{3}H]tryptamine, creatine sulfate (13.8 Ci/mmol) (both from Amersham Pharmacia Biotech), and (b) 50 μM glutamate was replaced by 50 μM GABA or 50 μM serotonin.

Monitoring IPF and Fodrin contents in the synaptosome

IPF and fodrin were incorporated into the synaptosome as described above, except for the omission of [^{3}H]glutamate. The IPF-or fodrin-containing synaptosomes described above were incubated for 10 min at 30°C, followed by centrifuged at 10 000 g_{max} for 10 min. The pellet was washed twice and resuspended in 1.5 mL of 6 mM Tris–maleate (pH 8.3) for 45 min to lyse synaptosomes, and centrifuged at 10 000 g_{max} for 10 min. The resulting supernatant was concentrated by using a vacuum centrifuge (~5-fold), and subjected to sodium dodecyl sulfate (SDS)/6% polyacrylamide gel electrophoresis, followed by electrophoretic transfer onto the nitrocellulose membrane (BioRad, Hercules, CA, USA); the transfer buffer contained 20% methanol, 192 nM glycine, and 25 mM Tris–HCl, pH 8.3. Blots were incubated with polyclonal antibodies raised against the N-terminal decapeptide of IPF (YHRFKELSTL) or of α-fodrin (MPSGVKVLE) and were visualized by enhanced chemiluminescence (ECL), according to the manufacturer's instructions (Amersham Pharmacia Biotech). Chemiluminescent blots were exposed to Hyperfilm ECL for 10 min at room temperature. The primary polyclonal antibodies to the N-terminal decapeptides of IPF and α-fodrin were raised in rabbits (Research Genetics, Huntsville, AL, USA); the former was shown to selectively recognize IPF and α-fodrin in a freshly prepared brain homogenate, as well as to interact with purified IPF and α-fodrin, as judged by Western blots probed with horseradish peroxidase (Amano *et al*. in preparation).

Subcellular fractionation

Subcellular fractions of rat brain were obtained by a modification of the procedure described in detail for canine cerebral cortex (Ueda *et al*. 1979). Briefly, five forebrains from 150–200 g Sprague-Dawley rats were homogenized in 10 volumes of 0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM EDTA, 1 mM EGTA, 3 mM benzamidine, and 0.3 mM phenylmethylsulfonyl fluoride (PMSF). Forebrains were dispersed by 10 strokes with a Dounce homogenizer (Thomas C270) rotating at 800 r.p.m. Differential centrifugation produced a nuclear and cell debris pellet (P1) (10 min, 1400 g_{ave}), a crude mitochondrial plus synaptosomal pellet (P2) (10 min, 13 800 g_{ave}), a microsomal pellet (P3) (60 min, 105 000 g_{ave}), and the final perikaryon supernatant (S1). The P1 pellet was washed once with the homogenization buffer. The P2 pellet was suspended in a minimal volume of homogenization buffer and osmotically shocked by addition of 9 volumes of ice cold 6 mM Tris–maleate, pH 8.1, containing 0.3 mM PMSF. After 45 min on ice, the osmotically shocked P2 fraction was centrifuged (20 min, 32 800 g_{ave}) to yield a plasma membrane/mitochondrial/myelin pellet (P4) and a supernatant containing synaptic vesicles (SV) and synaptosomal cytosol (S2). Crude synaptic vesicles (SV) and synaptosomal cytosol (S2) were separated by centrifugation (60 min, 105 000 g_{ave}). The crude synaptic vesicle fraction (SV) was suspended in 0.32 M homogenization buffer and subjected to centrifugation (120 min, 64 700 g_{ave}) on a discontinuous sucrose gradient consisting of equal volumes of 0.4 M, 0.6 M, and 0.8 M sucrose. Following centrifugation, five fractions were recovered and contained: the top 0.32 M sucrose sample layer and the 0.32 M/0.4 M sucrose interface (fraction A), 0.4 M sucrose layer and the 0.4 M/0.6 M sucrose interface (fraction B), 0.6 M sucrose layer and the 0.6/0.8 M sucrose interface (fraction C), 0.8 M sucrose layer (fraction D), and pelleted material at the bottom of the tube (fraction E). Each fraction A–D was diluted to 0.25 M sucrose and centrifuged (60 min, 105 000 g_{ave}) to pellet synaptic vesicles and membrane fragments. Resulting pellets were suspended in a minimal volume of homogenization buffer (0.32 M sucrose), assayed for protein content, and stored at −80°C. Fraction B had previously been shown to be rich in synaptic vesicles (Ueda *et al*. 1979). The amount of protein in each fraction was determined by the Bradford method (Pierce Coomassie Protein Assay Reagent) using bovine serum albumin as protein standard.

Western blotting of subcellular fractions

Proteins in the subcellular fractions obtained above were separated by SDS polyacrylamide gel (6%) electrophoresis (Laemmli 1970) and electrophoretically transferred onto the 0.45 μ nitrocellulose membrane (BioRad). The electrophoretic transfer buffer contained 15.6 mM Tris base, 120 mM glycine, and 0.1% SDS, pH 8.3. Electrophoretic transfer was conducted for 1 h at 100 V (constant voltage) in a BioRad mini trans-blot electrophoretic transfer cell. Nitrocellulose blots were blocked with 5% non-fat dried milk dissolved in TBS (20 mM Tris–HCl, 0.5 M NaCl, pH 8.3). Nitrocellulose blots were incubated with a primary antibody followed by an alkaline phosphase-conjugated goat antirabbit IgG antibody (Sigma). Unbound antiserum or alkaline phosphatase-conjugated secondary antibody was removed by 3 sequential washes with TBS containing 0.1% Tween 20. IPF antisera were raised in rabbits by immunization with the synthetic decapeptide corresponding to the N-terminal sequence of IPF (Özkan *et al*. 1997). BCIP and NBT were utilized as the substrate mixture for detection of bound alkaline phosphatase-conjugated secondary antibody (BioRad).

Results

Synaptosomes retain exocytotic ability after brief freeze treatment in DMSO

Synaptosomes, which had been frozen for 3 min in the presence of the cryoprotectant DMSO, followed by thawing at 1–4°C, were tested for the ability to exocytose the neurotransmitter glutamate. Fig. 1 shows K^+-evoked [^{3}H] glutamate release from synaptosomes frozen and thawed in the presence or absence of DMSO. Synaptosomes which underwent the freeze/thaw cycle in the presence of DMSO exhibited marked Ca^{2+}-dependent, K^+-evoked [^{3}H]glutamate release during 20- and 40-s incubation at

