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Modulation of glutamate release by a nitric oxide/cyclic GMP-dependent pathway

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Abstract

The mechanism by which changes in cyclic GMP (cGMP) regulate glutamate release was investigated in rat cerebrocortical nerve terminals. The elevation of cGMP levels by inhibition of cGMP-phosphodiesterase with 2-*o*-propoxy-phenyl-8-azapurin-6-one (zaprinast) reduced the Ca^{2+}-dependent glutamate release evoked by depolarization with 30 mM KCl or 1 mM 4-aminopyridine. The nitric oxide (NO) donor *S*-nitroso-*N*-acetylpenicillamine also enhanced cGMP and reduced glutamate release. In addition, the membrane-permeable analogs 8-bromoguanosine 3′:5′-cyclic monophosphate (8-Br-cGMP) and *N*,2′-*o*-dibutyrylguanosine (dbcGMP) at 10 μM also mimic glutamate release inhibition. The reduction in glutamate release was observed with no modifications in the ATP/ADP ratio, and was reversed in the presence of the protein kinases inhibitor {*N*-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide, HCl} (H-8). Interestingly, higher concentrations of dbcGMP (1 mM) abolished the inhibition observed with low concentrations although no facilitation was observed. This finding seems to indicate the existence of a dual role for cGMP in the control of glutamate exocytosis.

Keywords: Glutamate release; Synaptosome; Nitric oxide (NO); cGMP; Protein kinase G; Zaprinast

1. Introduction

Nitric oxide (NO) plays an important role in signal transduction in the central nervous system (Schuman and Madison, 1994; Garthwaite and Boulton, 1995). In target cells NO has been shown to activate several signal transduction pathways including soluble guanylate cyclase to increase cyclic GMP (cGMP) levels (Garthwaite, 1991). The involvement of the NO/cGMP pathway in the modulation of glutamatergic neurotransmission has been demonstrated in slice preparations from the hippocampus (O'Dell et al., 1991; Zhuo et al., 1993, 1994a,b; Broome et al., 1994; Boulton et al., 1994), and in cultured neurons (Arancio et al., 1995). Although in these studies a presynaptic action of NO in the release process has been suggested, it is not known how the NO/cGMP pathway controls glutamate exocytosis.

Nerve terminals contain NO-sensitive guanylate cyclase (Kamisaki et al., 1995) but the role of the NO/cGMP pathway in the modulation of glutamate release in nerve terminals is not clear, since either potentiation (Montague et al., 1994), inhibition (Kamisaki et al., 1995) or no effect (Kamisaki et al., 1994) has been reported. One source of complication in these studies is that NO may inhibit mitochondrial respiration (Bolaños et al., 1994) with the subsequent decrease in ATP which would enhance the release of cytoplasmic glutamate through reversal of the glutamate carrier (Sánchez-Prieto and González, 1988; Kauppinen et al., 1988; Rubio et al., 1991) while low ATP would inhibit the exocytotic Ca^{2+}-dependent release (Sánchez-Prieto et al., 1987; Kauppinen et al., 1988). In this study we have altered the intrasynaptosomal cGMP by three different means: (i) inhibition of cGMP-phosphodiesterase with zaprinast, (ii) activation of guanylate cyclase with NO donors and with (iii) the use of cGMP membrane-permeable analogs. In these three conditions we have observed an inhibition of glutamate exocytosis, neither related to a decrease in ATP levels nor to a reduction in the entry of Ca^{2+} in the nerve terminals, but consistent with a protein kinase G-dependent inhibition of a delayed component of release likely to account for vesicle mobilization.

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2. Materials and methods

2.1. Materials

S-Nitroso-*N*-acetylpenicillamine and {*N*-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide, HCl} (H-8) were from Calbiochem (San Diego, CA, USA). Fura-2 acetoxymethyl ester was from Molecular Probes (Eugene, OR, USA). (*RS*)-3,5-Dihydroxyphenylglycine (DHPG) was purchased from Tocris Neuramin. The cGMP kit was from Amersham (UK) and the ATP monitoring reagent was from Biorbit (Turku, Finland). 2-*o*-Propoxy-phenyl-8-azapurin-6-one (zaprinast) was a generous gift from Rhône-Poulenc Rorer (UK). This compound was also obtained from Sigma (St. Louis, MO, USA). All other reagents were from Sigma.

2.2. Synaptosomal preparation

Cerebral cortices from male adult (2–3 months) Wistar rats were isolated and homogenized in medium containing 320 mM sucrose, 0.5 mM EDTA and 5 mM 2-{[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]-amino} ethane sulfonate (TES), pH 7.4. The homogenate was spun for 5 min at $900 \times g$ at 4°C and the supernatants spun again for 10 min at $17\,000 \times g$. From the pellets thus formed, the white loosely compacted layer containing the majority of synaptosomes was gently resuspended in a medium containing 250 mM sucrose and 5 mM TES, pH 7.4 (10 ml/4 cortices) and the protein content was determined by the Biuret method. Pellets containing 1 mg of protein were stored on ice. Synaptosomes remained fully viable when stored as pellets for at least 6 h after preparation as judged by the extent of KCl- and 4-aminopyridine-evoked glutamate release. The P_2 fraction of cerebrocortical synaptosomes exhibited an extent of Ca^{2+}-dependent release comparable in absolute terms to that obtained with synaptosomes purified either by Ficoll or Percoll gradients.

2.3. Glutamate release

Glutamate release was assayed by on-line fluorimetry as described previously (Nicholls et al., 1987). Synaptosomal pellets were resuspended (0.67 mg/ml) in incubation medium containing 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 10 mM glucose and 20 mM TES buffer pH 7.4, and preincubated at 37°C for 1 h in the presence of 16 μM fatty-acid-free bovine serum albumin to bind any free fatty acids released from synaptosomes during the preincubation (Herrero et al., 1991). The synaptosomes were then washed by centrifugation at $12\,000 \times g$ for 1 min and resuspended (0.67 mg/ml) in incubation buffer without bovine serum albumin. An aliquot (1 ml) was transferred to a stirred cuvette containing 1 mM $NADP^+$, 50 U glutamate dehydrogenase and 1.33 mM $CaCl_2$ or 200 nM free Ca^{2+} and the fluorescence of NADPH followed in a Perkin Elmer LS-50 luminiscence spectrometer at excitation and emission wavelengths of 340 and 460 nm, respectively. Some experiments were performed in the presence of adenosine deaminase (1 U/mg) to remove adenosine and the tonic activation of adenosine receptors. Control release was not altered by the presence of the $GABA_B$ receptor antagonist phaclofen at 500 μM, thus ruling out tonic inhibition by γ-aminobutyric acid (GABA). Traces were calibrated by the addition of 2 nmol of glutamate at the end of each assay. Data points were obtained at 2-s intervals and corrected for Ca^{2+}-independent release. The Ca^{2+}-dependent release was calculated by subtracting the release obtained for 5 min at 200 nM free $[Ca^{2+}]$ from the release at 1.33 mM $CaCl_2$. Total synaptosomal glutamate determined with Triton X-100 (0.1%) was found to be 20.5 ± 1.1 nmol of glutamate/mg of protein.

