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Role of Arachidonic Acid and Glutamate in the Formation of Inositol Phosphates Induced by Noradrenalin in Striatal Astrocytes

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SUMMARY

The noradrenalin-evoked production of [^{3}H]inositol phosphates in mouse striatal astrocytes in primary culture appeared to be the result of the combined stimulation of α_1- and α_2-adrenergic receptors. Indeed, the noradrenalin (100 μM) response was only partially reproduced by a maximally effective concentration of methoxamine (100 μM), a selective agonist of α_1-adrenergic receptors. In addition, the noradrenalin (100 μM)-induced production of [^{3}H]inositol phosphates, which was completely suppressed by the α_1-adrenergic antagonist prazosin (1 μM), was also partially inhibited by yohimbine, a selective antagonist of α_2-adrenoceptors (maximum inhibition = −57 ± 11%, measured in the presence of 10 μM yohimbine; six experiments). Finally, UK14,304, a selective α_2-adrenergic agonist that was ineffective alone, enhanced the methoxamine-evoked production of [^{3}H] inositol phosphates (EC_{50} = 86 ± 21 nM; three experiments). These results suggest that the stimulation of α_1-adrenergic receptors is required for the α_2-adrenergic receptor-mediated enhancement of phospholipase C activity. The increased production of [^{3}H]inositol phosphates resulting from the stimulation of α_2-adrenergic receptors involved pertussis toxin-sensitive G proteins ($G_{i/o}$) and depended on extracellular calcium. As shown using the fluorescent dye indo-1, noradrenalin (100 μM) induced a long-lasting increase in cytosolic calcium in striatal astrocytes. Moreover, noradrenalin (100 μM) stimulated [^{3}H]arachidonic acid release from these cells. These two latter responses may result from synergistic effects due to the combined stimulation of α_1- and α_2-adrenergic receptors, because they were inhibited by either prazosin (1 μM) or yohimbine (10 μM). Finally, the noradrenalin-evoked production of [^{3}H]inositol phosphates seems to result partly from an inhibition by arachidonic acid of glutamate uptake into astrocytes, leading to the stimulation of glutamate metabotropic receptors coupled to phospholipase C. Indeed, the α_2-adrenergic component of the noradrenalin response was suppressed by either enzymatic removal of external glutamate or addition of 2-amino-3-phosphonopropionic acid (1 mM), an antagonist of glutamate metabotropic receptors that blocked the glutamate-evoked production of [^{3}H]inositol phosphates in striatal astrocytes, and was reproduced by the direct application of either glutamate or an inhibitor of glutamate uptake, β-methyl-DL-aspartic acid.

Activation of β-adrenergic receptors by noradrenalin has been shown to modulate several astrocytic functions, such as glycogenolysis and the release of neurotrophic substances or neuromediators (1). Binding studies have demonstrated that astrocytes also possess α_1- and α_2-adrenergic receptors (2). Stimulation of α_1- and α_2-adrenergic receptors in astrocytes induces the formation of inositol phosphates (3, 4) and the inhibition of adenylate cyclase, respectively (5). However, as recently suggested by Wilson and Minneman (6), synergistic effects mediated by both α-adrenergic receptor subtypes seem to be involved in the noradrenalin-evoked production of inositol phosphates in cultured astrocytes from the rat brain.

We have previously shown that 2-chloroadenosine (acting at A_1 purinergic receptors) or somatostatin enhances the α_1-adrenergic receptor-evoked formation of inositol phosphates in mouse striatal astrocytes (7, 8). Additional experiments suggested that a cascade of events were involved in the 2-chloroadenosine- and somatostatin-mediated enhancement of the α_1-adrenergic response, i.e., the combined stimulation of α_1-adrenergic receptors and somatostatin or A_1 purinergic receptors stimulated the release of arachidonic acid, which in turn may have inhibited the uptake of glutamate spontaneously released from cultured astrocytes. The resulting increase in the concentration of external glutamate, responsible for the stimulation

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ABBREVIATIONS: AP_3, 2-amino-3-phosphonopropionic acid; AP_4, 2-amino-4-phosphonobutyric acid; AP_5, 2-amino-5-phosphonopentanoic acid; *t*-ACPD, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; PTX, *Bordetella pertussis* toxin; NMDA, *N*-methyl-D-aspartate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate.

of metabotropic receptors coupled to phospholipase C, accounts for the enhancing effects of either 2-chloroadenosine or somatostatin on the α_1-adrenergic receptor-mediated production of inositol phosphates (7, 8).

In the course of our study of the modulation of the α_1-adrenergic response in striatal astrocytes, we observed that the efficacy of noradrenalin in stimulating the production of inositol phosphates was greater than that of methoxamine, a selective α_1-adrenergic agonist. Because α_2-adrenoceptors are generally coupled to their effectors through a transduction system ($G_{\alpha i/o}$ proteins) identical to that associated with A_1 purinergic and somatostatin receptors, the stimulation of α_2-adrenergic receptors could also potentiate the α_1-adrenergic receptor-mediated activation of phospholipase C, through a mechanism identical to that involved in the effect of either 2-chloroadenosine or somatostatin. Therefore, the present study was undertaken to provide evidence for the occurrence of synergistic effects resulting from the stimulation of α_1- and α_2-adrenergic receptors in the production of inositol phosphates in striatal astrocytes and to elucidate the biochemical events involved in this phenomenon.

Materials and Methods

Chemicals. The chemicals used were purchased from the following sources: methoxamine, prazosin, L-glutamate, β-methyl-DL-aspartic acid, isoproterenol, propranolol, mepacrine, and unlabeled arachidonic acid from Sigma; yohimbine, UK14,304 [5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine], AP_3, AP_4, AP_5, and MK-801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine] from Cambridge Research Biochemicals Ltd. (Cambridge, UK); *t*-ACPD and DNQX from Tocris Neuramin (Bristol, England); noradrenalin and PTX from Calbiochem (France Biochem, Meudon, France); and adenosine deaminase and glutamate-pyruvate transaminase from Boehringer (Mannheim, Germany).

Primary cultures of striatal astrocytes. Primary cultures of striatal astrocytes were prepared as described previously (7). Briefly, striata were removed from 16-day-old Swiss mouse embryos (Iffa Credo, Lyon, France) and were mechanically dissociated with a fire-narrowed Pasteur pipette in serum-free medium. Cells were plated in 24- and 12-well Nunc culture dishes (2.5×10^5 and 5×10^5 cells/well, respectively) that had been previously coated with poly-L-ornithine (1.5 μg/ml, M_r 40,000; Sigma). The culture medium was composed of a 1/1 mixture of minimal essential medium and Ham's F12 nutrient (GIBCO, Paris, France), supplemented with 33 mM glucose, 2 mM glutamine, 13 mM sodium bicarbonate, 5 mM HEPES buffer, pH 7.4, and 10% NU-Serum (Collaborative Research, France). After 12 days *in vitro*, the culture medium was changed and cytosine arabinoside (1 μM; Sigma) was added for 24 hr, to prevent the formation of cell multilayers and the proliferation of microglial cells. Thereafter, the culture medium was changed every 3 days. Under these conditions, after 21 days *in vitro* >95% of the cells were immunostained for glial fibrillary acid protein. The remaining 5% of cells could be immature glioblasts or O2A progenitors, which are known not to be labeled by anti-glial fibrillary acid protein antibodies. Cultures were devoid of microglial cells and neurons, because no immunostaining was observed using the monoclonal anti-mouse macrophage antibody anti-MAC-1 (Serotec, France) or anti-neurofilament triplet antibodies (kindly provided by Dr. R. K. Liem, Columbia University, New York, NY), respectively (7).