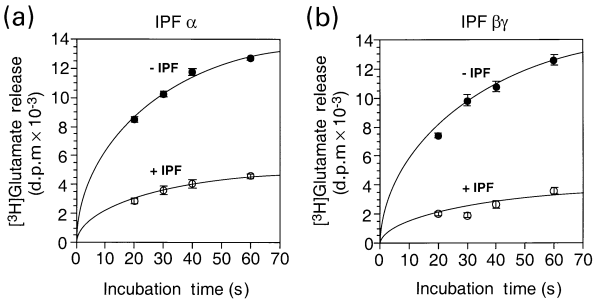
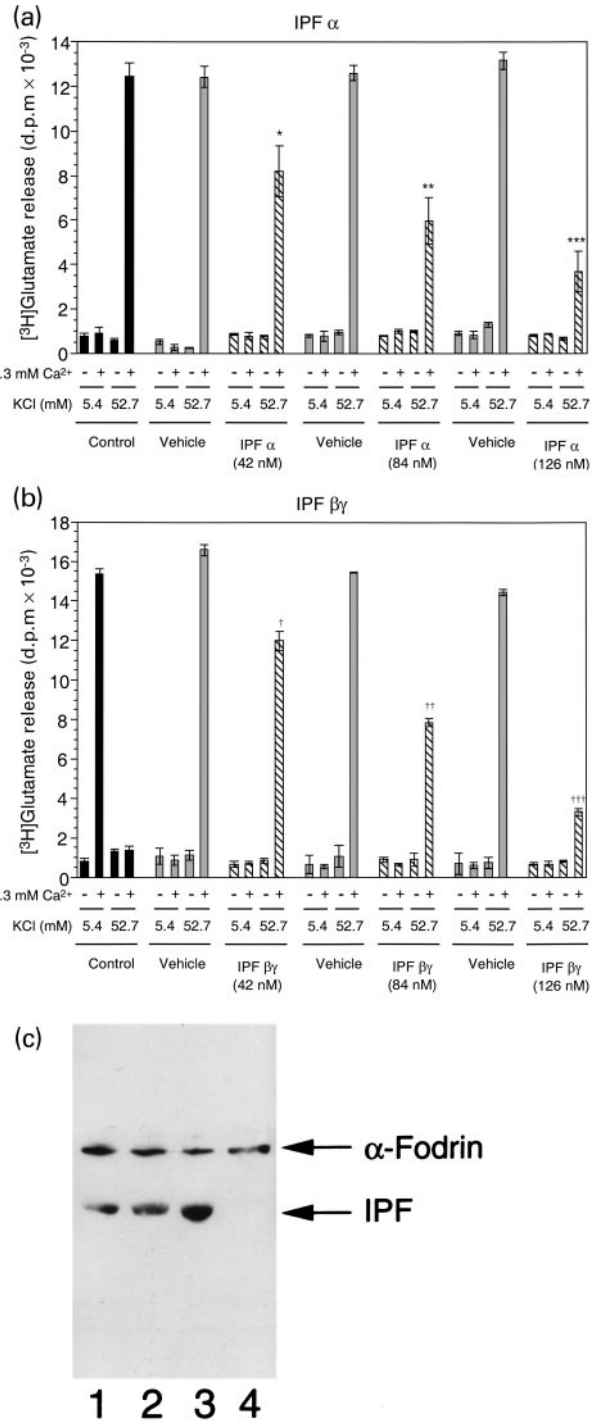


Fig. 3 Effect of IPF incorporated into synaptosomes on exocytotic glutamate release at various times. Incorporation into synaptosomes of Mono Q-purified IPFα was allowed at a concentration of 126 nM, as described in Materials and methods. In control experiments, an identical volume of an IPF-free solution containing 300 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethyl sulfonyl fluoride (PMSF), and 20 mM Tris–HCl, pH 7.6 (vehicle solution) was used. Glutamate release was allowed to proceed at 30°C for the various times indicated. Values at 0-s incubation have been subtracted from all data; the values subtracted were 5006 d.p.m. (– IPF; ●) and 3738 d.p.m. (+ IPF; ○) for (a), and 4639 d.p.m. (– IPF; ●) and 5230 d.p.m. (+ IPF; ○) for (b). The amount of [^{3}H] glutamate released during 60 s in the absence and presence of IPF represented 0.21 + 0.007% and 0.07 + 0.004% for (a), and 0.21 + 0.019% and 0.06 + 0.009% for (b), respectively, of the total amount of [^{3}H] glutamate added to load synaptosomes. The data represent the mean ± SEM of 3 independent experiments, using 3 separate synaptosome preparations.

Fig. 4 Glutamate release and intrasynaptosomal content of IPF as a function of added amount of IPF. (a,b) Glutamate release. Synaptosomes were permeabilized and resealed in the presence of various amounts of isolated IPF (0, 42, 84, and 126 nM for IPFα [a] or IPFβγ [b]) and subjected to [^{3}H]glutamate release for 40 s at 30°C, as described in Materials and methods. The value at 0-s incubation has been subtracted from all data. The values subtracted ranged from 4378 (IPFα, 126 nM) to 5485 (vehicle for 42 nM IPFα) d.p.m. for (a), and 4327 (IPFβγ, 84 nm) to 5485 (vehicle for 42 nM IPFβγ) d.p.m. for (b). The amounts of [^{3}H] glutamate released in the presence of Ca^{2+} and 52.7 mM KCl represented 0.16–0.17% for the vehicles in (a) and 0.16–0.18% for the vehicles in (b), respectively, of the total amount of [^{3}H] glutamate added to load synaptosomes. The data represent the mean ± SEM of 3 independent experiments, using 3 separate synaptosome preparations. Student's two-tailed *t*-test *p*-values (vs. vehicle): $*p = 0.027$; $**p = 0.004$; $***p = 0.0006$; $+p = 0.0002$; $++p < 0.0001$; $+++p < 0.0001$. (c) IPF content. Synaptosomes were subjected to permeabilization and resealing in the presence of purified IPF, 42 (lane 1), 84 (lane 2), and 126 (lane 3) nM or in its absence (lane 4), followed by washing as described in Materials and methods. Washed synaptosomes were incubated for 10 min at 30°C and lysed; intrasynaptosomal IPF was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/ECL, using IPF N-terminal decapeptide antibodies as the probe, as described in Materials and methods.

30°C. In the absence of Ca^{2+}, K^+-evoked [^{3}H]glutamate release was not observed at either incubation time. Little or no release of [^{3}H]glutamate was observed under non-depolarizing conditions, whether or not Ca^{2+} was present. These results were essentially the same observed with DMSO-untreated, unfrozen ('intact') synaptosomes (data not shown). In contrast, when synaptosomes were subjected to freeze/thaw treatment without DMSO, no K^+-evoked,



Ca^{2+}-dependent release of [^{3}H]glutamate was observed. These results indicate that the synaptosome can retain its primary functional property, namely, the ability to release a neurotransmitter (glutamate, in this case) in response to depolarization in the presence of Ca^{2+}, even when it is subjected to freeze/thaw treatment, so long as DMSO (e.g. 5%) is present during the freeze/thaw cycle. These observations are in agreement with those previously reported by Nichols *et al*. (1989), indicating that synaptosomes are permeabilized and resealed by freeze/thaw treatment in the presence of DMSO.

Time course of glutamate release from permeabilized/resealed synaptosomes

Fig. 2 shows that K^+-evoked, Ca^{2+}-dependent [^{3}H]glutamate release from the permeabilized and resealed synaptosome is dependent on incubation time. The Ca^{2+}-dependent, evoked release appears to begin to slow down at around 20 s under these conditions. In the absence of Ca^{2+}, K^+-induced depolarization elicited little if any release at any of the incubation times tested (Fig. 2a). In the absence of depolarization, Ca^{2+} caused little if any release of [^{3}H]glutamate (Fig. 2b).

