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Dual Action of Excitatory Amino Acids on the Metabolism of Inositol Phosphates in Striatal Neurons

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SUMMARY

Glutamate is able to stimulate inositol phosphate (IP) formation in striatal neurons in primary culture, mainly via an excitatory amino acid receptor of the quisqualate subtype. In the present study we show that carbachol (Carb)- (a cholinergic agonist), but not neurotensin or norepinephrine-induced IP production could be reduced by 40% when measured in the presence of Glu. The inhibition of the Carb response by Glu was dose dependent and reproduced by *N*-methyl-D-aspartate (NMDA). Quisqualate elicited an additive response with Carb. 2-Amino-5-phosphonovalerate (APV) completely reversed the NMDA-induced inhibition. APV had no significant effect on Glu- or kainate-induced inhibition. Therefore, striatal neurons contain at least three different excitatory amino acid receptors: a quisqualate receptor triggering the stimulation of IP metabolism, and an NMDA and a kainate receptor, both able to decrease the Carb-induced IP formation.

It is now well established that the stimulation of phosphoinositide turnover (1, 2) by a large number of hormone or neurotransmitter receptors results in the generation of two second messenger molecules: diacylglycerol and inositol-1,4,5-triphosphate (IP_3). Diacylglycerol activates a phospholipid-dependent protein kinase, termed protein kinase C (3, 4), whereas IP_3 has been shown to liberate Ca^{2+} from intracellular storing sites in permeabilized (5, 6) as well as in intact cell systems (7, 8). In view of the importance of the cellular physiological consequences of intracellular Ca^{2+} release, it can be expected that, in addition to neurotransmitters able to stimulate this pathway, there are others able to inhibit it. It has recently been reported that dopamine inhibits thyrotropin-releasing hormone- and angiotensin II-induced IP formation in pituitary cells in primary culture (9, 10). In rat hippocampal slices two groups have shown that EAAs inhibit neurotransmitter-induced IP formation (11, 12). The nature of the neurotransmitter involved is different in these reports. Baudry *et al.* (11) reported inhibition of the Carb-induced response and no effect of EAAs on the NE-induced response, whereas the response affected by EAAs in the study of Nicoletti *et al.* (12) was NE-induced stimulation with no effect on the Carb response. No explanation for these discrepancies is available for the moment. It seems that there are major differences for EAA-induced IP responses during the development of the CNS (12). During the main period of synaptogenesis in the hippocampus of newborn rats, glutamate stimulates the formation of IPs (12). This stimulatory response decreases thereafter to the low levels found in adult rats (12, 13). The inhibitory response, on the other hand, is only found in adult rats. In striatal neurons in primary culture we have reported (14) that glutamate stimulates IP formation via a receptor of the QA subtype. This response reaches its maximum value at the onset of synaptogenesis and declines thereafter. Here we report that, in addition to this stimulatory effect, glutamate is able to inhibit Carb-induced IP formation in striatal neurons. Since 93% of the cells are neurons in these cultures, one may assume that the responses obtained are of neuronal origin.

Materials and Methods

Agents used in this study were obtained from the following sources: QA, NMDA, and APV, Cambridge Research Biochemicals; NT, Bachem; and Carb, NE, Glu, and KA, Sigma.

Striatal neuronal cultures in serum-free medium. Cultured striatal neurons were prepared as previously described (15). Briefly, striata were removed from 14- to 15-day-old Swiss albino mouse embryos (Iffa Credo, Lyon, France) and mechanically dissociated with a

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ABBREVIATIONS: IP_3, inositol-1,4,5-triphosphate; IP, inositol phosphate; EAA, excitatory amino acid; Glu, glutamate; QA, quisqualate; KA, kainate; Carb, carbachol; NE, norepinephrine; NT, neurotensin; NMDA, *N*-methyl-D-aspartate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; QNB, quinuclidinyl benzilate; NMS, *N*-methylscopolamine; APV, 2-amino-5-phosphonovalerate; LDH, lactate dehydrogenase.

