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2-Chloroadenosine Potentiates the α_1-Adrenergic Activation of Phospholipase C through a Mechanism Involving Arachidonic Acid and Glutamate in Striatal Astrocytes

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In cultured striatal astrocytes, 2-chloroadenosine, an adenosine analog resistant to adenosine deaminase, although inactive alone, markedly potentiated the activation of phospholipase C induced by methoxamine, an α_1-adrenergic agonist. This effect was suppressed by antagonists of either A_1 adenosine or α_1-adrenergic receptors. An influx of calcium and two distinct G-proteins are involved in this phenomenon since the potentiating effect of 2-chloradenosine was suppressed in the absence of external calcium or when cells were pretreated with pertussis toxin. In addition, arachidonic acid is likely involved in this potentiating effect. This was shown first by examining the effects of inhibitors of phospholipase A2 or arachidonic metabolism, then by examining the action of arachidonic acid on the production of inositol phosphates in either the presence or absence of methoxamine, and finally by measuring the release of arachidonic acid. The sequential activation of phospholipase C and of protein kinase C is required for the 2-chloroadenosine–induced activation of phospholipase A2 since 2-chloroadenosine markedly stimulated phospholipase C activity in the absence of methoxamine when protein kinase C was activated by a diacylglycerol analog. Finally, the enhancing effect of 2-chloroadenosine on the methoxamine-evoked response seems to result from an inhibition of glutamate reuptake into astrocytes by arachidonic acid. Indeed, the potentiating effect of 2-chloroadenosine was suppressed when external glutamate was removed enzymatically and mimicked by either selective inhibitors of the glutamate reuptake process or direct application of glutamate.

Several studies indicate that adenosine modulates neuronal functions by acting both pre- and postsynaptically. Thus, adenosine has been shown either to inhibit synaptic transmission (Okada and Ozawa, 1980; Siggins and Shubert, 1981), to decrease the release of neurotransmitters (Harms et al., 1976; Vizie and Knoll, 1976; Dolphin and Pestwich, 1985), to hyperpolarize cell membranes by increasing potassium conductance (Trussel and Jackson, 1985), or to inhibit the induction of long-term potentiation (Dolphin, 1983). In addition, adenosine receptors are also present on astrocytes; these are mainly of the A_1 subtype and have been shown to be negatively coupled to adenylate cyclase (Ebersolt et al., 1983). Moreover, we have recently shown in rat striatal slices that 2-chloroadenosine, a stable analog of adenosine, potentiates the formation of inositol phosphates induced by the stimulation of α_1-noradrenergic or muscarinic receptors, also by acting on adenosine receptors of the A_1 subtype (El-Etr et al., 1989). These effects were also seen in cocultures of astrocytes and neurons from the striatum of embryonic mice (El-Etr et al., 1989). Nevertheless, 2-chloroadenosine did not enhance the α_1-adrenergic- or muscarinic-evoked activation of phospholipase C in pure neuronal primary cultures from the striatum, whereas the nucleoside enhanced the formation of inositol phosphates resulting from the activation of α_1-adrenergic receptors on pure populations of striatal astrocytes (El-Etr et al., 1989). These observations led us to conclude that (1) astrocytes are required for the potentiation by the nucleoside of the muscarinic-induced accumulation of inositol phosphates, a response that is of neuronal origin in the striatum, and (2) the initial biochemical events involved in this astrocytoneuronal interaction occur in astrocytes. Therefore, the present study was undertaken to determine the mechanism underlying the potentiation by 2-chloroadenosine of the α_1-adrenergic–induced production of inositol phosphates in cultured striatal astrocytes from the mouse embryo.

Materials and Methods

Cell cultures. Primary cultures of astrocytes were prepared as previously described (El-Etr et al., 1989). Striata were removed from 16-d-old Swiss mouse embryos (Iffa Credo) and were dissociated mechanically in serum-free medium. Cells were plated plated in 24- or 6-well Nunc culture dishes (2.5×10^5 or 8×10^5 cells per well, respectively), previously coated with poly-L-ornithine (1.5 μg/ml; MW, 40,000; Sigma). The culture medium was composed of Minimal Essential Medium and F-12 nutrient (GIBCO, Europe) supplemented with glucose (33 mM), glutamine (2 mM), $NaHCO_3$ (3 mM), HEPES (5 mM), and 10% NU-Serum® (Collaborative Research). Cells were cultured at 37°C for 18–20 d in a humidified atmosphere of 95% air and 5% CO_2. On day 12, the medium was changed and cytosine arabinoside (1 μM; Sigma) was added for 24 hr in order to avoid the formation of cell multilayers and the proliferation of microglial cells. Thereafter, the culture medium was changed twice a week. In these conditions, more than 95% of the cells were stained by the indirect immunofluorescence technique using a rabbit antibody against glial fibrillary acid protein (GFAP) (Dakopatts, Denmark). The remaining 5% of cells could be immature glioblasts, which are known to be unlabeled by GFAP antibodies (Eng, 1985). The cultures were free of microglial cells since no staining was observed using the monoclonal anti-mouse macrophage antibody (anti-MAC 1) (Serotec, France).