Measurement of [^{3}H]inositol phosphate production. Striatal astrocytes grown for 21 days in 24-well culture dishes were incubated for 24 hr in the presence of *myo*-[2-^{3}H]inositol (1 μCi/well, 17 Ci/mmol; Amersham, France). After three washes, cells were preincubated for 10 min in Krebs phosphate buffer (in mM: NaCl, 120; NaH_2PO_4, 15.6; KCl, 4.8; $MgSO_4$, 1.2; $CaCl_2$, 1.2; glucose, 33.3; pH 7.4) supplemented with LiCl (10 mM). Cells were then incubated for 20 min in the same medium in the presence of tested substances and adenosine deaminase (1 IU/ml), to prevent the effects of endogenous adenosine on inositol phosphate production (7). The incubation was stopped by successive additions of 200 μl of 0.1% Triton X-100/0.1 M NaOH, 500 μl of H_2O, and 200 μl of 0.1% Triton X-100/0.1 M HCl. [^{3}H]Inositol phosphates were then extracted and estimated as described previously (9).

Measurement of [^{3}H]arachidonic acid release. Striatal astrocytes, grown for 21 days in 12-well culture dishes, were incubated for 18–22 hr with [^{3}H]arachidonic acid (1 μCi/well, 200 Ci/mmol; Amersham). Cells were washed four times at 5-min intervals with 1 ml of Krebs phosphate buffer supplemented with 1 mg/ml fatty acid-free bovine serum albumin (Sigma) and were then incubated for 15 min in the presence of drugs. The incubation was stopped by removing the incubation medium, which was centrifuged at 300 × *g* for 5 min to eliminate nonadherent cells, and the supernatant was measured for radioactivity. High performance liquid chromatography analysis, performed as described previously (10), indicated that >95% of the radioactivity was recovered in a peak having the same retention time as authentic arachidonic acid.

Cytosolic calcium measurement. For cytosolic calcium measurements, cells were seeded on glass slides (3×10^6 cells/slide) that had been previously coated with poly-L-ornithine and were placed into 100-mm culture dishes. The intracellular calcium concentration was monitored by quantitative ratio imaging of the fluorescent calcium probe indo-1 (Molecular Probes Inc.). Cells were loaded for 60 min with 12 μM indo-1/acetoxymethyl ester in perfusion saline-HEPES buffer (in mM: HEPES, 20; glucose, 5.5; NaCl, 145; KCl, 5.5; $MgCl_2$, 0.9; $CaCl_2$, 1.1; pH 7.2). After loading, the glass slide was placed in a thermostable perfusion chamber, where cells were exposed to tested substances using a multichannel superfusion device. Cells were excited with a 75-W xenon light, filtered at 340 nm with a 10-nm-wide interferential filter. Excitation and emission spectra were separated by a 380-nm dichroic long-pass filter, and the emission spectra were then divided into two halves by a dichroic long-pass filter (opticals were obtained from Nikon and Hamamatsu, Japan). Two discriminant bands, at 400–410 nm and 470–480 nm, were selected from the two halves, and both fluorescent images were digitized (8 video frames/digitized image, permitting the recording of 1 image/sec). The camera dark noise was substracted from the recorded crude image (camera and digitizing system were from Hamamatsu). The cytosolic calcium concentration was calculated according to the equation described by Grynkiewicz *et al.* (11), with a dissociation constant for indo-1 (K_d) of 250 nM, as follows: $[Ca^{2+}] = K_d \times (F_{480f}/F_{480b}) \times (R - R_{min})/(R_{max} - R)$, where F_{480f} is the fluorescence of free indo-1, F_{480b} is the fluorescence of indo-1 bound to calcium, R is the ratio between fluorescence values measured at 405 and 480 nm, and R_{min} and R_{max} were determined in the presence of ionomycin (10 μM) and either EGTA (2 mM) or $CaCl_2$ (2 mM), respectively.

Statistical analysis. Statistical analyses were made with at least three independent experiments performed in triplicate, using variance analysis followed by Student's *t* tests; *p* values of <0.05 were considered significant.

Results

Involvement of α_1- and α_2-adrenergic receptors in the noradrenalin-induced production of [^{3}H]inositol phosphates in striatal astrocytes. As described in previous studies performed on cultured astrocytes from the rat brain (3, 4), noradrenalin stimulated the production of [^{3}H]inositol phosphates in striatal astrocytes from mouse embryos (Figs. 1 and 2). This effect seems to be mediated by both α_1- and α_2-adrenergic receptors, because it was completely suppressed by the selective α_1-adrenergic antagonist prazosin (1 μM) and partially inhibited by yohimbine (10 μM), an α_2-adrenergic antagonist (Fig. 1). Yohimbine probably acts at α_2-adrenergic

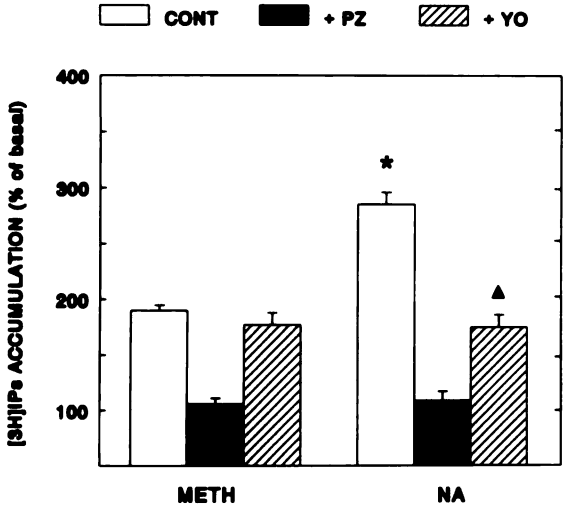

Fig. 1. Involvement of α_1- and α_2-adrenergic receptors in the noradrenalin-stimulated production of [^{3}H]inositol phosphates in striatal astrocytes. Striatal astrocytes in primary culture were incubated for 20 min with drugs added at the following concentrations: noradrenalin (*NA*), 100 μM; methoxamine (*METH*), 100 μM; yohimbine (*YO*), 10 μM; prazosin (*PZ*), 1 μM. The formation of [^{3}H]inositol phosphates (*[3H]IPs*) was measured as indicated in Materials and Methods. Results, expressed as a percentage of basal [^{3}H]inositol phosphate formation (7650 ± 884 dpm/well), are the means ± standard errors of values obtained in a typical experiment performed in triplicate. Five other experiments yielded identical results. Yohimbine and prazosin did not alter [^{3}H]inositol phosphate synthesis when they were added alone. *, Significantly different ($p < 0.01$) from the production of [^{3}H]inositol phosphates measured in the presence of methoxamine (CONT). ▲, Significantly different ($p < 0.01$) from the formation of [^{3}H]inositol phosphates triggered by noradrenalin alone. CONT, control.

receptors, because it partially inhibited the noradrenalin-stimulated formation of inositol phosphates in a saturable manner and with a relatively high potency (IC_{50} = 0.34 ± 0.18 μM; mean ± standard error of three independent experiments, deduced from Eadie-Hofstee plots) (Fig. 3).