IPF inhibits [^{3}H] glutamate release from synaptosomes

To investigate the effect of IPF on depolarization-induced Ca^{2+}-dependent release of glutamate, IPF was introduced into synaptosomes as described in Materials and methods. The amount of [^{3}H]glutamate released from IPFα-treated synaptosomes was about 65% smaller than that from control synaptosomes, at all incubation times tested (Fig. 3a). Since IPFβγ, a mixture of IPFβ and IPFγ presumably produced during IPF purification, also inhibited [^{3}H] glutamate uptake into isolated synaptic vesicles with almost identical potency (Özkan *et al*. 1997; also in this study), we have examined the effect of introduction of IPFβγ into synaptosomes on glutamate release. As shown in Fig. 3(b), IPFβγ reduced [^{3}H]glutamate release from synaptosomes throughout the incubation period. The IPFβγ-induced inhibition of [^{3}H]glutamate release at 20, 30, 40 and 60 s incubation was 73, 81, 75 and 72%, respectively.

Concentration-dependence of inhibition by IPF

Fig. 4(a) shows the effect of increasing the concentration of IPFα on [^{3}H]glutamate release. IPFα reduced Ca^{2+}-dependent, K^+-evoked [^{3}H]glutamate release from synaptosomes in a dose-dependent manner. The IPFα-induced inhibition of [^{3}H]glutamate release at 42, 84, and 126 nM was 26, 43 and 61%, respectively. These are calculated concentrations in the medium during the freeze/thaw and do not necessarily represent actual IPFα concentrations in the permeabilized and resealed synaptosomes; the latter may be lower than the former. This preparation of IPF(α or βγ) caused 50% inhibition of vesicular glutamate uptake into

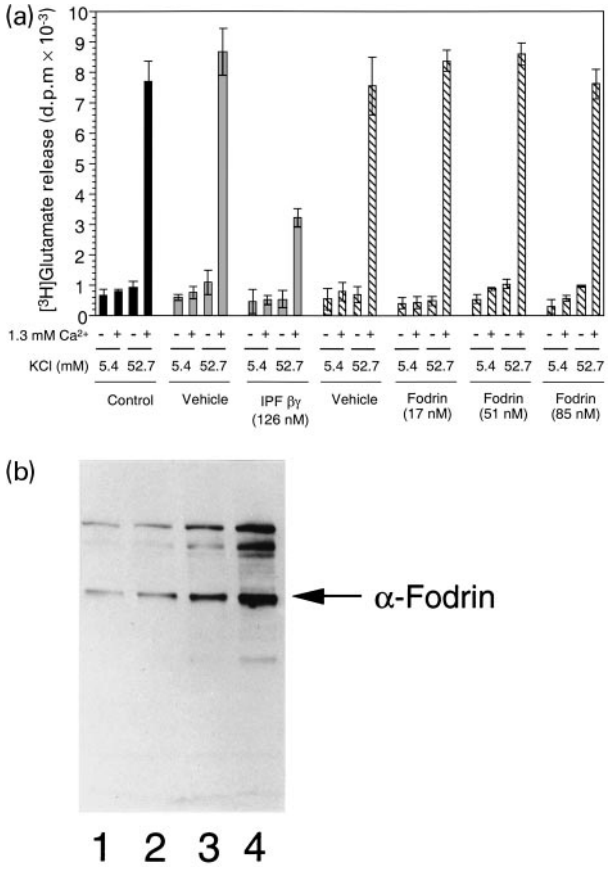


Fig. 5 Glutamate release and intrasynaptosomal content of α-fodrin as a function of added amount of fodrin. (a) Glutamate release. Synaptosomes were permeabilized and resealed in the presence of various amounts of isolated fodrin (0, 17, 51 and 85 nM) or of IPFβγ (0 and 126 nM as positive control), and subjected to [^{3}H]glutamate release for 40 s at 30°C, as described in Materials and methods. Fodrin was purified in a solution consisting of 10 mM NaH_2PO_4, 0.2 mM NaEDTA, 1 mM NaN_3 and 0.4 mM dithiothreitol, pH 7.5. The vehicle is the medium described above, used for the isolation of fodrin. The value at 0 s has been subtracted from all data. The values subtracted ranged from 2876 (control) to 3961 (fodrin, 17 nM) dpm. The amounts of [^{3}H] glutamate released in the presence of Ca^{2+} and 52.7 mM KCl for the IPFβγ and fodrin vehicles represented 0.14 + 0.016% and 0.12 + 0.016%, respectively, of the total amount of [^{3}H] glutamate added to load synaptosomes. The data represent the mean ± SEM of 3 independent experiments, using 3 separate synaptosome preparations. (b) Fodrin content. Synaptosomes were subjected to permeabilization and resealing in the absence (lane 1) or presence of purified fodrin, 17 nM (lane 2), 51 nM (lane 3), and 85 nM (lane 4), as described in Materials and methods, followed by the treatment described in the Fig. 4 legend. α-Fodrin and immunoreactive processed α-fodrin were detected by SDS-PAGE/ECL, using the α-fodrin N-terminal decapeptide antibodies as the probe.

