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Ionotropic glutamate receptors regulating labeled acetylcholine release from rat striatal tissue in vitro: possible involvement of receptor modulation in magnesium sensitivity

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Abstract

This study evaluated the role of glutamate ionotropic receptors on the control of [^{3}H]acetylcholine ([^{3}H]ACh) release by the intrinsic striatal cholinergic cells. [^{3}H]-choline previously taken up by chopped striatal tissue and converted to [^{3}H]ACh, was released under stimulation by glutamate, *N*-methyl-D-aspartate (NMDA), kainate and a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA). Experiments were conducted in the absence of choline uptake inhibitors or acetylcholinesterase inhibitors. A paradigm of two stimulations was employed, the first in control conditions and the second after 9 min of perfusion with the test agents MK-801, 2-amino-5-phosphonopentanoic acid (AP-5), tetrodotoxin (TTX), 6,7-dinitroquinoxaline-2,3-dione (DNQX), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-[f]quinoxaline-7-sulfonamide (NBQX), glycine and magnesium. Our results support that (1) in the absence of Mg^{2+}, NMDA is the most effective agonist to stimulate [^{3}H]ACh release from striatal slices (2) magnesium effectively antagonized kainate and AMPA stimulation suggesting that at least part of the kainate and AMPA effects might be attributed to glutamate release (3) besides NMDA, kainate receptors showed a more direct involvement in [^{3}H]ACh release control based on the smaller dependence on Mg^{2+} and less inhibition by TTX and (4) stimulation of ionotropic glutamate receptors may induce long lasting biochemical changes in receptor/ion channel function since the effects of TTX and/or Mg^{2+} ions on [^{3}H]ACh release were modified by previous exposure of the tissue to agonists.
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Keywords: Acetylcholine; Glutamate; Striatum; Neurotransmitter release; Ionotropic receptors; Phosphorylation

1. Introduction

The corticostriatal and thalamostriatal glutamate projections are important players in the extrapyramidal motor system and a better understanding of glutamate control on acetylcholine release is essential for the understanding of therapeutic management of motor disorders involving the extrapyramidal system. While cortical afferents synapse mainly onto principal GABAergic projection neurons, thalamic afferents tend to synapse on the large aspiny cholinergic neurons (Lapper and Bolam, 1992). Large aspiny striatal neurons are thought to be the cholinergic interneuron given to their choline acetyl transferase (CAT) immunoreactivity (Shepherd, 1998). These cells are difficult to study by conventional electrophysiological methods because they are relatively rare (only 2% of the neurons in striatum), small and should be identified by neurochemical markers after electrophysiological evaluation. Therefore, in vitro acetylcholine release assay seems to be a practical and reliable method to evaluate the role of afferent projections that rely on the striatal cholinergic

Abbreviations: [^{3}H]ACh, [^{3}H] acetylcholine; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; AP-5, 2-amino-5-phosphonopentanoic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-[f]quinoxaline-7-sulfonamide; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; TTX, tetrodotoxin; MK-801, dizocilpine maleate or (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate

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neuron. In addition, the in vitro release procedure is particularly suitable to describe local effects of drugs avoiding diffusional barriers and feedback loops present in whole animal microdialysis experiments. Chopped striatal tissue preserves the local cellular organization allowing for the study of afferent synapses onto cholinergic cells. Our experiments were performed without cholinesterase inhibitors or choline uptake inhibitors that are usually employed in such studies. Although several studies have approached some aspects of glutamate-stimulated acetylcholine release, only a few papers explored systematically the specific role of ionotropic glutamate receptors in this process (Cepeda et al., 2001; Jin, 1997; Jin and Fredholm, 1994, 1997). This paper reports the results of experiments on the release of tritium-labeled acetylcholine derived from previous incubation with [^{3}H]-choline, stimulated by glutamate and selective agonists *N*-methyl-D-aspartate (NMDA), a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and kainate. The roles of other important agents as glycine and the antagonists magnesium, MK-801, 2-amino-5-phosphonopentanoic acid (AP-5), 6,7-dinitroquinoxaline-2,3-dione (DNQX), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydro-benzo[f]quinoxaline-7-sulfonamide (NBQX) as well as tetrodotoxin (TTX), a sodium channel blocker, were studied. The potency of the agonists was established and sub maximal concentrations were employed to evaluate the effect of antagonists and glycine.

