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Modulation of dopamine and noradrenaline release and of intracellular Ca^{2+} concentration by presynaptic glutamate receptors in hippocampus

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1 We studied the release of [^{3}H]-dopamine and [^{3}H]-noradrenaline (NA) from hippocampal synaptosomes induced by glutamate receptors and the associated Ca^{2+} influx through Ca^{2+} channels. The release of tritiated neurotransmitters was studied by use of superfusion system and the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined by a fluorimetric assay with Indo-1 as a probe for Ca^{2+}.

2 Presynaptic glutamate receptor activation induced Ca^{2+}-dependent release of [^{3}H]-dopamine and [^{3}H]-NA from rat hippocampal synaptosomes. Thus, L-glutamate induced the release of both neurotransmitters in a dose-dependent manner ($EC_{50} = 5.62\,\mu M$), and the effect of 100 μM L-glutamate was inhibited by 83.8% in the presence of 10 μM 6-cyano-7-nitroquinoxaline-2,3-dioxine (CNQX), but was not affected by 1 μM (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine (MK-801).

3 Other glutamate receptor agonists also stimulated the Ca^{2+}-dependent release of [^{3}H]-dopamine and [^{3}H]-NA as follows: N-methyl-D-aspartate (NMDA), at 200 μM, released $3.65 \pm 0.23\%$ of the total ^{3}H catecholamines, and this effect was inhibited by 81.2% in the presence of 1 μM MK-801; quisqualate (50 μM), S-α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) (100 μM) or kainate (100 μM) released $1.57 \pm 0.26\%$, $1.93 \pm 0.17\%$ and $2.09 \pm 0.22\%$, of the total ^{3}H catecholamines, respectively.

4 The ionotropic glutamate receptor agonist, AMPA, induced an increase in the $[Ca^{2+}]_i$ which was inhibited by 58.6% in the presence of 10 μM CNQX. In contrast, the increase in $[Ca^{2+}]_i$ due to stimulation by glutamate was not sensitive to CNQX or MK-801.

5 Nitrendipine, at 1 μM, did not inhibit the neurotransmitter release induced by AMPA, but, both 0.5 μM ω-conotoxin GVIA (ω-CgTx) and 100 nM ω-Aga IVA reduced catecholamine release to $49.03 \pm 3.79\%$ and $46.06 \pm 10.51\%$ of the control, respectively. In the presence of both toxins the release was reduced to $12.58 \pm 4.64\%$ of the control.

6 The results indicate that activation of presynaptic glutamate receptors of the NMDA and non-NMDA type induces the release of [^{3}H]-dopamine and [^{3}H]-NA from rat hippocampal synaptosomes and that the release induced by AMPA involves the activation of N- and P-type Ca^{2+} channels which allow the influx of Ca^{2+} that triggers the ^{3}H catecholamines release.

Keywords: Hippocampus; catecholamines release; presynaptic glutamate receptors; calcium; synaptosomes; voltage-sensitive calcium channels

Introduction

The exocytotic release of neutrotransmitters depends on the Ca^{2+} influx into the cell (for review see: Carvalho, 1982; Augustine, 1987; Smith & Augustine, 1988; Knight *et al.*, 1989). The influx of Ca^{2+} is mediated by Ca^{2+} channels activated upon excitation of the nerve terminal, providing a mechanism for the coupling between stimulation and neurosecretion (Tsien *et al.*, 1988, 1991).

Voltage-sensitive Ca^{2+} channels (VSCCs) are activated by membrane depolarization and two groups of VSCC's can be distinguished on the basis of the different sensitivities to membrane depolarization. Low voltage-sensitive calcium channels or T-Type VSCCs are transiently activated by small decreases in membrane potential. High voltage-sensitive calcium channels are VSCCs which are activated after large depolarizations of the membrane. Three different types of high VSCCs can be distinguished on the basis of their sensitivity to specific antagonists. The L-type Ca^{2+} channels are a group of channels inhibited by dihydropyridines, like nitrendipine (Tsien *et al.*, 1991). The N-type Ca^{2+} channels are blocked by ω-conotoxin GVIA (ω-CgTx) (Kerr & Yoshikami, 1984; Tsien *et al.*, 1991). The P-type VSCCs are inhibited by the polyamine FTX (Llinás *et al.*, 1989) or by the peptide ω-Aga IVA (Mintz *et al.*, 1992b) which is a component of the venom of the funnel web spider, *Agenelopsis asperta*.

The excitatory neurotransmitter L-glutamate acts both at the postsynaptic and presynaptic membranes by interaction with its receptors. Glutamate receptors may be ionotropic, allowing the influx of ions through a receptor channel (Zorumski & Thio, 1992; Ruzicka & Jhamandas, 1993), or G protein-coupled metabotropic receptors, which are coupled to several second messenger systems (Schoepp & Conn, 1993). The metabotropic receptors are coupled to phospholipase C (Nicoletti *et al.*, 1986; Sugiyama *et al.*, 1987) and the activated phospholipase C catalyzes the hydrolysis of phosphatidyl inositols and produces inositol phosphate which releases Ca^{2+} from internal stores (Zorumski & Thio, 1992). The metabotropic receptor is activated by ACPD and also by quisqualate (Zorumski & Thio, 1992). The ionotropic glutamate receptors are the N-methyl-D-aspartate (NMDA), S-α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) and kainate receptors (Zorumski & Thio, 1992; Ruzicka & Jhamandas, 1993). The NMDA receptor is very permeable to Ca^{2+} (MacDermott *et al.*, 1986; Iino *et al.*, 1990), reversibly blocked by Mg^{2+} (Nowak *et al.*, 1984) and potentiated by the co-agonist, glycine (Johnson & Ascher,

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1987; Kemp & Leeson, 1993). As compared to the NMDA receptor, AMPA and kainate receptors are less permeable to Ca^{2+} (Iino *et al.*, 1990; Ruzicka & Jhamandas, 1993). The AMPA- and kainate-induced depolarization activates VSCCs which allows the influx of Ca^{2+}, coupling the stimulation to neurosecretion (Ruzicka & Jhamandas, 1993).

The effectiveness of the Ca^{2+} entering the nerve terminal in releasing of neurotransmitters, following presynaptic glutamate receptors activation, depends on the spatial localization of the receptors and the Ca^{2+} channels in the active zones (Smith & Augustine, 1988). In this work we studied the role of presynaptic glutamate receptors of rat hippocampal synaptosomes in inducing the release of [^{3}H]-dopamine and [^{3}H]-noradrenaline ([^{3}H]-NA) and we determined which high voltage-sensitive Ca^{2+} channels mediate the coupling between glutamate receptor activation and catecholamine release in synaptosomes isolated from rat hippocampus.