When methoxamine, a selective α_1-adrenergic agonist, was used instead of noradrenalin, the maximal production of inositol phosphates (187 ± 8% of basal inositol phosphate production; six experiments) was similar to that induced by noradrenalin in the presence of yohimbine and was insensitive to the α_2-adrenergic antagonist (Figs. 1 and 2). Most importantly, UK14,304, a selective α_2-adrenergic agonist that was ineffective alone, enhanced the methoxamine (100 μM)-evoked production of [^{3}H]inositol phosphates (EC_{50} = 86 ± 21 nM; three experiments) (Fig. 4a). Moreover, the increase in [^{3}H]inositol phosphate production induced by methoxamine (100 μM) in the presence of a maximally effective concentration of UK14,304 (1 μM) was of similar amplitude as that induced by noradrenalin (Fig. 4b). Finally, UK14,304 (1 μM) did not potentiate the noradrenalin response and, as expected, the potentiating effect of UK14,304 on the methoxamine-evoked production of [^{3}H] inositol phosphates was totally antagonized by 10 μM yohimbine (Fig. 4b).

β-Adrenergic receptors did not contribute to the noradrenalin response in striatal astrocytes. Indeed, propranolol (10 μM), a β-adrenergic antagonist, did not alter the effect induced by noradrenalin (100 μM), and the methoxamine (100 μM)-evoked increase in [^{3}H]inositol phosphate formation was not enhanced by isoproterenol (1 μM), a selective β-adrenergic agonist (Table 1).

Interestingly, the dose-response curves for methoxamine suggested that two α_1-adrenoceptor subtypes are responsible for the formation of inositol phosphates in striatal astrocytes (Hill coefficient, 0.47 ± 0.04; three experiments) (Fig. 2). Indeed, the Eadie-Hofstee plot deduced from the methoxamine dose-response curves revealed two components, one of high potency (EC_{50} = 0.11 ± 0.04 μM; three experiments) and low efficacy (32 ± 11% increase in [^{3}H]inositol phosphate production; three experiments) and the other of low potency (EC_{50} = 6.6 ± 2.8 μM; three experiments) and high efficacy (61 ± 23%) (Fig. 2). Similarly, the dose-response curves for noradrenalin in the absence as well as the presence of yohimbine suggested that several α_1-adrenoceptors contribute to the noradrenalin response (Hill coefficients, 0.78 ± 0.05 and 0.40 ± 0.09, respectively) but the differences in potency and efficacy of both

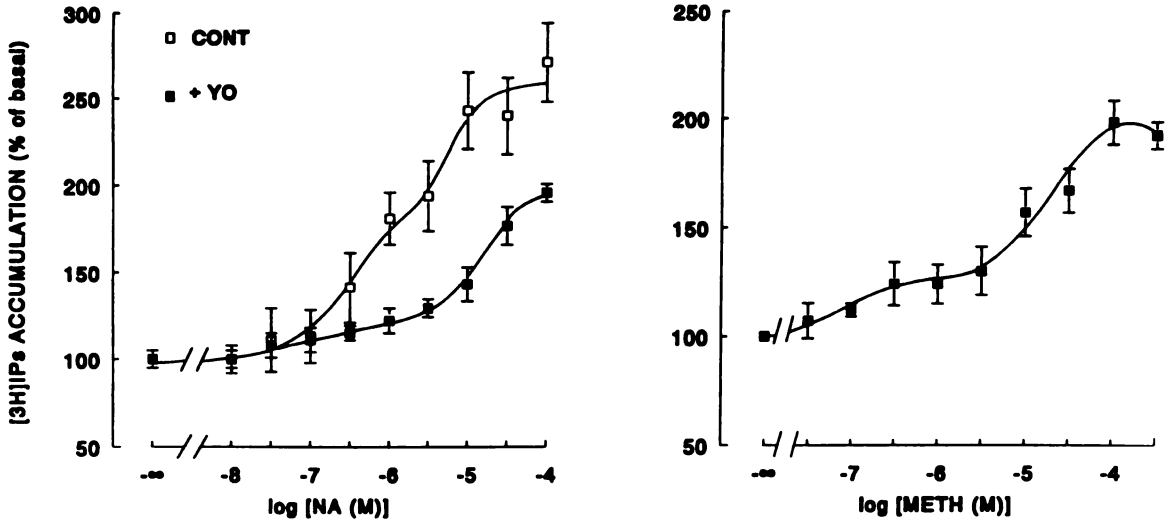

Fig. 2. Stimulation of [^{3}H]inositol phosphate production by increasing concentrations of noradrenalin and methoxamine. Striatal astrocytes were incubated for 20 min with increasing concentrations of noradrenalin (*NA*) [in the absence (*CONT*) or presence of 10 μM yohimbine (*YO*)] (*left*) or methoxamine (*METH*) (*right*). Results, expressed as a percentage of basal [^{3}H]inositol phosphate (*[3H]IPs*) formation (5270 ± 510 dpm/well), are the means ± standard errors of values obtained in a typical experiment performed in triplicate. Two other experiments provided similar results.

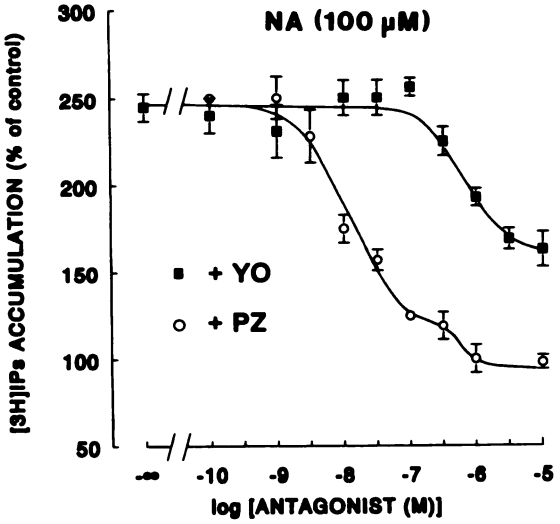

Fig. 3. Inhibition of the noradrenalin-stimulated accumulation of [^{3}H] inositol phosphates by increasing concentrations of prazosin and yohimbine. Astrocytes were incubated for 20 min with 100 μM noradrenalin (*NA*) and the indicated concentrations of prazosin (○) or yohimbine (■). Results, expressed as a percentage of basal [^{3}H]inositol phosphate (*[3H]IPs*) formation (8088 ± 366 dpm/well), are the means ± standard errors of values obtained in a typical experiment performed in triplicate. Two other experiments performed independently yielded similar results.

putative α_1-adrenergic components were less pronounced than in the presence of methoxamine. Finally, the dose-response curves for prazosin also suggested the involvement of two α_1-adrenoceptors in the noradrenalin response (Hill coefficient, 0.77 ± 0.05; three experiments).

Altogether, these results suggest that the stimulation of α_2-adrenergic receptors potentiates the production of inositol phosphates resulting from the activation of α_1-adrenergic receptors in striatal astrocytes. Comparison of the responses induced by increasing concentrations of noradrenalin in the presence and absence of yohimbine also suggests that the effects mediated by the two α_1-adrenergic receptor subtypes are enhanced by the activation of α_2-adrenoceptors.