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fire-narrowed Pasteur pipette in serum-free medium. Cells were plated at 0.8–1.0 × 10^6 cells/ml in 12-well (1 ml/well) or 6-well (2 ml/well) Costar (Cambridge, MA) culture dishes, previously coated successively with poly-L-ornithine (1.5 μg/ml, M_r = 40,000; Sigma) and culture medium containing 10% fetal calf serum. After withdrawing the last coating solution, cells were seeded in serum-free medium (5 μCi/ml *myo*-^{3}H-inositol; New England Nuclear) composed of: a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 nutrient (Gibco Europe, Paris, France) and including glucose (0.6%), glutamine (2 mM), and sodium bicarbonate (3 mM), all obtained from Sigma. In the place of serum, a defined hormone and salt mixture that included insulin (25 μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (60 μM), and selenium salt (30 μM), all from Sigma, was added. Using this technique, these cultures were immunocytochemically (with antibodies to neurofilament and glial fibrillary acidic proteins) and morphologically (with transmission electron microscopy) defined as purified (>93%) neurons (15, 16).

Inositol phosphate formation. Inositol phosphate formation in striatal neurons was examined as previously described (14). After 11–14 days *in vitro*, the culture medium was replaced with PBS buffer [composition (mM): NaCl, 137; KCl, 2.7; Na_2HPO_4, 8; KH_2PO_4, 1.5; $MgCl_2$, 0.5; $CaCl_2$, 0.45] and neurons were incubated for 10 min with 10 mM LiCl in order to block IP degradation (17–19). Agents were added at the indicated concentrations. The incubation temperature was 37°. The reaction was stopped after 30 additional minutes by replacement of the incubation medium by 5% PCA. The IPs were extracted as described by Bone *et al.* (20) and separated by ion exchange chromatography as described by Berridge *et al.* (21). The elution procedure was simplified by applying 24 ml of 1 M ammonium formate/0.1 M formic acid buffer immediately after the 60 mM sodium formate/5 mM disodium tetraborate elution in order to elute IP_1, IP_2 and IP_3 in a unique fraction.

Results

An extensive screening of putative neurotransmitters indicated that the most potent substances stimulating the IP response in striatal neurons were NT, NE, Carb (a muscarinic cholinergic agonist), and Glu. In order to examine possible interactions between the IP response of these four substances, saturating concentrations were tested alone or in pairs (Fig. 1). All pair combinations between NT, NE, and Carb resulted in clearly additive responses. This suggests that the respective receptors are localized on different cell types or different parts of the same cell as, for example, soma, dendrites, or nerve terminals. However, in combination with other agonists, glutamate had distinct effects on the IP response, depending on the co-agonist used. 1) The simultaneous application of Glu and NT resulted in an additive IP response. 2) The effects of Glu and NE were apparently not additive. The level of the IP response induced by the two compounds together corresponded to that induced by NE alone. Several hypotheses could explain this fact. The simplest is that both receptors are localized on the same cell and were using the same signal transduction system. Another explanation would be that there is a partial inhibition of one response by the other and that the remaining responses were additive. 3) The result of the combined application of Glu and Carb indicated that Glu inhibits about 45% (Fig. 1) of the IP response produced by Carb.

This inhibition was concentration dependent (Fig. 2) with a concentration required for half-maximal inhibition (IC_{50}) of 24 μM. NMDA, a selective agonist of one of the Glu receptor subtypes (22, 23), mimics the effect of Glu (IC_{50} = 56 μM), whereas QA does not. In fact, the QA-induced IP response was additive with the IP response evoked by 1 mM Carb except at very high concentrations of QA (10^{-3} M). The *dotted curve* in Fig. 2 would represent the expected theoretical accumulation of IPs if the stimulatory effects of Carb and QA had been strictly additive. The less than additive response observed at 1

