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Increased Presynaptic Protein Kinase C Activity and Glutamate Release in Rats with a Prenatally Induced Hippocampal Lesion

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Abstract

We have previously shown that protein kinase C (PKC) activity is up-regulated in nerve terminals of animals that have been subjected to targeted cellular ablation of cortical and hippocampal neurons by treatment with methylazoxymethanol (MAM), which results in impaired long-term potentiation (LTP) and cognitive deficit. In this study we investigated the consequences of increased membrane-bound PKC in the regulation of release of glutamate, the major excitatory transmitter involved in LTP. We show that nerve terminals of MAM-treated rats show higher PKC activity, as monitored by the *in situ* phosphorylation of B-50/GAP-43, in both basal and phorbol ester-stimulated conditions. In these animals, hippocampal nerve endings release a greater amount of glutamate than those of controls, both in basal conditions and when synaptosomes are stimulated with KCl or 3,4-diaminopyridine. The potentiation observed in MAM-treated rats was counteracted by the PKC blocker H-7 and the clostridial tetanus toxin. On the contrary, GABA release was not significantly up-regulated, either in basal or in depolarization-evoked conditions. Therefore our data show that the increase in synaptosomal PKC activity is paralleled by increased glutamate but not GABA release in this animal model. Whether this reflects specific up-regulation of membrane PKC activity in glutamatergic terminals or an alteration in the regulation of glutamate release remains to be determined.

Introduction

An activity-dependent change in synaptic strength, referred to as synaptic plasticity, seems to be responsible for modifications in the function of specific neuronal circuits underlying various forms of learning and memory. The best characterized example of activity-dependent synaptic plasticity in a vertebrate system occurs at glutamatergic synapses in the CA1 region of the hippocampus: high-frequency stimulation of these terminals induces long-term potentiation (LTP) of the efficacy of the junction between Schaeffer collaterals and pyramidal cells (for review see Bliss and Collingridge, 1993). Although the precise molecular mechanisms responsible for this long-lasting change have not yet been clarified, it is now accepted that synaptic plasticity has both a presynaptic and a postsynaptic component (Kullmann and Siegelbaum, 1995), involves the activation of different protein kinases (Kennedy, 1994) and, on the presynaptic side, probably an increase in neurotransmitter release (Malenka, 1994; Malgaroli *et al.*, 1995).

Changes in the activity of the Ca^{2+}/phospholipid-dependent protein kinase (PKC) and the Ca^{2+}/calmodulin-dependent kinase type II have been reported to occur after LTP, and elegant knock-out experiments have also confirmed their important role *in vivo* (Silva *et al.*, 1992; Abeliovich *et al.*, 1993).

However, how the dynamic regulation of these enzymes interplays in such a complex scenario to affect synaptic transmission on both pre- and postsynaptic sides is not completely understood. The involvement of PKC in the process of transmitter release has been postulated by many investigators (Chandler and Lesley, 1989; Herrero *et al.*, 1992a). *In vitro* experiments have shown that activators of PKC, such as phorbol esters, greatly stimulate the Ca^{2+}-dependent release of various transmitters, including glutamate (Herrero *et al.*, 1992b; Coffey *et al.*, 1993; Terrian *et al.*, 1993). This effect was observed when synaptosomes were exposed to 4-aminopyridine, a situation mimicking the invasion of nerve terminals by repetitive action potentials (Tibbs *et al.*, 1989; Barrie *et al.*, 1991). Moreover, phosphorylation of PKC substrates that are selectively localized in nerve terminals, such as B-50/GAP-43 and myristoylated alanine-rich protein kinase-C substrate, has been related to neurotransmitter release (Dekker *et al.*, 1989; Coffey *et al.*, 1994).

Whether changes in PKC activity reflect changes in synaptic transmission also *in vivo* was addressed in the experiments described here, using an animal model in which the presynaptic PKC system is permanently and constitutively hyperactivated in both cortex and hippocampus (Di Luca *et al.*, 1995).

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These animals were obtained by prenatal exposure to methylazoxymethanol acetate (MAM), a potent antiproliferative agent (Johnston and Coyle, 1982) showing peculiar selectivity for actively dividing neuroepithelial cells (Cattaneo *et al.*, 1995). Since cortical and hippocampal neurons enter their final mitotic division around gestational day 15 (Bayer *et al.*, 1993), the injection of a single dose of MAM in pregnant rats at gestational day 15 induces dose-dependent hypoplasia of the cortex and hippocampus in 100% of the offspring. When adult, these animals show cognitive deficits (Di Luca and Cattabeni, 1991) and lack of induction of LTP in the CA1 region of hippocampal slices (Ramakers *et al.*, 1993). Interestingly, we have shown that these neuroanatomical and electrophysiological abnormalities have their molecular counterpart in a consistent redistribution of PKC in synaptosomes of the cortex and hippocampus, the brain areas most affected by prenatal treatment with MAM. Indeed, the amount of PKC localized in the membrane compartment of synaptosomes is significantly increased, and this increase is paralleled by an equivalent decrease in PKC in the cytosolic compartment. This modified translocation is common to all PKC isoforms measured in synaptosomes, i.e. α and β (Ca^{2+}-dependent) and ε (Ca^{2+}-independent) isoenzymes (Caputi *et al.*, 1996). In addition, PKC substrates specifically localized at the presynaptic level, such as B-50/GAP-43, show a higher degree of phosphorylation in basal conditions (Di Luca *et al.*, 1995). Moreover, we have previously shown that the change in B-50/GAP-43 phosphorylation, as measured by a *post hoc* phosphorylation assay, is detectable in these animals as early as postnatal day 8 and is still present after 9 months, indicating that the alteration in PKC redistribution is not a transient phenomenon but persists throughout the life of the animal (Di Luca *et al.*, 1993).