Preliminary experiments in the presence of magnesium showed that radioactivity released by potassium up to 20 mM is completely calcium-dependent (results not shown). Other studies have demonstrated the correspondence between total tritium outflow and [^{3}H]ACh release, total radioactivity representing mostly labeled choline derived from calcium-dependent release (Fredholm, 1990a,b; Richardson and Szerb, 1974).

2. Methods

2.1. Drugs and reagents

MK-801, *N*-methyl-D-aspartate and kainate were from RBI-Sigma, USA; 2-amino-5-phosphonopentanoic acid, tetrodotoxin, 6,7-dinitroquinoxaline-2,3-dione, a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide were from Tocris; UK, glycine and glutamate were from Sigma. [^{3}H]methyl choline (specific activity 3.3 PBq/mol) was from New England Nuclear (USA). All other reagents were of analytical grade. The concentrations of antagonists were based on literature data from similar in vitro experiments.

2.2. Release experiments

Labeled neurotransmitter release experiments were carried out as described elsewhere (Camillo et al., 2001; Troncone et al., 1995). Briefly, in each experiment three adult male Wistar rats were killed by decapitation and the striata were dissected and placed in ice-cold Krebs–Ringer bicarbonate buffer (KRB) with the following composition: NaCl (118 mM), $NaHCO_3$ (25 mM), KCl (4.8 mM), $CaCl_2$ (1.2 mM), KH_2PO_4 (1.2 mM), and glucose (10 mM), pH 7.3 and constant gassing with CO_2/O_2 was maintained throughout the experiment. Striatal tissue was then chopped twice in a Mcllwain tissue shopper set to cut at 250 μm to form prisms. Chopped tissue was pooled, dispersed with a pipette and washed twice in 20 ml ice-cold KRB and transferred to a beaker containing 3 ml KRB with 0.055 μM of [^{3}H]methyl choline and maintained at 37 °C for 20 min to allow for the uptake. Tissue was then filtered and washed twice with normal ice cold KRB and distributed in 10 superfusion chambers with internal volume of 0.25 ml. Superfusion was performed at a rate of 0.25 ml/min with a 10 channel peristaltic pump, during 60 min in order to achieve a stable baseline of [^{3}H]ACh release. After this time, three successive baseline samples were collected from each chamber at 3-min intervals with an adapted custom-made sample collector. Superfusion with stimulating agent followed in the fourth interval and lasted 2 min. A second identical stimulus was performed in the 11th interval and six more samples were collected afterwards. In the 18th interval, tissue was perfused with 0.1 M HCl for two periods of 3 min to induce release of the total amount of [^{3}H]ACh still present in the tissue.

Superfusion with antagonists was started in the eighth interval and was maintained until the 14th interval in the experimental samples. $MgSO_4$ 1.2 mM was used in specific experiments as described below. Results are expressed as fractional release, i.e. percent of [^{3}H]neurotransmitter released over the total contained in the tissue at the time of release. The effects of drugs were evaluated initially by the fractional release profile compared to the control sample profile and afterwards by comparing the values of B_2/B_1 and S_2/S_1 (B_n stands for basal release here named “spontaneous” for practical purposes). S_1 is the release obtained by stimulation in control conditions and S_2 is the stimulated release obtained in the presence of antagonists. Spontaneous releases in the presence of antagonist (B_2) and control spontaneous release (B_1) were used to evaluate the effects of antagonists on spontaneous release using the B_2/B_1 ratio. S_n is calculated subtracting B_n from the total release obtained under stimulation. B_1 corresponds to the mean of the three samples that preceded S_1 stimulation and the last fractional release before S_2 was considered as B_2. Radioactivity was measured by scintillation spectrometry corrected by external standard counting performed in a Beckman 6500 scintillation counter and standard toluene, triton, 2,5-diphenyloxazole (PPO) and 1,4-bis[5-phenyl-2-oxazolil]-benzene (POPOP) cocktail.

Experimental protocols were submitted to the ethical committee for the use of experimental animals of the Butantan Institute and found in agreement to the rules of the Brazilian

college of animal experimentation (protocol number: 068/2002).