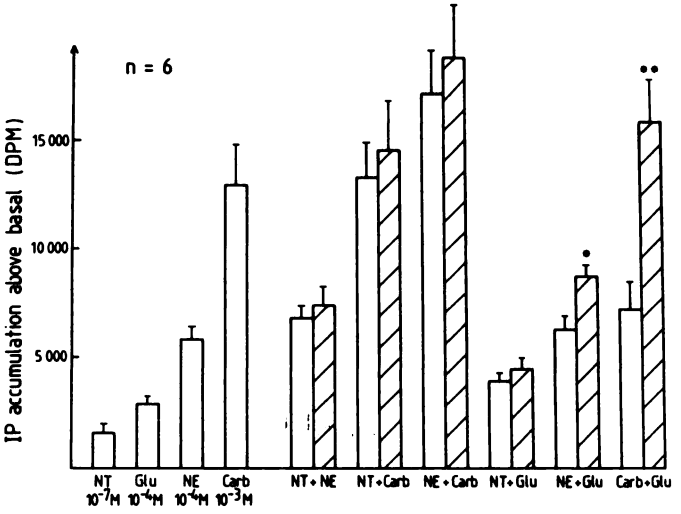

Fig. 1. IP formation in response to combinations of agonists. Drugs were added at the following concentrations (M): NT, 10^{-7}; Glu, 10^{-4}; NE, 10^{-4}; and Carb, 10^{-3}. Results are means, expressed in dpm above a basal value of 2636 dpm ± 267 dpm, ± SE of six independent experiments on separate culture preparations, performed in duplicate. ▨, value of IP formation expected if the two responses were strictly additive. Expected and experimental responses were significantly different (*, $p < 0.05$ and **, $p < 0.01$) for the combined application of NE + Glu and Carb + Glu as determined with Student's *t* test.

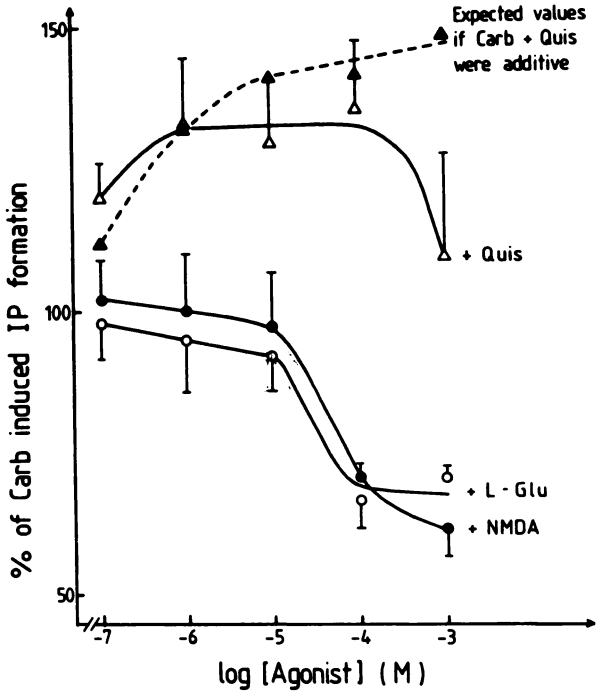

Fig. 2. Concentration-response curves for EAA inhibition of Carb-induced stimulation of IP formation. Cells were exposed to 10^{-3} M Carb and increasing concentrations of the indicated amino acids. Results were means, expressed as percentage of the response induced by 10^{-3} M Carb ± SE of four independent experiments for NMDA and L-Glu and three independent experiments for QA (*Quis*) on separate culture preparations, performed in duplicate. – – –, the values of IP formation expected if the Carb and QA responses were strictly additive.

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mM was not due to an autoinhibition of the QA-evoked IP response since the stimulation of IP formation by QA alone remains at its maximum value up to a concentration of 1 mM (Fig. 3). The most likely explanation is that QA, at high concentrations, may activate an EAA receptor which inhibits cholinergic induced IP formation.

Since neither NMDA (10^{-4} and 10^{-3} M) nor Glu (10^{-4} and 10^{-3} M) reduced total tritiated QNB or NMS binding to intact striatal neurons, and neither EAA significantly altered Carb displacement of tritiated QNB or NMS binding, we can exclude that NMDA or Glu blocks the muscarinic receptor.

The actions of the NMDA receptor-specified antagonist APV on the different EAA-induced responses are shown in Table 1. APV had no effect on Carb-, Glu-, and KA-induced IP formation but completely inhibited the slight NMDA effect. The NMDA-induced inhibition was completely reversed by APV ($p < 0.01$). But the inhibitory effects of Glu and KA were not significantly touched by this antagonist.

At the end of some of the experiments of Table 1 we determined the content of the cytosolic enzyme lactate dehydrogenase (LDH) in the sonicated supernatants (Table 2). Already, under basal and Carb-stimulated conditions significant amounts of LDH could be detected. But the addition of NMDA further increased the LDH concentrations in the supernatant.

