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Protein kinase C activity blocks neuropeptide Y-mediated inhibition of glutamate release and contributes to excitability of the hippocampus in status epilepticus

Ana P. Silva,*[,‡] Joana Lourenço,[‡] Sara Xapelli,[‡] Raquel Ferreira,[‡] Heidi Kristiansen,[§] David P. D. Woldbye,[§] Catarina R. Oliveira,[†,‡] and João O. Malva[†,‡,1]

*Institute of Pharmacology and Therapeutics and [†]Institute of Biochemistry, Faculty of Medicine, University of Coimbra, Coimbra, Portugal; [‡]Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal; [§]Laboratory of Neuropsychiatry, Rigshospitalet University Hospital and Department of Pharmacology, University of Copenhagen, Denmark

ABSTRACT **The unbalanced excitatory/inhibitory neurotransmitter function in the neuronal network afflicted by seizures is the main biochemical and biophysical hallmark of epilepsy. The aim of this work was to identify changes in the signaling mechanisms associated with neuropeptide Y (NPY)-mediated inhibition of glutamate release that may contribute to hyperexcitability. Using isolated rat hippocampal nerve terminals, we showed that the KCl-evoked glutamate release is inhibited by NPY Y_2 receptor activation and is potentiated by the stimulation of protein kinase C (PKC). Moreover, we observed that immediately after status epilepticus (6 h postinjection with kainate, 10 mg/kg), the functional inhibition of glutamate release by NPY Y_2 receptors was transiently blocked concomitantly with PKC hyperactivation. The pharmacological blockade of seizure-activated PKC revealed again the Y_2 receptor-mediated inhibition of glutamate release. The functional activity of PKC immediately after status epilepticus was assessed by evaluating phosphorylation of the AMPA receptor subunit GluR1 (Ser-831), a substrate for PKC. Moreover, NPY-stimulated [^{35}S]GTPγS autoradiographic binding studies indicated that the common target for Y_2 receptor and PKC on the inhibition/potentiation of glutamate release was located downstream of the Y_2 receptor, or its interacting G-protein, and involves voltage-gated calcium channels.—Silva, A. P., Lourenço, J., Xapelli, S., Ferreira, R., Kristiansen, H., Woldbye, D. P. D., Oliveira, C. R., Malva, J. O. Protein kinase C activity blocks neuropeptide Y-mediated inhibition of glutamate release and contributes to excitability of the hippocampus in status epilepticus. *FASEB J.* 21, 000–000 (2007)**

Key Words: Y_2 receptor · nerve terminal · seizures · phosphorylation

NEUROPEPTIDE Y (NPY) HAS BEEN IMPLICATED in several centrally mediated physiological functions such as regulation of circadian rhythms, body temperature, sexual behavior, blood pressure, appetite, and neuroendocrine secretions (1–3). This peptide has also been shown to modulate anxiety-related disorders and cognitive functions such as learning and memory (4), and it has been widely suggested to be involved in epileptogenesis and epilepsy (5–7). Moreover, the broad physiological actions of NPY are transduced by at least five cloned NPY receptors subtypes (Y_1, Y_2, Y_4, Y_5, and y_6), which belong to the G-protein-coupled receptor superfamily (8). NPY and NPY Y_1, Y_2, and Y_5 receptors are particularly abundant in the hippocampus, where they play a major role in regulating glutamate release (3, 9).

Endogenous NPY seems to play a major role in regulating seizure activity, and this is supported by the massive increase of NPY levels under epileptic conditions in both inhibitory interneurons and excitatory granule cells (5, 10). Moreover, seizure-related increase in NPY expression is accompanied by modified levels of NPY receptor subtypes in the hippocampus (11), and it seems that more than one receptor subtype could be responsible for mediating the antiepileptic effects of NPY, but clearly Y_2 and/or Y_5 receptors are key players (10, 12, 13). It was reported that NPY-deficient mice have increased susceptibility to seizures induced by pentylenetetrazol or kainate and that seizure activity was reduced in rat models of NPY overexpression (14–17). Furthermore, NPY acting *via* Y_2 receptors can inhibit excitatory synaptic transmission (18, 19), the release of glutamate (20, 21), and epileptiform activity (10, 22) in hippocampal slices. NPY was also found to inhibit kainate-induced seizures *via* Y_5 receptors (13, 23), but recently El Bahh and collaborators (12) demonstrated that Y_2 receptors play a key role in the

[1]Correspondence: Center for Neuroscience and Cell Biology, Institute of Biochemistry, Faculty of Medicine, University of Coimbra, 3004–504 Coimbra, Portugal. E-mail: jomalva@fmed.uc.pt

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antiepileptic properties of NPY in the hippocampus. Recently, we observed that in different stages of the epileptogenic process, the ability of different NPY receptors to modulate the release of glutamate is changed (21). We suggested that in the acute phase of epilepsy, a transient loss of NPY-mediated inhibition of glutamate release might be associated with hyperexcitability of the hippocampal neuronal network and seizure activity. After the acute phase, an adaptation of the NPYergic modulation of glutamate release was identified and the inhibitory effect of NPY was again close to control levels (21).

It is well established that protein kinase A- and C-mediated substrate phosphorylation is implicated in a broad spectrum of neuronal events, including neuronal differentiation, process outgrowth, axonal regeneration (24–26), glutamate-induced neurotoxicity (27), and neurotransmitter release (28, 29). It has also been shown that protein kinases act on exocytotic machinery (30) and modulate ion channels (29, 31) and receptor activity/function (32).

Taken together, the current data indicate that NPY receptors clearly play a role in normal physiological conditions, as well as in response to pathological hyperactivity in the hippocampus (6, 21, 33, 34). Yet despite the role of these receptors already mentioned, there is little consensus about the intracellular signaling pathway (or pathways) that can interfere with their function under pathological conditions such as epilepsy.

MATERIALS AND METHODS

Rodent model of temporal lobe epilepsy

Male Wistar rats 6–7 wk of age were used. Kainate (KA; Ocean Produce International, Shelburne, Nova Scotia, Canada) was dissolved in a maximum volume of 500 μl of sterile 0.9% NaCl and injected intraperitoneally (i.p.) at a dosage of 10 mg/kg body wt. The control group of animals was injected with the same volume of saline (21). Rats were observed for at least 3 h and their behavior was rated as described previously (35). All the animals used in the present work achieved status epilepticus and were sacrificed 6 h or 24 h after KA injection.

All procedures involving experimental animals were performed in accordance with European Community guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Preparation of rat hippocampal synaptosomes

A partially purified synaptosomal fraction (P_2) was isolated from hippocampi or from hippocampal subregions CA1, CA3, and dentate gyrus (DG) of male Wistar control or epileptic rats (sacrificed 6 h or 24 h after KA injection), essentially as described for brain cortex (36), with some modifications (21, 37, 38). The hippocampi were homogenized in 0.32 M sucrose, 10 mM HEPES-Na, pH 7.4, using a Thomas B-Potter homogenizer (Thomas Scientific, Swedesboro, NJ, USA) and centrifuged at 3000 *g* for 2 min. The pellet obtained was resuspended, followed by sedimentation at the same speed. The combined supernatants were spun for 12 min at 14,600 *g* and a P_2 pellet was obtained. The upper white layer of the pellet containing synaptosomes (39) was removed with a small spoon and resuspended in the same sucrose medium used before.