2.3. Statistics

Statistical analysis employed ANOVA followed by Dunnet's test to compare antagonist treated samples to a control group. A value of $P \leq 0.05$ was assumed for significance limit. Statistical analysis was performed with the software GraphPad Prism. EC_{50} values were obtained fitting the values to a sigmoidal dose–response curve with variable slope, identical to the four-parameter logistic equation (top, bottom, $\log EC_{50}$, and slope). Values of CI_{95} were also obtained and equivalence between the EC_{50s} of the agonists was evaluated considering the overlap between averages of $CI_{95} \pm 20\%$ and respective errors, as described elsewhere (Sigle et al., 2003).

3. Results

3.1. Agonist-stimulated release of ACh

These experiments aimed at determining whether glutamate, NMDA, kainate, and AMPA were capable of stimulating [^{3}H]ACh release from striatal tissue and establish a submaximal concentration of each agonist for the subsequent studies employing antagonists. For illustrative purposes Fig. 1 depicts a typical experiment and the samples used to calculate B_1, B_2, S_1 and S_2, as well as the perfusion of experimental drugs/conditions. A stable spontaneous release of [^{3}H]ACh was attained after 40 min of superfusion. After 60 min of superfusion, the amount of labeled transmitter released in 3 min corresponded to $1.09 \pm 0.15\%$ (average ±S.D.) of the total radioactivity present in the sample (fractional release). Under stimulation fractional release

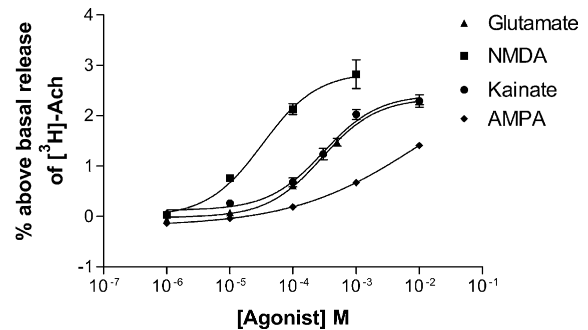

Fig. 2. Effect of agonists on the release of [^{3}H]acetylcholine from striatal chopped tissue superfused in Krebs–Ringer-bicarbonate buffer without magnesium. Error bars represent S.E.M. of 2–13 values per point. Values of R^2 were 0.95, 0.89, 0.89, and 0.96 for glutamate, NMDA, kainate, and AMPA, respectively.

was $2.39 \pm 0.57\%$ for 500 μM glutamate; $3.41 \pm 0.61\%$ for 100 μM NMDA; $1.89 \pm 0.21\%$ for 300 μM kainate and $1.47 \pm 0.28\%$ for 1000 μM AMPA. Concentration–response curves are presented in Fig. 2. AMPA was the least effective releaser with an EC_{50} of about 1134 μM (CI_{95} = 712.5 to 1805 μM) while glutamate and kainate had similar EC_{50s} of 287 μM (CI_{95} = 201.2 to 409.5 μM) and 299 μM (CI_{95} = 190.6 to 471.2 μM), respectively, although equipotence cannot be assumed with an error probability below 5%, as described above in statistics. NMDA was the most potent releaser with EC_{50} of 33 μM (CI_{95} = 15.7 to 67.8 μM). [^{3}H]acetylcholine release became very unstable in the absence of Ca^{2+} and Mg^{2+} rendering impossible to assess the calcium-dependence of the release.

3.2. Effect of antagonists on glutamate-stimulated ACh release

In order to confirm the pharmacological identity of the receptors involved, the following series of experiments was