Measurement of ^{3}H-inositol phosphate formation. Cells, grown in 24-

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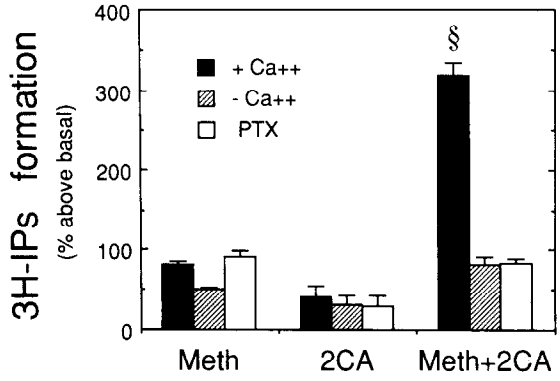

Figure 1. The potentiation by 2-chloroadenosine of methoxamine-evoked accumulation of ^{3}H-inositol phosphates requires external calcium and involves two G-proteins. ^{3}H-inositol phosphate (*IPs*) formation was measured as described in Materials and Methods. Methoxamine (*Meth*; 25 μM) and 2-chloroadenosine (*2CA*; 10 μM) were added alone or together (*Meth+2CA*) into the incubation medium in the presence of either 1.2 mM $CaCl_2$ (+ Ca^{++}) or 5 mM EGTA (− Ca^{++}). The absence of external calcium or the pretreatment of striatal astrocytes with PTX (0.1 μg/ml for 18 hr) completely suppressed the 2-chloroadenosine–evoked potentiation of the methoxamine response. None of these treatments modified the basal production of ^{3}H-inositol phosphates. Results, expressed as a percentage above basal value, are the means ± SEM of values obtained in a typical experiment performed in triplicate. §, significant different ($p < 0.01$) from ^{3}H-inositol phosphate production induced by methoxamine alone. Two other independent experiments yielded similar results.

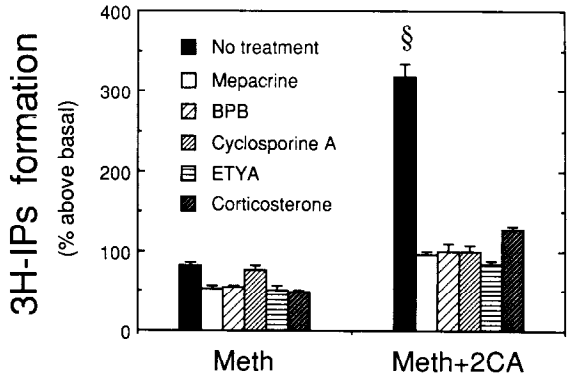

Figure 2. Inhibitors of the enhancing effect of 2-chloroadenosine on methoxamine-evoked formation of ^{3}H-inositol phosphates (*IPs*). Striatal astrocytes were preincubated for either 20 min with mepacrine (100 μM), 4-*p*-bromophenacyl bromide (*BPB*; 10 μM), or cyclosporine A (8 μM), 2.5 hr with ETYA (20 μM) or 24 hr with corticosterone (0.1 μM), and then incubated in the presence of each of these compounds and either methoxamine alone (*Meth*; 25 μM) or both methoxamine (25 μM) and 2-chloroadenosine (10 μM) (*Meth+2CA*). None of these treatments significantly modified the basal production of ^{3}H-inositol phosphates. Results, expressed as in Figure 1, are the means ± SEM of values obtained in a typical experiment performed in triplicate. §, significantly different ($p < 0.01$) from ^{3}H-inositol phosphate production induced by methoxamine alone. Two other independent experiments yielded similar results.