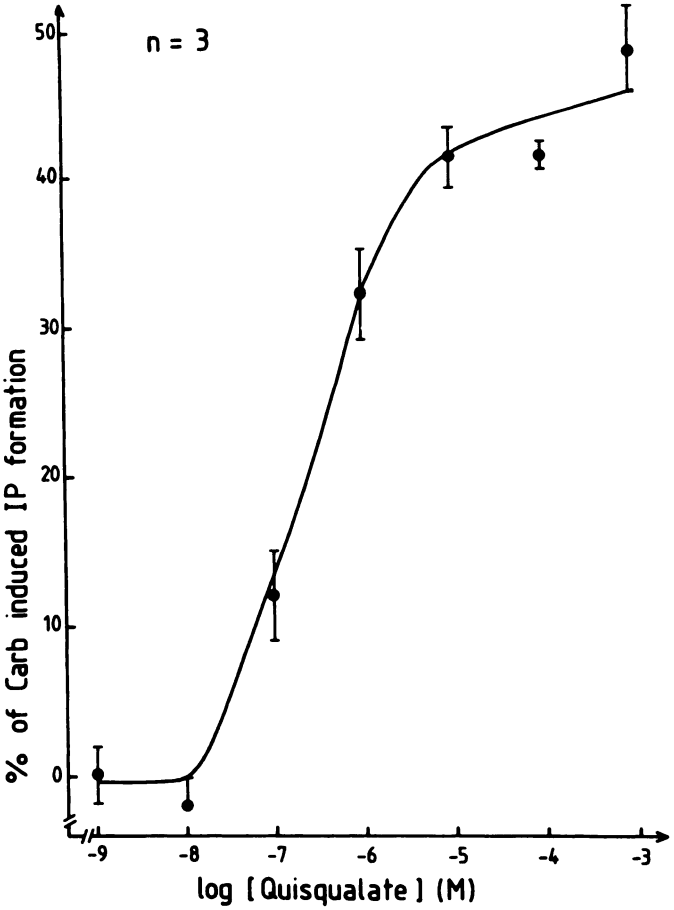

Fig. 3. Concentration-response curve for QA-induced stimulation of IP formation. Cells were exposed to increasing concentrations of QA. Results were means, expressed as percentage of the response induced by 10^{-3} M Carb ± SE of three independent experiments on separate culture preparations performed in duplicate.

TABLE 1

Effects of APV on EAA-induced inositol phosphate responses

APV (10^{-4} M) was added together with Li^+ 10 min prior to agonist addition. Reaction was stopped 90 min later by addition of 5% PCA. The concentrations of agonists used were (M): Carb, 10^{-3}; NMDA, 10^{-4}; Glu, 10^{-4}; KA, 10^{-4}. Results are expressed as percentage of the maximal Carb response and are the means ± standard errors of the number of independent determinations indicated in parentheses. The mean value of the basal was 759 ± 68 dpm in the absence and 868 ± 61 dpm in the presence of APV. One hundred per cent of Carb stimulation corresponded to 4065 ± 269 dpm. The significance of the inhibitions compared to the 100% Carb value were determined by a Student's *t* test.

Drug	Control	APV
Carb	100 ± 5 (12)	93 ± 5 (6)
NMDA	11 ± 2 (6)	−1 ± 2 (6)
Glu	21 ± 8 (6)	28 ± 5 (6)
KA	6 ± 2 (6)	6 ± 2 (6)
Carb + NMDA	68 ± 6 (11)[a]	122 ± 24 (6)
Carb + Glu	49 ± 5 (6)[a]	62 ± 6 (6)
Carb + KA	66 ± 9 (6)[b]	70 ± 7 (6)

[a] $p < 0.001$.
[b] $p < 0.01$.

TABLE 2

LDH contents of the sonicated supernatants at the end of incubations in some of the experiments shown in Table 1

Results were expressed as percentage of the total neuronal LDH contents. LDH activity was determined as described in Ref. 34.

Drug	Control	APV
None	19.7 ± 1.3	11.7 ± 0.1
Carb	26.0 ± 1.4	11.8 ± 0.1
NMDA	33.0 ± 1.8	12.5 ± 0.6
Carb + NMDA	37.5 ± 4.4	12.2 ± 0.1

APV reduced the LDH content of the supernatant in all four conditions to the same value.