In this study we investigated the consequences of the *in vivo* increased PKC activity in regulating the release of glutamate, the major excitatory transmitter involved in LTP, and of GABA. Specifically, the following questions were addressed: (i) is it possible to further stimulate PKC translocation to membranes of synaptosomes in MAM-treated rats? (ii) is the basal or depolarization-evoked transmitter release increased in the hippocampus of MAM-treated rats? (iii) does additional PKC translocation still induce a similar increase in neurotransmitter release in controls and MAM-treated rats?

Materials and methods

Animal treatments

Pregnant Sprague–Dawley rats (Charles River, Calco, Italy) received a single intraperitoneal injection of 25 mg/kg MAM (Sigma) diluted in sterile saline, or of vehicle alone, at gestational day 15. Litters were born on day 22 or 23 of gestation. No alterations of gestational parameters were observed, in agreement with previous results (Balduini *et al.*, 1991). Twenty-one days after birth, pups were weaned and housed under standard conditions with water and food *ad libitum*. At 2 months of age the animals were killed by decapitation and the hippocampus was rapidly dissected, weighed and processed.

Synaptosome preparation

Synaptosomes were prepared from the hippocampus of control and MAM-treated rats and purified by Percoll–sucrose density gradient centrifugation according to Dunkley *et al.* (1988). The tissue was homogenized in 14 volumes of 0.32 M sucrose, adjusted to pH 7.4 with NaOH, and centrifuged (5 min, 1000 *g*, at 0–4°C). Aliquots (2 ml) of the supernatant were layered on a discontinuous four-step Percoll gradient comprising 1.8 ml each of 23, 15, 10 and 3% Percoll in sucrose (vol/vol), adjusted to pH 7.4 with HCl, and centrifuged at 32 000 *g* for 5 min. The third fraction (10–15% interface) was removed and pooled in each experiment. For release experiments, the pooled fractions were washed once with 10 ml of physiological medium of the following composition (mM): NaCl, 125; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1; $NaHCO_3$, 22; glucose, 10 (aeration with 95% O_2 and 5% CO_2); pH 7.2–7.4. Synaptosomes were collected at 15 000 *g* for 15 min. For Western blot analysis and *in situ* phosphorylation experiments, purified synaptosomes were collected in a medium with a different composition, as detailed below.

Transmitter release

Identical aliquots of the synaptosome suspension (ranging between 0.25 and 0.5 mg protein in the different experiments) were distributed on 0.65 μm micropore filters placed at the bottom of 20 parallel superfusion chambers maintained at 37°C (Raiteri *et al.*, 1974). Superfusion was then started with standard medium at the rate of 0.5 ml/min, and continued for a total of 48 or 52 min. After an initial 36 min period to equilibrate the system, three separate fractions of the superfusate were successively collected according to the following scheme: a first fraction (basal release) lasting 3 min [4 min when using 3,4-diaminopyridine (3,4-DAP)], a second fraction (evoked release) of 6 min (8 min when using 3,4-DAP), and the last fraction (basal release) of 3 min (4 min when using 3,4-DAP). A 90 s period of stimulation using 12 or 30 mM KCl (NaCl substituting for an equimolar concentration of KCl) or 10 μM 3,4-DAP was applied after collecting the first fraction. When used, Ca^{2+}-free medium was introduced 10 min before depolarization. 4β-Phorbol dibutyrate (PDBu; 0.1 μM) or 10 μM 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) was introduced 10 min before depolarization. In a set of experiments synaptosomes were incubated (1 h, 37°C) in the presence or absence of 50 nM tetanus toxin, before starting superfusion.

Glutamate and GABA determination

Collected fractions and superfused filters were analysed for their endogenous transmitter content. The amount of endogenous glutamate or GABA released or remaining in the synaptosomes after superfusion was measured by high-performance liquid chromatography after precolumn derivatization with *o*-phthalaldehyde and fluorimetric detection. Amino acid resolution was obtained using a C18 reverse-phase chromatography column (Sep-pack cartridge; Waters, Millford, OH; 10 cm × 4.6 mm, 3 μm) and a three-solvent discontinuous gradient, from 23% methanol in 0.1 M acetate buffer, pH 6, to 46% methanol in 0.1 M acetate buffer, pH 5.8, in 22 min at a flow rate of 0.9 ml/min. The depolarization-evoked release was estimated by subtracting the basal release from the release evoked in the 6 min (8 min in the experiments with 3,4-DAP).

Subcellular localization of PKC

Purified synaptosomes were incubated in the absence or presence of increasing concentrations of PDBu (10^{-9} to 10^{-5} M). The incubation was carried out at 30°C and stopped after 7 min. The reaction was blocked by addition of cold Krebs buffer containing 10 mM EGTA. Synaptosomes were collected and then resuspended in lysis buffer according to Shearman *et al.* (1991). The soluble and membrane fractions were then prepared and collected as previously described (Di Luca *et al.*, 1995). PKC was recognized in these fractions by Western blot analysis using a polyclonal antibody (Upstate Biotechnology, Lake Placid, NY; dilution 1:1000). Antigen–antibody complexes were detected by enhanced chemiluminescence (ECL; Amersham).