2.4. Cytoplasmic free Ca^{2+}

Cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_c$, was measured with fura-2 acetoxymethyl ester. Synaptosomes were resuspended (2 mg/ml) in incubation medium with 16 μM bovine serum albumin in the presence of 1.33 mM $CaCl_2$ and 5 μM fura-2-acetoxymethyl ester and incubated at 37°C for 20 min. After fura-2 acetoxymethyl ester loading, synaptosomes were pelleted and resuspended in fresh incubation medium without bovine serum albumin (0.67 mg/ml). An aliquot (1.5 ml) was transferred to a stirred cuvette containing 1.33 mM $CaCl_2$ and fluorescence was monitored at 340 and 510 nm. Data points were collected at 0.5-s intervals. The cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_c$, was calculated using the equations previously described (Grynkiewicz et al., 1985).

2.5. Intrasynaptosomal cGMP measurements

Synaptosomes (2 mg/ml) were resuspended in incubation medium with 16 μM bovine serum albumin and incubated at 37°C. After 5 min, 1.33 mM $CaCl_2$ was added, followed by zaprinast or *S*-nitroso-*N*-acetylpenicillamine 2 min later. After 5 min, 500 μl aliquots were taken and added to 150 μl of 1 M $HClO_4$/50 mM EDTA. In the experiments with depolarized nerve terminals, 4-aminopyridine was added 5 min after zaprinast or *S*-nitroso-*N*-acetylpenicillamine and aliquots taken 5 s after depolarization with 4-aminopyridine. Samples were maintained in ice for 20 min and then centrifuged at $12\,000 \times g$ for 1 min in a bench microfuge. The supernatants were collected and neutralized with a solution of 3 M KOH/1.5 mM triethanolamine and centrifuged again, and the cGMP content of the supernatants was determined using a commercial [^{3}H]cyclic GMP radioimmunoassay kit from Amersham International (UK).

2.6. cAMP determination

Synaptosomes were resuspended (2 mg/ml) in incubation medium with 16 μM bovine serum albumin and preincubated at 37°C for 5 min. Preincubations were made exactly as for cGMP determinations. 500 μl aliquots were taken and added onto 150 μl of 1 mM $HClO_4$/50 mM EDTA. The samples were shaken and placed in ice for 20 min. Then the samples were centrifuged at $12\,000 \times g$ for 1 min in a bench microfuge. The supernatants were neutralized with a solution of 3 M KOH and 1.5 M triethanolamine. The supernatants were collected and the cAMP content was estimated by radioimmunoassay with a commercially available cAMP kit from Amersham International (UK).

2.7. Determination of the ATP and ADP content

Synaptosomes (0.67 mg/ml) were resuspended in incubation medium and incubation performed under the same conditions as described for cGMP. After incubation, aliquots (100 μl) were mixed with 150 μl of 1 M $HClO_4$/50 mM EDTA at 0°C and neutralized with 3 M KOH in 1.5 M triethanolamine. ATP and ADP were measured with a luciferin/luciferase reagent (Biorbit, Turku, Finland) according to Kauppinen and Nicholls (1986b). 57 μl of the reagent were added to 143 μl of a medium containing 100 mM Tris, 2 mM EDTA, 5 mM $MgCl_2$, 6 mM dithiothreitol and 2 mM KCl, pH 7.75. After 5 min incubation at 25°C, samples (10 μl) were added and the ATP content determined by the increase in luminiscence. In the determination of the ADP content 2 mM phosphoenolpyruvate and 2 U of pyruvate kinase were added and maintained in the dark to allow the reaction to occur. After this time, a further increase in luminiscence was measured due to ADP. Finally, the increases in luminiscence were calibrated by the addition of a standard of ATP (20 pmol) at the end of each assay.

2.8. Kinetic analysis

The enzymatic detection of glutamate produces a delay between the actual increase in glutamate concentration due to release and the increase in fluorescence signal due to NADPH formation. To correct for this delay, the data points of the NADPH produced from the Ca^{2+}-dependent release were fitted according to a biphasic model in which a fast and a slow components were considered (Herrero et al., 1996b).

3. Results

The depolarization of nerve terminals opens the voltage-gated Ca^{2+} channels coupled to the exocytotic machinery and induces the release of glutamate. Depolarization in the absence of Ca^{2+} also releases cytoplasmic glutamate via a Ca^{2+}-independent pathway mediated by the reversal of the plasma membrane glutamate transporter. For this reason, in order to estimate the extent of the Ca^{2+}-dependent release, the release observed in the presence of EGTA (Fig. 1A) was subtracted from that observed in the presence of 1.33 mM $CaCl_2$. The Ca^{2+}-dependent release evoked by 1 mM 4-aminopyridine over a period of 5 min depolarization was 3.07 ± 0.27 nmol of glutamate/mg of protein ± S.D. ($n = 16$) ($100 \pm 9.0\%$). The cGMP-phosphodiesterase inhibitor zaprinast (Beavo and Reifsnyder, 1990) at 100 μM added 5 min prior to depolarization reduced release in the presence of Ca^{2+} but did not alter that observed in the presence of EGTA (Fig. 1A). Thus, zaprinast reduced the extent of Ca^{2+}-dependent release to $45.0 \pm 6.5\%$ of control ($n = 15$) (Fig. 1B). The cGMP membrane-permeable analogs 8-bromoguanosine 3′:5′-cyclic monophosphate (8-Br-cGMP) and *N*,2′-*o*-dibutyrylguanosine 3′:5′-cyclic monophosphate (dbcGMP) both at 10 μM when added 1 and 5 min prior to depolarization also reduced the extent of the Ca^{2+}-dependent release to $61.5 \pm 4.7\%$ ($n = 4$) and $62.9 \pm 3.5\%$ ($n = 3$) of control, respectively. It is also shown in Fig. 1B that the nitric oxide (NO) donor *S*-nitroso-*N*-acetylpenicillamine at 100 μM added 5 min prior to depolarization reduced the release to $72.7 \pm 5.1\%$ ($n = 15$). The addition of *S*-nitroso-*N*-acetylpenicillamine, that was depleted from its NO content by keeping the solution exposed to light, was without effect on glutamate release (data not shown). Unfortunately, we could not test the NO donor, 3-morpholinosydnomine (SIN-1) in release experiments due to quenching of the fluorescent signal.