Coronal slices of hippocampus (800 μm-thick) were prepared for isolation of the synaptosomes from hippocampal subregions (CA1, CA3, and DG) of nonepileptic and epileptic rats (6 h and 24 h after KA injection). In each slice, the fimbria and the subiculum were separated from the rest of the slice under stereomicroscopic observation. CA3 subslices were obtained by separation from CA1 and DG, and the last separation (CA1 from DG) was performed through the hippocampal sulcus (38). The pooled subslices were homogenized in the sucrose medium using a Thomas AA-Potter, transferred to Eppendorf tubes, and centrifuged as described for the isolation of whole hippocampal synaptosomes. The protein concentration was determined by the Biuret method (40) for glutamate release experiments, and the synaptosomes were stored as drained pellets containing 1 mg of protein.

Preparation of hippocampal lysates

For Western blot analysis, the hippocampi or hippocampal subregions (CA1, CA3, and DG) of male Wistar control or epileptic rats (sacrificed 6 h or 24 h after KA injection) were removed as described in the foregoing section. Then the tissue was individually homogenized with 40 strokes in a glass homogenizer in 50 mM Tris-HCl, 0.5% Triton X-100, supplemented with 100 μM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol (DTT), 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 5 μg/ml pepstatin A (all from Sigma Chemical Co., St. Louis, MO, USA), pH 7.4, at 4°C. Protein concentration was determined by the bicinchoninic acid method (41).

Measurement of glutamate release

The release of endogenous glutamate was followed using a continuous fluorimetric assay as described previously (42), with some modifications (37, 38). Synaptosomes (1 mg protein) were preincubated for 20 min at 37°C in the following medium (in mM): 132 NaCl, 1 KCl, 1 $MgCl_2$, 1.2 H_3PO_4, 0.1 $CaCl_2$, 10 glucose, 10 HEPES-Na, pH 7.4, with 0.1% fatty acid-free BSA. After this period, NPY or Y_1, Y_2, or Y_5 receptor agonists ([Leu^{31}, Pro^{34}]NPY, NPY(13–36) or NPY(19–23)-(Gly^1, Ser^3, Gln^4, Thr^6, AL^{31}, Aib^{32}, Gln^{34})-PP, respectively) (Bachem AG, Bubendorf, Switzerland), antagonists [BIBP3226 (Peninsula Labs, Belmont, CA, USA), BIIE0246 (kindly provided by Dr. Henri Doods, Boehringer Ingelheim Pharma KG, Germany) and L-152,804 (Tocris, Bristol, UK), respectively], and/or PKA and PKC activator/inhibitor [8-Br-cAMP/H-89 and phorbol myristate acetate (PMA)/bisindoylmaleimide I (BIS), respectively] (all from Sigma Chemical Co.), were added to the incubation medium for an additional 10 min. Synaptosomes were then centrifuged at 15,800 *g* and resuspended in 1 ml of the same medium without BSA containing 1 mM $CaCl_2$. The suspension was transferred to a stirred acrylic cuvette, maintained at 37°C, followed by the addition of 1 mM $NADP^+$, 50 U of purified glutamate dehydrogenase, and again NPY receptor agonists or antagonists. Fluorescence was measured using a Perkin-Elmer model LS-5B luminescence spectrometer (Perkin-Elmer Ltd, Beaconsfield, Buckinghamshire, England) at the excitation and emission wavelengths of 340 nm and 460 nm, respectively, with excitation and emission slits of 5 nm and 10 nm, respectively. The data were collected at 0.5 s intervals and at the end of each experiment, 2.5 nmol of L-glutamate was

added as a calibration to allow quantification of released glutamate.

Glutamate release was monitored for 11 min and synaptosomes were stimulated 4 min after the beginning of each experiment with 15 mM KCl or 5 μM ionomycin.

Western blot analysis

Twenty-five micrograms of protein from total hippocampus or from hippocampal subregions were separated by SDS-PAGE on 7.5% acrylamide/bisacrilamide gels, using a Bicine/SDS-based electrophoresis buffer (pH 8.3), and transferred onto PVDF membranes (750 mA, 50 min at 4°C in a solution containing 10 mM CAPS and 10% methanol, pH 11.0) (43). Membrane blocking was performed for 1 h at room temperature in Tris-buffered saline containing 5% low-fat milk and 0.1% Tween 20. Primary antibodies raised against GluR1 and phospho-GluR1 (S831) (both from Upstate, Barcelona, Spain) were applied overnight at 4°C and were detected using alkaline phosphatase conjugated secondary antibodies. Immunoblots were visualized using the Enhanced ChemiFluorescence detection reagent and a Versa Doc 3000 imaging system (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry

Rats were deeply anesthetized with sodium pentobarbital (120 mg/kg, i.p.) and perfused transcardially first with PBS, then fixed with 4% paraformaldehyde in PBS. Brains were removed and coronal sections (20 μm) were cut on a cryostat. Brain sections placed on gelatin-coated glass slides were washed with 0.05 M Tris-buffered saline (TBS) for 15 min. Afterward the sections were sequentially incubated with 1% Triton three times for 15 min each, 10% FBS for 30 min and with antiphospho-GluR1 (S831) for 2 days at 4°C (1:200; Upstate, Barcelona, Spain). After 30 min at room temperature, sections were washed again three times in 1% Triton for 15 min each, incubated for 1 h with anti-rabbit Alexa 488 (1:200; Molecular Probes, Leiden, The Netherlands), and stained with Hoescht 33342 (Molecular Probes) for 5 min. Finally, sections were washed (TBS), mounted with DakoCytomation fluorescent mounting medium (Dako, Glostrup, Denmark), coverslipped, and examined in a Fluorescence Microscope (Zeiss Axioskop 2 Plus).

NPY stimulated [^{35}S]GTPγS binding autoradiography

The brains obtained from control and epileptic rats were cut in coronal sections (20 μm) at –20°C, thaw mounted onto Superfrost slides, and dried on a hot plate. Tissue sections were stored at –80°C until further processing.