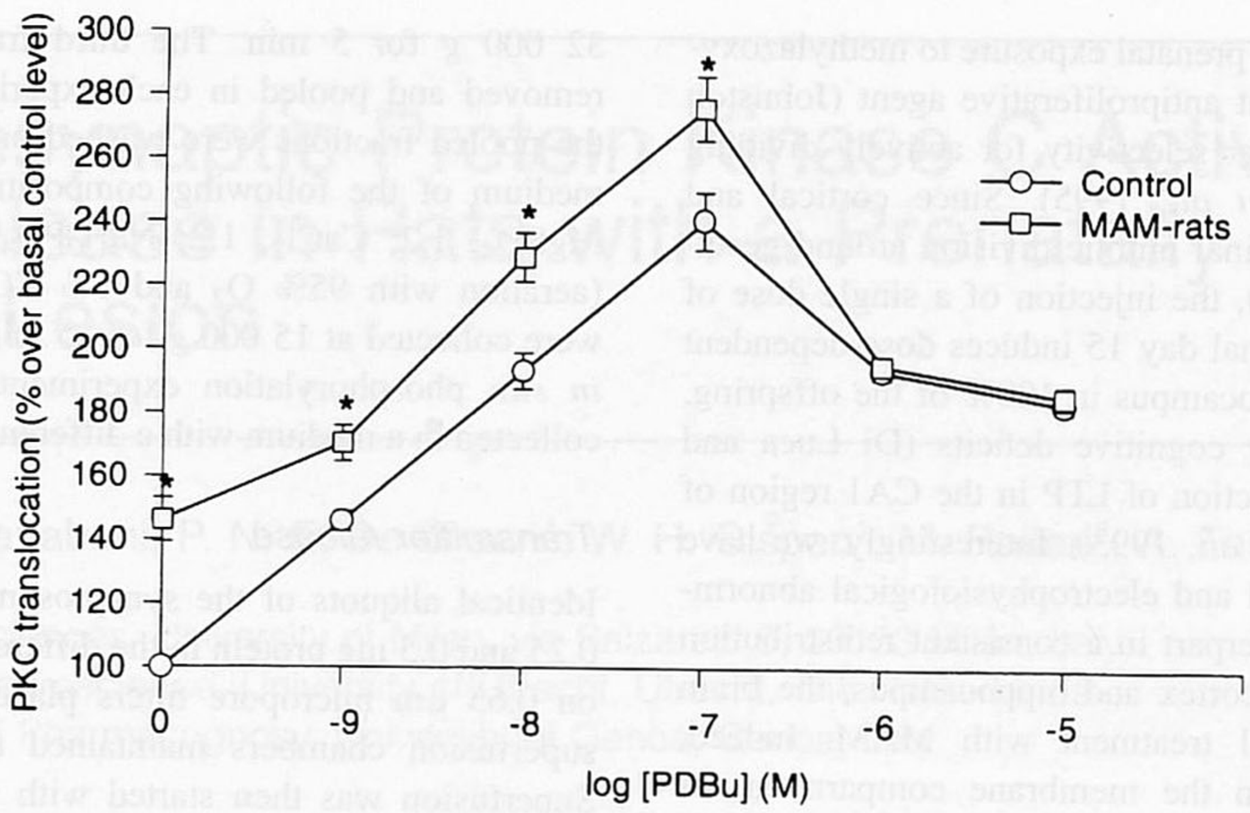

FIG. 1. Membrane-bound PKC in hippocampal synaptosomes from control and MAM-treated rats following exposure to increasing concentrations of PDBu. Synaptosomes were exposed for 7 min to concentrations of PDBu as indicated. Data are expressed as percentage of PKC translocation with respect to controls in basal conditions. Six independent experiments on pooled tissues of four control and eight MAM-treated rats to obtain an equivalent amount of protein were performed. $*P < 0.001$, MAM-treated rats versus controls.

In situ B-50 phosphorylation

The synaptosomal preparation obtained from the hippocampus of control and MAM-treated rats was diluted in Krebs buffer of the following composition: 124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose and 26 mM $NaHCO_3$, saturated with CO_2/O_2 5%/95%, pH 7.4, to a protein concentration of 1 μg/μl and labelled with 2 μCi of ^{32}P (3000 Ci/mmol; Amersham) per microgram of protein at 34°C for 60 min. Synaptosomes were then incubated for 7 min at 30°C in the absence or presence of 10^{-7} M PDBu or in high-potassium buffer. The reaction was stopped with 10 μl denaturing solution containing 3% sodium dodecyl sulphate, 5% mercaptoethanol, 10% glycerol and 0.002% bromophenol blue in 62.5 mM Tris, pH 6.8. *In situ* B-50 phosphorylation was measured by quantitative immunoprecipitation as previously described (De Graan *et al.*, 1989; anti B-50 serum: 8521, dilution 1:200).

Quantitative analysis and statistical analysis

Quantitative analysis of Western blots of membrane-bound PKC and of phosphorylated and immunoprecipitated B-50/GAP-43 was performed by computer-assisted imaging (NIH-Image, developed by Dr Wayne Rasband, NIH, Bethesda, MD)

Statistical evaluation was performed using one-way analysis of variance and Student's *t post hoc* comparison tests.