In parallel experiments we have tested that the compounds that reduce the Ca^{2+}-dependent release of glutamate evoked by 4-aminopyridine, namely zaprinast, *S*-nitroso-*N*-acetylpenicillamine, dbcGMP and 8-Br-cGMP, do not alter the Ca^{2+}-independent release. Thus, the release evoked by 1 mM 4-aminopyridine in the presence of EGTA over 5 min was 1.0 ± 0.25 nmol of glutamate/mg protein in control synaptosomes and 0.95 ± 0.27 and 1.04 ± 0.30 nmol/mg protein in the presence of zaprinast and *S*-nitroso-*N*-acetylpenicillamine, respectively. The Ca^{2+}-independent release in the presence of the cGMP analogs dbcGMP both at 10 μM and 1 mM and 8-Br-cGMP at 10 μM was 1.02 ± 0.28, 1.04 ± 0.27 and 0.99 ± 0.30 nmol of glutamate/mg protein, respectively.

Depolarization of nerve terminals with a high concentration of KCl also promotes Ca^{2+}-dependent release of glutamate, but in contrast to 4-aminopyridine, KCl-induced depolarization does not involve tetrodotoxin-sensitive action potentials (Tibbs et al., 1989). The extent of Ca^{2+}-dependent release induced by KCl was 3.45 ± 0.21 nmol of glutamate/mg of protein ($100 \pm 6.2\%$) ($n = 9$) (Fig. 1C and D). Zaprinast inhibited the release to $45.7 \pm 5.0\%$ ($n = 9$), while 8-Br-cGMP and dbcGMP as well as *S*-nitroso-*N*-acetylpenicillamine reduced the Ca^{2+}-dependent

release to 72.8 ± 3.5% ($n = 4$), 75.3 ± 3% ($n = 2$) and 72.3 ± 3.5% ($n = 6$), respectively.

It has been previously reported that the inhibition of hippocampal synaptic transmission by zaprinast and *S*-nitroso-*N*-acetylpenicillamine is prevented by the adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxantine (DPCPX), suggesting the involvement of adenosine in the process (Broome et al., 1994). However, in parallel experiments not shown in this paper, we found that the addition of adenosine deaminase (1 U/mg) 1 h prior to depolarization to remove extrasynaptosomal adenosine and the tonic activation of adenosine receptors (Dunwiddie and Hoffer, 1980) modified neither control release nor the inhibition caused by zaprinast.

Glutamate exocytosis is an ATP-dependent process not only on account of the fact that the active transport of the amino acid into synaptic vesicles uses the electrochemical gradient of H^+ generated by a H^+-ATPase (Disbrow et al., 1982; Naito and Ueda, 1983; Maycox et al., 1988) but also because some steps of the exocytosis-endocytosis cycle require ATP (Söllner and Rothman, 1994). It could well be, therefore, that the inhibition of glutamate exocytosis is the consequence of a decrease in energy levels in the nerve terminal. In this regard, it has been reported that NO