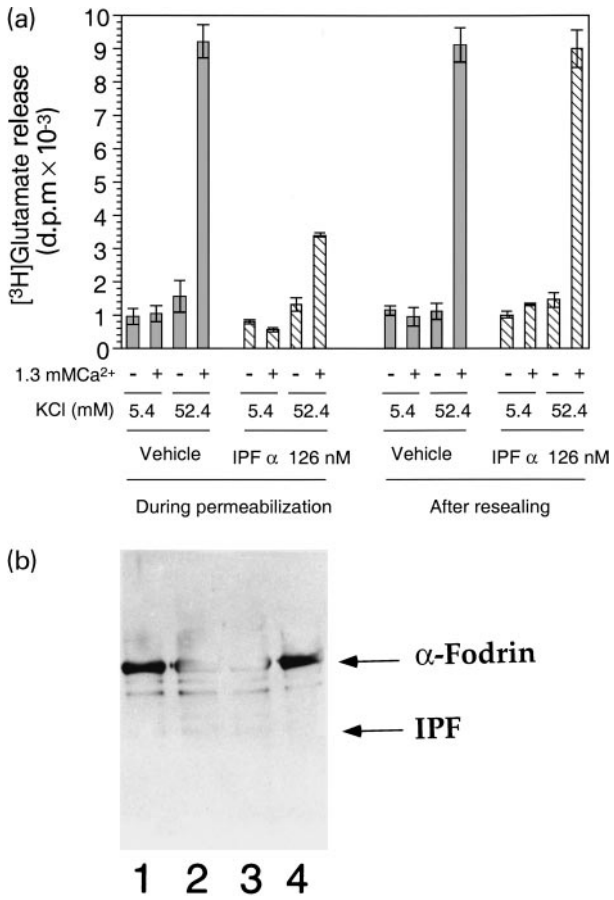


Fig. 6 Effects of extrasynaptosomal IPF on [^{3}H]glutamate release from permeabilized/resealed synaptosomes and on intrasynaptosomal IPF content. (a) Glutamate release. Synaptosomes were first subjected to permeabilization (without IPF), resealing, washing, incubation at 30°C for 10 min, and two final washings, and suspended in BSS, as described in Materials and methods. IPFα was then added to give a final concentration of 126 nM (*after resealing*). Aliquots (250 μL) were assayed for [^{3}H]glutamate release as described in Materials and methods. In parallel positive control experiments, synaptosomes were permeabilized and resealed in the presence of IPFα 126 nM, further processed, and aliquots (250 μL) analyzed for [^{3}H]glutamate release, in the same manner described in the Fig. 4 legend. The value at 0 s has been subtracted from all data. The values subtracted were 3264 d.p.m. (vehicle) and 3549 d.p.m. (IPFα, 126 nM), for 'during permeabilization', and 3456 d.p.m. (vehicle) and 3396 d.p.m. (IPFα, 126 nM) for 'after permeabilization'. The amount of [^{3}H] glutamate released in the presence of Ca^{2+} and 52.4 mM KCl for both vehicles represented 0.15% of the total amount of [^{3}H] glutamate added to load synaptosomes. The data represent the mean ± SEM of 3 independent experiments, using 3 separate synaptosome preparations. (b) Intrasynaptosomal content of IPF. IPFα was added to the resealed synaptosomes just prior to the [^{3}H] glutamate release period, to give final concentrations of 0 (lane 1), 42 nM (lane 2), 84 nM (lane 3), and 126 nM (lane 4), as described above. Synaptosomes were lysed; the lysate was concentrated and analyzed for IPF, as described in the Fig. 4 legend.

isolated synaptic vesicles at 42 nM (data not shown). This is comparable to the IPF potency on release; the apparent, though small, discrepancy could be due to potential incomplete equilibration of IPF concentration between the outside and inside of the synaptosome during the freeze/thaw period. IPF did not affect [^{3}H]glutamate release in the absence of Ca^{2+} under depolarizing conditions or basal release. Similar dose-dependent inhibition of [^{3}H]glutamate release was also observed with IPFβγ (Fig. 4b). In order to confirm that IPF was indeed introduced into synaptosomes by freeze/thaw treatment, the synaptosomal cytosol fraction was assayed for IPF by Western blot with anti-IPF N-terminal peptide antiserum. As shown in Fig. 4(c), the intrasynaptosomal content of IPF was increased as a function of the amount of IPF present in the medium during the freeze/thaw period. In the absence of added IPF, IPF was hardly detected. This could be due to its leakage into the larger volume of the medium, as a result of the freeze/thaw-induced permeabilization and resealing. These results support the notion that an increase in IPF within the synaptosome leads to a decrease in the amount of [^{3}H] glutamate released by the exocytotic mechanism.

Fodrin introduction into synaptosomes does not alter [^{3}H]glutamate release

Sequence analysis of IPF suggests that IPF is produced by at least two steps of proteolytic cleavage, from the α subunit of fodrin, a major cytoskeletal protein (Özkan *et al*. 1997). IPF thus formed has the N-terminal sequence distinct from that of α-fodrin; the C-terminal sequence of IPF is yet to be determined. In contrast to IPF, neither isolated fodrin nor α-fodrin exhibited inhibitory activity in glutamate uptake into isolated synaptic vesicles (data not shown). Here, isolated fodrin was incorporated into synaptosomes by the same procedure used for introduction of IPF into synaptosomes. Its effect on [^{3}H]glutamate release from these synaptosomes was then compared with the effect of incorporation of IPF determined in parallel experiments. As shown in Fig. 5(a), introduction of isolated fodrin had no effect on [^{3}H]glutamate release even at the highest concentration tested (85 nM), in contrast to IPFα, which exhibited substantial inhibition at this concentration (see Fig. 4a). At 126 nM, IPFα and IPFβ caused similar degrees of inhibition in the experiments shown in Figs 4(a) and 5(a). Moreover, introduction of isolated α-fodrin (at 293 nM, calculated based upon the molecular weight of 284 000) into synaptosomes led to no inhibition of glutamate release (data not shown). Fig. 5(b) indicates that fodrin is incorporated into synaptosomes in a concentration-dependent manner. Fodrin's inability to affect [^{3}H] glutamate release is therefore not due to its failure to enter the synaptosome. The two prominent bands above the α-fodrin band probably represent aggregated forms of α-fodrin.

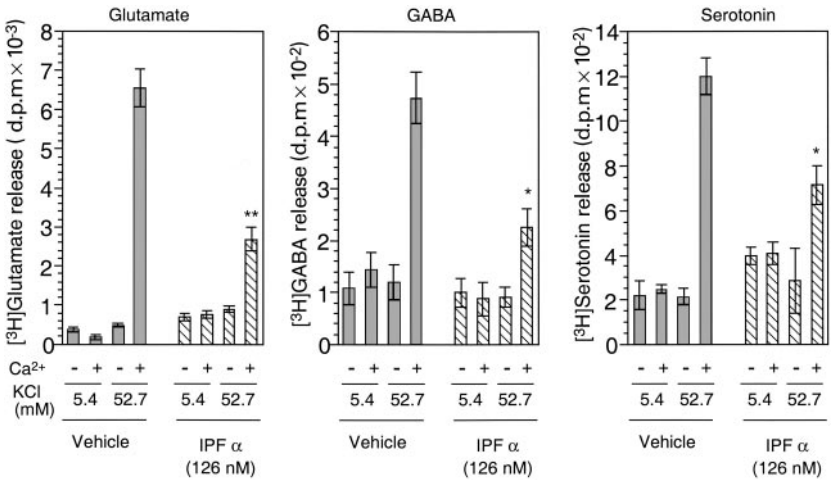


Fig. 7 Neurotransmitter specificity of IPF. The assay conditions for GABA and serotonin release were the same described for the glutamate release assay, except that [^{3}H]glutamate was replaced by 7.5 μCi of [^{3}H]GABA or 5-hydroxy[^{3}H]tryptamine, creatine sulfate, and 50 μM glutamate by 50 μM GABA or 50 μM 5-hydroxytryptamine, creatine sulfate. Synaptosomes were permeabilized and resealed in the absence or presence of IPFα 126 nM and then subjected to the release assay as described in the Fig. 4 legend. The value at 0 s has been subtracted from all data. The values subtracted were 3504 d.p.m. (vehicle) and 3500 d.p.m. (IPFα) for glutamate; 930 d.p.m. (vehicle) and 920 d.p.m. (IPFα) for GABA; and 1743 d.p.m. (vehicle) and 1851 d.p.m. (IPFα) for serotonin. The amounts of [^{3}H] glutamate released in the presence of Ca^{2+} and 52.7 nM KCl, in the absence of IPFα, for the glutamate, GABA, and serotonin represented 0.11 + 0.008%, 0.022 + 0.0004%, and 0.083 + 0.007%, respectively, of the total amount of [^{3}H] glutamate, GABA, and serotonin added to load synaptosomes. The data represent the mean ± SEM of 3 independent experiments, using 3 separate synaptosome preparations. *Two-tailed *p*-values: $^*p = 0.0024$; $^{**}p = 0.016$.

Extrasynaptosomal IPF has no effect on [^{3}H] glutamate release

In order to confirm that IPF affects glutamate release intrasynaptosomally as opposed to extrasynaptosomally, synaptosomes were first permeabilized, resealed, washed and then subjected to glutamate release in the presence or absence of IPF (126 nM) under the four conditions described in the Fig. 4 legend. Under these conditions, IPF had no effect on glutamate release from synaptosomes (Fig. 6a). Fig. 6(b) shows that IPF was hardly entrapped into synaptosomes when added after synaptosomes had been resealed. These results indicate that IPF does not act from the exterior of the synaptosome but rather functions within the synaptosome.