Results

PKC can be further translocated in hippocampal synaptosomes of MAM-treated rats

Figure 1 shows that in MAM-treated rats the proportion of PKC bound to the membrane compartment of hippocampal synaptosomes in basal conditions was increased by 46% when compared with controls, confirming previous observation (Di Luca *et al.*, 1995).

Despite this profound alteration in the basal compartmentalization of the kinase in nerve terminals, incubation with phorbol ester at up to 10^{-7} M was still able to produce a dose-dependent translocation of PKC to the membrane compartment (Fig. 1). In addition, the percentage increase in translocation in synaptosomes of controls (140.2 ± 8.93.2% versus basal control level) and MAM-treated rats (175.8 ± 10.12% versus basal MAM level) was quantitatively comparable and not statistically different at the maximal PDBu concentration. The effect of PDBu on PKC translocation, maximal at 10^{-7} M, showed a decrease at higher concentrations, indicating down-regulation of the kinase in both experimental groups, as previously reported by others (Oda *et al.*, 1991).

The treatment with 10^{-7} M PDBu was also able to induce an increase in the phosphorylation of the presynaptic substrate B-50/GAP-43 as measured by *in situ* phosphorylation. Table 1 shows the results of densitometric analysis of B-50/GAP-43 phosphorylated and immunoprecipitated from hippocampal synaptosomes from control and MAM-treated rats under basal conditions and after stimulating intact synaptosomes with either PDBu or high-K^+ buffer. These experiments confirm that the basal phosphorylation of this protein is increased in treated animals when compared with controls (by 43.8 ± 8.3%), and reveal that treatment with PDBu and K^+ depolarization are able to increase B-50 phosphorylation in both control and treated animals, indicating that PKC translocation indeed results in increased kinase activity in these animals.

TABLE 1. Phosphorylation of B-50/GAP-43 in synaptosomes from hippocampus of control and MAM-treated rats under basal and K^+-stimulated conditions

Condition	Control rats	MAM-treated rats
Basal	100 ± 8	144 ± 8[a]*
PDBu (10^{-7} M)	230 ± 64[a]**	242 ± 40[b]**
K^+ (30 mM)	227 ± 7[a]**	288 ± 49[b]**

[a]Percentage (±SD) of basal control value.
[b]Percentage (±SD) of basal value for MAM-treated rats.
$*P < 0.05$ versus basal control.
$**P < 0.001$ versus respective basal value.

Basal and K^+-evoked glutamate but not GABA release is increased in MAM-treated rats

Basal and K^+-evoked glutamate release was evaluated in purified synaptosomes from the hippocampus of control and MAM-treated rats.