Discussion

The present results indicate that the selective agonists NMDA and KA were far more potent than QA in inhibiting Carb-induced IP formation. The NMDA effect was completely reversed by APV. Thus, it seems that EAAs can block the Carb effect via their action at the NMDA and KA receptor subtypes, whereas EAA action at the QA receptor subtype appears responsible for the stimulation of IP formation in the same culture of striatal neurons (14). QA was the agonist which exhibited the most pronounced difference in EC_{50} values for the two types of responses. Its EC_{50} for the stimulatory receptor was less than 1 μM (Fig. 3 and Ref. 14), but higher than 1 mM for the inhibitory receptor (Fig. 2). This difference explains the observation of the almost additive effect seen for QA and Carb when using low doses of QA but the non-additive effects seen with higher doses of QA (Fig. 2). NMDA was only a partial agonist of the stimulatory response ($EC_{50} = 15$ μM, $E_{max} = 43\%$ of maximal Glu effect, Ref. 14), whereas it was as efficient as Glu for the inhibitory one. The EC_{50} of NMDA for the inhibitory receptor was therefore similar to its EC_{50} for the stimulatory receptor. The affinities reported in binding studies were similar to the EC_{50} reported here (24), as were the EC_{50} values reported in Na^+ efflux experiments (25, 26).

NMDA and KA with much lower stimulatory responses than Glu were not more potent than Glu in inhibiting Carb-induced IP formation. On the other hand, since Glu is an agonist of all

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EAA receptors, it should inhibit via KA and NMDA receptors but potently stimulate via QA receptors. This is a very complex situation and careful studies have to be carried out to characterize the interactions between different EAA receptors.

In rat hippocampal slices, two groups recently reported the presence of an EAA receptor capable of inhibiting Carb- or NE-induced formation of IPs (11, 12). These inhibitory receptors were, as in striatal neurons, of the NMDA and KA subtypes, although the affinity for glutamate was about 60 times lower in hippocampal slices (11) than in our system. Since Carb and NE have been shown to stimulate IP formation both in glial cells (27, 28) and neurons (this study), it is not possible to determine the cellular localization of the inhibitory effects reported by Baudry *et al.* (11) and Nicoletti *et al.* (12). Our results suggest that the inhibitory effect of EAAs on neurotransmitter-induced IP formation is a neuronal effect at least in the striatum.

Two interesting observations remain to be discussed. The first one is that NMDA and KA inhibit the cholinergic IP response (Fig. 2) and stimulate, although weakly, basal IP formation in striatal neurons (Table 1). It remains to be determined if these two effects are mediated through two different NMDA/KA receptor subtypes. A great number of neurotransmitters are known to have opposite effects on second messenger systems via different receptor types. One classical example is dopamine which stimulates and inhibits cyclic AMP production through D_1 and D_2 dopaminergic receptors in striatal neurons (29). Other possibilities are that the same receptor is able to mediate directly both effects or that one effect is the consequence of the other. Further studies will show which explanation is the closest to reality. The second observation to be discussed is the fact that the inhibition by NMDA of the cholinergic IP response was obtained in the presence of Mg^{2+}, whereas biochemical (30) as well as electrophysiological (31, 32) responses to NMDA are generally blocked by Mg^{2+}. At least two explanations can be proposed. One is that the NMDA receptor involved in the response described here is different from the one generally studied. The other is that cholinergic receptors depolarized the neurons and therefore suppressed the Mg^{2+} blockade (31, 32) of the NMDA response.

The mechanism of the inhibitory effects of EAAs is still unknown. It is well established that long-term stimulation with Glu, NMDA, and KA can have neurotoxic effects (33). We therefore determined the amount of the cytosolic enzyme LDH in the supernatant at the end of the experiments shown in Table 1 (Table 2). The results obtained suggest that neurotoxic actions of the EAA receptors could be at the origin of the inhibitory effects described. Our results would then indicate that these EAA receptors are localized in striatal neurons mainly on acetylcholine-sensitive neurons. But in order to respond clearly to the question whether the EAA-induced inhibitions of the IP response are due to their neurotoxic effects, detailed microscopic analysis has to be done. We were currently investigating this problem.

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