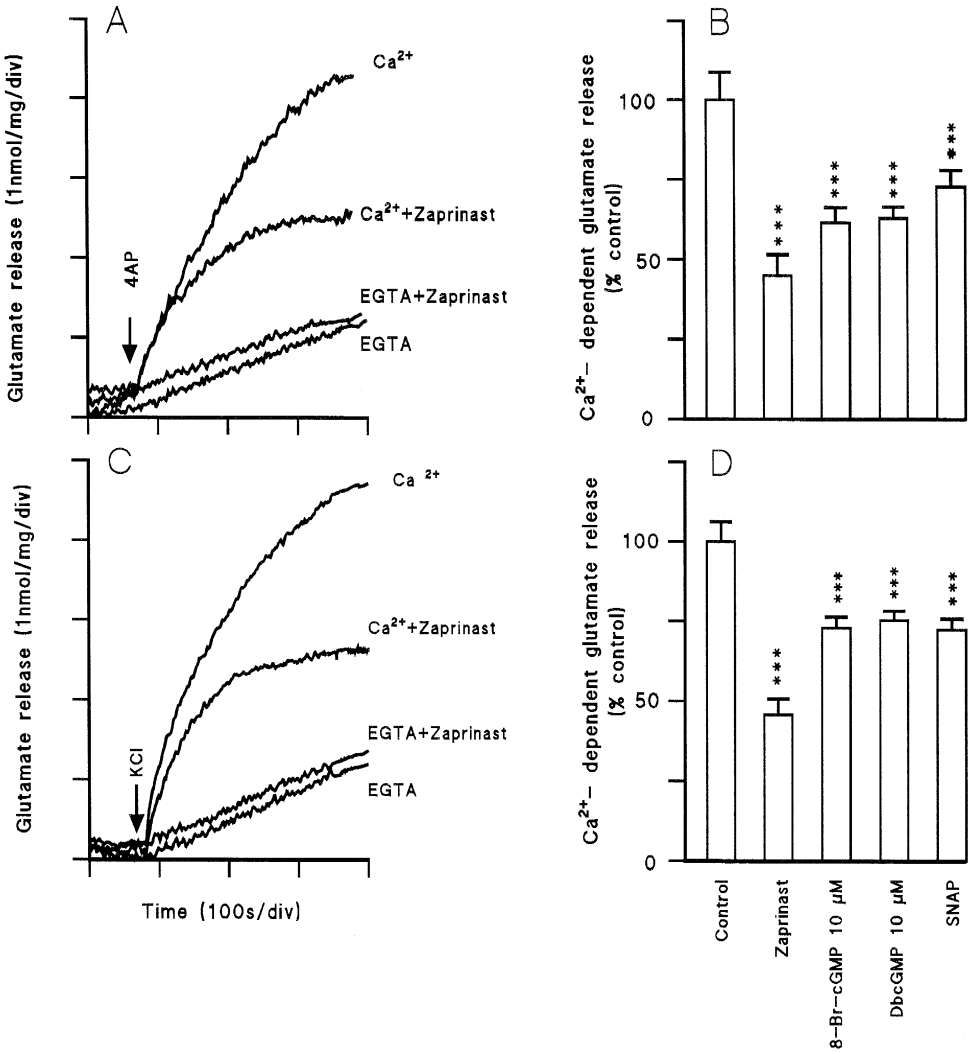

Fig. 1. Inhibition of the Ca^{2+}-dependent release of glutamate by zaprinast, cGMP analogs and *S*-nitroso-*N*-acetylpenicillamine (SNAP). Glutamate release was evoked by depolarization of synaptosomes with 1 mM 4-aminopyridine (4AP) (A) or 30 mM KCl (C) either in the presence of 1.33 mM $CaCl_2$ (Ca^{2+}) or in medium at a free Ca^{2+} concentration of 200 nM (EGTA), both in the absence and in the presence of 100 μM zaprinast added 5 min before depolarization. Calcium-dependent glutamate release was determined by subtracting the release obtained, after 5 min depolarization, at a free calcium concentration of 200 nM from that at 1.33 mM $CaCl_2$. Extent of Ca^{2+}-dependent release evoked by 1 mM 4-aminopyridine (B) or 30 mM KCl (D) in the absence (control) or in the presence of zaprinast (100 μM), dbcGMP (10 μM), *S*-nitroso-*N*-acetylpenicillamine (100 μM) which were added 5 min before depolarization, or in the presence of 8-Br-cGMP (10 μM) added 1 min prior to 4-aminopyridine or KCl. Each trace is the mean of data obtained from at least nine preparations of synaptosomes. Statistical significance was calculated using Student's *t*-test with respect to the control value. $^{***}P < 0.001$; NS, not significant.

Table 1
The incubation of synaptosomes with zaprinast or *S*-nitroso-*N*-acetylpenicillamine does not alter ATP and ADP levels

	ATP (nmol/mg protein)	ADP (nmol/mg protein)	ATP/ADP
Control	1.183 ± 0.109	0.258 ± 0.059	4.585 ± 0.084
	$n = 9$	$n = 9$	$n = 9$
1 μM zaprinast	1.227 ± 0.057	0.247 ± 0.012	4.888 ± 0.032
	$n = 3$ NS	$n = 3$ NS	$n = 3$
10 μM zaprinast	1.062 ± 0.038	0.233 ± 0.078	4.558 ± 0.058
	$n = 4$ NS	$n = 4$ NS	$n = 4$
100 μM zaprinast	1.192 ± 0.169	0.278 ± 0.019	4.228 ± 0.094
	$n = 11$ NS	$n = 11$ NS	$n = 11$
100 μM *S*-nitroso-*N*-acetylpenicillamine	1.204 ± 0.107	0.286 ± 0.016	4.210 ± 0.0615
	$n = 4$ NS	$n = 4$ NS	$n = 4$
2 mM iodoacetate + 10 μM rotenone	0.083 ± 0.012	0.268 ± 0.033	0.310 ± 0.022
	$n = 5$ [a]	$n = 5$ [a]	$n = 5$

Synaptosomes (0.67 mg/ml) in incubation medium in the presence of 1.33 mM $CaCl_2$, were preincubated during 5 min with increasing concentrations of zaprinast or *S*-nitroso-*N*-acetylpenicillamine (100 μM). Then, synaptosomes were depolarized with 1 mM 4-aminopyridine and aliquots taken to determine the ATP and ADP content as described in Section 2. In the experiments with metabolic inhibitors, 2 mM iodoacetate and 10 μM rotenone were added 10 min before depolarization. Results in nmol/mg of protein are means ± S.D. of number of data shown in the table. Statistical significance was calculated using Student's *t*-test with respect to the control value. [a] $P < 0.001$; NS, not significant.

donors may inhibit mitochondrial respiration (Bolaños et al., 1994). For these reasons, we determined the ATP and ADP levels in nerve terminals. The data in Table 1 show that the ATP and ADP as well as the ATP/ADP ratios were not modified by zaprinast (1-100 μM) or the NO donor *S*-nitroso-*N*-acetylpenicillamine (100 μM) when compared to control synaptosomes. These data therefore clearly indicate that the inhibition of glutamate release observed is not related to changes in the energetic status of the nerve terminals. However, a significant decrease in ATP levels was observed with metabolic inhibitors. Thus, Table 1 also shows that the addition of iodoacetate and rotenone to inhibit glycolysis (Kauppinen and Nicholls, 1986a) and respiration (Kauppinen and Nicholls, 1986b), respectively, strongly depressed ATP levels.

To establish whether the changes in glutamate release are due to the alterations in cGMP we determined the levels of this nucleotide in the synaptosomal preparation. Basal levels of cGMP in polarized cerebrocortical nerve terminals in the presence of 1.33 mM $CaCl_2$ were 1.12 ± 0.56 pmol/mg of protein ± S.D. ($n = 16$) (Fig. 2A). The preincubation of synaptosomes for 5 min with the cGMP-