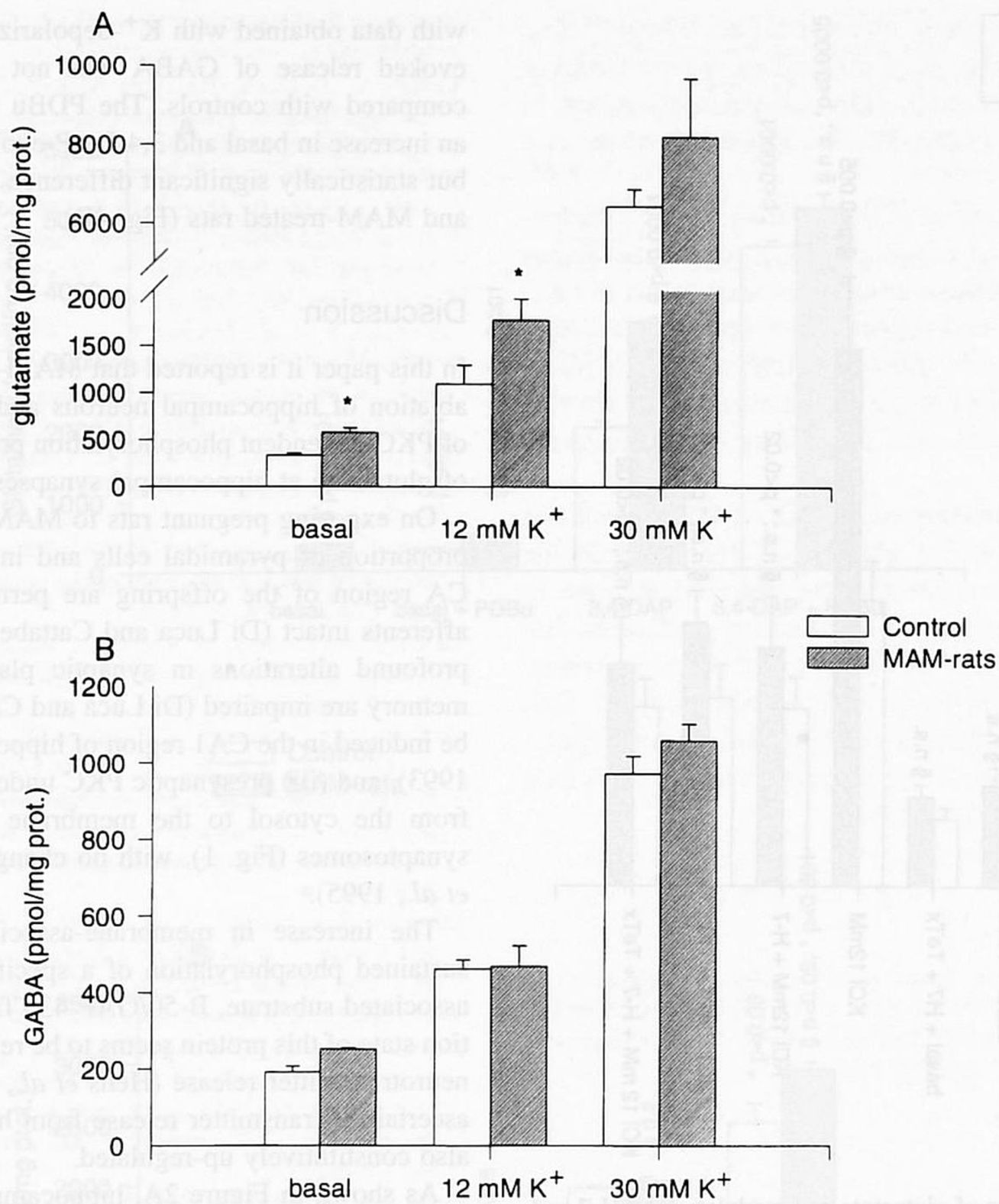

FIG. 2. Basal and K^+-evoked release of glutamate (A) and GABA (B) from hippocampal synaptosomes of control and MAM-treated rats. Synaptosomes were prepared and purified as described under Materials and methods. Depolarization was obtained by superfusion with a 90 s pulse with 12 or 30 mM KCl, as indicated. Results are the means ± SEM of at least three independent experiments. $*P < 0.01$ versus controls.

Figure 2A shows that the basal release of glutamate in hippocampal synaptosomes of MAM-treated rats was increased by ~73% compared with control animals. In these conditions, the Ca^{2+}-dependent basal glutamate release represented 58 ± 8 and 64 ± 5% of the total spontaneous release in control and MAM-treated rats respectively. In fact the basal release was 332 ± 23 and 575 ± 50 pmol/mg protein in control and MAM-treated rats respectively ($P < 0.001$, $n = 8$), and the basal release in Ca^{2+}-free medium was 191 ± 27 (controls) and 370 ± 29 pmol/mg protein (MAM-treated rats; $P < 0.005$, $n = 5$).

When synaptosomes were superfused with 12 mM KCl a larger increase in total glutamate release in MAM-treated rats compared with control animals (control: mean ± SD, 1088 ± 205 pmol/mg protein; MAM-treated rats: 1771 ± 225 pmol/mg protein, $P < 0.01$, $n = 4$) was observed (Fig. 2A). Under these experimental conditions the depolarization-evoked overflow of glutamate was almost totally (>85%) dependent on the presence of extracellular calcium (data not shown). Superfusion of synaptosomes with 30 mM KCl induced a further increase in total glutamate release. In this condition, however, statistically non-significant differences were observed between the two experimental groups. Interestingly the Ca^{2+}-dependent glutamate overflow evoked by 30 mM KCl was 32 ± 14%, and 20 ± 3% in control and MAM-treated rats respectively, since hyperdepolarization (pmol/mg protein) was 6430 ± 860 for controls, 8200 ± 1480 for MAM-treated rats ($n = 5$) in Ca^{2+} containing medium, and in Ca^{2+} free medium it was 4370 ± 208 for controls and 6570 ± 1120 for MAM-treated rats ($n = 4$).

GABA release was also measured in the same synaptosomal preparation, and the results are reported in Figure 2B. Levels of basal and K^+-evoked GABA release were not statistically different in control and MAM-treated rats.

The total amounts of glutamate and GABA measured in intact, non-stimulated synaptosomes were similar in control and MAM-treated rats (glutamate, 41.2 ± 9.5 nmol/mg protein in controls and 46.9 ± 11.4 nmol/mg protein in MAM-treated rats; GABA, 4.78 ± 1.8 and 4.81 ± 1.3 respectively), suggesting that the observed increase in glutamate release was not due to a different content of glutamate in the nerve terminals of MAM-treated rats.

Figure 3 shows that the PKC inhibitor H-7 decreased the glutamate overflow induced by 12 mM KCl in MAM-treated rats. In the presence of H-7 a significant difference between two experimental groups was no longer observed.

In an attempt to differentiate between the vesicular and cytosolic pools of glutamate, experiments were performed in which synaptosomes were incubated with the vesicular clostridium toxin, tetanus toxin. At 50 nM the toxin inhibited glutamate overflow in MAM-treated rats by ~50%. Concomitant exposure to both tetanus toxin and H-7 did not further modify glutamate overflow (Fig. 3).