Involvement of two G proteins in the noradrenalin-evoked production of [^{3}H]inositol phosphates. The enhancement of inositol phosphate production mediated by α_2-adrenergic receptors seems to involve G proteins sensitive to PTX ($G_{\alpha i1-3}$ or $G_{\alpha o1-2}$), because the noradrenalin-induced response was smaller and became insensitive to yohimbine in astrocytes that had been pretreated for 18 hr with PTX (0.1 or 1 μg/ml), compared with that observed in untreated cells (Fig. 5). In contrast, the methoxamine response was completely insensitive to PTX (7, 8). Assuming that the formation of inositol phosphates linked to the stimulation of α_1-adrenergic receptors involves G proteins, these results suggest that the noradrenalin-evoked response involves at least two G proteins, sensitive (α_2 component) and insensitive (α_1 component) to PTX.

Role of extracellular calcium. As demonstrated for the potentiating effects of 2-chloroadenosine and somatostatin on the α_1-adrenergic response (7, 8), the enhanced production of inositol phosphates resulting from the combined stimulation of α_2-adrenoceptors required the presence of calcium in the extracellular medium. Indeed, in the absence of external calcium the noradrenalin response was markedly decreased, reaching a level identical to that observed in the presence of methoxamine, and

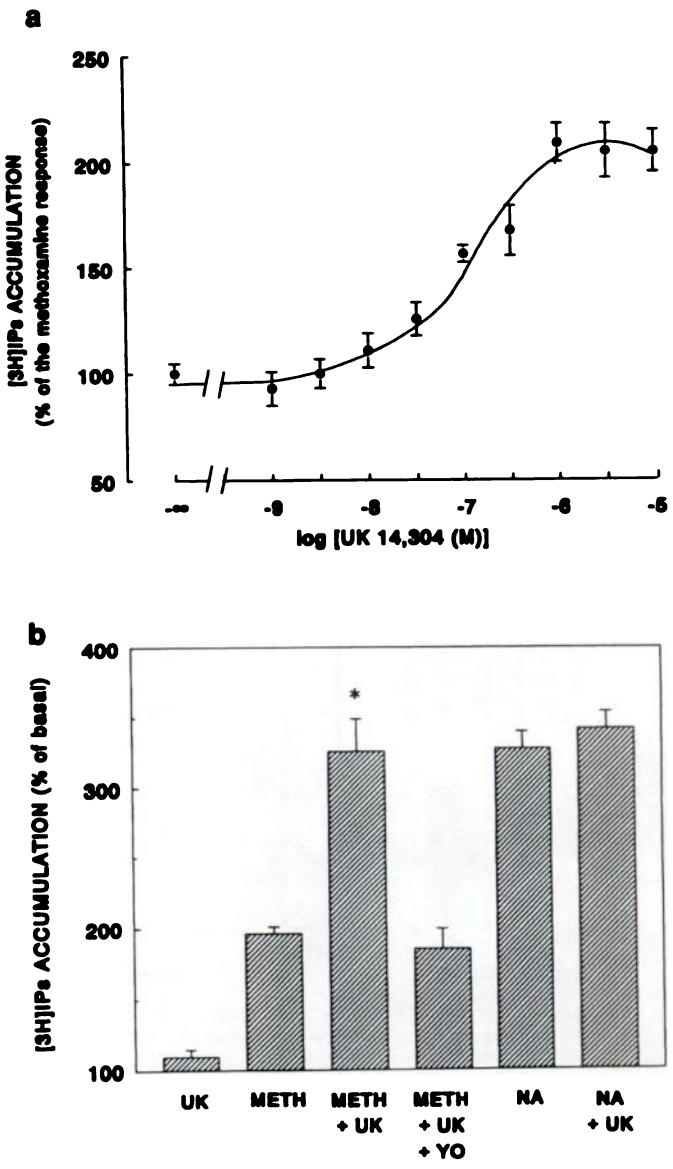

Fig. 4. Effects of the α_2-adrenergic agonist UK14,304 on the methoxamine- and noradrenalin-evoked production of [^{3}H]inositol phosphates in striatal astrocytes. a, Striatal astrocytes were incubated for 20 min in the presence of 100 μM methoxamine and the indicated concentrations of UK14,304. When used alone, UK14,304 (up to 10 μM) did not alter the basal accumulation of [^{3}H]inositol phosphates (*[3H]IPs*) (5570 ± 384 dpm/well). Methoxamine increased the basal production of [^{3}H]inositol phosphates by 94 ± 7%. b, Agonists and antagonists were used at the following concentrations: methoxamine (*METH*), 100 μM; noradrenalin (*NA*), 100 μM; UK14,304 (*UK*), 1 μM; yohimbine (*YO*), 10 μM. Results are the means ± standard errors of values obtained in a typical experiment performed in triplicate. Two other experiments provided similar results. *, Significantly different ($p < 0.01$) from the production of [^{3}H]inositol phosphates measured in the presence of methoxamine alone.

was insensitive to yohimbine (Fig. 6). It must be noted that the α_1-adrenergic response was also decreased in the absence of extracellular calcium.

Additional information on the role of calcium in the noradrenalin-evoked production of inositol phosphates was obtained by measuring the effects of noradrenalin on the cytosolic calcium concentration using indo-1 as a calcium dye. Noradrenalin (100 μM) induced a biphasic increase in cytosolic calcium in all astrocytes tested (70 cells tested), with a calcium

TABLE 1

Role of β-adrenergic receptors in the production of [^{3}H]inositol phosphates in astrocytes

Striatal astrocytes were incubated with agonists and antagonists added at the following concentrations: noradrenaline, 100 μM; methoxamine, 100 μM; isoproterenol, 1 μM; propranolol, 10 μM. Results, expressed as a pecentage of basal [^{3}H]inositol phosphate production (5790 ± 650 dpm/well), are the means ± standard errors of triplicate determinations performed in a typical experiment. Two other independent experiments yielded identical results

Treatment	[^{3}H]inositol phosphate accumulation
	% of basal
Noradrenaline	386 ± 26
Propranolol	104 ± 8
Noradrenaline + propranolol	399 ± 34
Methoxamine	221 ± 4
Isoproterenol	112 ± 9
Methoxamine + isoproterenol	226 ± 24