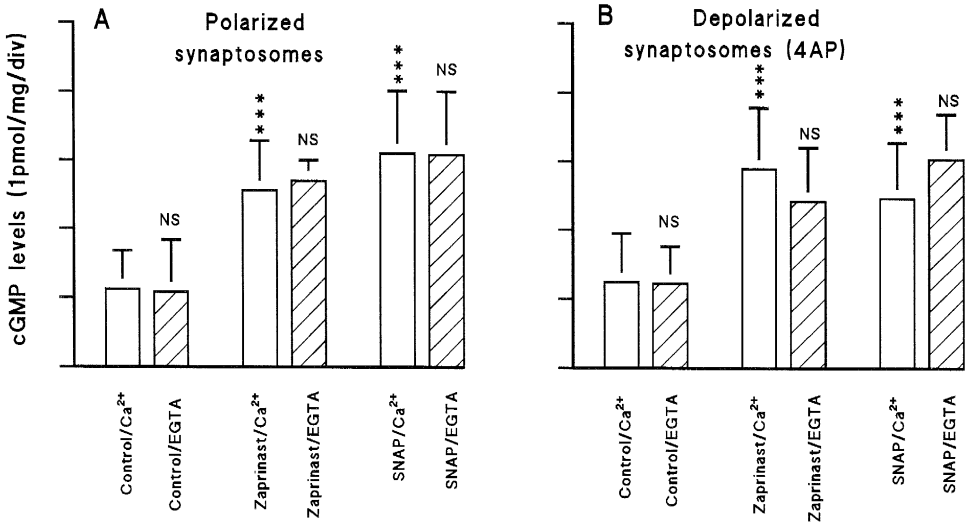

Fig. 2. Intrasynaptosomal cGMP levels. The cGMP content of cerebrocortical nerve terminals was determined in polarized synaptosomes (A) and in synaptosomes depolarized with 1 mM 4-aminopyridine (4AP) (B) both in the presence of 1.33 mM $CaCl_2$ (Ca^{2+}) or 200 nM free Ca^{2+} (EGTA). In polarized synaptosomes samples for cGMP content were taken 5 min after the addition of zaprinast (100 μM) or *S*-nitroso-*N*-acetylpenicillamine (SNAP) (100 μM). In experiments with depolarized synaptosomes the nerve terminals were preincubated with zaprinast (100 μM) or *S*-nitroso-*N*-acetylpenicillamine (100 μM) for 5 min and then samples were taken 5 s after depolarization with 1 mM 4-aminopyridine. Results are the means ± S.D. of data obtained from at least ten preparations of synaptosomes. Statistical significance was calculated using Student's *t*-test. The statistical significance shown above the Ca^{2+} bars was calculated with respect to control values, while that above the EGTA bars was estimated with respect to the equivalent Ca^{2+} value.

phosphodiesterase inhibitor, zaprinast, or with the NO donor, *S*-nitroso-*N*-acetylpenicillamine, significantly increased cGMP levels (Fig. 2A). In parallel experiments we tested that zaprinast did not change the cAMP levels which were 13.80 ± 0.31 pmol of cAMP/mg of protein in control and 13.51 ± 1.12 in the presence of zaprinast. In order to mimic release conditions, we also estimated cGMP 5 s after the depolarization of nerve terminals with 4-aminopyridine. Depolarization did not alter cGMP in the control or in the presence of zaprinast (Fig. 2B), whereas a moderate although not significant decrease in cGMP was observed in the presence of *S*-nitroso-*N*-acetylpenicillamine. Since 4-aminopyridine increases the intrasynaptosomal concentration of Ca^{2+} (Tibbs et al., 1989), we also determined cGMP in the presence of EGTA in order to establish whether the decrease in cGMP in the presence of the NO donor was due to the stimulation by Ca^{2+} of the cGMP hydrolysis (Mayer et al., 1992). Fig. 2 shows that the substitution of Ca^{2+} for EGTA does not significantly alter cGMP levels.

To obtain direct evidence that the increase in cGMP was responsible for the inhibition of glutamate release we produced dose-response curves for zaprinast both in cGMP production and glutamate release effect. Zaprinast at 10 μM slightly alters both cGMP levels and glutamate release while maximal effects in both parameters were obtained at 100 μM (Fig. 3). We could not test the effect of 1 mM in release experiments due to quenching of the fluorescent signal.

One possibility is that the rise in cGMP activates cGMP-dependent protein kinases. To assess the involvement of a cGMP-dependent protein kinase in the inhibition of glutamate release we applied the protein kinases inhibitor H-8, which, at low concentrations, is highly effective in inhibiting cyclic nucleotide-dependent protein kinases (Hidaka et al., 1984). The data in Fig. 4 show that preincubation for 30 min with H-8 at 10 μM, while having no effect in control release (data not shown), was able to reverse the inhibitory effect of zaprinast.

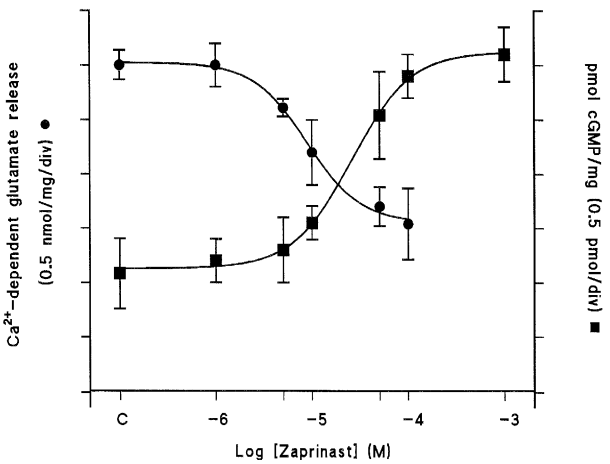

Fig. 3. The inhibition of glutamate release by zaprinast correlates with the increase in cGMP levels. Glutamate release was initiated by the addition of 1 mM 4-aminopyridine (4AP) in the absence or in synaptosomes preincubated for 5 min with increasing concentrations of zaprinast. The Ca^{2+}-dependent release (●) was calculated by subtracting release, after 5 min depolarization, at a free Ca^{2+} concentration of 200 nM from release at 1.33 mM $CaCl_2$. cGMP levels (■) were determined in aliquots taken 5 s after depolarization in the presence of 1.33 mM $CaCl_2$. Results are means ± S.D. of data obtained from at least three preparations of synaptosomes.

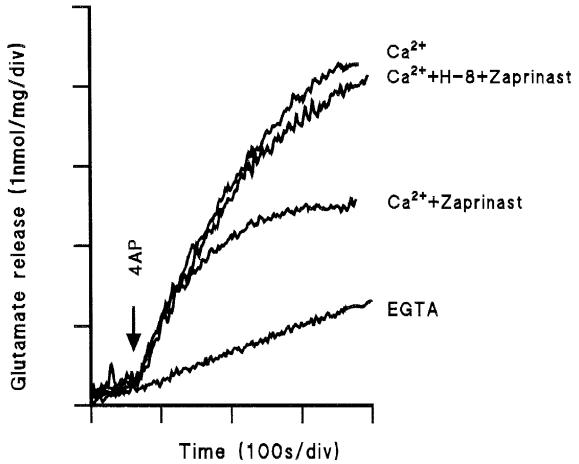

Fig. 4. The protein kinase inhibitor H-8 reverses the inhibition of release induced by zaprinast. Glutamate release was evoked by depolarization of synaptosomes with 1 mM 4-aminopyridine (4AP). The protein kinase inhibitor H-8 at 10 μM and zaprinast at 100 μM were added 30 and 5 min prior to depolarization, respectively. Traces are means of data obtained from three preparation of synaptosomes.