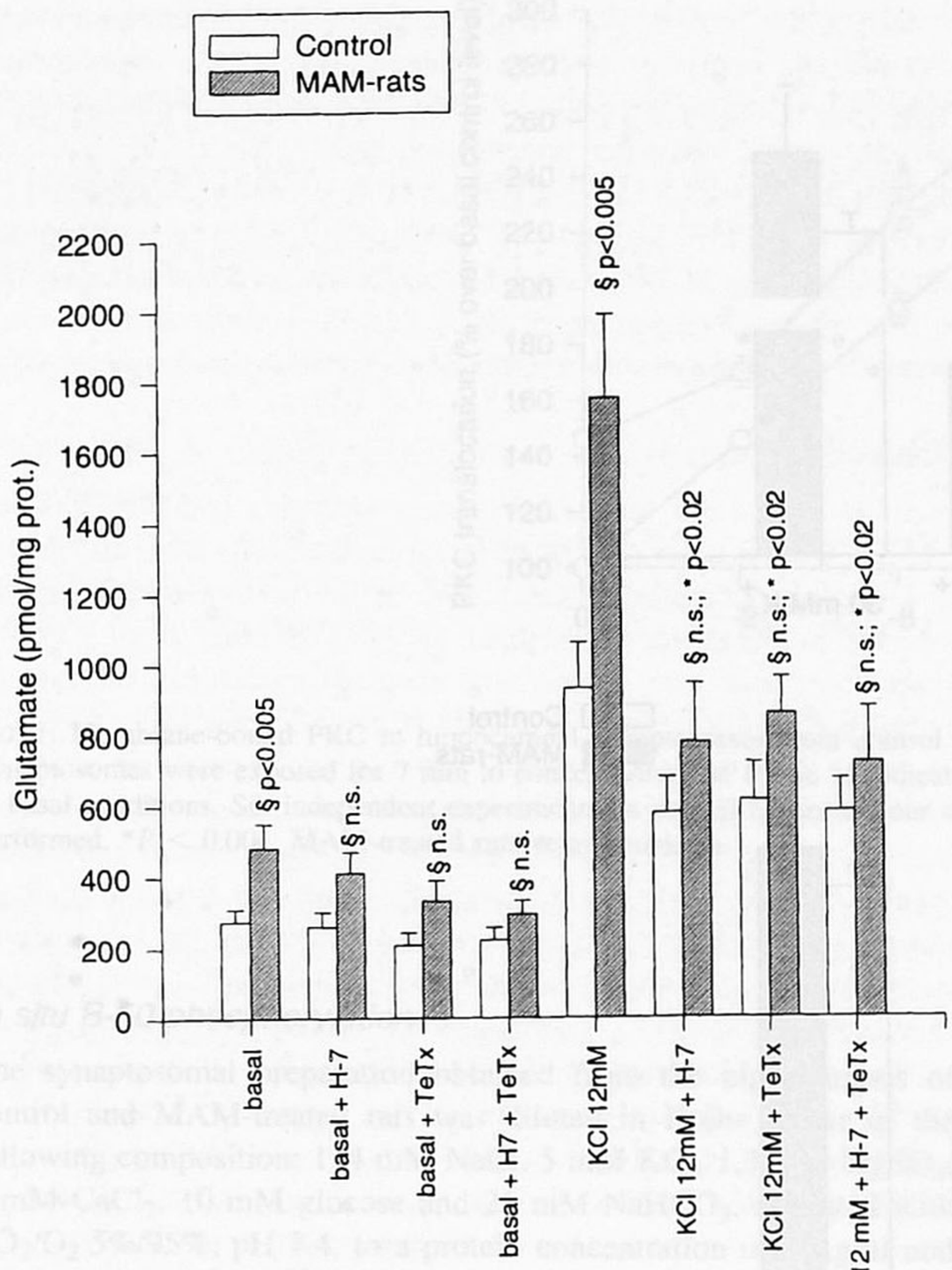

FIG. 3. Basal and K^+-evoked release of glutamate and modulation by H-7 and tetanus toxin in hippocampal synaptosomes obtained from control and MAM-treated rats. A 90 s depolarization pulse was applied at the end of the first fraction collected; 10 μM H-7 was introduced 10 min before depolarization; 50 nM tetanus toxin was applied in incubation 1 h at 37°C before starting superfusion. Results are the means ± SEM of at least three independent experiments. §, versus respective controls; *versus depolarization-evoked release within the same experimental group.

Modulation of PKC activity partially affects 3,4-DAP-induced glutamate release in MAM-treated rats

To further demonstrate a modulatory role of PKC in glutamate release in this system, we superfused synaptosomes with PDBu in the absence or presence of 3,4-DAP. Ten minutes of exposure of synaptosomes to 10^{-7} M PDBu led to an increase in basal glutamate release; the potentiation observed, however, was markedly different in the two experimental groups (Fig. 4A; increase over basal release in controls was 69.5 ± 3.1% and that in MAM-treated rats was 38.4 ± 0.89%), suggesting that under the basal condition glutamate release in MAM-treated rats is already potentiated.

Depolarization with 3,4-DAP, like K^+-depolarization, evoked a larger glutamate release in MAM-treated rats than in control animals (Ca^{2+}-dependent release in both control and MAM-treated rats ranged between 90 and 95%). When 3,4-DAP-evoked glutamate release was measured in synaptosomes previously exposed to PDBu, the potentiation appeared to be less pronounced in synaptosomes obtained from MAM-treated animals compared with control animals (controls, 125.9 ± 3.4%; MAM-treated rats, 43.9 ± 1.1%; Fig. 4A).

Experiments were also performed to evaluate the possible modulatory effect of PKC on 3,4-DAP-evoked GABA release. In agreement with data obtained with K^+-depolarization, both basal and 3,4-DAP-evoked release of GABA was not affected in MAM-treated rats compared with controls. The PDBu treatment was able to produce an increase in basal and 3,4-DAP-evoked GABA release, and a slight but statistically significant difference was observed between controls and MAM-treated rats (Fig. 4B).