A slightly modified method of Sim and collaborators (44) was used. Briefly, sections were air dried for 30 min at room temperature (RT) before being rehydrated in assay buffer A (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl; pH 7.4) for 10 min (RT). Subsequently, a 20 min (RT) preincubation was performed in assay buffer B (assay buffer A+0.2 mM DTT, 2 mM GDP, 1 μM DPCPX (1,3-dipropyl-8-cyclopentylxanthine), 0.5% BSA (and NPY receptor antagonist, if applicable), followed by incubation in assay buffer B + 40 pM [^{35}S]GTPγS (1250 Ci/mmol, Perkin-Elmer, Denmark), 3 μM NPY, and/or NPY receptor antagonists or PKC activator/inhibitor (Sigma Chemical Co.) for 2 h at 25°C. In each experiment, basal binding was determined by omitting the application of NPY receptor ligands and nonspecific binding by applying 10 μM unlabeled GTPγS (Perkin-Elmer, Norwalk, CT, USA). The incubation was terminated by 2 × 5 min washing in ice-cold 50 mM Tris-HCl buffer (pH 7.4), followed by a brief rinse in cold deionized water. Sections were subsequently dried and exposed to Kodak BioMax MR film for 4 days together with ^{14}C standards (Amersham Life Sciences, Piscataway, NJ, USA). The films were developed in Kodak D19 developer. Optical densities were measured bilaterally over the dorsal CA1 (pyramidal layer, strata oriens, and radiatum) and CA3 (pyramidal layers, strata oriens, radiatum, and lucidum) as well as DG (molecular layer) using computer-assisted image analysis (Scion Image® analysis program). Right and left side values were averaged per section and used to calculate the mean of each animal. Background measurements immediately adjacent to each brain section were subtracted from each measurement before calculations. The percent of stimulation above basal level was calculated as [(Stim.–Basal)/Basal]*100%.

The following compounds were used for these experiments: NPY (3 μM, Bachem AG, Bubendorf, Switzerland), BIBP3226 (30 μM; Bachem AG), BIIE0246 (30 μM; Tocris Cookson Ltd., UK), L-152,804 (30 μM; Tocris) PMA (300 nM), and BIS (3 μM). The NPY Y_2 receptor population was determined using NPY in the presence of NPY Y_1 (BIBP3226) and NPY Y_5 (L-152,804) receptor antagonists. Total blocking of the NPY stimulation was obtained adding a mixture of BIBP3226, BIIE246, and L-152,804.

Statistical analysis

The data are expressed as means ± SE. Statistics were performed using an ANOVA (one-way ANOVA), followed by Dunnett's or Bonferroni's post-tests, as indicated in the figure legends.

RESULTS

Modulation of glutamate release by NPY receptors in rat hippocampal synaptosomes: cross-talk with PKA or PKC pathway

We have shown that the activation of Y_1, Y_2, or Y_5 receptors with selective agonists inhibited KCl-evoked glutamate release in hippocampal synaptosomal preparations and that these inhibitory effects were prevented by their selective NPY receptor antagonists (38, 45). Moreover, we also showed that the inhibition induced by NPY (endogenous agonist, full sequence) was due to the activation of Y_2 receptors and not Y_1 or Y_5 subtypes (45). With the present study, we intended to clarify the possible involvement of PKA and/or PKC in the inhibitory effect mediated by NPY receptors under control and epileptic conditions.

In hippocampal synaptosomes prepared from control rats, the total KCl-evoked release of glutamate was 0.9 ± 0.04 nmol glutamate/mg protein/min, and the inhibitory effect mediated by NPY (**Fig. 1*A, B***) or by Y_1, Y_2, Y_5 receptor agonists (**Fig. 2*A–C***) was not significantly modified by either activation of PKA with the cAMP analog 8-bromo-cAMP (500 μM 8-Br-cAMP) or by inhibition with H89 (1 μM). The inhibition of glutamate release induced by NPY or by the activation of Y_1, Y_2, Y_5 receptor plus 500 μM 8-Br-cAMP was 61.5 ± 6.6%, 63.0 ± 3.6%, 64.7 ± 3.5%, or 68.6 ± 4.0% of control, respectively, and the PKA activator by itself was without effect (91.5±4.9% of control). Similar results were

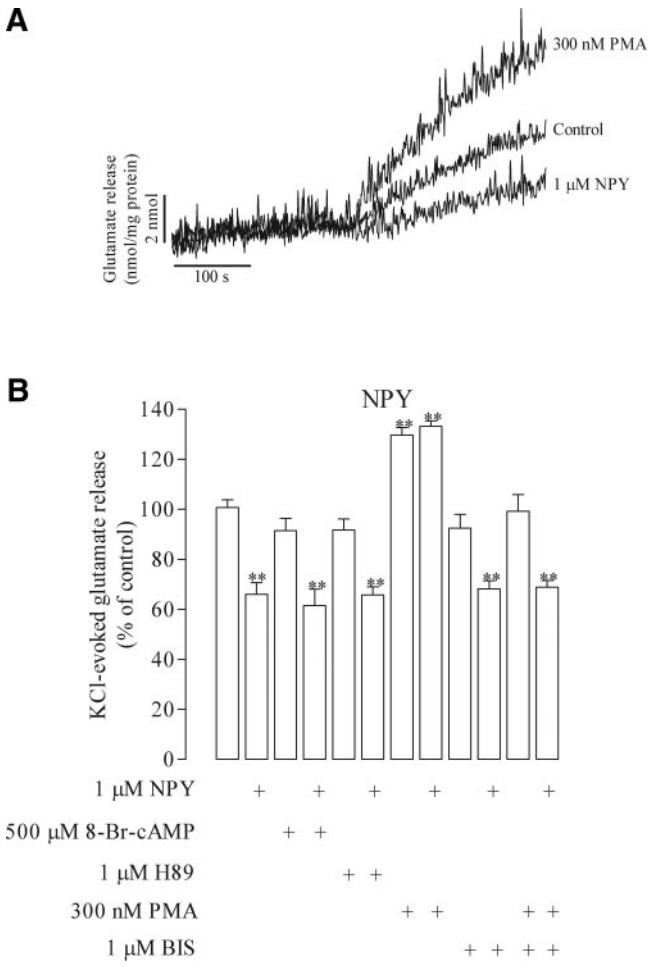

Figure 1. Representative recording of the effect of NPY (1 μM) or PMA (300 nM) (*A*) on the 15 mM KCl-evoked glutamate release in hippocampal synaptosomes (1 mM Ca^{2+} present in the external medium). Quantitative analysis of the effect of NPY (1 μM) (*B*) on glutamate release from hippocampal synaptosomes depolarized with 15 mM KCl in the absence or presence of PKA activator (500 μM 8-bromo-cAMP)/inhibitor (1 μM H89) or PKC activator (300 nM PMA)/inhibitor (1 μM BIS). Results represent the mean ± SE of 4 to 9 independent experiments in different synaptosomal preparations. $^{**}P < 0.01$, Dunnett's post-test, statistical significance when compared with control (KCl stimulation).

obtained in the presence of 1 μM NPY (Fig. 1*B*), 1 μM [Leu^{31}, Pro^{34}]NPY (Fig. 2*A*), 300 nM NPY(13–36) (Fig. 2*B*), or 1 μM NPY(19–23)-(Gly^{1}, Ser^{3}, Gln^{4}, Thr^{6}, AL^{31}, Aib^{32}, Gln^{34})-PP (Fig. 2*C*) plus 1 μM H89 as follows: 65.8 ± 3.3%, 59.3 ± 3.1%, 63.8 ± 1.8%, 70.4 ± 3.5% of control, respectively. Again, the inhibitor of PKA (H89) by itself had no effect (91.8±4.3% of control). All these observations allow us to conclude that PKA was not interfering with the inhibition of glutamate release mediated by NPY receptor activation.