A preliminary account of this work has been presented at the 14th International Society for Neurochemistry Meeting (Malva *et al.*, 1993).

Methods

Preparation of synaptosomes

A crude synaptosomal fraction (P_2) was isolated from hippocampi of two month-old male Wistar rats, essentially as described previously (McMahon *et al.*, 1992), with some modifications. Hippocampi were homogenized in 9 volumes of 0.32 M sucrose, 5 mM HEPES-Tris, pH 7.4, by using a Thomas B homogenizer, and centrifuged at 3000 g_{max} for 2 min. The pellet so obtained was washed by resuspension in approximately the original volume of the sucrose solution and centrifuged as above. The combined supernatants were centrifuged at 14600 g_{max}, for 12 min, and a P_2 pellet was obtained. The whiter and loosely packed layer of the pellet, which contains the majority of the synaptosomes, was carefully isolated with a spatula and resuspended in 0.32 M sucrose, 5 mM HEPES-Tris, pH 7.4, at a protein concentration of about 10 mg ml^{-1}. Aliquots of 1 mg of protein were diluted in 1 ml of sucrose medium and pelleted in a microfuge at 15800 g_{max}, for 2 min. The drained pellets were stored on ice and used within 3 h. Protein concentration was determined by the biuret method (Layne, 1957).

$[Ca^{2+}]_i$ measurements

Loading of synaptosomes with Indo-1 was carried out essentially as described previously (Duarte *et al.*, 1991). Synaptosomes (3.5 mg ml^{-1}) were incubated with 3 μM Indo-1-acetoxymethyl ester (Indo-1/AM) in a medium containing (mM): NaCl 132, KCl 1, $MgCl_2$ 1.4, $CaCl_2$ 100, H_3PO_4 1.2 fatty acid-free bovine serum albumin (BSA) 1 mg ml^{-1}, glucose 10 and HEPES-Na 10, pH 7.4, for 20 min, at 25°C, followed by 10 min at 30°C. The loading was stopped by pelleting the synaptosomes at 15800 *g*, for 10 s, and the supernatant, containing the nonhydrolyzed probe, was discarded. The synaptosomal pellet was resuspended (0.5 mg ml^{-1}) in a medium containing (mM): NaCl 132, KCl 1, $MgCl_2$ 1, glucose 10 and HEPES-Na 10, pH 7.4, and transferred into the cuvette. Fluorescence measurements were carried out at 30°C during stirring, in a Spex Fluoromax spectrofluorometer, at two emission wavelengths of 410 nm and 485 nm with a single excitation wavelength of 335 nm, using 5 nm slits. The maximal fluorescence ratio was obtained upon addition of 2 μM ionomycin, in the presence of 1 mM $CaCl_2$, and the minimal ratio was determined by adding 40 μl of 0.5 M EGTA solution at pH 10. The fluorescence intensities were converted into $[Ca^{2+}]_i$ values by using the calibration equation for double emission wavelength measurements and taking the dissociation constant of the Indo-1/Ca^{2+} complex at 250 nM (Grynkiewicz *et al.*, 1985). In all experiments, the synaptosomes were preincubated for 300 s before stimulation, in the presence or in the absence of drugs.

^{3}H-catecholamine release by synaptosomes

The uptake and release of ^{3}H catecholamines was performed as follows: synaptosomes were loaded (0.75 mg ml^{-1}) with 100 nM dopamine, containing 5 μCi of [^{3}H]-dopamine nmol^{-1}, in Na^+ medium (NaCl 132 mM, KCl 1 mM, $MgCl_2$ 1.4 mM, H_3PO_4 1.2 mM, $CaCl_2$ 100 μM, glucose 10 mM and HEPES-Tris 10 mM, pH 7.4) containing nialamide 12.5 μM, ascorbic acid 0.1% and fatty acid-free BSA 1 mg ml^{-1}. The loading of synaptosomes proceeded for 15 min at 30°C and was stopped by pelleting the synaptosomes at 15800 *g* for 30 s. The supernatant was discarded and the pellet resuspended in Na^+ medium (1.5 mg ml^{-1}). Samples of the synaptosomal suspension containing 0.75 mg of protein were aspirated and collected onto Whatman GF/B filters mounted in a superfusion system as described previously (Raiteri *et al.*, 1974). Synaptosomes were washed for 15 min with Na^+ medium containing 12.5 μM nialamide and 0.1% ascorbic acid, at a flow rate of 0.8 ml min^{-1}, using a peristaltic pump. By this time a stable basal release of ^{3}H catecholamines was obtained and the sample collection was started. Each sample was collected for 1 min and the total experiment proceeded for 10 min. All drugs used were added 1 min before starting the sample collection and the stimulation of synaptosomes was initiated at minute 3. At the end of the experiment, all the filters containing synaptosomes were dried and transferred into scintillation vials. The radioactivity present in the samples and in the filters was determined in vials after addition of a scintillation cocktail (Universol, ICN laboratories), using a liquid scintillation spectrometer (Packard, Tri-Carb 2000) provided with a d.p.m. correction programme.

Some of the results are presented as ^{3}H-catecholamines released in percentage of the total ^{3}H-catecholamines accumulated. To determine the ^{3}H-catecholamines effectively released upon stimulation, the following calculations were made: from the amount of ^{3}H-catecholamines released in each sample, the average value of basal release was subtracted. All individual values above basal release were summed and the obtained value was considered as the ^{3}H-catecholamines released by the stimulus. The total ^{3}H-catecholamines accumulated was calculated as the sum of ^{3}H-catecholamines released by stimulation of synaptosomes and the ^{3}H-catecholamines still present in the filters at the end of the experiment.

Catecholamine analysis by h.p.l.c.-e.d.

Dopamine, NA and dihydroxyphenylacetic acid (DOPAC) content of synaptosomes was compared in untreated synaptosomes (endogenous catecholamines) and in synaptosomes loaded with unlabelled dopamine in the conditions described in the previous section for the loading with tritiated dopamine. In both cases, 2 mg of synaptosomal protein was pelleted in order to determine the content of catecholamines. The obtained pellets were resuspended in 0.5 ml of a solution consisting of 10% acetic acid and 0.4 M perchloric acid. The samples were kept at −20°C for later high performance liquid chromatography (h.p.l.c.) analysis (Duarte *et al.*, 1993).