In nerve terminals there is a facilitatory pathway for glutamate release that involves the synergistic activation of protein kinase C both by the diacylglycerol generated by a metabotropic glutamate receptor sensitive to (*RS*)-3,5-dihydroxyphenylglycine (DHPG) and by exogenous arachidonic acid (Herrero et al., 1992). The potentiation of the release by this pathway has been shown to be the consequence of an increase in the depolarization and in the subsequent entry of Ca^{2+} in the nerve terminal (Barrie et al., 1991; Herrero et al., 1992). We found, as shown in Fig. 5, that the inhibition by zaprinast is still observed in synaptosomes after the activation of the facilitatory pathway with DHPG and arachidonic acid. One possible explanation for this result is that cGMP inhibits release in a step located down-stream of the entry of Ca^{2+}. Nevertheless, to better understand the mechanism of cGMP inhibition, we determined the changes in the cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_c$, induced by depolarization with the Ca^{2+} indicator fura-2 acetoxymethyl ester (Grynkiewicz et al., 1985).

A reduction in the entry of Ca^{2+} in nerve terminals has been shown to be responsible for the inhibition of glutamate release that follows the activation of presynaptic receptors such as adenosine A_1 (Barrie and Nicholls, 1993), and L-AP4-sensitive metabotropic glutamate receptors (Herrero et al., 1996a). It is shown in Fig. 6, however, that

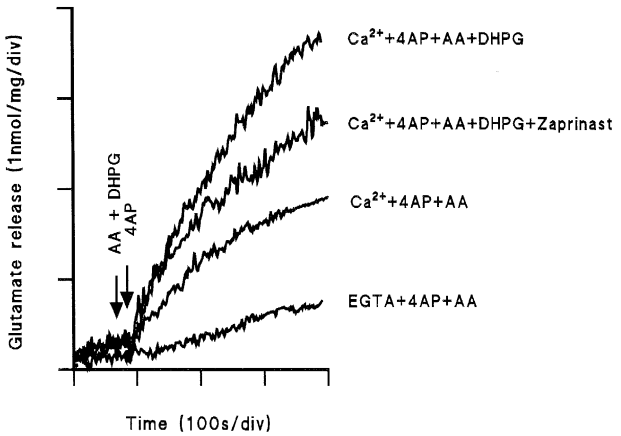

Fig. 5. The facilitation of glutamate release induced by the mGlu receptor agonist DHPG and arachidonic acid (AA) fails to overcome the inhibitory effect of zaprinast. Synaptosomes (0.67 mg/ml) were incubated for 1 h in incubation medium with bovine serum albumin and then washed by centrifugation (see Section 2) and resuspended again in incubation medium but in the absence of bovine serum albumin. Glutamate release was induced by depolarization of synaptosomes with 50 μM 4-aminopyridine (4AP). Arachidonic acid at 2 μM, alone or in combination with DHPG (100 μM), was added just prior to depolarization. In the experiments with zaprinast (100 μM) the cGMP-dependent phosphodiesterase inhibitor was added 5 min prior to depolarization. Traces show computer-generated means of data obtained from three preparations of synaptosomes.

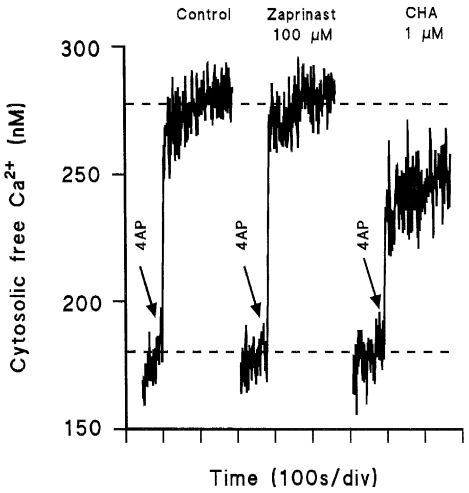

Fig. 6. Zaprinast does not reduce the depolarization-evoked increase in $[Ca^{2+}]_c$. The cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_c$, was determined as described in Section 2 in the presence of adenosine deaminase (1 U/mg) added 25 min prior to depolarization. Synaptosomes were depolarized by the addition of 1 mM 4-aminopyridine (4AP) to control synaptosomes, to synaptosomes preincubated with zaprinast (100 μM) for 5 min and to synaptosomes in the presence of cyclohexyladenosine (CHA) (1 μM) added 1 min prior to depolarization. Results are the mean of data obtained from six preparations of synaptosomes.

zaprinast did not modify the 4-aminopyridine-induced rise in the fura-2 acetoxymethyl ester response. The absence of effect by zaprinast in the fura-2 signal is in contrast with the reduction in the depolarization-evoked rise in $[Ca^{2+}]_c$ observed in the presence of the adenosine A_1 receptor agonist cyclohexyladenosine. Thus, it seems that a reduction in Ca^{2+} in the nerve terminals is not responsible for the cGMP-dependent inhibition of glutamate release.

The release trace in the presence of zaprinast shows that the inhibition by phosphodiesterase inhibitor is apparent in the second release phase but not in the first minute of release (Fig. 1A, Ca^{2+} + zaprinast). In order to study this more thoroughly we analyzed the release trace. The enzymatic detection of glutamate produces a delay between the actual increase in glutamate concentration due to release and the increase in fluorescence signal. To correct for this delay, we modelled glutamate release as a two-step process: a step like instantaneous release, and an exponentially fading slow phase (Herrero et al., 1996b). Using this protocol we found (Fig. 7 and Table 2) that the extent of the fast component of release is not altered by the presence of zaprinast, while a strong reduction of the slow component is observed in the presence of the phosphodiesterase inhibitor.