Discussion

In this paper it is reported that MAM-treated rats, carrying a targeted ablation of hippocampal neurons and a constitutive hyperactivation of PKC-dependent phosphorylation processes, show increased release of glutamate at hippocampal synapses.

On exposing pregnant rats to MAM at gestational day 15, a large proportion of pyramidal cells and interneurons in the hippocampal CA region of the offspring are permanently knocked out, leaving afferents intact (Di Luca and Cattabeni, 1991). These animals show profound alterations in synaptic plasticity, since (i) learning and memory are impaired (Di Luca and Cattabeni, 1991), (ii) LTP cannot be induced in the CA1 region of hippocampal slices (Ramakers *et al.*, 1993), and (iii) presynaptic PKC undergoes a profound redistribution from the cytosol to the membrane compartment in hippocampal synaptosomes (Fig. 1), with no change in its total amount (Di Luca *et al.*, 1995).

The increase in membrane-associated PKC is paralleled by a sustained phosphorylation of a specific presynaptic and membrane-associated substrate, B-50/GAP-43 (Table 1). Since the phosphorylation state of this protein seems to be relevant for its role in modulating neurotransmitter release (Hens *et al.*, 1993a, b), it was of interest to ascertain if transmitter release from hippocampal synaptosomes was also constitutively up-regulated.

As shown in Figure 2A, hippocampal nerve endings from MAM-treated rats release a greater amount of glutamate than those of controls, both in basal and in stimulated conditions. The possible causal relationship between this phenomenon and the reported up-regulation of PKC activity in these animals is suggested by the results obtained with the PKC blocker H-7, which indeed decreases the KCl-evoked release in MAM-treated rats to levels similar to those of controls. Moreover, the preferential involvement of the vesicular pool of glutamate in the effect observed in MAM-treated rats seems to be indicated by the lack of potentiation when synaptosomes are pretreated with tetanus toxin, a clostridial toxin which selectively cleaves the synapse-associated protein synaptobrevin, thus inhibiting vesicular transmitter release (Schiavo *et al.*, 1992). Furthermore, in the presence of tetanus toxin H-7 failed to exert any additional effect on glutamate release. Indeed the effect of pretreatment with 50 nM tetanus toxin is different between synaptosomes of control and MAM-treated rats, being higher in the latter case. This result might suggest an increase in the number of vesicles or vesicular glutamate in MAM-treated rats, which might account for the increased glutamate release observed both in basal and in depolarization-evoked conditions, whereas this parameter seems to be unchanged in GABAergic nerve terminals.

Since it has been suggested that tetanus toxin provides a useful tool to discriminate between docked and non-docked vesicles involved in neurotransmitter release (McMahon *et al.*, 1992; Pellegrini *et al.*, 1994), our data suggest that in MAM-treated rats there might be an imbalance between the different pools of glutamatergic vesicles. An alteration in the various steps involved in vesicle recycling can also account for the discrepancies in glutamate release observed in MAM-treated rats.

The total glutamate contents in synaptosomes from control and MAM-treated rats are similar. It is interesting to note in this context