Dopamine and DOPAC analysis was performed as follows: the samples were injected into a 15 × 0.4 cm Spherisorb ODS-1 column with 5 μm particle size and eluted at a flow rate of 1 ml min^{-1} with a mobile phase consisting of 50 mM NaH_2PO_4, 1.24 mM heptanesulphonic acid, 0.24 mM EDTA, 10% methanol, pH 2.9. The electrochemical detector used was an ESA model Coulochem II.

The same samples were used to quantify NA levels in hippocampal synaptosomes by h.p.l.c. Samples were injected

in a column Ultrasphere ODS, 4.7 × 75 mm with 3 μm particle size. The mobile phase was 50 mM NaH_2PO_4, 0.02% trichloroacetic acid and 14% acetonitrile, pH 3.5 (Levine, 1986). The flow rate of the mobile phase and the conditions for electrochemical detection were the same used for dopamine and DOPAC analysis.

Analysis of released [^{3}H]-NA, [^{3}H]-dopamine and [^{3}H]-DOPAC by h.p.l.c.-e.d.

Hippocampal synaptosomes were loaded as described previously in this paper, but with the following modifications. The loading proceeded in the same Na^+ medium (1 mg ml^{-1} with 100 nM dopamine, containing 20 μCi of [^{3}H]-dopamine $nmol^{-1}$. One sample (1 min) of the basal release and another sample during the first min of stimulation, were collected. To each sample acetic acid (10% final concentration) and perchloric acid (0.4 M final concentration) were added, and the samples were stored at −20°C for latter h.p.l.c.-e.d. analysis (Duarte *et al.*, 1993).

The samples were injected in a column Ultrasphere ODS, 4.7 × 7.5 mm with 3 μm particle size with the same mobile phase and flow conditions as described in the previous section for NA quantification. The injected samples were prepared as following: 186 μl of tritiated sample were mixed with cold standards containing 100 pmol dopamine, 100 pmol NA and 50 pmol DOPAC, at a final volume of 200 μl. A volume of 100 μl of this mixture was injected into the column, using the appropriated loop. At the exit of the analytical cell, five of each 2 min samples were collected into scintillation vials and the radioactivity determined as previously described. The radioactivity in each sample was compared with the chromatogram of the cold standards (see Figure 1).

Materials

Nitrendipine was obtained from Research Biochemical International, Natick, U.S.A. The ω-conotoxin GVIA (ω-CgTx) was from Peninsula Laboratories Inc., Belmont, CA, U.S.A. The ω-AGA IVA was from Scientific Marketing Associates, U.K. and 6-nitro-7-cyano-quinoxaline-2,3-dione (CNQX) was obtained from NOVO, Nordisk, Denmark. (+)-5-Methyl-16,11-dihydro-5H-dibenzo [a,d] cyclohepten-5,10-imine maleate (MK-801) was purchased from Merck Sharp & Dohme Research Lab., Rahway, N.J., U.S.A. S-α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) was obtained from Tocris Neuramin, Bristol, England. The acetoxymethyl ester of Indo-1 (Indo-1/AM) was obtained from Molecular Probes Inc. Eugene, OR, U.S.A. Ionomycin and fatty acid-free bovine serum albumin (BSA) were from Calbiochem-Boehringer Corp., San Diego, U.S.A. [7,8-^{3}H]-dopamine hydrochloride (40–60 Ci $mmol^{-1}$) was purchased from Amersham Laboratories, Buckinghamshire, England. All other reagents were from Sigma Chemical Co., St. Louis, MO, U.S.A. or from Merck-Schuchard, Germany. Stock solutions of Indo-1/AM, ionomycin, nitrendipine and CNQX were prepared in dimethyl sulphoxide.

Other methods

Results are presented as means ± s.e.mean of the number of experiments indicated. Statistical significance was determined by Student's two-tailed *t* test.

Results

Endogenous content of NA, dopamine and DOPAC in hippocampal synaptosomes

The hippocampal synaptosomes contain endogenous NA (2.94 ± 0.22 pmol mg^{-1} protein), dopamine (0.69 ± 0.16 pmol mg^{-1} protein) and small amounts of DOPAC (0.029 ± 0.027 pmol mg^{-1} protein) (Table 1). When we loaded the synaptosomes with exogenous dopamine, as described in Methods, we observed that the total content of NA was not significantly different from the content of endogenous NA (2.91 ± 0.21 pmol mg^{-1} protein) ($P > 0.05$). However, after the loading with dopamine, the synaptosomal content of this catecholamine increased to 4.40 ± 0.78 pmol mg^{-1} protein. The content of DOPAC in the synaptosomes observed after loading with exogenous dopamine was also significantly higher (2.28 ± 0.41 pmol mg^{-1} protein) (Table 1) than the content of the endogenous DOPAC in the synaptosomes.

Release of ^{3}H-catecholamines by hippocampal synaptosomes loaded with [^{3}H]-dopamine

After loading the hippocampal synaptosomes with [^{3}H]-dopamine, we observed that the main component of the tritium release in basal conditions was DOPAC (68.11 ± 2.17% of basal release), but also some dopamine (23.13 ± 1.89% of basal release) and NA (8.76 ± 0.64% of basal release) were released in the same conditions (Figure 1). However, after stimulation with L-glutamate, AMPA or KCl, the amount of DOPAC released did not increase as compared to basal conditions, some NA was released, and we observed a large increase in the tritium efflux with the retention time characteristic of dopamine (Figure 1). Thus, the increase in ^{3}H catecholamines release due to stimulation with 100 μM L-glutamate, 100 μM AMPA, 5 mM KCl or 30 mM KCl was mainly due to the release of [^{3}H]-dopamine (68.43 ± 4.03%, 74.05 ± 2.66, 69.53 ± 4.17 and 71.35 ± 3.19%, respectively of the total tritium released), but a significant part of [^{3}H]-NA was also released in the same conditions (31.58 ± 4.03%, 25.95 ± 2.66%, 30.48 ± 4.17% and 28.65 ± 3.19%, respectively). Thus, this methodology allow us to study in the same sample the release of both NA and dopamine induced by stimulating the glutamate receptors, although the major neurotransmitter in the efflux medium is dopamine (Figure 1c).

Stimulation of ^{3}H-catecholamines release from hippocampal synaptosomes by glutamate receptors agonists

Synaptosomes obtained from rat hippocampus released ^{3}H-catecholamines in response to stimulation with L-glutamate in a dose-dependent manner (Figure 2). Superfusion with medium containing 1 mM Ca^{2+} released 0.658 ± 0.171% ($n = 9$) of the total ^{3}H-catecholamines accumulated. The addition of L-glutamate released ^{3}H-catecholamines in a Ca^{2+}-dependent process, with the EC_{50} determined as 5.6 μM and the maximum ^{3}H-catecholamines release of 2.17% of the total tritium accumulated (Figure 2b). The release induced by L-glutamate was dependent on extracellular Ca^{2+}, since we did not observe [^{3}H]-catecholamines release in the absence of added Ca^{2+} (Figure 2a).