PKC-mediated substrate phosphorylation has been implicated in a broad spectrum of neuronal events, including neurotransmitter release (28). Indeed, activation of PKC by phorbol esters leads to an increase in neurotransmitter release from nerve terminals of hippocampal slices (46), from nerve terminals in the spinal cord (47), and from isolated nerve terminals from mammalian brain (48, 49). In agreement with these studies, the activation of PKC with 300 nM PMA induced an increase in KCl-evoked glutamate release to 129.7 ± 3.0% of control (Figs. 1, 2), which was blocked by the PKC inhibitor (BIS) to 99.3 ± 6.6% of control (Figs. 1*B*, 2). By itself, 1 μM BIS did not induce any significant effect (92.5±5.4% of control). Moreover, the inhibitory effect induced by 1 μM [Leu^{31}, Pro^{34}]NPY (Fig. 2*A*) or 1 μM NPY(19–23)-(Gly^{1}, Ser^{3}, Gln^{4}, Thr^{6}, AL^{31}, Aib^{32}, Gln^{34})-PP (Fig. 2*C*) was not affected by the simultaneous activation of PKC (100.0±7.3% or 93.8±8.0% of control, respectively), keeping in mind that PMA by itself increased glutamate release to 129.7 ± 3.0% of control. However, this was not the case concerning NPY or Y_2 receptor activation, since in the simultaneous presence of NPY or Y_2 receptor agonist plus PMA, glutamate release was 133.2 ± 2.2% (Fig. 1*B*) or 133.8 ± 2.9% (Fig. 2*B*) of control, respectively. Moreover, the inhibitory effect mediated by 1 μM NPY (66.0±4.7% of control) or 300 nM NPY(13–36) (71.0±1.2% of control) was recovered in the presence of the PKC inhibitor to 68.9 ± 2.6% or 65.0 ± 5.4% of control, respectively (Fig. 1*B*, Fig. 2*B*). The PKC inhibitor by itself (1 μM BIS) did not affect the inhibition induced by Y_1 (Fig. 2*A*), Y_2 (Fig. 2*B*), Y_5 (Fig. 2*C*) receptor agonists or NPY (Fig. 1*B*) (75.8±2.9%, 67.0±5.5%, 63.6±3.4%, or 68.2±3.2% of control, respectively). These results show that, under control conditions, the pharmacological activation of PKC blocks the inhibition of glutamate release mediated by the activation of Y_2 receptors.

Involvement of PKC in the modulation of glutamate release by Y_2 receptors in hippocampal synaptosomes obtained from epileptic rats

Several studies have demonstrated significant alterations in PKC expression and subcellular distribution in the hippocampus after KA treatment (50, 51). To investigate the role of PKC in the inhibition of glutamate release induced by Y_2 receptor activation under epileptic conditions, we injected rats with 10 mg/kg KA (i.p.), a well-established model of epileptogenesis, as described in Materials and Methods. Using synaptosomes obtained from rats sacrificed 6 h postinjection, we observed that the total KCl-evoked release of glutamate was 1.1 ± 0.03 nmol glutamate/mg protein/min and that none of the agonists alone or in the presence of the PKC activator had an inhibitory effect (**Fig. 3*A***). The values obtained with 1 μM NPY, 300 nM NPY(13–36), 1 μM NPY plus 300 nM PMA, or 300 nM NPY(13–36) plus 300 nM PMA were 94.1 ± 3.3%, 93.4 ± 3.3%, 93.7 ± 3.1%, or 96.0 ± 4.5% of control, respectively (Fig. 3*A*). Moreover, 1 μM BIS did not modify the KCl-evoked glutamate release (108.0±4.0% of control); in contrast to what happened under control conditions, 300 nM PMA by itself had no effect (100.2±3.9% of control) (Fig. 3*A*). However, after inhibition of PKC

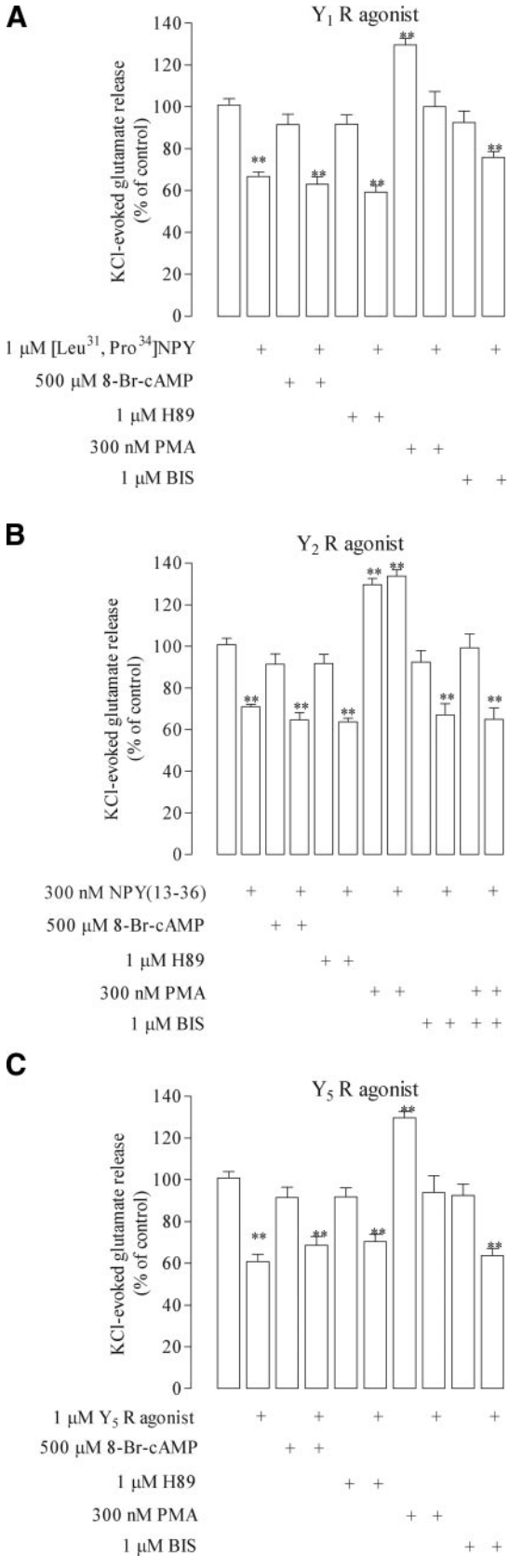

Figure 2. Involvement of PKA or PKC on the inhibition of glutamate release mediated by NPY Y_1, Y_2, or Y_5 receptor activation in rat hippocampal synaptosomes. Quantitative analysis of the effect of 1 μM [Leu^{31}, Pro^{34}]NPY (*A*), 300 nM NPY(13–36) (*B*), 1 μM NPY(19–23)-(Gly^1, Ser^3, Gln^4, Thr^6, AL^{31}, Aib^{32}, Gln^{34})-PP (*C*) on glutamate release from hippocampal synaptosomes depolarized with 15 mM KCl in the absence or in the presence of PKA activator (500 μM 8-bromo-cAMP)/inhibitor (1 μM H89) or PKC activator (300 nM PMA)/inhibitor (1 μM BIS). Results represent the mean ± SE of 4 to 9 independent experiments in different synaptosomal preparations. $^{**}P < 0.01$, Dunnett's post-test, statistical significance when compared with control (KCl stimulation).