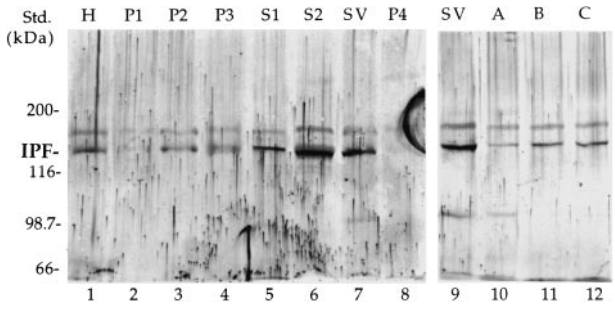


Fig. 8 IPF immunoblot of rat brain subcellular fractions. Each subcellular fraction (100 μg of protein) was subjected to SDS polyacrylamide gel (6%) electrophoresis, transferred to a nitrocellulose membrane, and immunoblotted with an antiserum raised against IPF N-terminal decapeptide. Lane 1, homogenate (H); Lane 2, nuclear and cell debris pellet (P1); Lane 3, synaptosomal and mitochondrial pellet (P2); Lane 4, microsomal pellet (P3); Lane 5, perikaryon cytosol supernatant (S1); Lane 6, synaptosomal cytosol (S2); Lanes 7 and 9, crude synaptic vesicles (SV); Lane 8, plasma membrane/mitochondria/myelin fraction (P4); Lane 10, crude synaptic vesicle subfraction A (0.32 M sucrose); Lane 11, crude synaptic vesicle subfraction B (0.40 M sucrose); Lane 12, crude synaptic vesicle subfraction C (0.60 M sucrose).

Neurotransmitter specificity of IPF action

IPF has been shown to inhibit not only accumulation of glutamate into isolated synaptic vesicles but also accumulation of GABA (Özkan *et al*. 1997), suggesting that IPF may not specifically regulate glutamate storage *in vivo*. In order to gain insight into this matter, we have determined the effect of IPF incorporation into synaptosomes on the subsequent exocytotic release of GABA and serotonin (a representative monoamine neurotransmitter). As shown in Fig. 7, IPFα also reduced depolarization-induced, Ca^{2+}-dependent release of GABA and serotonin, although the magnitude of these effects was somewhat smaller than the effect on glutamate release. IPFα-induced inhibition of exocytotic release of [^{3}H]glutamate, [^{3}H]GABA, and [^{3}H]serotonin was 71, 58 and 54%, respectively. These

results raise the possibility that IPF could affect vesicular storage of multiple neurotransmitters *in vivo*.

Subcellular distribution of IPF

In order for IPF to play a physiological role as a vesicular neurotransmitter storage inhibitor, IPF would have to occur in the nerve ending. To address this issue, a freshly prepared rat brain homogenate was subfractionated and each subcellular fraction analyzed for IPF. Fig. 8 shows that IPF ($M_r = 138\,000$) is present in the rapidly prepared homogenate, indicating that it occurs in the intact cell; this rules out the possibility that IPF is a purification artifact. Of interest, the Figure also shows that IPF, of all the subcellular fractions tested, is most concentrated in the synaptosomal cytosol fraction. It was hardly detected in the nuclear fraction and the plasma membrane/mitochondrian/myelin fraction derived from the P2 fraction. However, significant amounts of IPF were found in the purified synaptic vesicle fraction (Fig. 8, lane 11); this is consistent with high affinity of IPF for synaptic vesicles, as judged by its high potency in vesicular glutamate/GABA uptake inhibition (Özkan *et al*. 1997).

Discussion

Neural communication is thought to be most effectively modulated at the level of synaptic transmission. A variety of evidence indicates that there are diverse modes of presynaptic as well as postsynaptic modulation. Presynaptic regulation results in an alteration in the amount of neurotransmitter released from the nerve terminal per given period of time. This regulation can be achieved by modulating (a) voltage-dependent calcium channels (Dolphin 1990; Miller 1990; Baskys and Malenka 1991; Barrie and Nicholls 1993; Colmers and Bleakman 1994; Dittman and Regehr 1996; Stefani *et al*. 1996; Takahashi *et al*. 1996); (b) potassium channels (Kandel and Schwartz 1992; Augustine 1990; Herrero *et al*. 1992; Robitaille and Charlton 1992); (c) the Na^+/Ca^{2+} exchanger (Gleason *et al*. 1994; Bouron and Reuter 1996); (d) the nicotinic acetylcholine receptor non-selective cation channel (McGehee *et al*. 1995); (e) the cyclic nucleotide-gated channel (Rieke and Schwartz 1994; Savchenko *et al*. 1997); (f) transition from the neurotransmitter pool reserve state to the ready-release state, or priming process (Bittner and Holz 1992; Greengard *et al*. 1993; Neher and Zucker 1993; Smith *et al*. 1998); and (g) the final exocytotic release process (Prince and Stevens 1992; Scholz and Miller 1992; Silinsky and Solsona 1992; Chavez-Noriega and Stevens 1994; Capogna *et al*. 1996; Dittman and Regehr 1996; Trudeau *et al*. 1996; Kinney *et al*. 1998), not to mention neurotransmitter synthesis. In addition, it is conceivable that the amount of neurotransmitter to be released could be altered by varying the synaptic vesicleís transmitter storage activity or capacity. Relatively little attention had been paid to this aspect of the endogenous regulation of neurotransmission.

Observations presented in this communication provide evidence that endogenous substances in the nerve terminal, such as IPF, might play a role in regulating neurotransmitter release by inhibiting neurotransmitter accumulation into synaptic vesicles. We have previously provided evidence that isolated IPF potently inhibits glutamate uptake into isolated synaptic vesicles; IPF is devoid of inhibitory activity toward ATP-independent glutamate uptake as well as toward Na^+-dependent glutamate uptake (Özkan *et al*. 1997). In the present study, we have loaded synaptic vesicles within the synaptosomes with [^{3}H] glutamate, using conditions optimal for glutamate uptake into isolated synaptic vesicles (i.e. in the presence of Mg-ATP and a low millimolar chloride) in the absence or presence of IPF, and measured the subsequent [^{3}H] glutamate release. We have shown that IPF leads to a concentration-dependent reduction of exocytotic release of glutamate over time when introduced, together with [^{3}H] glutamate, into synaptosomes.

The potency of IPF in release ($IC_{50} = \sim 84$ nM) was found to be in general agreement with that in glutamate uptake into isolated synaptic vesicles ($IC_{50} = 26$ nM). This would argue for the notion that the IPF-induced decrease in the amount of exocytotically released glutamate is mediated by inhibition by IPF of vesicular glutamate uptake. IPF does not bear the actin-binding domain of fodrin; IPF is within the N-terminal half of α-fodrin (Özkan *et al*. 1997) and actin binds to the N-terminal region of β-fodrin (Karinch *et al*. 1990). This rules out the possibility that the inhibitory effect of IPF on release is mediated by an alteration in the interaction with actin. The role of actin in mediating IPF-induced inhibition is also ruled out since incorporation into synaptosomes of purified fodrin, which is capable of interacting with actin (Goodman *et al*. 1995), failed to affect [^{3}H] glutamate release. However, alternative possibilities, such as the effect on other vesicle priming steps and the final release step, in addition to or instead of vesicular storage of transmitters, are not ruled out at present. It also remains to be determined whether synaptic vesicle glutamate content is reduced within the IPF-containing synaptosomes.