Table 1 Catecholamine levels in synaptosomes from rat hippocampus, determined by high performance liquid chromatography (h.p.l.c.)

	A	*B*
DOPAC	0.03 ± 0.03 ($n = 6$)	2.28 ± 0.41 ($n = 6$)*
NA	2.94 ± 0.22 ($n = 6$)	2.91 ± 0.21 ($n = 6$)
Dopamine	0.69 ± 0.16 ($n = 5$)	4.40 ± 0.78 ($n = 5$)*

A, Endogenous content of dihydroxyphenylacetic acid (DOPAC), noradrenaline (NA) and dopamine. **B** Contents of DOPAC, NA and dopamine observed after the loading of the synaptosomes with exogenous dopamine as described in the Methods Section. The values are expressed as pmol mg^{-1} protein.
*Significantly higher than endogenous, $P < 0.05$.

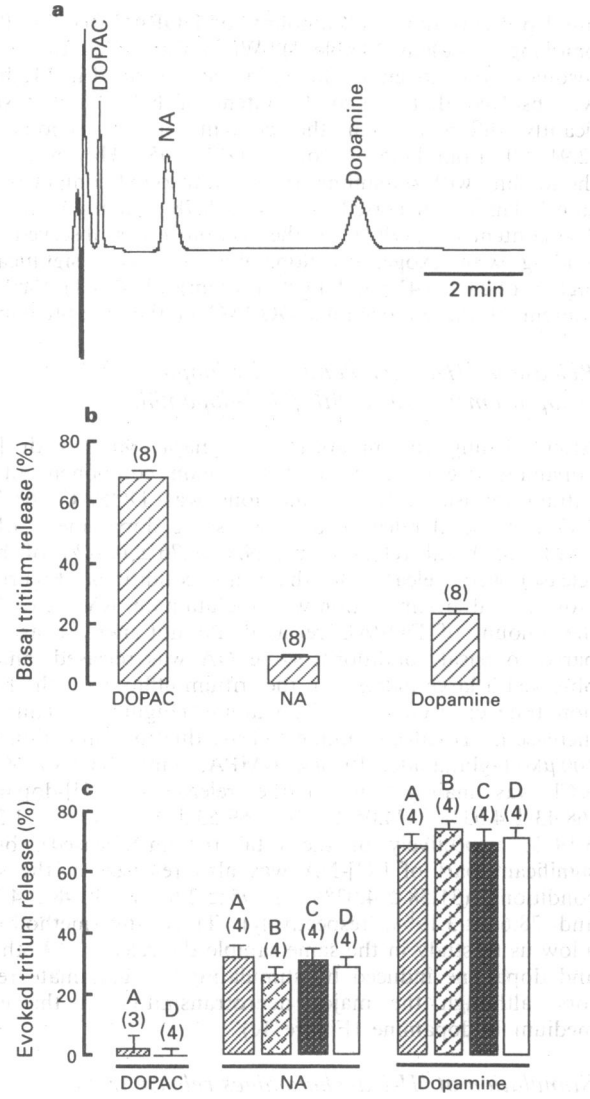

Figure 1 Release of tritiated catecholamines by hippocampal synaptosomes loaded with [^{3}H]-dopamine. (a) H.p.l.c. chromatogram of cold standards of 50 pmol of dihydroxyphenylacetic acid (DOPAC), 100 pmol noradrenaline (NA) and 100 pmol dopamine. (b) Separation of tritiated DOPAC, NA and dopamine released in basal conditions by h.p.l.c. The tritiated samples were supplemented with cold standards of DOPAC, NA and dopamine, and the 2 min samples correspondent to each standard were collected and the radioactivity counted. (c) Tritiated NA and dopamine released by stimulation of synaptosomes with 100 μM L-glutamate (A), 100 μM AMPA (B), 5 mM KCl (C) or 30 mM KCl (D). Only NA and dopamine release was evoked by the stimulation of the synaptosomes, whereas the release of DOPAC was the same before or after the stimulation. The results are the mean value ± s.e.mean of 4 to 8 experiments performed in duplicate. For abbreviations in this and subsequent figure legends, please see text.

We also observed that agonists of the ionotropic glutamate receptors induced the release of ^{3}H-catecholamines (Figures 2 and 3). Thus, the extent of tritium release by 100 μM AMPA was 1.93 ± 0.17% ($n = 9$) ($EC_{50} = 10.9$ μM and maximum ^{3}H-catecholamines release = 2.09%), and by 100 μM kainate was 2.09 ± 0.22% ($n = 6$) ($EC_{50} = 4.9$ μM and maximum ^{3}H-catecholamines release = 2.22%) (Figures 2b and 3b). Quisqualate (50 μM) released 1.57 ± 0.26% ($n = 5$) of the total ^{3}H accumulated. We also observed that NMDA, in the presence of 5 μM glycine and in the absence of added Mg^{2+}, was effective in releasing ^{3}H-catecholamines. Thus, NMDA (200 μM) released 3.65 ± 0.23% ($n = 13$) of the total ^{3}H-catecholamines (Figure 3).

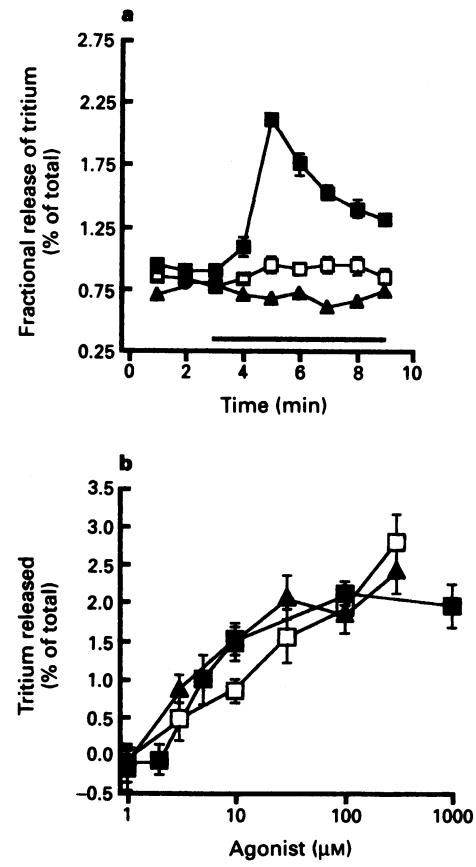

Figure 2 Effect of L-glutamate (Glu) on the release of ^{3}H-catecholamines in rat hippocampal synaptosomes. (a) Fractional release induced by Ca^{2+} (1 mM) alone (□), by 100 μM L-glutamate in medium without Ca^{2+} (▲), or by 10 μM L-glutamate (■) in the presence of 1 mM Ca^{2+}. The results are the mean value ± s.e.mean of 4 to 9 experiments performed in duplicate. (b) Dose-response curves for the release of ^{3}H-catecholamines induced by L-glutamate (■), AMPA (□) or kainate (▲). The curves were determined by the mean value ± s.e.mean of 4 to 5 independent experiments performed in duplicate.