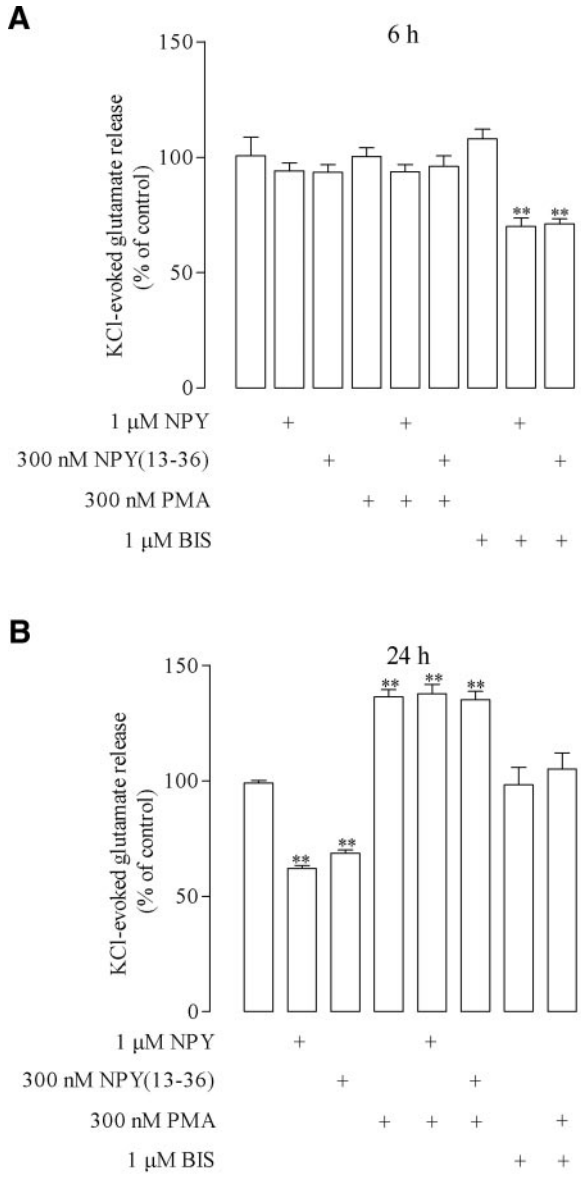

Figure 3. Involvement of PKC on the inhibition of glutamate release mediated by NPY or Y_2 receptor activation in epileptic rats. Quantitative analysis of the effect of 1 μM NPY or 300 nM NPY(13–36) on glutamate release evoked by 15 mM KCl depolarization in hippocampal synaptosomes obtained from epileptic rats sacrificed 6 h (*A*) or 24 h (*B*) postinjection. Status epilepticus was induced in rats after injection with KA (10 mg/kg) as described in Material and Methods. Results represent the mean ± SE of 3 to 7 independent experiments in different synaptosomal preparations. $^{**}P < 0.01$, Dunnett's post-test, statistical significance when compared with control (KCl stimulation).

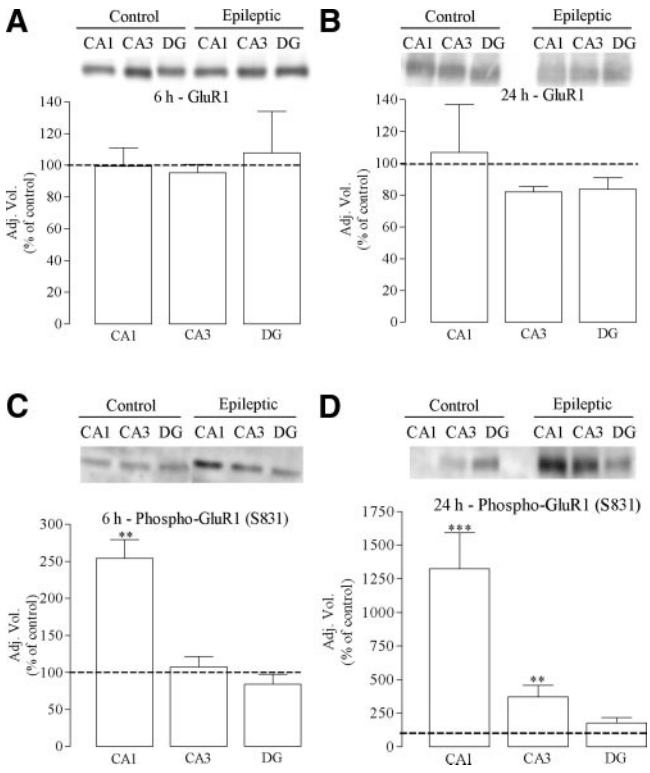

Figure 4. Western blot analysis of the changes in GluR1 (*A, B*) or phospho-GluR1 (*C, D*) levels in hippocampal subregions (CA1, CA3, DG) of control rats (injected with saline) and epileptic rats sacrificed 6 h (*A, C*) or 24 h (*B, D*) after KA injection (10 mg/kg, i.p.). Results are expressed as mean percentage of control ± SE of 3 or 4 independent experiments. ***$P < 0.001$, **$P < 0.01$. Bonferroni's post-test, statistical significance when compared with CA1, CA3, or DG from control rats.

with 1 μM BIS, we again observed a significant inhibitory effect caused by 1 μM NPY (70.0±3.6% of control) or by 300 nM NPY(13–36) (71.0±2.3% of control) (Fig. 3*A*). Taking in consideration that 6 h after kainate administration cells can be in a depolarized state and still have increased calcium levels, we performed additional experiments in which we observed that in synaptosomes obtained from epileptic rats sacrificed 6 h postinjection, the P-/Q-type VGCC blocker ω-agatoxin IVA (100 nM) inhibited glutamate release to 51% of control (data not shown), similar to what we observed earlier in control animals (45). These results show that the functional release of glutamate is fully functional in epileptic rats and is not blocked due to increased levels of presynaptic calcium.