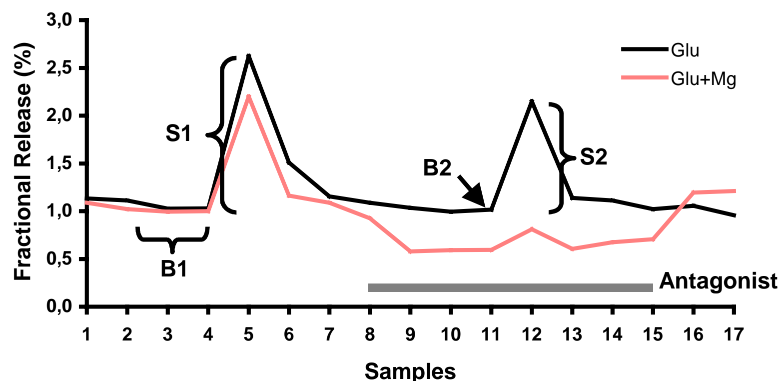

Fig. 1. Typical profile obtained in experiments of labeled neurotransmitter release from striatal chopped tissue. Two stimulations produced by agonists (in this case glutamate 500 μM, black line) at sample 4 and 11 are depicted and the experimental samples (gray line) were perfused with KRB plus magnesium from sample 8–15. Lines are the average of two samples perfused in identical conditions. The delay in the elution of radioactive samples was calibrated such that most of the radioactivity released during the stimulation in sample 4 could be collected in sample 5 and so forth. Brackets point the S_1 and S_2 in the control samples, while a bracket and an arrow point the values used to calculate B_1 and B_2, respectively. In experimental samples, the equivalent points were used.

performed. To study the effects of antagonists on [^{3}H]ACh release stimulated by agonists, the following sub maximal concentrations were chosen based on the curves above: glutamate (500 μmol/l), NMDA (100 μmol/l), kainate (300 μmol/l), and AMPA (1000 μmol/l).

When glutamate was employed as stimulating agent, magnesium, TTX, and AP-5 significantly reduced both spontaneous and stimulated releases. Glycine had no effect on [^{3}H]ACh release in the concentration of 1 μM.

3.3. Effect of antagonists on NMDA-stimulated ACh release

When NMDA was used as stimulating agent, magnesium, TTX, AP-5 and MK-801 reduced stimulated release but only magnesium, TTX and MK-801 affected spontaneous release. Glycine had no effect on spontaneous or NMDA-stimulated [^{3}H]ACh release.

3.4. Effect of antagonists on kainate-stimulated ACh release

When kainate was used as stimulating agent, magnesium, TTX, and AP-5 decreased stimulated release but only TTX reduced spontaneous release. Glycine had no effect on spontaneous or kainate-stimulated [^{3}H]ACh release.

3.5. Effect of antagonists on AMPA-stimulated ACh release

When AMPA was used as stimulating agent magnesium, TTX, DNQX, and NBQX were used as antagonists and all four reduced the stimulated [^{3}H]ACh release. Again, only TTX reduced spontaneous release.

3.6. Effects of TTX and Mg^{2+} on spontaneous and agonist-stimulated ACh release

Comparing the S_2/S_1 values, TTX significantly reduced glutamate-stimulated release by 31%, NMDA by 64%, kainate by 60%, and AMPA by 76%. It is worth mentioning that B_2 represents the effect of drugs (TTX, Mg^{2+}, and others) on a recently stimulated tissue. Comparing B_2/B_1 values in TTX experiments, spontaneous release was also reduced by 42% in the experiments where glutamate was employed as stimulus, NMDA by 37%, kainate by 28% and AMPA by only 16%. On the other hand, Mg^{2+} inhibited about 66% of the release stimulated by kainate, 76% for AMPA, while this inhibition amounted to 80% for glutamate- and 98% for NMDA-stimulated releases. Regarding spontaneous release, tissue previously stimulated by glutamate or NMDA was rendered sensitive to Mg^{2+} and significant decreases in B_2/B_1 were detected (−42 and −32%, respectively). Such magnesium-induced decreases in spontaneous release were not observed after Kainate and AMPA stimulations.

4. Discussion

The absence of cholinesterase inhibitors and choline uptake blockers allowed us to evaluate the [^{3}H]ACh release process in a more physiological condition. It is well established that after release, the inactivation of acetylcholine is made mostly by enzymatic breakdown and that the uptake process is also very important in removing choline from the synaptic cleft. The EC_{50} values for the glutamate agonists calculated from our data are in agreement with those obtained by other authors (Cepeda et al., 2001; Jin, 1997; Jin and Fredholm, 1994, 1997). Eventually, the absence of these drugs allowed for the detection of modifications in magnesium and TTX sensitivity as discussed below. Using electrically evoked release and in the presence of hemicholinium-3, TTX was capable of completely abolish NMDA-, AMPA,- and glutamate-stimulated release of [^{3}H]ACh, kainate-stimulated release was not inhibited while 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) completely abolished AMPA- and kainate-stimulated release of [^{3}H]ACh (Jin and Fredholm, 1994). Also, the experiments performed by these authors employed a double-labeling protocol in which both dopamine and ACh releases were assayed simultaneously and they observed that TTX also blocked completely the release of dopamine, demonstrating that depolarization is involved in this process but the authors stress that no conclusion may be drawn from their data about the sub cellular location of the receptors since synaptosomal preparations were susceptible to TTX blockade as well. Since we used agonists of ionotropic glutamate receptors as stimulus, our results seem to be more suitable for speculations on sub cellular receptor location.