Glutamate-induced release of ^{3}H-catecholamines is due to receptor activation

It has been described in various cell models that L-glutamate increases the $[Ca^{2+}]_i$ in part due to glutamate transport, which can cause membrane depolarization, and activate VSCCs (Heinonen *et al.*, 1989; McMahon *et al.*, 1989). Therefore, we investigated whether the observed Ca^{2+}-dependent release of ^{3}H-catecholamines induced by L-glutamate was mediated through the L-glutamate transport or by activation of presynaptic glutamate receptors.

We observed that the increase of the hippocampal synaptosomal $[Ca^{2+}]_i$ due to 100 μM L-glutamate was not inhibited by 10 μM CNQX or 1 μM MK-801 (Figure 4a). However, the 100 μM AMPA-induced increase in the $[Ca^{2+}]_i$ was strongly inhibited by 10 μM CNQX (58.6% inhibition) ($P < 0.05$) (Figure 4b). Interestingly, when we studied the effects of L-glutamate or AMPA on the release of ^{3}H-catecholamines, we observed that the release induced by L-glutamate or by AMPA was inhibited by CNQX (Figure 5a and b), but, in the presence of 1 μM MK-801, the observed release of [^{3}H]-catecholamines induced by 100 μM L-glutamate was not significantly different from the control ($P > 0.05$), indicating that the release evoked under these conditions did not involve the activation of NMDA receptors. However, in the presence of 10 μM CNQX, the release induced by 100 μM L-glutamate was reduced by 83.8% (Figure 5a), indicating

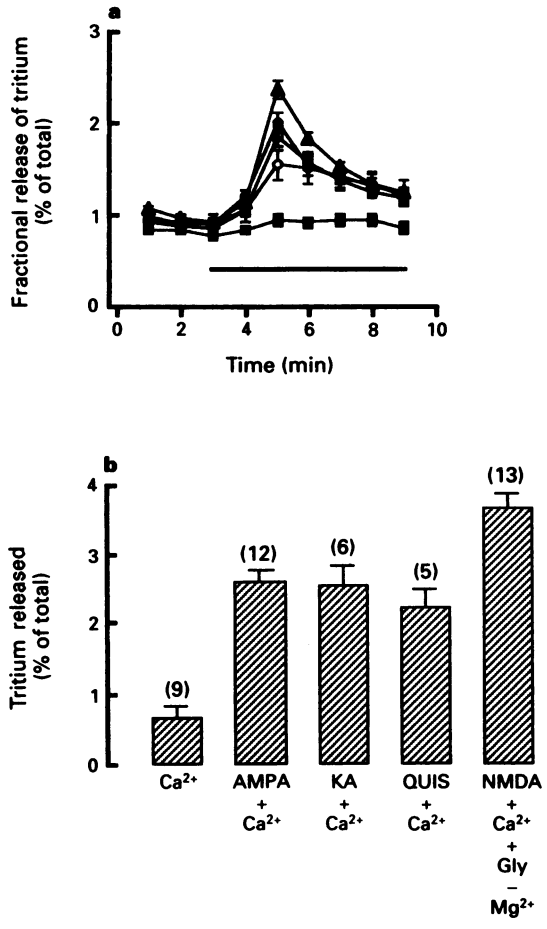

Figure 3 ^{3}H-catecholamine release induced by L-glutamate receptor agonists. (a) Fractional release induced by Ca^{2+} (1 mM) alone (■) or by 100 μM AMPA (□), 100 μM kainate (●), 50 μM quisqualate (○), in the presence of 1 mM Ca^{2+}; or 200 μM NMDA in the absence of Mg^{2+} and in the presence of 1 mM Ca^{2+} (▲). (b) ^{3}H-catecholamines released by L-glutamate receptor agonists (applied from min 3 to 9) as a percentage of the total tritium accumulated. The results are the mean value ± s.e.mean of 5 to 13 experiments performed in duplicate.

activation of ionotropic non-NMDA glutamate receptors coupled to [^{3}H]-dopamine and [^{3}H]-NA release. Further evidence that presynaptic non-NMDA glutamate receptors activation release ^{3}H-catecholamines was obtained in a study in which we observed that a specific non-NMDA glutamate receptor agonist (AMPA) induced the release of ^{3}H-catecholamines and that this release was significantly inhibited ($P<0.05$) by 10 μM CNQX (61.2% inhibition) (Figure 5b).

As reported above, we did not observe any inhibition of the 100 μM L-glutamate induced [^{3}H]-dopamine or [^{3}H]-NA release by 1 μM MK-801 (Figure 5a). However, we observed that 200 μM NMDA in the presence of 5 μM glycine, and in the absence of added Mg^{2+}, induced the release of 3.65 ± 0.23% ($n = 13$) of the total tritiated neurotransmitter accumulated (Figures 3 and 5c). The release of ^{3}H-catecholamines induced by NMDA was inhibited by 81.2% by 1 μM MK-801 (Figure 5c).

AMPA induced release of ^{3}H-catecholamines is mediated by N- and P-type VSCCs

The ionotropic glutamate receptors activated by AMPA allow the influx of cations, inducing membrane depolarization (Ruzicka & Jhamandas, 1993) which may activate VSCCs, allowing the influx of Ca^{2+} which may trigger the release of several neurotransmitters. In this work we observed that the L-type VSCCs blocker nitrendipine (Tsien *et al.*, 1988; 1991) did not inhibit the release of [^{3}H]-dopamine or [^{3}H]-NA, but the N-type calcium channel blocker, ω-CgTx (Kerr & Yoshikami, 1984; Reynolds *et al.*, 1986) inhibited the release of [^{3}H]-catecholamines to 49.03 ± 3.79% of the control whereas, the ω-Aga IVA, a blocker of the P-type VSCCs (Mintz *et al.*, 1992a,b) reduced the release of ^{3}H-catecholamines to 46.06 ± 10.51% the control. When both blockers were perfused simultaneously, the release of ^{3}H-catecholamines was inhibited to 12.76 ± 4.64% ($n = 4$) of the control. The release observed in the presence of ω-CgTx, in the presence of ω-Aga IVA or with both toxins were significantly different from the release observed in the absence of VSCC blockers ($P<0.05$) (Figure 6).