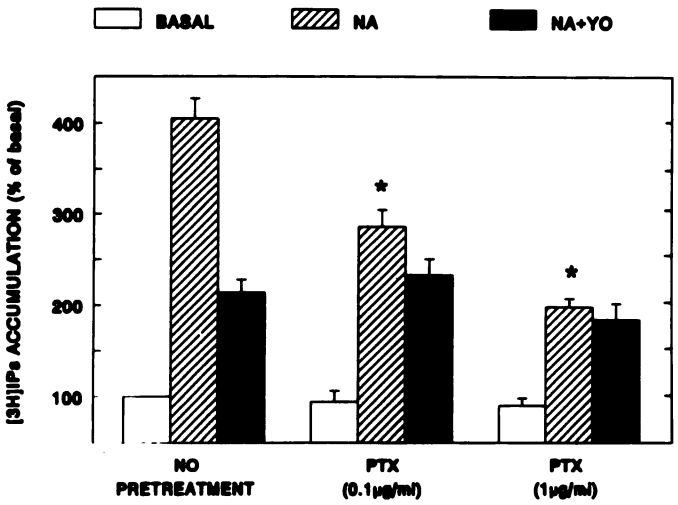

Fig. 5. Involvement of two G proteins in the noradrenalin-evoked production of [^{3}H]inositol phosphates. Where indicated, striatal astrocytes were incubated for 18 hr with PTX (0.1 or 1 μg/ml). Cells were then exposed for 20 min to noradrenalin (*NA*) (100 μM) in the absence or presence of yohimbine (*YO*) (10 μM). Results, expressed as a percentage of basal [^{3}H]inositol phosphate (*[3H]IPs*) formation (8280 ± 355 dpm/well), are the means ± standard errors of triplicate determinations performed in a typical experiment. Two other experiments provided similar results. *, Significantly different ($p < 0.05$) from the accumulation of [^{3}H]inositol phosphates evoked by noradrenalin in untreated cells.

peak reaching a maximal value of 1500 ± 42 nM in <3 sec, followed by a long-lasting elevation of calcium decreasing progressively to a level of 290 ± 19 nM, estimated 5 min after the onset of the noradrenalin application (Fig. 7). However, as already described with other agonists (10, 12), in about 25% of the cells tested oscillations were observed during the plateau phase, with the mean value being in the same range as that of nonoscillating cells and the frequency varying from 2 to 7 oscillations/min, up to 30 min (data not shown). Prazosin (1 μM) totally suppressed the increase in cytosolic calcium induced by noradrenalin (data not shown). In the presence of yohimbine, the peak phase of the noradrenalin response was markedly diminished (790 ± 38 nM; 49 cells tested) and its long-lasting effect was totally suppressed (Fig. 7, *upper*). Interestingly, in the absence of external calcium only the peak phase of the noradrenalin response persisted, and its amplitude was similar to that of the response induced in the presence of yohimbine and external calcium (data not shown). When α_1-adrenergic

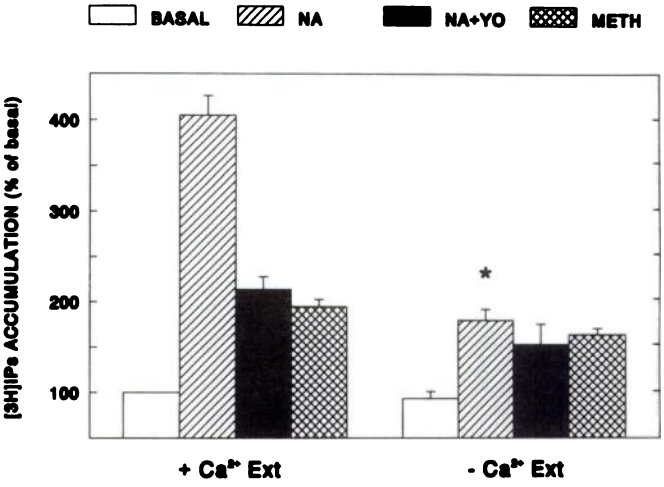

Fig. 6. Role of external calcium in the α_1- and α_2-adrenergic receptor-evoked production of [^{3}H]inositol phosphates. Striatal astrocytes were exposed to drugs, added to incubation medium containing either 1.2 mM Ca^{2+} (+ *Ca^{2+} Ext*) or 5 mM EGTA (– *Ca^{2+} Ext*), at the following concentrations: noradrenalin (*NA*), 100 μM; methoxamine (*METH*), 100 μM; yohimbine (*YO*), 10 μM. Results, expressed as a percentage of basal [^{3}H]inositol phosphate (*[3H]IPs*) formation (7480 ± 725 dpm/well), are the means ± standard errors of triplicate determinations performed in a typical experiment. Two other experiments provided similar results. *, Significantly different ($p < 0.01$) from the noradrenalin-evoked accumulation of [^{3}H]inositol phosphates in the presence of external Ca^{2+}.

receptors were selectively stimulated by methoxamine, a response similar to that evoked by the combined application of noradrenalin and yohimbine was observed in about 75% of the cells tested, i.e., a peak phase (840 ± 160 nM; 70 cells tested) followed by a rapid decrease (regular or sometimes oscillating) in cytosolic calcium, with the basal level (92 ± 14 nM) being recovered in <3 min (Fig. 7, *lower*). Altogether, these results demonstrate that the co-stimulation of α_1- and α_2-adrenergic receptors induces a prolonged influx of calcium in striatal astrocytes, a phenomenon identical to that observed in the presence of methoxamine and either somatostatin or 2-chloroadenosine (8, 10).

Role of arachidonic acid and glutamate. We previously demonstrated that the combined activation of α_1-adrenoceptors and A_1 purinergic or somatostatin receptors stimulates the release of arachidonic acid from striatal astrocytes (7, 8). In addition, the stimulation of α_2-adrenergic receptors in transfected Chinese hamster ovary cells stimulated arachidonic acid release (13) or potentiated the ATP-stimulated release of this fatty acid (14). In both cases, the α_2-adrenoceptor-mediated response required the presence of external calcium and involved a PTX-sensitive G protein. Moreover, in sympathetic postganglionic neurons, by acting at α_2-adrenergic receptors, noradrenalin induced production of prostaglandins resulting from the stimulation of phospholipase A_2 (15). Similarly, in striatal astrocytes that had been preincubated for 18 hr with [^{3}H] arachidonic acid, noradrenalin (100 μM) stimulated the release of [^{3}H]arachidonic acid (Fig. 8). This effect seems, again, to be mediated by both α_1- and α_2-adrenergic receptors, because it was inhibited by either prazosin (1 μM) or yohimbine (10 μM) (Fig. 8). The α_2-adrenergic receptor-mediated enhancement of [^{3}H]inositol phosphate production may be linked to the release of arachidonic acid resulting from the activation of phospholipase A_2 by noradrenalin. Indeed, mepacrine (100 μM), a nonselective inhibitor of phospholipase A_2, slightly decreased both