The addition of 1.2 mM magnesium to the perfusion medium almost eliminated glutamate- and NMDA-stimulated [^{3}H]ACh release. A significant reduction in spontaneous release was observed only in these experiments suggesting that other regulatory mechanisms may be involved. Hence, Mg^{2+} ions also inhibited in different degrees the release stimulated by kainate and AMPA, as can be observed in Table 1. As mentioned in Section 3, Mg^{2+} was ineffective in reducing spontaneous [^{3}H]ACh release when striatal tissue was subjected to a previous stimulation by kainate or AMPA. Distinct biochemical changes in the release machinery may take place after NMDA, kainate or AMPA stimulation and account for these results. It is now well accepted that NMDA and possibly other ionotropic glutamate receptors are phosphorylated and dephosphorilated by kinases and phosphatases (Bayer and Schulman, 2001; Hatt, 1999; Sigel, 1995) and calcineurin (Paul et al., 2003). The neurotransmitter release assay has been successfully employed in this kind of study (Dohovics et al., 2003) and provided results that could be considered very close to the physiological/functional process, opposed to the rather biochemical nature of the in vitro protein–protein interaction or protein phosphorylation studies.

Table 1
Effect of glutamate receptor agonists and antagonists on the release of [^{3}H]ACh by striatal chopped tissue in the absence of magnesium (unless otherwise stated)

Condition	B_2/B_1	n	Δ (%)	S_2/S_1	n	Δ (%)
Glu 500 μM	0.98 ± 0.11	17		0.74 ± 0.13	17	
Glu 500 μM + Mg^{2+} 1.2 mM	0.57 ± 0.04	4	−42*	0.15 ± 0.05	4	−80*
Glu 500 μM + Gly 1 μM	1.13 ± 0.13	4	+15	0.69 ± 0.11	4	−7
Glu 500 μM + TTX 1 μM	0.57 ± 0.11	3	−42*	0.51 ± 0.21	3	−31*
Glu 500 μM + AP-5 100 μM	0.89 ± 0.05	6	−9*	0.59 ± 0.19	6	−20*
NMDA 100 μM	0.87 ± 0.11	28		0.80 ± 0.10	19	
NMDA 100 μM + Mg^{2+} 1.2 mM	0.59 ± 0.06	4	−32*	0.02 ± 0.03	4	−98*
NMDA 100 μM + Gly 1 μM	0.79 ± 0.07	6	−9	0.73 ± 0.12	4	−9
NMDA 100 μM + TTX 1 μM	0.55 ± 0.04	4	−37*	0.29 ± 0.11	4	−64*
NMDA 100 μM + AP-5 100 μM	0.80 ± 0.06	6	−8	0.18 ± 0.04	5	−78*
NMDA 100 μM + MK-801 100 μM	0.62 ± 0.09	6	−29*	0.02 ± 0.03	5	−98*
Kainate 300 μM	0.83 ± 0.10	20		0.85 ± 0.22	14	
Kainate 300 μM + Mg^{2+} 1.2 mM	0.88 ± 0.05	4	+6	0.29 ± 0.16	2	−66*
Kainate 300 μM + Gly 1 μM	0.86 ± 0.04	4	+4	0.64 ± 0.26	4	−25
Kainate 300 μM + TTX 1 μM	0.60 ± 0.04	4	−28*	0.34 ± 0.12	4	−60*
Kainate 300 μM + AP-5 100 μM	0.89 ± 0.07	4	+7	0.07 ± 0.09	2	−92*
Kainate 300 μM + MK-801 100 μM	0.76 ± 0.11	6	−8	0.85 ± 0.46	4	0
AMPA 1 mM	0.83 ± 0.08	16		0.88± 0.37	13	
AMPA 1 mM + Mg^{2+} 1,2 mM	0.77 ± 0.08	6	−7	0.21 ± 0.10	4	−76*
AMPA 1 mM + TTX 1 μM	0.70 ± 0.11	6	−16*	0.21 ± 0.15	5	−76*
AMPA 1 mM + DNQX 100 μM	0.79 ± 0.11	6	−5	0.07 ± 0.07	4	−92*
AMPA 1 mM + NBQX 100 μM	0.68 ± 0.11	4	−18	0.11 ± 0.14	3	−88*