These results suggest that most of the Ca^{2+}-dependent release of [^{3}H]-dopamine and [^{3}H]-NA, due to activation of

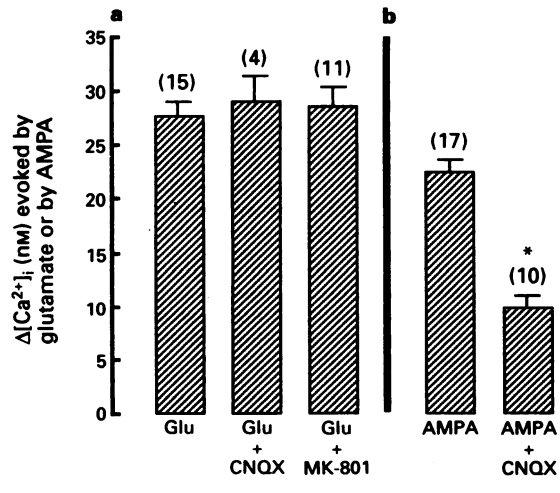

Figure 4 Increases in the $[Ca^{2+}]_i$ concentration in hippocampal synaptosomes induced by 100 μM L-glutamate or 100 μM AMPA. (a) Increase in the $[Ca^{2+}]_i$ induced by L-glutamate (Glu) is not sensitive to either 10 μM CNQX or 1 μM MK-801. (b) AMPA-induced increase in the $[Ca^{2+}]_i$ is sensitive to 10 μM CNQX. The results are the mean ± s.e.mean of 4 to 17 experiments. Significantly lower than control: $^*P<0.05$.

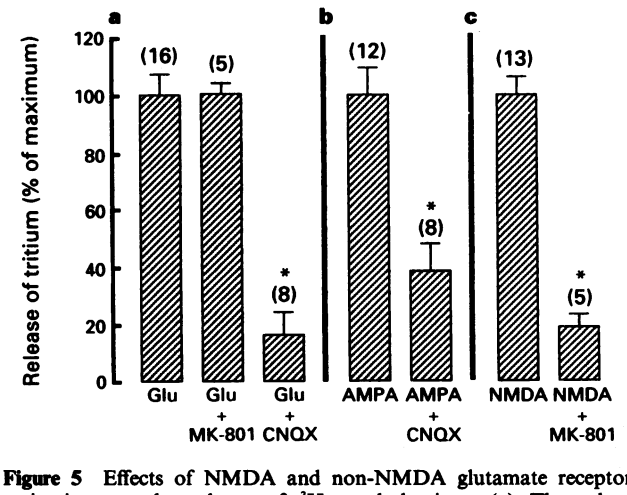

Figure 5 Effects of NMDA and non-NMDA glutamate receptors activation on the release of ^{3}H-catecholamines. (a) The release induced by 100 μM L-glutamate is inhibited by 10 μM CNQX and is not sensitive to the NMDA receptor antagonist MK-801 (1 μM). (b) The release of ^{3}H-catecholamines evoked by 100 μM AMPA is inhibited by 10 μM CNQX. (c) The release induced by 200 μM NMDA, in medium without Mg^{2+} and in the presence of 1 mM Ca^{2+} and 5 μM glycine, is inhibited by 1 μM MK-801. The results are the mean value ± s.e.mean of 5 to 16 experiments performed in duplicate. Significantly lower than control: $^*P<0.05$.

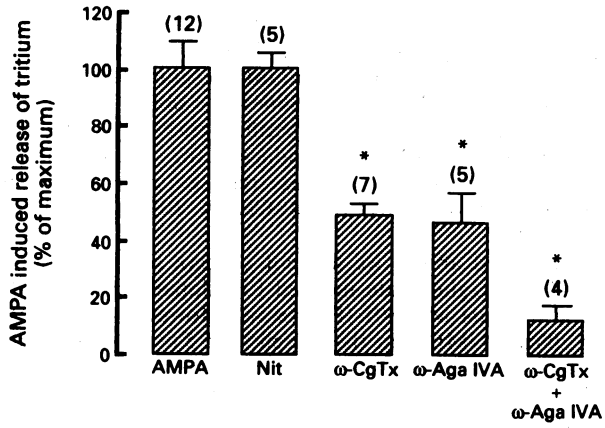

Figure 6 Characterization of VSCCs which couple the activation of the AMPA receptors with the release of ^{3}H-catecholamines. Addition of 100 μM AMPA in the absence or in the presence of nitrendipine (1 μM), ω-CgTx (0.5 μM), ω-Aga IVA (100 nM), or both ω-CgTx (0.5 μM) and ω-Aga IVA (100 nM). The results are the mean value ± s.e.mean of 4 to 12 experiments performed in duplicate. Significantly lower than control, $*P<0.05$.

presynaptic receptors with AMPA, is a consequence of activation of VSCCs due to membrane depolarization induced by activation of the AMPA receptors (Figures 5 and 6).

Discussion

Dopaminergic and noradrenergic terminals are present in the hippocampus (Wyss *et al.*, 1979; Lopes da Silva *et al.*, 1990). It was shown previously that synaptosomes isolated from rat hippocampus are endowed with endogenous dopamine and NA, and that they release catacholamines in response to depolarization (Verhage *et al.*, 1992). However, in spite of the presence of dopamine in the hippocampus, the physiological role of this neurotransmitter in this area of the brain is still unclear (Lopes da Silva *et al.*, 1990).

We have determined the endogenous content of NA and dopamine in the crude synaptosomal fraction of the hippocampus used in this work. The NA content of these synaptosomes is about four fold higher than the dopamine content (Table 1), in agreement with previous observations showing much higher levels of NA than of dopamine in purified hippocampus synaptosomes (Verhage *et al.*, 1992). However, we find a dopamine content about 3.5 fold higher than that reported (Verhage *et al.*, 1992). We also found that the NA content was about 18.3 fold lower than the levels described earlier (Verhage *et al.*, 1992). These differences are probably due to different isolation methods used for preparing the synaptosomes. Apparently, we used a crude synaptosomal fraction which is much richer in dopamine and poorer in NA than the purified synaptosomes used by Verhage *et al.* (1992).