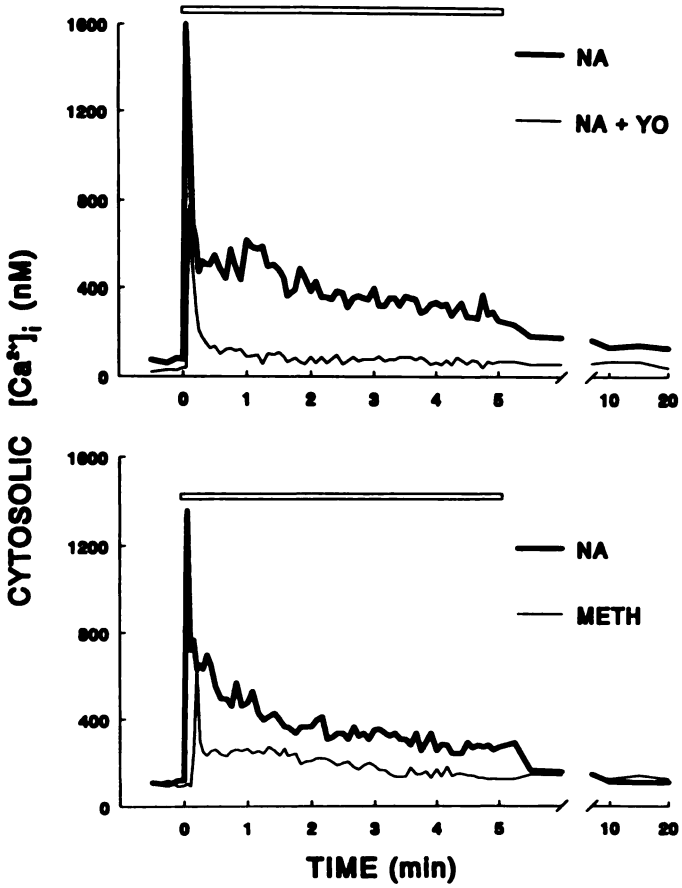

Fig. 7. Synergistic effects of α_1- and α_2-adrenergic receptor stimulation on the cytosolic calcium concentration. Variations of the cytosolic calcium concentration (expressed in nM) in response to noradrenalin (*NA*) (100 μM) alone or in the presence of yohimbine (*YO*) (10 μM) (*upper*) or methoxamine (*METH*) (100 μM) (*lower*) were measured using the fluorescent probe indo-1. Data correspond to recordings of single cells exibiting responses representative of those displayed by at least 35 other cells chosen at random in five different coverslips and subjected to identical treatments.

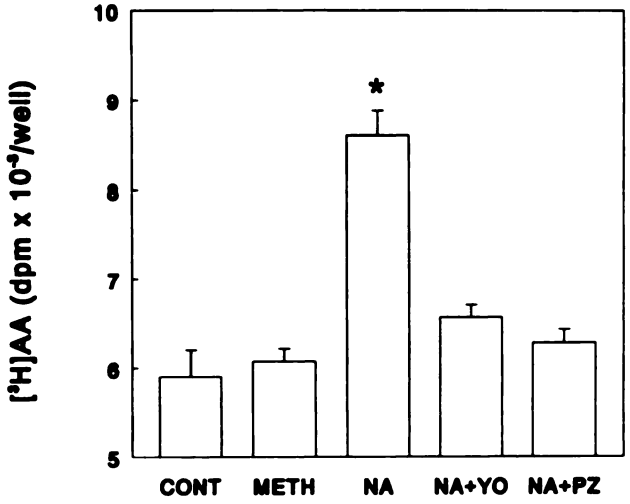

Fig. 8. Synergistic effects of α_1- and α_2-adrenergic receptor stimulation on the release of [^{3}H]arachidonic acid from striatal astrocytes. Striatal astrocytes were preincubated for 18 hr with [^{3}H]arachidonic acid (*[^{3}H] AA*) as described in Materials and Methods. Cells were then incubated for 15 min with drugs used at the following concentrations: methoxamine (*METH*), 100 μM; noradrenalin (*NA*), 100 μM; prazosin (*PZ*), 1 μM; yohimbine (*YO*), 10 μM. Prazosin and yohimbine, when added alone, did not significantly alter [^{3}H]arachidonic acid release from astrocytes (data not shown). Results are the means ± standard errors of triplicate determinations performed in a typical experiment. Two other experiments yielded similar results. *, Significantly different ($p < 0.05$) from basal [^{3}H] arachidonic acid release (CONT). CONT, control.

basal and α_1-adrenergic receptor-mediated [^{3}H]inositol phosphate formation but strongly inhibited the noradrenalin response, which reached a level similar to those of the responses induced by methoxamine or noradrenalin in the presence of yohimbine (Table 2).

Arachidonic acid has been shown to inhibit glutamate uptake into glial cells (16, 17). Moreover, this amino acid is spontaneously released from astrocytes in culture (18). Therefore, arachidonic acid released under the combined stimulation of α_1- and α_2-adrenoceptors could be responsible for the accumulation of external glutamate. Moreover, glutamate (300 μM) was shown to stimulate [^{3}H]inositol phosphate formation in striatal astrocytes. The involvement of ionotropic receptors in the glutamate-evoked production of [^{3}H]inositol phosphates can be ruled out, because neither agonists nor antagonists of NMDA and AMPA/kainate receptors reproduced or inhibited, respectively, the glutamate response (Tables 3 and 4). A metabotropic receptor seems to be responsible for the glutamate-evoked increase in [^{3}H]inositol phosphate production in striatal astrocytes, because this effect was totally suppressed by AP_3 (1 mM) (Table 4), a known antagonist of these glutamatergic receptor subtypes (19). However, metabotropic glutamate receptors responsible for the formation of [^{3}H]inositol phosphates

TABLE 2

Possible involvement of phospholipase A_2 in the α_2-adrenergic-stimulated production of [^{3}H]inositol phosphates in striatal astrocytes

The effects of mepacrine (100 μM) on the accumulation of [^{3}H]inositol phosphates evoked by methoxamine (100 μM) and noradrenaline (100 μM), in the presence or absence of yohimbine (10 μM), are represented. When tested, mepacrine was added to the cells 10 min before and during the incubation period. Results, expressed as a percentage of basal [^{3}H]inositol phosphate production (7770 ± 650 dpm/well), are the means ± standard errors of triplicate determinations performed in a typical experiment. Two other independent experiments yielded identical results

Treatment	[^{3}H]inositol phosphate accumulation	
	Control	Mepacrine
	% of basal	
None	100 ± 7	87 ± 9
Methoxamine	221 ± 19	184 ± 9
Noradrenaline	405 ± 21	222 ± 12[a]
Noradrenaline + yohimbine	214 ± 15	214 ± 6

[a] Significantly different ($p < 0.05$) from the noradrenaline response measured in the absence of mepacrine.

in astrocytes seem to be distinct from those present in neurons, because *t*-ACPD (up to 1 mM), a selective agonist of the neuronal metabotropic receptors (20), was almost ineffective in astrocytes (Table 3). The stimulation of metabotropic receptors present on astrocytes, resulting from the accumulation of external glutamate, could therefore account for the α_2-adrenergic receptor-mediated enhancement of phospholipase C activity.

The involvement of glutamate in the response evoked by the stimulation of α_2-adrenoceptors is supported by the following observations. 1) The glutamate (300 μM)-evoked response was additive with that induced by methoxamine (100 μM) alone or by noradrenalin (100 μM) in the presence of yohimbine (10 μM) (Fig. 9). In addition, in the presence of both glutamate and methoxamine (used at maximally effective concentrations) the production of [^{3}H]inositol phosphates was similar to that

TABLE 3

Effects of glutamate receptor agonists on the production of [^{3}H] inositol phosphates in striatal astrocytes

Cells were incubated for 20 min in the presence of several agonists of glutamate ionotropic and metabotropic receptors, used at the following concentrations: glutamate, 300 μM; quisqualate, 100 μM; *t*-ACPD, 1 mM; kainate, 100 μM; AMPA, 100 μM; NMDA, 100 μM. Results, expressed as a percentage of basal [^{3}H]inositol phosphate production (6240 ± 520 dpm/well), are the means ± standard errors of triplicate determinations performed in a typical experiment. Two other independent experiments yielded identical results

Agonist	[^{3}H]inositol phosphate accumulation
	% of basal
Glutamate	202 ± 6[a]
Quisqualate	205 ± 4[a]
t-ACPD	117 ± 6
Kainate	98 ± 4
AMPA	94 ± 8
NMDA	100 ± 4

[a] Significantly different ($p < 0.01$) from basal inositol phosphate production.