The results obtained 24 h post-injection were similar to the control situation (Fig. 3*B*). The total KCl-evoked release of glutamate was 1.1 ± 0.1 nmol glutamate/mg protein/min; 1 μM NPY or 300 nM NPY(13–36) inhibited KCl-evoked glutamate release to 62.0 ± 1.4% or 68.6 ± 1.4% of control, respectively (Fig. 3*B*). Again, PMA by itself potentiated the release of glutamate to 136.3 ± 3.1% of control, and in the presence of NPY or the Y_2 receptor agonist, glutamate release was 137.8 ± 4.0% or 135.0 ± 3.7% of control, respectively. Moreover, as observed under control conditions, 1 μM BIS by itself was without effect (98.2±7.6% of control) and completely blocked the potentiation of glutamate release induced by PMA (105.0±7.0% of control) (Fig. 3*B*). Based on these results, we suggest that at 6 h postinjection PKC is highly active and occludes the inhibition of glutamate release mediated by Y_2 receptors.

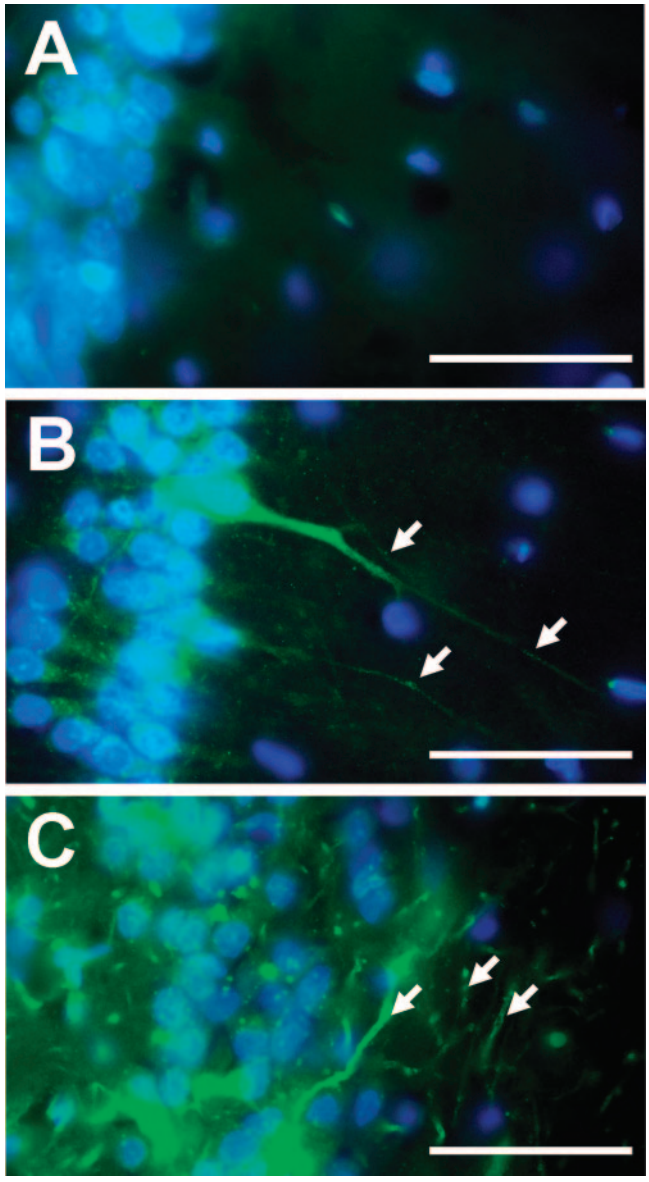

Figure 5. Representative fluorescence microscopy images of phospho-GluR1 (S831) immunoreactivity in the CA1 pyramidal cell layer of the hippocampus of control rats (*A*) or epileptic rats injected with KA (10 mg/kg) and sacrificed 6 h (*B*) or 24 h (*C*) postinjection. There is an increase of immunoreactivity against phospho-GluR1 (S831) in animals with seizures. At 6 h postinjection (*B*), punctuate-like labeling is clearly visible, suggesting a synaptic localization (arrows), whereas after 24 h (*C*) immunoreactivity is distributed along neurites (arrows). Brain slices (20 μm) were labeled with antiphospho-GluR1 (S831) (green) and stained with Hoescht 33342 (blue). Scale: 50 μm.

TABLE 1. *Effect of PKC activator (PMA) or inhibitor (BIS) on NPY-stimulated [^{35}S]GTPγS binding in adult control and epileptic rat hippocampal subregions*[a]

	Percentage increase above basal [^{35}S]GTPγS binding (mean±SE, n = 6–8)						
	NPY				Y_2[b]		
Region	+PMA	+BIS	+PMA	+BIS	+PMA	+PMA	+BIS
CA1							
Control	63 ± 6	51 ± 6	66 ± 7	66 ± 11	33 ± 3	31 ± 9	45 ± 5
Epileptic	82 ± 9	58 ± 13	74 ± 14	78 ± 18	41 ± 5	38 ± 18	59 ± 10
CA3							
Control	70 ± 12	57 ± 9	75 ± 11	74 ± 12	56 ± 10	54 ± 13	58 ± 13
Epileptic	74 ± 11	59 ± 9	72 ± 12	74 ± 15	63 ± 12	48 ± 10	73 ± 13
DG							
Control	41 ± 5	33 ± 5	45 ± 7	48 ± 9	0	0	2 ± 5
Epileptic	57 ± 8	45 ± 8	52 ± 9	57 ± 9	10 ± 9	1 ± 13	18 ± 7

[a]Control rats were injected with saline (0.9% NaCl); epileptic seizures were induced with KA (10 mg/kg, i.p. injection) and rats were sacrificed 6 h postinjection. Brain sections were processed as described in Materials and Methods. CA1, regio-superior cornu ammonis; CA3, regio-inferior cornu ammonis; DG, dentate gyrus. [b]The NPY Y_2 receptor population was determined using NPY in the presence of NPY Y_1 (BIBP3226) and NPY Y_5 (L-152,804) receptor antagonists.

GluR1 and phospho-GluR1 (S831) levels in hippocampal subregions of control and epileptic rats

The results described above suggest that in the acute phase of epilepsy, PKC may be strongly activated and that this effect can be associated with the loss of Y_2 receptor-mediated inhibition of glutamate release. To assess whether PKC is active under these conditions, we decided to investigate the levels of phospho-GluR1 (S831) as an indication of PKC activity. AMPA receptors containing the GluR1 subunit may be regulated by extracellular signals acting through PKC (52, 53). Indeed, PKC phosphorylates Ser-831 of the GluR1 subunit (54) and can contribute to modulate synaptic transmission. Thus, we evaluated the levels of GluR1 and phospho-GluR1 in hippocampal subregions of control and epileptic rats.

In hippocampal subregions CA1, CA3, and DG, GluR1 levels at 6 h or 24 h postinjection were not significantly different from those of control (**Fig. 4*A, B***). In contrast, phospho-GluR1 (S831) levels robustly increased at 6 h (253.7±25.3% of control), and even more pronounced 24 h postinjection in the CA1 subregion (1325.6±267.4% of control) (Fig. 4*C, D*). No significant effects were seen in the DG at any time point after KA injection (Fig. 4*C, D*).

In the CA1 subregion, we observed an increase of immunoreactivity against phospho-GluR1 (S831) in animals with seizures (**Fig. 5**). At 6 h postinjection (Fig. 5*B*), punctuate-like labeling was clearly visible, suggesting a synaptic localization, whereas after 24 h the immunoreactivity was distributed along neurites (Fig. 5*C*).