We used h.p.l.c.-e.d. to analyze the nature of the ^{3}H-catecholamines released by hippocampal synaptosomes previously loaded with [^{3}H]-dopamine. In the hippocampus the catecholamine nerve terminals are less than 1% of the total nerve terminals (Lopes da Silva *et al.*, 1990), and the endogenous content of either NA or dopamine is too low to allow the study of endogenous catecholamines release stimulated by presynaptic glutamate receptors. Thus, we decided to use [^{3}H]-dopamine loaded synaptosomes since the high sensitivity of the tritium detection allowed us to detect very small amounts of neurotransmitters released.

Monoamine oxidase can transform dopamine in DOPAC, in an enzymatic reaction inhibited by nialamide. We observed in our preparation that after loading the synaptosomes with exogenous dopamine, the synaptosomal content of DOPAC increased (Table 1). We concluded that, in spite of the presence of nialamide throughout the experiment, some dopamine was metabolized to DOPAC. In this work, we also observed that DOPAC is released in basal conditions and that the increase in ^{3}H-catecholamines release observed after synaptosomal stimulation was mainly due to the release of dopamine and, to a smaller extent, to release of NA (Figure 1). After loading the synaptosomes with dopamine, we observed that the NA content of hippocampal synaptosomes did not increase (Table 1). However, we observed that after stimulation of the synaptosomes some [^{3}H]-NA was released, indicating that in hippocampal synaptosomes some [^{3}H]-dopamine can be transported into noradrenergic terminals and then be converted in [^{3}H]-NA (Figure 1).

The increase in [^{3}H]-NA release observed after [^{3}H]-dopamine loading and the finding that NA content of the synaptosomes did not increase after loading with dopamine may be explained by the rapid turnover of NA, which allows the conversion of [^{3}H]-dopamine in [^{3}H]-NA without affecting total NA levels. During the loading of hippocampal synaptosomes with [^{3}H]-dopamine the neurotransmitter can be transported into both dopaminergic and noradrenergic nerve terminals. In the dopaminergic nerve terminals this catecholamine is stored in dopaminergic synaptic vesicles (Henry & Scherman, 1989; Moriyama *et al.*, 1993), whereas in noradrenergic terminals the [^{3}H]-dopamine is transported into the nerve terminal and is accumulated in the noradrenergic synaptic vesicles where it is transformed to [^{3}H]-NA. In other brain regions with high ratios NA/dopamine, experimental evidence suggested that dopamine can also be accumulated into noradrenergic terminals and converted into NA (Kelly *et al.*, 1985; Carboni *et al.*, 1990).

We observed that the majority of the ^{3}H-catecholamines released after stimulation of the synaptosomes with 30 mM KCl (71.4 ± 3.2%), 5 M KCl (69.5 ± 4.2%), 100 μM L-glutamate (68.4 ± 4.0%) or 100 μM AMPA (74.1 ± 2.7%), was in fact [^{3}H]-dopamine, but that a significant portion of the ^{3}H-catecholamines was released as [^{3}H]-NA (28.7 ± 3.2% for 30 mM KCl, 30.5 ± 3.2% for 5 mM KCl, 31.6 ± 4.0% for 100 μM L-glutamate and 26.0 ± 2.7% for 100 μM AMPA). L-Glutamate induced the release of [^{3}H]-dopamine and [^{3}H]-NA from rat hippocampal synaptosomes in a process which depends on extracellular Ca^{2+} (Figure 2). The dependence on external Ca^{2+} of the dopamine release induced by depolarization (Raiteri *et al.*, 1978; Drapeau & Blaustein, 1983) is due to the requirement of Ca^{2+} for exocytosis, since this neurotransmitter is stored in synaptic vesicles within the synaptic terminal (Henry & Scherman, 1989; Moriyama *et al.*, 1993).

The release of ^{3}H-catecholamines induced by 100 μM L-glutamate in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+} was reduced by 83.8% by 10 μM CNQX, but it was insensitive to 1 μM MK-801 (Figure 5). The insensitivity of the L-glutamate induced [^{3}H]-dopamine and [^{3}H]-NA release to the NMDA receptor inhibitor, MK-801, probably reflects the blocking of the NMDA receptor channel by Mg^{2+} (Nowak *et al.*, 1984; Zorumski & Thio, 1992), and the depolarization which occurs after the non-NMDA receptors activation probably was not sufficient to remove the Mg^{2+} from the NMDA receptor channel. Another possibility is the localization of the NMDA and non-NMDA receptors in different synaptosomes, which could explain the finding that the activation of the non-NMDA receptors was not sufficient to release the NMDA channel from the blockade due to Mg^{2+}.

The 100 μM L-glutamate induced change in $[Ca^{2+}]_i$, which reflects the influx of Ca^{2+} following stimulation of synaptosomes, was insensitive to 10 μM CNQX or 1 μM MK-801 (Figure 4). The insensitivity of the L-glutamate induced increase in the $[Ca^{2+}]_i$ to the non-NMDA glutamate receptor antagonist, CNQX (Honoré *et al.*, 1988; Zorumski & Thio, 1992; Figure 4a), or to the NMDA glutamate receptor antagonist, MK-801 (Zorumski & Thio, 1992; Figure 4a) is compatible with the idea that L-glutamate transport through its carrier may lead to Ca^{2+} influx due to depolarization which activates VSCCs. These results are in agreement with pre-

vious observations that D-aspartate increases the $[Ca^{2+}]_i$ in synaptosomes from the guinea-pig cerebral cortices (McMahon *et al.*, 1989). However, the release of ^{3}H-catecholamines induced by glutamate does not appear to be mediated by a carrier-dependent process, since it is sensitive to CNQX. This observation indicates that presynaptic non-NMDA glutamate receptors mediate the release of ^{3}H-catecholamines in this preparation. We also observed that both NMDA and non-NMDA glutamate receptor agonists induced the release of ^{3}H-catecholamines (Figure 3). The release induced by 100 μM AMPA was sensitive to 10 μM CNQX and the release induced by 200 μM NMDA was inhibited by 1 μM MK-801 (Figure 5). These results are consistent with both presynaptic non-NMDA and NMDA receptor activation mediating the Ca^{2+}-dependent ^{3}H-catecholamine release.