TABLE 4

Effects of glutamate receptor antagonists on glutamate-evoked [^{3}H]inositol phosphate production in striatal astrocytes

Astrocytes were incubated for 20 min in the presence of glutamate (300 μM) and the following antagonists: AP_3 (1 mM), AP_4 (1 mM), DNQX (100 μM), AP_5 (100 μM), and MK-801 (1 μM). None of these antagonists significantly altered the basal [^{3}H] inositol phosphate level (5760 ± 890 dpm/well). Results are the means ± standard errors of triplicate determinations performed in a typical experiment. Two other independent experiments yielded identical results

Antagonist	[^{3}H]inositol phosphate accumulation
	% of 300 μM glutamate response
AP_3	6 ± 5[a]
AP_4	105 ± 14
DNQX	98 ± 8
AP_5	112 ± 9
MK-801	99 ± 7

[a] Significantly different ($p < 0.01$) from glutamate-evoked [^{3}H]inositol phosphate production.

evoked by noradrenalin alone (i.e., by the combined stimulation of α_1- and α_2-adrenoceptors) (Fig. 9). 2) A known inhibitor of glutamate uptake, β-methyl-DL-aspartic acid (1 mM), enhanced the responses induced either by methoxamine alone or by the combined application of noradrenalin and yohimbine, whereas the effect evoked by noradrenalin alone remained unchanged (Fig. 9). 3) The component of the noradrenalin-evoked production of inositol phosphates mediated by α_2-adrenoceptors was selectively inhibited when external glutamate was enzymatically removed by glutamate-pyruvate transaminase (10 IU/ml) (Fig. 10), which converts glutamate into α-ketoglutarate and alanine in the presence of high concentrations of pyruvate (21). As expected, the production of [^{3}H]inositol phosphates evoked by either glutamate (300 μM), β-methyl-DL-aspartic acid (1 mM), or exogenous arachidonic acid (20 μM) was also suppressed in the presence of this enzyme (data not shown). 4) Finally, the effect mediated by α_2-adrenergic receptors was selectively inhibited in the presence of AP_3 (1 mM) (Fig. 10), whereas it remained unchanged in the presence of DNQX (100 μM), AP_5 (100 μM), or MK-801 (1 μM) (data not shown).

Discussion

The present study indicates that in mouse striatal astrocytes the formation of inositol phosphates induced by noradrenalin results from the combined stimulation of α_1- and α_2-adrenergic

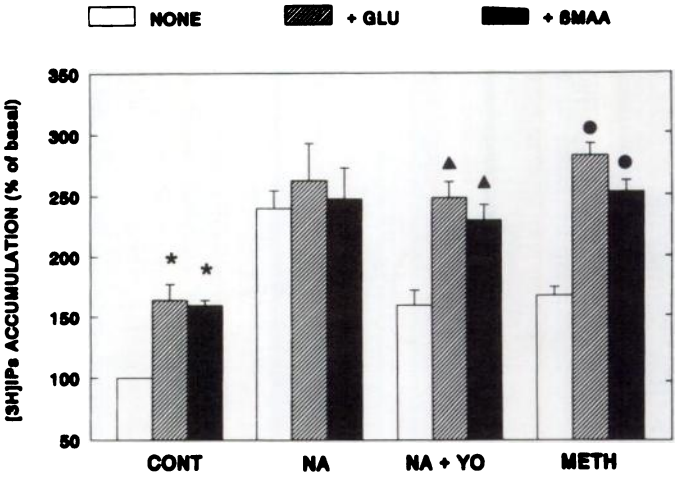

Fig. 9. Additivity of the α_1-adrenergic and glutamatergic stimulation of [^{3}H]inositol phosphate accumulation. Striatal astrocytes were incubated with either methoxamine (*METH*) (100 μM), noradrenalin (*NA*) (100 μM), or noradrenalin plus yohimbine (*YO*) (10 μM), in the absence or presence of either glutamate (*GLU*) (300 μM) or the glutamate uptake inhibitor β-methyl-DL-aspartic acid (*βMAA*) (1 mM). Results, expressed as a percentage of basal [^{3}H]inositol phosphate (*[3H]IPs*) production (8640 ± 710 dpm/well), are the means ± standard errors of triplicate determinations performed in a typical experiment. Two other independent experiments yielded similar results. *, Significantly different ($p < 0.05$) from basal [^{3}H]inositol phosphate accumulation. ▲, Significantly different ($p < 0.05$) from the accumulation of [^{3}H]inositol phosphates measured in the presence of noradrenalin plus yohimbine. ●, Significantly different ($p < 0.05$) from the production of [^{3}H]inositol phosphates triggered by methoxamine alone. CONT: Production of [^{3}H]inositol phosphates in the absence of α_1-adrenergic agonist.

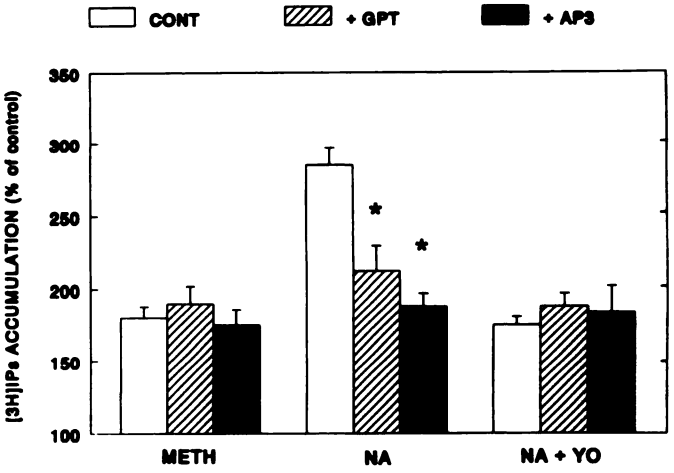

Fig. 10. Role of glutamate in the α_2-adrenergic receptor-mediated production of [^{3}H]inositol phosphates. Striatal astrocytes were incubated with either methoxamine (*METH*) (100 μM), noradrenalin (*NA*) (100 μM), or noradrenalin plus yohimbine (*YO*) (10 μM), in the absence (CONT) or presence of glutamate-pyruvate transaminase (*GPT*) (10 IU/ml) and pyruvate (1 mM) or AP_3 (1 mM). Basal [^{3}H]inositol phosphate accumulation was not altered in the presence of either glutamate-pyruvate transaminase or AP_3, suggesting that, in the absence of agonist, the level of external glutamate was too low to stimulate inositol phosphate formation. Results, expressed as a percentage of basal [^{3}H]inositol phosphate (*[3H]IPs*) production (9550 ± 630 dpm/well), are the means ± standard errors of triplicate determinations performed in a typical experiment. Two other independent experiments yielded similar results. *, Significantly different ($p < 0.05$) from the accumulation of [^{3}H]inositol phosphates triggered by noradrenalin alone. CONT, control.

receptors. This is in agreement with the results of Wilson and Minneman (6), showing that synergistic effects linked to the combined stimulation of α_1- and α_2-adrenergic receptors are involved in the noradrenalin-evoked production of inositol phosphates in cultured astrocytes from rat brain. Four main observations support the involvement of α_2-adrenoceptors in the noradrenalin-evoked response in striatal astrocytes. 1) The efficacy of noradrenalin in stimulating the formation of inositol phosphates was higher than that of the selective α_1-adrenergic agonist methoxamine. 2) Yohimbine, a specific antagonist of α_2-adrenergic receptors, partially inhibited the noradrenalin response without altering that of methoxamine. 3) The noradrenalin response was reproduced by the coapplication of both methoxamine and UK14,304, a specific α_2-adrenergic agonist. 4) Finally, PTX selectively inhibited the yohimbine-sensitive component of the noradrenalin response, whereas the effect evoked by methoxamine remained unchanged. These results are in agreement with the usual involvement of PTX-sensitive G proteins in the coupling of α_2-adrenoceptors to their effectors. Therefore, the strong inhibition by PTX of the noradrenalin-induced accumulation of [^{3}H]inositol phosphates previously observed in astrocytes from rat brain (22) could be due, at least in part, to the implication of α_2-adrenergic receptors in this response.