Characterization of the intracellular mechanisms by which PKC activity blocks NPY-mediated inhibition of glutamate release

To study both the distribution and functionality of the NPY receptors under control and epileptic conditions, we performed functional autoradiographic studies. **Table 1** and **Fig. 6*A–C*** show levels of [^{35}S]GTPγS binding in CA1, CA3, and DG after stimulation with 3 μM NPY. This effect was completely blocked by a mixture of Y_1, Y_2, and Y_5 receptor antagonists (30 μM BIBP3226+30 μM BIIE0246+30 μM L-152,804) (Fig. 6*D*). Activation of the Y_2 receptor subtype (3 μM NPY+30 μM BIBP3226+30 μM L-152,804) only induced an increased binding signal above basal binding in the CA1 and CA3 subregions, correlating with the binding pattern observed for the Y_2 receptor in traditional autoradiographic studies (55) (Table 1; Fig. 6*E*). Moreover, the PKC activator (PMA) and/or inhibitor (BIS) did not significantly alter the total NPY receptor or Y_2 receptor-mediated [^{35}S]GTPγS binding under either control or epileptic conditions (6 h postinjection) (Table 1; Fig. 6). These results suggest that the function of the NPY receptors is similar under control *vs.* epileptic conditions. Moreover, PKC does not seem to directly modulate the activity of NPY receptors, suggesting that the PKC target is located downstream the receptor level.

We previously showed that none of the NPY receptor agonists used currently could reduce ionomycin-induced glutamate release (45). Accordingly, in **Fig. 7** we observed that PMA by itself (100.0±3.9% of control) or in the presence of NPY (86.5±2.3% of control) or NPY(13–36) (93.8±2.5% of control) did not significantly potentiate or inhibit glutamate release, in contrast to what happened when we stimulated glutamate release with KCl (Fig. 1*B*, Fig. 2*B*); 1 μM BIS or 1 μM BIS + 1 μM NPY had no effect (data not shown). Since ionomycin promotes calcium influx and glutamate release independently of voltage-gated Ca^{2+} channels (VGCCs) activity, the present results suggest that PKC is acting on VGCCs, increasing Ca^{2+} conductance and thereby occluding the inhibitory effect of Y_2 receptors.

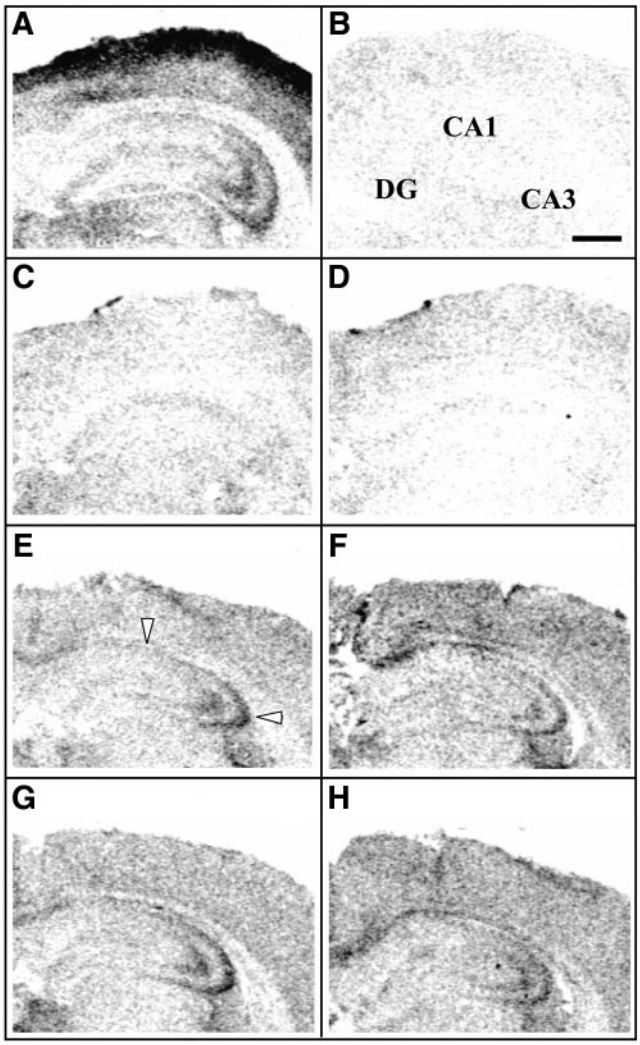

Figure 6. Representative images of [^{35}S]GTPγS functional binding in the rat hippocampal CA1, CA3, and DG at 6 h after i.p. injection of saline (0.9% NaCl) or KA (10 mg/kg). Visualization of all NPY receptor subtypes (Y_1, Y_2, Y_5) with 3 μM NPY-stimulated binding (*A*), nonspecific binding (*B*), basal binding (*C*), and total block of NPY-stimulated binding (3 μM NPY+30 μM Y_1, Y_2, Y_5 antagonists) (*D*) in saline-treated rats. Y_2 receptor functional binding (3 μM NPY+30 μM Y_1, Y_5 antagonists) did not differ in CA1 and CA3 (arrows) between saline- (*E*) and KA-treated rats (*F*). No functional Y_2 receptor binding was detected in the DG. The presence of PKC activator (300 nM PMA) did not affect Y_2 receptor-mediated functional binding in either saline- (*G*) or KA-injected rats (*H*).

DISCUSSION

In previous studies, we and others have shown that NPY receptors modulate Ca^{2+} influx (38, 56), K^+ currents (57, 58), and glutamate release (38, 59) from neuronal cells. Moreover, NPY inhibits excitatory neurotransmission in the hippocampus (18), exogenous administration of NPY prominently suppresses limbic seizure activity induced by KA (15), and NPY-deficient mice develop more severe seizures in response to KA, suggesting that NPY may act as an endogenous anticonvulsant agent. So, since NPY affects glutamatergic synaptic transmission and neuronal excitability (5, 10, 12), it is of great interest to investigate the functional role of the NPY system in epileptic seizures. Indeed, little is known about the signaling mechanism (mechanisms) that underlie NPY receptor effects under epileptic conditions.