Interestingly, NMDA (200 μM) is the glutamate receptor agonist which induces the highest release of ^{3}H-catecholamines (Figure 3). This result apparently is contradictory to the observation that NMDA is the least effective glutamate receptor agonist in elevating the $[Ca^{2+}]_i$ (not shown). Probably, the local Ca^{2+} concentrations attained in the active zones (Smith & Augustine, 1988) after the NMDA receptors activation are sufficient to induce ^{3}H-catecholamine release, without changing the bulk Ca^{2+} concentrations to any great extent. Another possible explanation is that only a subpopulation of synaptosomes respond to NMDA stimulation, inducing a large efflux of ^{3}H-catecholamines, without causing large average changes in the $[Ca^{2+}]_i$ of all the synaptosomes.

Glutamate receptors modulate the release of several neurotransmitters and neuromodulators (Ruzicka & Jhamandas, 1993). The ionotropic NMDA receptor stimulation was reported to induce the release of NA from rat hyppothalamus slices, but had no effect on the release of dopamine in this preparation (Blandina *et al.*, 1992). The presence of presynaptic NMDA receptors able to induce the release of NA was also demonstrated in synaptosomes isolated from rat cerebral cortex (Fink *et al.*, 1990). In the hippocampus, the presence of presynaptic glutamate receptors which modulate the release of NA was shown for both NMDA receptors (Pittaluga & Raiteri, 1990; Wang *et al.*, 1992) and non-NMDA receptors (Pittaluga & Raiteri, 1992; Wang *et al.*, 1992). Glutamate receptors also modulate the release of dopamine in rat mesencephalic cells in culture by both NMDA and non-NMDA subtypes (Mount *et al.*, 1990). Presynaptic glutamate receptors activated by kainate (Desce *et al.*, 1991; Wang, 1991), by AMPA (Desce *et al.*, 1991; Wang *et al.*, 1991; Desce *et al.*, 1992) or by NMDA (Krebs *et al.*, 1991; Wang, 1991; Desce *et al.*, 1992) also modulate the release of dopamine from synaptosomes isolated from the rat striatum.

The present work specifically addressed the mechanisms involved in the coupling between presynaptic glutamate receptor activation and catecholamine release. We showed that after stimulating hippocampal synaptosomes with glutamate or AMPA, an increase in the $[Ca^{2+}]_i$ was observed (Figure 4), due to presynaptic glutamate receptors stimulation. We further characterized the involvement of VSCCs in the Ca^{2+} influx coupled to dopamine and NA release. Thus, we observed that, nitrendipine, which blocks the L-type VSCCs (Tsien *et al.*, 1988; 1991) did not affect the AMPA induced catecholamines release (Figure 6), indicating that L-type calcium channels do not mediate the influx of Ca^{2+} following AMPA receptor activation. It was previously observed that 1 μM nitrendipine does not inhibit significantly the release of dopamine from cultured foetal rat ventral mesencephalon evoked by glutamate receptor agonists (Chadieu *et al.*, 1992), also indicating that the L-type VSCCs are not activated following glutamate receptor activation. However, it was observed in the same work that the activation of the L-type calcium channels by Bay K 8644 induces the release of [^{3}H]-dopamine and that this activation could be blocked by nitrendipine (Chadieu *et al.*, 1992).

The N-type calcium channels blocker, ω-CgTx (Kerr & Yoshikami, 1984; Reynolds *et al.*, 1986), strongly inhibited the 100 μM AMPA induced release of ^{3}H-catecholamines (Figure 6), and the same extent of inhibition by ω-CgTx was also observed on the elevation of the $[Ca^{2+}]_i$ induced by AMPA (not shown), indicating that the N-type VSCCs are important in mediating the effects between AMPA receptor activation and neurotransmitter release. It was shown previously that ω-CgTx inhibits the release of noradrenaline and 5-hydroxytryptamine, as well as the Ca^{2+} influx, in synaptosomes isolated from rat hippocampus or striatum (Reynolds *et al.*, 1986). Furthermore, the ability of ω-CgTx in inhibiting the KCl evoked release of NA and dopamine as well as the influx of Ca^{2+}, was shown to depend on stimulus intensity (Keith *et al.*, 1993; Turner *et al.*, 1993).

A dihydropyridine and ω-CgTx insensitive class of VSCCs, which is inhibited by FTX (Llinás *et al.*, 1989) and by ω-Aga IVA (Mintz *et al.*, 1992a,b) the P-type calcium channels, have been shown to be particularly important in mammalian nerve terminals (Mintz *et al.*, 1992a,b; Turner *et al.*, 1992; 1993; Tareilus *et al.*, 1993). We observed in the present study that the ^{3}H-catecholamines release induced by AMPA was also strongly inhibited by ω-Aga IVA (Figure 6). This observation indicates that P-type VSCCs are important in mediating the influx of Ca^{2+} following AMPA receptor activation, which induced the release of ^{3}H-catecholamines. We observed that ω-Aga IVA also inhibited the AMPA-induced increase in the $[Ca^{2+}]_i$ as well as the KCl induced increase in the $[Ca^{2+}]_i$ (not shown). When we applied ω-CgTx and ω-Aga IVA simultaneously, we observed that the inhibition of ^{3}H-catecholamine release was almost complete, supporting the idea that the influx of Ca^{2+} following AMPA receptor stimulation occurred mainly through N- and P-type VSCCs. Other authors have reported effects of ω-Aga IVA on $^{45}Ca^{2+}$ influx into synaptosomes (Mintz *et al.*, 1992b; Tareilus *et al.*, 1993). The release of glutamate by rat cortical or striatal synaptosomes is also sensitive to ω-Aga IVA (Turner *et al.*, 1992; 1993), also indicating the presence of P-type VSCCs in glutamatergic terminals. The release of dopamine in rat striatal synaptosomes induced by depolarization with KCl or 4-aminopyridine is inhibited by blockers of the P-type VSCCs (Turner *et al.*, 1993). A synergistic inhibition of KCl evoked [^{3}H]-dopamine release was observed by combining the effects of ω-Aga IVA and ω-CgTx, suggesting the coexistence of P- and N-type calcium channels in dopaminergic terminals of the striatum (Turner *et al.*, 1993).

In summary, we showed here that presynaptic glutamate receptors modulate the release of [^{3}H]-dopamine and [^{3}H]-NA in rat hippocampal synaptosomes, since glutamate receptors agonists were effective in releasing these catecholamines in a Ca^{2+}-dependent manner. The AMPA induced release of ^{3}H-catecholamines is mediated by VSCC activation of the N- and P-type, which allows the influx of Ca^{2+} and therefore the exocytosis of synaptic vesicles. These observations are in agreement with the current idea that the function of the N-type calcium channels can be closely related to the release of catecholamines (Reynolds *et al.*, 1986; Tsien *et al.*, 1988), and with the coexistence of both N- and P-type VSCCs in dopaminergic terminals (Turner *et al.*, 1993).

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