Values represent the mean ± S.D. of n chambers. Δ (%) represents the percent change compared to control value. Statistical analysis performed by ANOVA followed by Dunnet's test as described in text. In experiments with glutamate, B_2/B_1, $F = 17.95$ and $F_{crit} = 2.53$; S_2/S_1, $F = 16.23$ and $F_{crit} = 2.53$. In experiments with NMDA, B_2/B_1, $F = 115.03$ and $F_{crit} = 2.34$; S_2/S_1, $F = 126.8$ and $F_{crit} = 2.53$. In experiments with kainate, B_2/B_1, $F = 6.501$ and $F_{crit} = 2.53$; S_2/S_1, $F = 5.929$ and $F_{crit} = 2.62$. In experiments with AMPA, B_2/B_1, $F = 3.209$ and $F_{crit} = 2.69$; S_2/S_1, $F = 12.40$ and $F_{crit} = 2.74$.

* $P \leq 0.05$.

Tetrodotoxin was employed in an attempt to identify the sub-cellular distribution of these receptor subtypes on the striatal cholinergic cell. According to our results, AMPA-stimulated [^{3}H]ACh release was the most sensitive to TTX showing a reduction of 76% of the stimulated release, suggesting that this receptor could be located on dendrites or cell body of the large aspiny striatal neuron. NMDA and Kainate stimulations were the second most inhibited (64 and 60%, respectively) suggesting that these receptors could be rather homogeneously distributed on the cholinergic cell. On the other hand, spontaneous release after previous agonist stimulation showed an opposite pattern of sensitivity to TTX blockade of sodium channels. In the extreme case, although statistically significant, a previous exposure to AMPA seems to decrease the effect of TTX on spontaneous [^{3}H]ACh release. These effects could alternatively be explained by biochemical modifications of sodium channels by phosphorylation/dephosphorylation as have been proposed by other authors (Catterall, 2000; Choe and McGinty, 2001; Schiffmann et al., 1998) following the same rationale proposed above for the effects of Mg^{2+} on spontaneous release.

Glycine has been described as a co-agonist of glutamate on NMDA receptors. In order to assess this facilitating effect, we used glycine in a series of experiments. Glycine failed to increase [^{3}H]ACh release induced by the agonists. Although controversial, since the effective concentration to potentiate NMDA receptor is below the micromolar range, we assumed that our preparation should not be considered free of endogenous glycine. Therefore, the stimulation obtained in the absence of added glycine corresponds already to the maximal effect attainable and the addition of 1 μM glycine resulted in no additional increment.

The giant aspiny cell, recognized as the striatal cholinergic interneuron, expresses a peculiar set of ionotropic glutamate receptors. NMDA receptors are composed by NR1 and NR2 subunits with the latter presenting A to D variants. Recent studies demonstrated that choline acetyl transferase-positive (CAT) cells in rat and human striatum express NR1, NR2B, and also NR2D subunits (Chen et al., 1996; Kuppenbender et al., 2000; Standaert et al., 1999). These studies emphasize the absence of the NR2A subunit and the functional consequence of this. The presence of the NR2A subunit generates a receptor more dependent on glycine (Nankai et al., 1995) and the lack of effect of added glycine observed in our experiments support this observation. Also, the antagonist selectivity profile of NMDA receptors with the above subunit organization fits well to our results obtained in the TTX, Mg^{2+} and MK-801 experiments, since a receptor containing the NR2C subunit would be insensitive to magnesium. On the other hand, MK-801 was effective in our experiments since we used a rather high concentration (Bodi et al.,

1995). To build a better picture of subunit composition of the NMDA receptors studied here based on MK-801 sensitivity, the IC_{50} values should be evaluated (Nankai et al., 1998).