An inhibition of glutamate release by cGMP has also been observed in nerve terminals from the cerebellum (Kamisaki et al., 1995). In addition, zaprinast and NO donors have been reported to inhibit synaptic transmission in area CA_1 of the rat hippocampus (Boulton et al., 1994). However, an inhibition of glutamate release by the NO/cGMP pathway is not consistent with the role for NO as a retrograde messenger able to enhance glutamate release at glutamatergic nerve terminals since a moderate

Table 2
Zaprinast inhibits a slow component of the 4-aminopyridine-induced Ca^{2+}-dependent release

	Fast component (nmol glutamate/mg protein)	Slow component (nmol glutamate/mg protein)	Total (nmol glutamate/mg protein)
Control (1 mM 4AP)	0.630 ± 0.030	2.573 ± 0.034	3.203 ± 0.032
	$n = 5$	$n = 5$	$n = 5$
100 μM zaprinast	0.642 ± 0.025	1.581 ± 0.036	2.223 ± 0.028
	$n = 5$ NS	$n = 5$ [a]	$n = 5$ [a]

The fast and slow components of the Ca^{2+}-dependent release were calculated by kinetic analysis of the release traces as described in Section 2. Total release was the sum of the fast and slow components. The Ca^{2+}-dependent release, calculated as the difference in release observed in medium with 1.33 mM $CaCl_2$ and at a free Ca^{2+} concentration of 200 nM, was induced by the addition of 1 mM 4-aminopyridine in the absence (control) and after 5 min preincubation with 100 μM zaprinast. Results are means ± S.D. of data from 5 independent experiments. Statistical significance was calculated using Student's *t*-test with respect to the control value. [a] $P < 0.001$; NS, not significant.

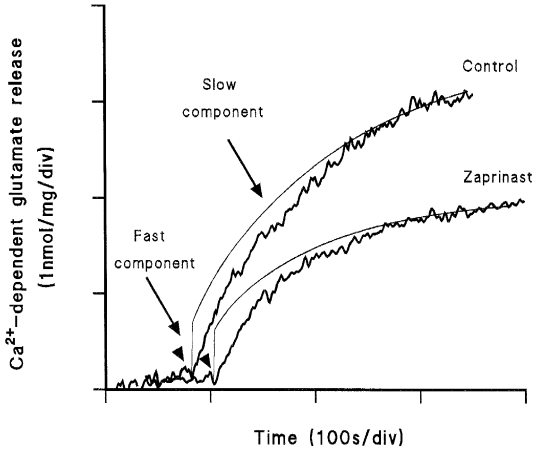

Fig. 7. Zaprinast reduces the slow but not the fast component of glutamate release. The fluorescence traces show the Ca^{2+}-dependent release initiated by the addition of 1 mM 4-aminopyridine (4AP) (arrowheads) both to control and to synaptosomes incubated with zaprinast (100 μM) which was added 5 min prior to depolarization. The modelized glutamate release components are also shown superimposed onto the actual fluorescent trace. The initial step increase (fast component) represents g_0, followed by a gradual release of the slow component, g_1 (Herrero et al., 1996b). The delay between glutamate release and detection by fluorescence increase is evident. Experimental data points fall within 99% confidence limits when fitted to this biphasic model (Herrero et al., 1996b)

increase in cGMP levels seems to be responsible for the inhibition of release found in this paper as well as for the reduction of synaptic transmission found by Boulton et al. (1994). We decided to test the effects of high levels of cGMP on release using the analog dbcGMP. The data presented in Fig. 8A show that dbcGMP inhibited the release at 10^{-7} to 10^{-4} M but at 10^{-3} M dbGMP the inhibitory effect was abolished. The absence of inhibition by high concentrations of cGMP was not altered by the presence of the cyclic nucleotide-dependent protein kinase inhibitor H-8 (Fig. 8B), suggesting that the reversion of glutamate inhibition by high cGMP does not involve H-8-sensitive protein kinase activity. If high cGMP reverses the inhibition of glutamate release, the addition of combinations of zaprinast, *S*-nitroso-*N*-acetylpenicillamine and suboptimal concentrations of dbcGMP should also reverse the inhibition of release. Fig. 8C shows that combinations of 10 μM dbcGMP either with 100 μM zaprinast or 100 μM *S*-nitroso-*N*-acetylpenicillamine significantly reversed the inhibition of release caused by the addition of these compounds alone.

4. Discussion

The results presented in this paper demonstrate that the increase in the cGMP levels in nerve terminals induces the depression of glutamate release by a mechanism which seems to imply the activation of a cGMP-dependent kinase. We have altered cGMP levels by three independent means: (i) with the specific inhibitor of the cGMP-dependent phosphodiesterase zaprinast, (ii) by activation of the guanylyl cyclase with the NO donor *S*-nitroso-*N*-acetyl-

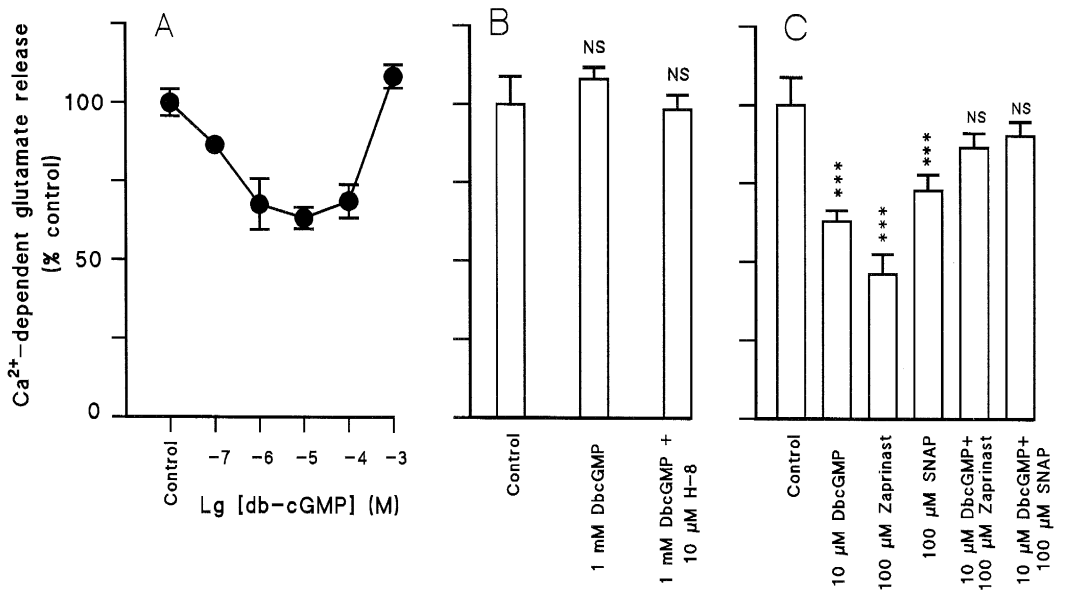

Fig. 8. Dual effect of dbcGMP in the Ca^{2+}-dependent release of glutamate. (A) Glutamate release was induced by depolarization of nerve terminals with 1 mM 4-aminopyridine (4AP) in the absence (Control) and in the presence of different concentrations of dbcGMP added 5 min prior to depolarization. (B) Ca^{2+}-dependent release in control and in synaptosomes incubated with 1 mM dbcGMP both in the absence and in the presence of 10 μM H-8 added 30 min prior to depolarization. (C) Effects of dbcGMP (10 μM), zaprinast (100 μM), *S*-nitroso-*N*-acetylpenicillamine (SNAP) (100 μM) or combinations of dbcGMP (10 μM) with zaprinast or *S*-nitroso-*N*-acetylpenicillamine. All compounds were added 5 min prior to depolarization. Results are means of data obtained from at least three preparations of synaptosomes. Statistical significance was calculated using Student's *t*-test with respect to the control value. $^{***}P < 0.001$; NS, no significance.