In common with some other presynaptic metabotropic receptors (60), the observed inhibitory effect mediated by NPY Y_1, Y_2, and Y_5 receptors did not work through a cyclic AMP-dependent mechanism as it was not influenced by PKA activator (8-Br-cAMP) or by its inhibitor (H89). In fact, in our study we observed that the activation or inhibition of PKA failed to modify inhibition of glutamate release mediated by any of the NPY receptors. In contrast, the role of PKC activation in regulation of presynaptic function in general, and glutamate release in particular, is well established in synaptosomal studies (29, 60). In the present work, we observed that the activation of PKC by a phorbol ester (PMA) induced an increase of glutamate release. In fact, it is well established that the activation of PKC by phorbol esters leads to an increase in neurotransmitter release from rat hippocampal slices (46), and mammalian brains synaptosomes (29, 48), and also potentiates excitatory postsynaptic currents (EPSCs) (61). NPY- or Y_2 receptor-mediated inhibition of glutamate release under control conditions was abolished following PKC activation. In contrast, when using synaptosomes obtained from epileptic rats 6 h postinjection, we observed no NPY receptor-mediated inhibition of glutamate release (21). Moreover, phorbol esters did not affect glutamate release, in contrast to what was observed 24 h postinjection and in controls. These results suggest that PKC can be highly active following status epilepticus and somehow blocks the inhibition of glu-

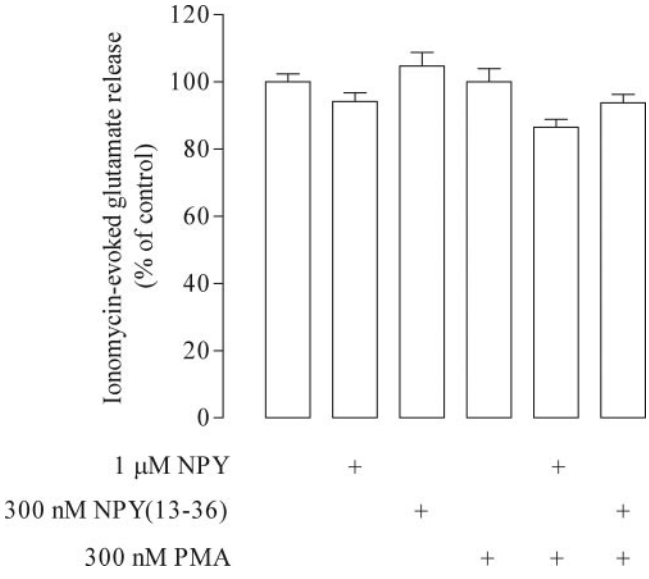

Figure 7. Lack of effect of NPY, NPY(13–36), and PMA on glutamate release evoked by 5 μM ionomycin in control rat hippocampal synaptosomes. Results represent the mean ± SE of 4 to 8 independent experiments in different synaptosomal preparations.

tamate release induced by Y_2 receptor activation. Another possible interpretation for our results is the inhibition of PKC effects by NPY acting through Y_1 and Y_5 receptors. As we show in the present study, in epileptic rats (6 h post-kainate injection), NPY is not efficient at inhibiting the release of glutamate; this effect is similar to that observed after stimulation of Y_2 receptors but different from the results obtained in the presence of Y_1 or Y_5 agonists (21). The effect of NPY under basal conditions and epileptic ones (24 h postinjection) is mediated mainly through Y_2 receptors, but after status epilepticus (6 h), when PKC is active, Y_2 receptor activity is not linked to the efficient inhibition of glutamate release. However, whether or not Y_1 and Y_5 receptor activity can shortcut the effect of PKC in stimulating the release of glutamate is an alternative and attractive hypothesis. Future studies will determine whether in epileptic rats (6 h) the blockade of Y_2 receptors relieve the predominant effect of NPY at Y_2 receptors, making Y_1 and Y_5 receptors sensitive to NPY (as we previously reported in 45), and whether in this condition NPY can efficiently block the stimulatory effect of PKC.

It is clear that status epilepticus induces alterations in the subcellular distribution of individual PKC isoforms in a temporally and regionally specific manner (62). In fact, we observed that by inhibiting PKC in synaptosomes isolated 6 h postinjection with KA, Y_2 receptors recovered their inhibitory effect on glutamate release. Moreover, analyzing the expression levels of phospho-GluR1 (S831), it is evident there is an increase of PKC activity after status epilepticus. This increase was significant in the CA1 subregion, in agreement with other studies that show a differential activity of PKC among brain regions or subregions (62). Also, Hussain and Carpenter (63) showed that the presence and function of PKC are age dependent and different between Schaffer collateral-CA1 and Mossy fibers-CA3 pathways within the normal hippocampus. With the present data, we cannot form definitive conclusions about the mechanisms contributing to the differences observed in the functional release of glutamate between 6 h and 24 h. We can, however, suggest that other mechanisms able to shortcut/silent the stimulatory effect of PKC on glutamate release (*i.e.*, increased activity of phosphatases) may be functional at 24 h but not at 6 h postinjection. It is important to keep in mind that phospho-GluR1 was used as a marker PKC activity, but this approach cannot lead to conclusions about the spatial and temporal activity of PKC at presynaptic sites, especially important for the control of voltage-gated calcium channels.

As already mentioned, several actions have been attributed to PKC, including not only the enhancement of Ca^{2+} currents and elevation of cytosolic-free Ca^{2+} resulting in increased neurotransmitter release (64), inhibition of K^+ channels (31), and activation of exocytotic machinery downstream of Ca^{2+} influx (30), but also regulation of activity of NMDA receptors (65) or α_{1d}-adrenergic receptors (66). However, the mechanism (or mechanisms) whereby PKC blocks the inhibitory effect of Y_2 receptors is unknown. Therefore, we first tried to investigate whether PKC was acting directly on the receptors or at the G-protein level. Our results indicated that this might not be the case, in contrast to what is known to happen with several other receptors (65). Indeed, the [^{35}S]GTPγS binding assay we used to assess the function of Y_2 receptors gave similar results under control and epileptic conditions with or without the pharmacological activation of PKC with phorbol ester, indicating that these receptors are not functionally directly regulated by PKC. So the precise mechanism (or mechanisms) underlying the blockage of Y_2 receptors are yet to be determined, but given that modulation of ion channel activity is considered one of the major target mechanisms, it is plausible that a good target candidate for PKC could be the voltage-gated Ca^{2+} channels (VGCCs). In previous studies performed by us and others, it was shown that NPY receptors modulate (inhibit) the $[Ca^{2+}]_i$ response in the hippocampus, and this was mainly due to the inhibition of different VGCCs (45). If PKC is acting on VGCCs by increasing Ca^{2+} conductance, as we suggested in the present study and as described by others (29), it is plausible to suggest that this mechanism may account for the cross-talk between PKC and Y_1 (29) or PKC and Y_2 (present study) receptor function. A major unbalance of the excitatory/inhibitory transmission occurs in status epilepticus and causes a major shift toward hyperexcitability, contributing to the generalization of seizure activity and to tonic-clonic convulsions. Our present demonstration that PKC contributes to the increased release of glutamate and at the same time, blocks a key inhibitory modulator (Y_2 receptor) may help to highlight the impact of the PKC/NPY cross-talk in status epilepticus.

In summary, the present findings show a transient loss of NPY- and Y_2 receptor-mediated inhibition of glutamate release following status epilepticus. We also show that PKC is highly active and may contribute to occlude the functional effects of Y_2 receptors in inhibiting glutamate release. Since Y_2 receptors are essential for NPY regulation of limbic seizures originating in the hippocampus, understanding the cell signaling that underlies its effect under control and epileptic conditions may be quite useful to find potential new treatments for temporal lobe epilepsy. FJ

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