Since discovery of the miniature endplate potential at the neuromuscular junction and formulation of the quantal release theory (Fatt and Katz 1952; Del Castillo and Katz 1954), much study had focused on alteration of quantal content (quantal number) and its mechanism. We postulate that IPF might function as an endogenous regulator of this quantal release. Recent evidence points to the notion that quantal size is variable and that vesicular neurotransmitter content can be altered (Doherty *et al*. 1984; Whitton *et al*. 1986; Bekkers *et al*. 1990; Van der Kloot 1991; Stevens 1993). At the neuromuscular synapse, quantal size can be changed under certain conditions, such as prolonged

stimulation, an increase in intracellular Ca^{2+}, exposure to acetylcholine agonists and hypertonic solutions, as well as exposure to vesamicol, a potent inhibitor of the vesicular acetylcholine transporter. More recently, Brailoiu and Van der Kloot (1996) have shown that acetylcholine quantal size is reduced by introduction of acetylcholine esterase or the choline acetyltransferase inhibitor bromoacetylcholine into motor nerve endings. In the central nervous system, Pothos *et al*. (1998) have provided direct evidence that the number of dopamine molecules per releasable quantum from mid-brain dopaminergic neurons is increased by exposure either to a glial-derived neurotrophic factor (GDNF) or to the dopamine precursor L-dihydroxyphenylalanine (L-DOPA).

IPF may represent the first example of an endogenous substance which can intracellularly control quantal size of amino acid neurotransmitters, not by regulating their synthesis, but by modulating their uptake into synaptic vesicles. Controlling quantal size may be important in preventing neurotransmitter overflow onto neighboring synapses and/or abnormally prolonged activation of postsynaptic receptors, as well as in modulating build-up of the threshold potential required for triggering the action potential.

Evidence presented here indicates that, under *in vitro* conditions, IPF introduced into the synaptosome is capable of decreasing the amount of exocytotically released glutamate, GABA and serotonin. This suggests that IPF can inhibit uptake of monoamine transmitters, as well as of glutamate and GABA, into synaptic vesicles within synaptosomes. This is somewhat at odds with previous work which demonstrated that IPF has little effect on catecholamine uptake into dense-core granules isolated from chromaffin cells (Özkan *et al*. 1997). Amino acid neurotransmitters are stored in clear, small synaptic vesicles, whereas monoamine transmitters, including serotonin, are stored in both clear vesicles and dense-core vesicles (Maley *et al*. 1990; Bruns and Jahn 1995; Johnson and Yee 1995). These observations, taken together, raise the possibility that several, if not all, types of *clear* vesicles may share a common IPF binding site or protein, deficient in dense-core vesicles. Consistent with the proposed role of IPF as a presynaptic regulator of exocytotic glutamate release, specific content of IPF was found to be highest in the synaptic vesicle fraction of all brain subcellular fractions tested (Fig. 8). Whether IPF regulation occurs with multiple types of neurotransmitters *in vivo* is not known. It would depend upon whether IPF is present, for example, in the glutamatergic, GABAergic, or monoaminergic nerve terminal.

IPF is structurally related to, but functionally distinct from, the α subunit of the major cytoskeletal protein fodrin. In contrast to IPF, neither isolated fodrin nor its α subunit affects vesicular glutamate uptake or glutamate release. IPF differs from α-fodrin in its N-terminal sequence and presumably in its C-terminal sequence as well (Özkan *et al*. 1997). Based upon amino acid sequence analysis, it is thought that IPF is produced from α-fodrin via two or more steps of cleavage. However, how IPF is produced and its activity regulated remain to be elucidated.

Acknowledgements

This work was supported by grants from the National Institutes of Health (Javits Neuroscience Investigator Award NS 26884 and NS 36656) to TU, and by National Institute of Mental Health Postdoctoral Training Grant (MH 15794–18) to EDÖ. We thank Mary Roth for excellent assistance in the preparation of the manuscript.

References

Augustine G. J. (1990) Regulation of transmitter release at the squid giant synapse by presynaptic delayed rectifier potassium current. *J. Physiol.* **431**, 343–364.

Barrie A. P. and Nicholls D. G. (1993) Adenosine A_1 receptor inhibition of glutamate exocytosis and protein kinase C-mediated decoupling. *J. Neurochem.* **60**, 1081–1086.

Baskys A. and Malenka R. C. (1991) Agonists at metabotropic glutamate receptors presynaptically inhibit EPSCs in neonatal rat hippocampus. *J. Physiol.* **444**, 687–701.

Bekkers J. M., Richerson G. B. and Stevens C. F. (1990) Origin of variability in quantal size in cultured hippocampal neurons and hippocampal slices. *Proc. Natl Acad. Sci. USA* **87**, 5359–5362.

Bellocchio E. E., Reimer R. J., Fremeau R. T. Jr and Edwards R. H. (2000) Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science* **289**, 957–960.

Bennett V., Baines A. J. and Davis J. (1986) Purification of brain analogs of red blood cell membrane skeletal proteins: ankyrin, protein 4.1 (synapsin), spectrin, and spectrin subunits. *Meth. Enzymol.* **134**, 55–69.

Bittner M. A. and Holz R. W. (1992) Kinetic analysis of secretion from permeabilized adrenal chromaffin cells reveals distinct components. *J. Biol. Chem.* **267**, 16219–16225.

Bliss T. V. P. and Collingridge G. L. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature* **361**, 31–39.

Bouron A. and Reuter H. (1996) A role of intracellular Na^+ in the regulation of synaptic transmission and turnover of the vesicular pool in cultured hippocampal cells. *Neuron* **17**, 969–978.

Bradford H. F. (1995) Glutamate, GABA and epilepsy. *Prog. Neurobiol.* **47**, 477–511.

Brailoiu E. and Van der Kloot W. (1996) Bromoacetylcholine and acetylcholinesterase introduced via liposomes into motor nerve endings block increases in quantal size. *Pflugers Arch. – Eur. J. Physiol.* **432**, 413–418.

Bruns D. and Jahn R. (1995) Real-time measurement of transmitter release from single synaptic vesicles. *Nature* **377**, 62–65.

Bunney B. G., Bunney W. E. Jr and Carlsson A. (1995) Schizophrenia and glutamate, in *Psychopharmacology: the fourth generation of progress* (Bloom F. E. and Kupfer D. J., eds), pp. 1205–1214. Raven Press, New York.

Burger P. M., Mehl E., Cameron P. L., Maycox P. R., Baumert M., Lottspeich F., De Camilli P. and Jahn R. (1989) Synaptic vesicles

immuno-isolated from rat cerebral cortex contain high levels of glutamate. *Neuron* **3**, 715–720.

Calvert R., Bennett P. and Grazer W. (1980) Properties and structural role of the subunits of human spectrin. *Eur. J. Biochem.* **107**, 355–361.