penicillamine, (iii) by using the membrane-permeable analogs of cGMP, 8-Br-cGMP and dbcGMP. In all these cases the result was an inhibition of the Ca^{2+}-dependent release of glutamate evoked by depolarization. However, we found no evidence that the NO/cGMP pathway is capable of enhancing glutamate release as would be expected given the suggested role for NO as a retrograde messenger during long-term potentiation (LTP) (O'Dell et al., 1991). Nevertheless we did find a reversal of the cGMP inhibitory effect at higher concentrations of the permeable analog dbcGMP that suggests a dual role for the NO/cGMP pathway in the control of glutamate release.

An increase in the Ca^{2+}-independent neurotransmitter release by NO donors has been observed in hippocampal nerve terminals (Meffert et al., 1994, 1996). However, in our hands all compounds that enhance cGMP, including the NO donor *S*-nitroso-*N*-acetylpenicillamine, reduced the depolarization-evoked Ca^{2+}-dependent release of glutamate without altering the Ca^{2+}-independent release.

Several cyclic nucleotide phosphodiesterases have been shown to be sensitive to zaprinast (Merkel, 1993). However, a moderate reduction in cGMP levels by the increase in $[Ca^{2+}]_c$ induced by depolarization (Fig. 2B), together with the relatively high concentration of zaprinast required to enhance cGMP (Fig. 3) all suggest the presence of a Ca^{2+}/calmodulin-dependent phosphodiesterase (type I) in nerve terminals. The presence of neuronal Ca^{2+}/calmodulin-dependent phosphodiesterase is consistent with the inhibition of cGMP formation by Ca^{2+} in brain preparations (Olson et al., 1976; Knowles et al., 1989) including nerve terminals (Mayer et al., 1992).

An inhibition of glutamate exocytosis can be the result of an ATP depletion of nerve terminals caused by the dependence of glutamate exocytosis on ATP (Sánchez-Prieto et al., 1987; Kauppinen et al., 1988). However, no changes in ATP nor ADP were detected in conditions where an inhibition of release was observed. Another possibility is that inhibition of glutamate release by zaprinast is mediated by the release of endogeneous adenosine. This has been put forward as a possible explanation for the inhibitory effect of zaprinast in synaptic transmission (Broome et al., 1994). However, the ability of the phosphodiesterase inhibitor zaprinast to reduce glutamate release was maintained in the presence of adenosine deaminase (data not shown) which should prevent the tonic effects of endogeneous adenosine.

A decrease in Ca^{2+}-dependent glutamate release by the activation of the NO/cGMP-dependent pathway can also be the consequence of a depletion of synaptic glutamate vesicles. In this respect it has recently been found that NO donors reduce glutamate uptake into synaptic vesicles (Wolosker et al., 1996). However, the inhibition of glutamate uptake is not mimicked by dbcGMP and seems to be mediated by *N*-nitrosylation of sulfhydryl groups, while the inhibition of the Ca^{2+}-dependent release of glutamate is a cGMP-dependent process.

The inhibition of glutamate release observed with zaprinast or the NO donor *S*-nitroso-*N*-acetylpenicillamine was related to an increase in cGMP. Additionally, the membrane-permeable analogs 8-Br-cGMP and dbcGMP also mimicked the inhibitory effects on glutamate release. An inhibition of KCl-evoked glutamate release by the NO-cGMP pathway has also been found in synaptosomes from rat cerebellum (Kamisaki et al., 1995), although in this study the mechanism of the inhibition was not further investigated. Similarly, the inhibition of synaptic transmission in CA_1 hippocampal slices caused by zaprinast and NO donors has also been related to a moderate increase in cGMP levels (Boulton et al., 1994). Thus, it seems that the NO/cGMP-dependent pathway plays an inhibitory role in the control of glutamate release in nerve terminals from several brain areas.

The finding that the inhibitory effect of zaprinast does not alter the depolarization-evoked increase in the cytosolic concentration of $[Ca^{2+}]_c$, is consistent with the lack of effect of zaprinast in the fast-release component – which is highly dependent on a large increase in Ca^{2+} in the active zone (Goda and Stevens, 1994). By contrast, zaprinast inhibits a slow-release component that probably corresponds to the mobilization of vesicles from a reserve pool to the release site (Herrero et al., 1996b). The characterization of the two components of release (Herrero et al., 1996b) has shown that the fast component was resistant to the action of the Ca^{2+}-chelator BAPTA providing evidence that this component represents the release of vesicles docked at the plasma membrane (Herrero et al., 1996b). Thus, the close proximity between the release machinery and the Ca^{2+} channels in docked vesicles prevents BAPTA from competing efficiently with the exocytotic Ca^{2+} sensor. In contrast, the slow component is inhibited by BAPTA suggesting that this component represents the release of a pool of vesicles distal to the plasma membrane (Herrero et al., 1996b). Since zaprinast reduced the slow but not the fast component it is tentative to suggest that a slower vesicle mobilization in the presence of zaprinast is responsible for the inhibitory effect in glutamate release. Although the mechanism of mobilization of reserve vesicles is not fully understood, mobilization may involve the phosphorylation of synapsin I, the Ca^{2+}/calmodulin-dependent kinase II to free vesicles from its interaction with cytoskeleton elements (Valtorta et al., 1992). In this regard it is interesting to note that the majority of the phosphoproteins which are relatively specific substrates for protein kinase G in the forebrain are associated with phospholipids or the cytoskeleton (Wang and Robinson, 1995).

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