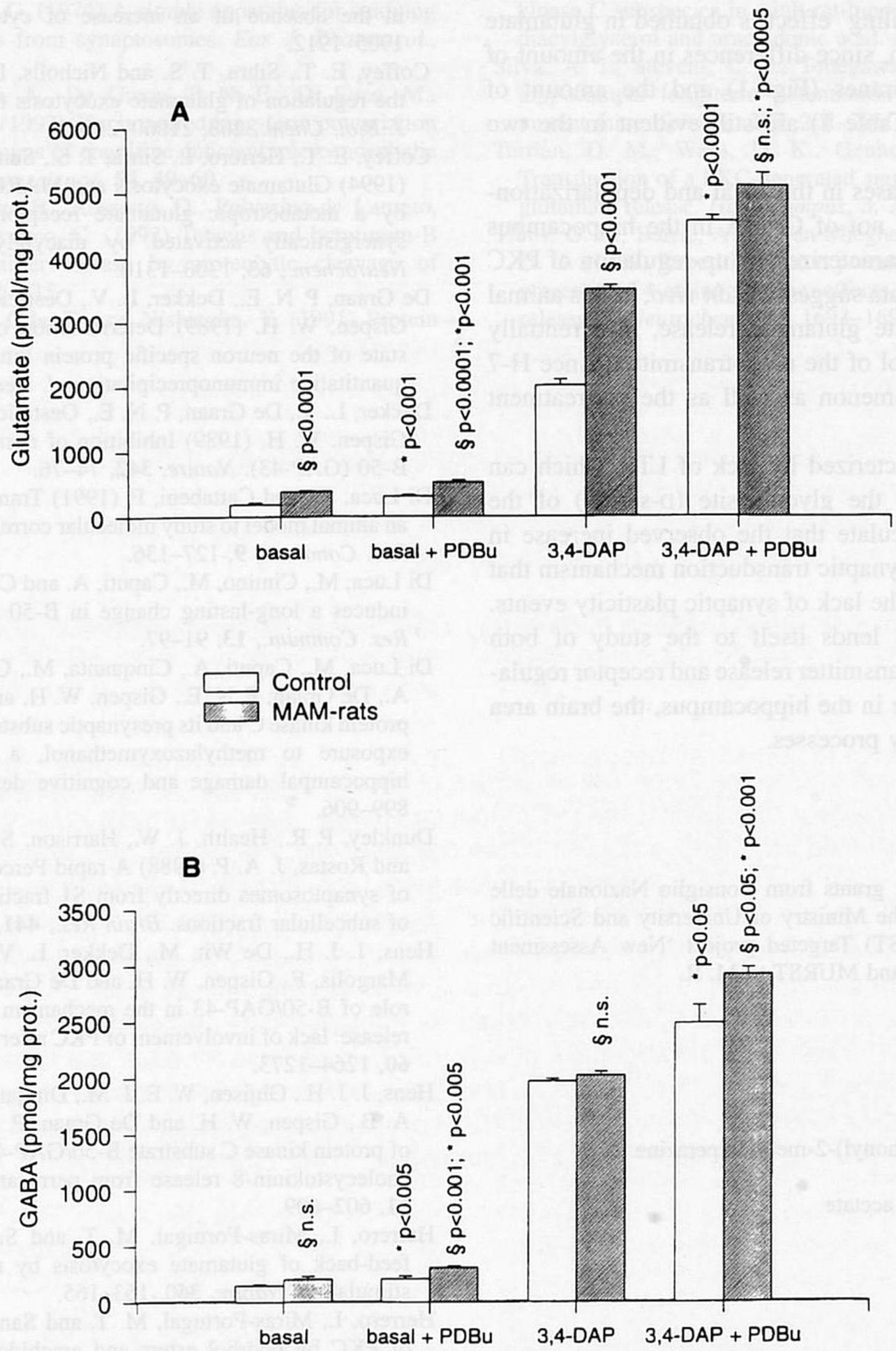

FIG. 4. Basal and 3,4-DAP-evoked of glutamate (A) and GABA (B) and modulation by PDBu from hippocampal synaptosomes obtained from control and MAM-treated rats. A 90 s depolarization pulse was applied at the end of the first fraction collected; 10^{-7} M PDBu was introduced 10 min before depolarization. See Materials and methods for other technical details. Data are means ± SEM of at least three experiments run in triplicate. §, versus respective controls; *versus non-PDBu-modulated release within the same experimental group

that the concentrations of synaptophysin, a major 38 kDa integral membrane protein present in small clear-core vesicles (Johnston *et al.*, 1989), are not changed in MAM-treated rats when compared with controls (Di Luca *et al.*, 1995).

Interestingly, GABA release is not significantly up-regulated in hippocampal synaptosomes from MAM-treated rats, under either basal or depolarized conditions. What determines this selectivity remains to be elucidated; our data, however, indicate that selective modulation exerted by PKC on glutamatergic but not on GABAergic terminals is unlikely, since PDBu, an activator of PKC, potentiates GABA and glutamate release alike (Fig. 3A, B).

Since MAM-treated rats show impairments in synaptic plasticity, as evidenced by the lack of LTP in the CA1 region of the hippocampus (Ramakers *et al.*, 1993) and deficits in several tests for learning and memory (Di Luca and Cattabeni, 1991), we tested whether this was due to the inability of nerve terminals of these animals to respond to stimuli known to induce PKC activation and neurotransmitter release in synaptosomes from normal animals. The results obtained by PDBu stimulation and by superfusing with K^+ show that the percentage increase over basal levels of both PKC activity (Fig. 1) and B-50/GAP-43 phosphorylation is similar in control and MAM-treated rats (Table 1), suggesting that the increased basal activity does not prevent its further activation in MAM-treated rats.

When glutamate release in the presence of 12 mM K^+ is considered, again the increase over basal release is similar in control and MAM-treated rats (Fig. 2A), suggesting that the system regulating glutamate release is not saturated at this K^+ concentration. Similarly, with 3,4-DAP the increase in glutamate release over basal was increased to a similar extent in both experimental groups (Fig. 3A).

Interestingly, the differences in glutamate release between control and MAM-treated rats are abolished with high K^+ (30 mM) or with 3,4-DAP in the presence of PDBu (Figs 2A and 3A), suggesting that

under maximal stimulation a 'ceiling' effect is obtained in glutamate release but not in PKC activation, since differences in the amount of PKC translocated to the membranes (Fig. 1) and the amount of B-50/GAP-43 phosphorylated (Table 1) are still evident in the two experimental groups.

In conclusion, we report increases in the basal and depolarization-evoked release of glutamate but not of GABA in the hippocampus of MAM-treated rats, a model characterized by up-regulation of PKC activity in nerve terminals. Our data suggest that *in vivo*, in this animal model, PKC is able to modulate glutamate release, preferentially interfering with the vesicular pool of the neurotransmitter, since H-7 is able to counteract this phenomenon as well as the pretreatment with tetanus toxin.

Since these animals are characterized by lack of LTP, which can be rescued by agents acting at the glycine site (D-serine) of the NMDA receptor, we might speculate that the observed increase in glutamate release alters the postsynaptic transduction mechanism that might be in turn responsible for the lack of synaptic plasticity events.

Therefore, this animal model lends itself to the study of both molecular mechanisms of neurotransmitter release and receptor regulation of the glutamatergic synapse in the hippocampus, the brain area involved in learning and memory processes.

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Abbreviations

3,4-DAP	3,4-diaminopyridine
H-7	1-(5-isoquinolinylsulphonyl)-2-methylpiperazine
LTP	long-term potentiation
MAM	methylazoxymethanol acetate
PDBu	4β-phorbol dibutyrate
PKC	protein kinase C

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