Capogna M., Gahwiler B. H. and Thompson S. M. (1996) Presynaptic inhibition of calcium-dependent and-independent release elicited with ionomycin, gadolinium, and α-latrotoxin in the hippocampus. *J. Neurophysiol.* **75**, 2017–2028.

Carlson M. D., Kish P. E. and Ueda T. (1989) Solubilization of the ATP-dependent vesicular glutamate uptake system and its reconstitution into liposomes. *J. Biol. Chem.* **264**, 7369–7376.

Chapman A. G. (1998) Glutamate receptors in epilepsy. *Prog. Brain Res.* **116**, 371–383.

Chavez-Noriega L. E. and Stevens C. F. (1994) Increased transmitter release at excitatory synapses produced by direct activation of adenylate cyclase in rat hippocampal slices. *J. Neurosci.* **14**, 310–317.

Choi D. W. and Rothman S. M. (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Ann. Rev. Neurosci.* **13**, 171–182.

Cidon S. and Sihra T. S. (1989) Characterization of a H^+-ATPase in rat brain synaptic vesicles. *J. Biol. Chem.* **264**, 8281–8288.

Collingridge G. L. and Bliss T. V. P. (1987) NMDA receptors – their role in long-term potentiation. *Trends Neurosci.* **10**, 288–293.

Colmers W. F. and Bleakman D. (1994) Effects of neuropeptide Y on the electrical properties of neurons. *Trends Neurosci.* **17**, 373–379.

Cotman C. W., Monaghan D. T., Ottersen O. P. and Storm-Mathisen J. (1987) Anatomical organization of excitatory amino acid receptors and their pathways. *Trends Neurosci.* **10**, 273–279.

Cotman C. W., Monaghan D. T. and Ganong A. H. (1988) Excitatory amino acid neurotransmission: NMDA receptors and Hebb-type synaptic plasticity. *Ann. Rev. Neurosci.* **11**, 61–80.

Coyle J. T. and Puttfarcken P. (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262**, 689–695.

Del Castillo J. and Katz B. (1954) Quantal components of the end-plate potential. *J. Physiol.* **124**, 560–573.

Dittman J. S. and Regehr W. G. (1996) Contributions of calcium-dependent and calcium-independent mechanisms to presynaptic inhibition at a cerebellar synapse. *J. Neurosci.* **16**, 1623–1633.

Doherty P., Hawgood B. J. and Smith I. C. (1984) Changes in miniature end-plate potentials after brief nervous stimulation at the frog neuromuscular junction. *J. Physiol.* **356**, 349–358.

Dolphin A. C. (1990) G protein modulation of calcium currents in neurons. *Ann. Rev. Physiol.* **52**, 243–255.

Fatt P. and Katz B. (1952) Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* **117**, 109–128.

Fonnum F. (1984) Glutamate: a neurotransmitter in mammalian brain. *J. Neurochem.* **42**, 1–11.

Fykse E. M., Christensen H. and Fonnum F. (1989) Comparison of the properties of γ-aminobutyric acid and L-glutamate uptake into synaptic vesicles isolated from rat brain. *J. Neurochem.* **52**, 946–951.

Gleason E., Borges S. and Wilson M. (1994) Control of neurotransmitter release from retinal amacrine cells by Ca^{2+} influx and efflux. *Neuron* **13**, 1109–1117.

Goodman S. R., Zimmer W. E., Clark M. B., Zagon I. S., Barker J. E. and Bloom M. L. (1995) Brain spectrin: of mice and men. *Brain Res. Bull.* **36**, 593–606.

Greengard P., Valtorta F., Czernik A. J. and Benfenati F. (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. *Science* **259**, 780–785.

Hartinger J. and Jahn R. (1993) An anion binding site that regulates the glutamate transporter of synaptic vesicles. *J. Biol. Chem.* **268**, 23122–23127.

Herrero I., Miras-Portugal M. T. and Sanchez-Prieto J. (1992) Positive feedback of glutamate exocytosis by metabotropic presynaptic receptor stimulation. *Nature* **360**, 163–166.

Hollmann M. and Heinemann S. (1994) Cloned glutamate receptors. *Ann. Rev. Neurosci.* **17**, 31–108.

Johnson M. D. and Yee A. G. (1995) Ultrastructure of electrophysiologically-characterized synapses formed by serotonergic raphe neurons in culture. *Neuroscience* **67**, 609–623.

Kandel E. R. and Schwartz J. H. (1992) Molecular biology of learning: modulation of transmitter release. *Science* **218**, 433–443.

Karinch A. M., Zimmer W. E. and Goodman S. R. (1990) The identification and sequence of the actin-binding domain of human blood cell β-spectrin. *J. Biol. Chem.* **265**, 11833–11840.

Kinney G. A., Emmerson P. J. and Miller R. J. (1998) Galanin receptor-mediated inhibition of glutamate release in the arcuate nucleus of the hypothalamus. *J.Neurosci.* **18**, 3489–3500.

Kish P. E. and Ueda T. (1991) Calcium-dependent release of accumulated glutamate from synaptic vesicles within permeabilized nerve terminals. *Neurosci. Lett.* **122**, 179–182.

Krueger B. K., Forn J. and Greengard P. (1977) Depolarization-induced phosphorylation of specific proteins, mediated by calcium ion influx, in rat brain synaptosomes. *J. Biol. Chem.* **252**, 2764–2773.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lobur A. T., Kish P. E. and Ueda T. (1990) Synaptic vesicular glutamate uptake: modulation by a synaptosomal cytosolic factor. *J. Neurochem.* **54**, 1614–1618.

Maley B. E., Engle M. G., Humphreys S., Vascik D. A., Howes K. A., Newton B. W. and Elde R. P. (1990) Monoamine synaptic structure and localization in the central nervous system. *J. Electron Microsc. Technical* **15**, 20–33.

Malgaroli A. and Tsien R. W. (1992) Glutamate-induced long-term potentiation of the frequency of miniature synaptic currents in cultured hippocampal neuron. *Nature* **357**, 134–139.

Maycox P. R., Deckwerth T., Hell J. W. and Jahn R. (1988) Glutamate uptake by brain synaptic vesicles. *J. Biol. Chem.* **263**, 15423–15428.

Maycox P. R., Hell J. W. and Jahn R. (1990) Amino acid neurotransmission: spotlight on synaptic vesicles. *Trends Neurosci.* **13**, 83–87.

McGehee D. S., Heath M. J. S., Gelber S., Devay P. and Role L. W. (1995) Nicotine enhancement of fast excitatory synaptic transmission in CNS by presynaptic receptors. *Science* **269**, 1692–1696.

McMahon H. T. and Nicholls D. G. (1991) The bioenergetics of neurotransmitter release. *Biochim. Biophys. Acta* **1059**, 243–264.

Mehta P. P., Battenberg E. and Wilson M. C. (1996) SNAP-25 and synaptotagmin involvement in the final Ca $(^{2+})$-dependent triggering of neurotransmitter exocytosis. *Proc. Natl Acad. Sci. USA* **93**, 10471–10476.

Meldrum B. (1991) Excitatory amino acid neurotransmitters in epilepsy. *Epilepsia* **32**, S1–S3.

Miller R. J. (1990) Receptor-mediated regulation of calcium channels and neurotransmitter release. *FASEB J.* **4**, 3291–3299.