Although α_2-adrenoceptors contribute to the noradrenalin-evoked accumulation of inositol phosphates, the blockade of α_1-adrenergic receptors by prazosin totally inhibited this response. Moreover, in agreement with a previous study (6), the selective α_2-adrenergic agonist UK14,304 did not increase the production of [^{3}H]inositol phosphates in astrocytes when added alone. These results suggest that the stimulation of α_1-adrenergic receptors exerts a permissive action on the effects mediated by α_2-adrenoceptors. Similarly, the stimulation of α_1-adrenergic receptors has been shown to exert a permissive action on the enhancement by 2-chloroadenosine or somatostatin of the synthesis of inositol phosphates in mouse striatal astrocytes (7, 8).

Two α_1-adrenergic receptor subtypes seem to contribute to the formation of inositol phosphates in striatal astrocytes. Indeed, the methoxamine dose-response curves revealed both high and low affinity components in the increased production of inositol phosphates. Similarly, the dose-response curves for noradrenalin and prazosin suggested the involvement of several α_1-adrenergic receptor subtypes in the production of [^{3}H]inositol phosphates in striatal astrocytes. Two α_1-adrenergic receptor subtypes (α_{1A} and α_{1B}) have already been identified by their pharmacological properties (23, 24). This heterogeneity of α_1-adrenoceptors was further confirmed by molecular cloning experiments, which allowed the identification of at least three different subtypes of α_1-adrenoceptors (so-called $\alpha_{1A/D}$, α_{1B}, and α_{1C}) (25–28). Although the affinities of methoxamine for the cloned α_{1A}- and α_{1B}-adrenoceptors (29) are compatible with the involvement of these two receptor subtypes in the formation of inositol phosphates in striatal astrocytes, additional experiments are required to precisely identify the α_1-adrenergic receptor subtypes involved in this response.

The α_2-adrenoceptor-evoked potentiation of the production of inositol phosphates resulting from the stimulation of α_1-adrenergic receptors seems to involve a cascade of events identical to those involved in the effects induced by adenosine and somatostatin (7, 8). 1) Indeed, the component of the noradrenalin response mediated by α_2-adrenoceptors required the presence of external calcium and may be subsequent to calcium influx. In fact, the pattern of the noradrenalin-evoked increase in cytosolic calcium was similar to that observed with the combined applications of methoxamine and 2-chloroadenosine or somatostatin, i.e., a mobilization of intracellular calcium followed by a calcium influx (8, 10). Interestingly, this calcium influx also resulted from the synergistic effects of the α_1- and α_2-adrenergic receptor stimulations, because it was suppressed by antagonists of each receptor subtype. As shown in a previous study (10), the prolonged calcium influx observed in striatal astrocytes may be linked to the release of arachidonic acid induced by noradrenalin, because it was reproduced by exogenous application of this fatty acid. The link between this sustained calcium entry and the enhancement of the peak phase of calcium increase induced by the combined stimulation of α_1- and α_2-adrenoceptors remains to be elucidated. A quite similar synergistic effect between the two α-adrenergic receptor subtypes has been shown to be involved in the noradrenalin-induced contraction of smooth muscle (30, 31), with the response mediated by α_2-adrenoceptors also resulting from an increased calcium influx (31). However, the role of arachidonic acid in this process had not been investigated. 2) The costimulation of α_1- and α_2-adrenoceptors was required to trigger the release of [^{3}H]arachidonic acid from striatal astrocytes, which may result from the stimulation of phospholipase A_2, an enzyme that is highly sensitive to the calcium concentration (32). Assuming that the stimulation of α_2-adrenoceptors activates phospholipase A_2, as demonstrated in other systems (13–15), this enzyme seems to be tonically inhibited in striatal astrocytes and its disinhibition may be linked to the activation of protein kinase C resulting from the α_1-adrenergic receptor-mediated stimulation of phospholipase C. This hypothesis is identical to that previously suggested for the responses evoked by 2-chloroadenosine or somatostatin (7, 8). Finally, the blockade of the α_2-adrenergic receptor-mediated enhancement of [^{3}H]inositol phosphate formation by mepacrine, a nonselective inhibitor of phospholipase A_2, suggests that this unsaturated fatty acid plays a role in the α_2 response. 3) In fact, arachidonic acid released under the combined stimulation of α_1- and α_2-adrenergic receptors leads to the accumulation of glutamate in the external medium, by blocking its re-uptake (16–17).

Several observations support the involvement of glutamate in the enhancing effect of α_2-adrenergic receptor stimulation on the noradrenalin-evoked production of inositol phosphates. 1) The α_2 component of the noradrenalin response was selectively inhibited by either glutamate-pyruvate transaminase or the presence of AP_3, an inhibitor of metabotropic glutamate receptors that blocked the glutamate-evoked increase in [^{3}H] inositol phosphate formation in striatal astrocytes. 2) The production of inositol phosphates induced by either β-methyl-DL-aspartic acid (an inhibitor of the high affinity and Na^+-dependent glutamate uptake) or glutamate was additive with that evoked by the stimulation of α_1-adrenergic receptors. 3) Finally, the noradrenalin response remained unchanged in the presence of either glutamate or β-methyl-DL-aspartic acid.

Therefore, our results are in agreement with a recent study providing evidence for high sensitivity of the glutamate uptake process in astrocytes to extracellular free arachidonic acid levels (17). In fact, arachidonic acid can be released from neurons in response to glutamate receptor stimulation (33, 34) or from astrocytes in response to ATP (35), endothelin (36), the com-

bined stimulation of α_1-adrenergic and A_1 purinergic or somatostatin receptors (7, 8), or the combined stimulation of α_1- and α_2-adrenergic receptors, as demonstrated in the present study.

As already indicated, by acting at β-adrenergic receptors noradrenalin modulates glycogenolysis (1) and the synthesis of neurotrophic substances (37) in astrocytes and stimulates the release of taurine from these cells (38). The present study suggests that by acting at α-adrenergic receptors noradrenalin stimulates the release of arachidonic acid from striatal astrocytes, which in turn could inhibit glutamate uptake into this cell population. Therefore, through this mechanism, noradrenalin may also contribute to the modulation of glutamatergic neurotransmission by astrocytes.

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