Striatal cholinergic cells also express AMPA receptors. These receptors are composed of subunits GluR1–4. Immunohistochemical and pharmacological studies have demonstrated that the striatal cholinergic cell expresses mainly GluR1 and GluR4 subunits and lack the GluR2/3 subunit, conferring to these receptors a low Ca^{2+} conductance and slow desensitization (Bernard et al., 1997; Buldakova et al., 1999; Martin et al., 1993). Other authors failed to detect AMPA receptors in cholinergic striatal cells by immunocytochemistry (Chen et al., 1996). In our experiments, AMPA behaved as a poor releaser. Results of the experiments employing antagonists suggested that AMPA may act via the release of endogenous glutamate since 80% of the AMPA stimulated release was inhibited by Mg^{2+}, TTX blocked 76% and DNQX blocked 65% of the stimulated release. These results suggest that the effect of AMPA could hardly be differentiated from those of glutamate in our experiments, suggesting the possible involvement of endogenous glutamate released by AMPA. This notion deserves a careful approach in future studies.

Kainate receptors are thought to be composed of GluR5/6/7 and the high affinity KA1 and KA2 (Bettler and Mulle, 1995; Chittajallu et al., 1999). Unfortunately the literature is unclear regarding the subunit composition of kainate receptors on the striatal cholinergic interneuron. Using immunohistochemistry, some authors were able to detect GluR6 and KA2 subunits as the prevalent configuration of kainate receptors in caudate-putamen (Chen et al., 1996). In mRNA amplification studies though, results are somewhat different suggesting that GluR5 and KA1 to be the most frequent pattern in striatal cholinergic cells (Chittajallu et al., 1999; Lilliu et al., 2002). Besides the original post-synaptic localization of kainate receptors, a presynaptic localization has been proposed, with an inhibitory action on the release of glutamate and GABA in several neural structures as hippocampus, cerebellum and striatum (Chittajallu et al., 1999). According to our results, kainate receptors seem to be more involved in ACh release in striatal tissue, corroborating other studies (Jin, 1997). This conclusion is further supported by the results obtained in magnesium experiments in which this ion was less effective in counteract kainate-stimulated [^{3}H]ACh release, suggesting a smaller contribution of endogenous glutamate acting through NMDA receptors.

Finally, in vivo microdialysis experiments also present some variable results. While NMDA receptors exert a clear stimulatory effect on ACh release (Anderson et al., 1994; Consolo et al., 1996; Ikarashi et al., 1998; Knauber et al., 1999), AMPA and kainate receptors are rather controversial. AMPA receptor stimulation inhibited ACh release, an effect completely reversed by bicuculine suggesting that AMPA induced GABA release and the latter inhibited ACh release (Giovannini et al., 1995). In the opposite way, in another microdialysis study quisqualate (AMPA agonist) induced ACh release that was completely blocked by TTX (Anderson et al., 1994; Rakovska et al., 2002). Whatever is the final effect of AMPA receptor stimulation in vivo, it seems that an intermediary step is involved. The role of kainate receptors in this process seems again somewhat neglected. All microdialysis studies employed acetyl cholinesterase inhibitors.

In conclusion, our results support that (1) in the absence of Mg^{2+}, NMDA receptors are the most effective glutamate receptors involved in the glutamate-stimulated [^{3}H]ACh release from rat striatal slices (2) magnesium, AP-5 (in kainate experiments) and TTX effectively antagonized kainate and AMPA stimulation suggesting that at least part of the kainate and AMPA effects might be attributed to glutamate release acting through NMDA receptors (3) kainate receptors showed a more direct involvement in [^{3}H]ACh release based on the smaller dependence on Mg^{2+} and less inhibition by TTX, the latter also pointing to a favored pre-synaptic location of these receptors, as well as the results showing the expression of kainate mRNA in cholinergic cells (4) stimulation of ionotropic glutamate receptors may induce long lasting biochemical changes in receptor/ion channel function modifying the effects of TTX and/or Mg^{2+} ions on [^{3}H]ACh release, in the same fashion as described elsewhere (Sigle et al., 2003) for human but not mouse cortical tissue. These authors detected an important activation of choline acetyl transferase activity in cortical tissue submitted to 10 or 20 mM K^+ depolarization. This complicates the interpretation of results of experiments involving the two-stimulation paradigm.

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