Moghaddam B. and Adams B. W. (1998) Reversal of phencyclidine effects by a group I metabotropic glutamate receptor agonist in rats. *Science* **281**, 1349–1352.

Monaghan D. T., Bridges R. J. and Cotman C. W. (1989) The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the central nervous system. *Ann. Rev. Pharmacol. Toxicol.* **29**, 365–402.

Moriyama Y. and Yamamoto A. (1995) Vesicular L-glutamate transporter in microvesicles from bovine pineal glands: driving

force, mechanism of chloride anion activation, and substrate specificity. *J. Biol. Chem.* **270**, 22314–22320.

Naito S. and Ueda T. (1983) Adenosine triphosphate-dependent uptake of glutamate into synaptic vesicles. *J. Biol. Chem.* **258**, 696–699.

Naito S. and Ueda T. (1985) Characterization of glutamate uptake into synaptic vesicles. *J. Neurochem.* **44**, 99–109.

Nakanishi S. (1992) Molecular diversity of glutamate receptors and implications for brain function. *Science* **258**, 597–603.

Neher E. and Zucker R. S. (1993) Multiple calcium-dependent processes related to secretion in bovine chromaffin cells. *Neuron* **10**, 21–30.

Ni B., Rostek P. R., Jr, Nadi N. S. and Paul S. M. (1994) Cloning and expression of a cDNA encoding a brain-specific Na^+-dependent inorganic phosphate cotransporter. *Proc. Natl Acad. Sci. USA* **91**, 5607–5611.

Nicholls D. G. (1989) Release of glutamate, aspartate, and γ-aminobutyric acid from isolated nerve terminals. *J. Neurochem.* **52**, 331–341.

Nicholls D. G. and Sihra T. S. (1986) Synaptosomes possess an exocytotic pool of Glutamate. *Nature* **321**, 772–773.

Nichols R. A., Wu W. C.-S., Haycock J. W. and Greengard P. (1989) Introduction of impermeant molecules into synaptosomes using freeze/thaw permeabilization. *J. Neurochem.* **52**, 521–529.

Özkan E. D. and Ueda T. (1998) Glutamate transport and storage in synaptic vesicles. *Jpn. J. Pharmacol.* **77**, 1–10.

Özkan E. D., Lee F. S. and Ueda T. (1997) A protein factor that inhibits ATP-dependent glutamate and γ-aminobutyric acid accumulation into synapticvesicles: purification and initial characterization. *Proc. Natl Acad. Sci. USA* **94**, 4137–4142.

Pothos E. N., Davila V. and Sulzer D. (1998) Presynaptic recording of quanta from midbrain dopamine neurons and modulation of the quantal size. *J. Neurosci.* **18**, 4106–4118.

Prince D. A. and Stevens C. F. (1992) Adenosine decreases neurotransmitter release at central synapses. *Proc. Natl Acad. Sci. USA* **89**, 8586–8590.

Rieke F. and Schwartz E. A. (1994) A cGMP-gated current can control exocytosis at cone synapses. *Neuron* **13**, 863–873.

Robitaille R. and Charlton M. P. (1992) Presynaptic calcium signals and transmitter release are modulated by calcium-activated potassium channels. *J. Neurosci.* **12**, 297–305.

Savchenko A., Barnes S. and Kramer R. H. (1997) Cyclic nucleotide-gated channels mediate synaptic feedback by nitric oxide. *Nature* **390**, 694–698.

Scholz K. P. and Miller R. J. (1992) Inhibition of quantal transmitter release in the absence of calcium influx by a G protein-linked adenosine receptor at hippocampal synapses. *Neuron* **8**, 1139–1150.

Silinsky E. M. and Solsona C. S. (1992) Calcium currents at motor nerve endings: absence of effects of adenosine receptor agonists in the frog. *J. Physiol.* **457**, 315–328.

Sladeczek F., Recasens M. and Bochaert J. (1988) A new mechanism for glutamate receptor action: phosphoinositide hydrolysis. *Trends Neurosci.* **11**, 545–549.

Smith C., Moser T., Xu T. and Neher E. (1998) Cytosolic Ca^{2+} acts by two separate pathways to modulate the supply of release-competent vesicles in chromaffin cells. *Neuron* **20**, 1243–1253.

Stefani A., Pisani A., Mercuri N. B. and Calabresi P. (1996) The modulation of calcium currents by the activation of mGluRs. *Mol. Neurobiol.* **13**, 81–95.

Stevens C. F. (1993) Quantal release of neurotransmitter and long-term potentiation. *Neuron* **10**, 55–63.

Storm-Mathisen J., Leknes A. K., Bore A. T., Vaaland J. L., Edminson P., Haug F.-M. S. and Ottersen O. P. (1983) First visualization of glutamate and GABA in neurons by immunocytochemistry. *Nature* **301**, 517–520.

Tabb J. S., Kish P. E., Van Dyke R. and Ueda T. (1992) Glutamate transport into synaptic vesicles. Roles of membrane potential, pH gradient, and intravesicular pH. *J. Biol. Chem.* **267**, 15412–15418.

Takahashi T., Forsythe I. D., Tsujimoto T., Barnes-Davies M. and Onodera K. (1996) Presynaptic calcium current modulation by a metabotropic glutamate receptor. *Science* **274**, 594–597.

Takamori S., Rhee J. S., Rosenmund C. and Jahn R. (2000) Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature* **407**, 189–194.

Tamura Y., Özkan E. D. and Ueda T. (1998) The inhibitory protein factor capable of reducing vesicular glutamate accumulation causes a decrease in exocytotic release of glutamate. *Soc. Neurosci. Abstract.* **24** (Part 2), 1570.

Trudeau L.-E., Emery D. G. and Haydon P. G. (1996) Direct modulation of the secretory machinery underlies PKA-dependent synaptic facilitation in hippocampus neurons. *Neuron* **17**, 789–797.

Ueda T. (1986) Glutamate transport in the synaptic vesicle, in *Excitatory amino acids* (Roberts P. J. Storm-Mathisen J. and Bradford H. F., eds), pp. 173–195. Macmillan, London.

Ueda T., Greengard P., Berzins K., Cohen R. S., Blomberg F., Grab D. J. and Siekevitz P. (1979) Subcellular distribution in cerebral cortex of two proteins phosphorylated by a cyclic AMP-dependent protein kinase. *J. Cell Biol.* **83**, 308–319.

Van der Kloot W. (1991) The regulation of quantal size. *Prog. Neurobiol.* **36**, 93–130.

Watkins J. C., Krogsgaard-Larsen P. and Honore T. (1990) Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. *Trends Pharmacol. Sci.* **11**, 25–33.

Whitton P. S., Marshall I. G. and Parsons S. M. (1986) Reduction of quantal size by vesamicol (AH5183), an inhibitor of vesicular acetylcholine storage. *Brain Res.* **385**, 189–192.

Wolosker H., de Souza D. O. and de Meis L. (1996) Regulation of glutamate transport into synaptic vesicles by chloride and proton gradient. *J. Biol. Chem.* **271**, 11726–11731.

Zalutsky R. A. and Nicoll R. A. (1990) Comparison of two forms of long-term potentiation in single hippocampal neurons. *Science* **248**, 1619–1624.