well dishes were incubated for 24 hr in the presence of *myo*-^{3}H-inositol (1 μCi/well, 17 Ci/mmol; Commissariat à l'Energie Atomique, France) added into the culture medium. The accumulation of ^{3}H-inositol phosphates was estimated as previously described (El-Etr et al., 1989). Cells were preincubated for 10 min in Kreb's 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). Effectors were then added for 20 min (except when otherwise stated) in the presence of adenosine deaminase (1 IU/ml; Boehringer-Mannheim) in order to prevent the effect of endogenous adenosine on phospholipase C activity. When stated, glutamate pyruvate transaminase (GPT; 10 IU/ml; Boehringer-Mannheim), which converts glutamate into α-ketoglutarate in the presence of a high concentration of pyruvate (1 mM), was added into the incubation medium in order to eliminate enzymatically external glutamate (O'Brien and Fischbach, 1986). The incubation was stopped by adding successively 0.1% Triton X-100/0.1 M NaOH (200 μl), 0.1% Triton X-100/0.1 M HCl (200 μl), and water (500 μl). Cells were then scraped and ^{3}H-inositol phosphates were extracted as previously described (El-Etr et al., 1989).

Measurement of ^{3}H-arachidonic acid release. Cells in 35 mm dishes were incubated for 18–22 hr with ^{3}H-arachidonic acid (1 μCi/ml, 200 Ci/mmol; CEA, France) added into the culture medium. After labeling, astrocytes were washed four times at 5 min intervals with 2 ml of a calcium-free Kreb's phosphate buffer containing fatty acid–free bovine serum albumin (1 mg/ml; Sigma) and adenosine deaminase (1 IU/ml; Boehringer-Mannheim), the last wash being made with the medium supplemented with 50 μM thimerosal (mercury-[(*O*-carboxyphenyl)thio]-ethyl sodium salt from Sigma), an inhibitor of acyl coenzyme A lysolecithin acyl transferase. Cells were then exposed for 20 min at 37°C to the agonists in 2 ml of the same medium supplemented with 1.2 ml $CaCl_2$. The incubation medium was then collected and centrifuged at 300× *g* for 10 min to remove nonadherent cells, and the supernatant was measured for radioactivity.

HPLC analysis: identification of arachidonic acid. Supernatants were acidified to pH 3.5 with formic acid, and 2 vol of acetone were added to precipitate proteins. After centrifugation, the acetonic phase was extracted twice with 2 vol of chloroform and the organic phase was evaporated under vacuum and injected onto a reverse-phase HPLC column (ultrasphere ODS, 3 μm, Beckman). Elution was performed (1 ml/min flow rate) with a gradient of acetonitrile in water, pH 3.5. Fractions were collected and counted for radioactivity.

Results

Involvement of calcium influx and of two G-proteins in the potentiation by 2-chloroadenosine of the methoxamine-evoked accumulation of inositol phosphates in striatal astrocytes

As previously reported, when striatal astrocytes are incubated in the presence of adenosine deaminase, the activation of α_1-adrenoreceptors by noradrenaline increases the accumulation of ^{3}H-inositol phosphates in the cells (mainly ^{3}H-inositol monophosphate, more than 90% of total ^{3}H-inositol phosphates formed), and this response is amplified by 2-chloroadenosine, an analog of adenosine resistant to adenosine deaminase (El-Etr et al., 1989). Similarly, in the present study, methoxamine (25 μM), a selective agonist of α_1-adrenoreceptors, increased the production of ^{3}H-inositol phosphates in striatal astrocytes, and this effect was strongly enhanced by the combined addition of 2-chloroadenosine (10 μM) (Fig. 1). Responses evoked by methoxamine either in the presence or absence of 2-chloroadenosine were completely suppressed by prazosin (1 μM), a specific antagonist of α_1-adrenoreceptors (methoxamine + prazosin, 104 ± 8% of control).

When used alone, 2-chloroadenosine (10 μM) induced a weak production of ^{3}H-inositol phosphates, which never exceeded 40% of the basal value (Fig. 1). This response and the marked potentiation by 2-chloroadenosine of the methoxamine-evoked production of ^{3}H-inositol phosphates were completely inhibited by the adenosine receptor antagonist 1,3-dipropyl-8-(2-amino-4-chlorophenyl)-xanthine (PACPX; 1 μM) (data not shown).

The potentiating action of 2-chloroadenosine seems to require an influx of calcium since it completely disappeared in the absence of external calcium whereas, in this condition, the methoxamine-induced production of ^{3}H-inositol phosphates was only partially decreased (Fig. 1). Purinergic and α_1-adrenergic receptors of striatal astrocytes are likely to be linked to different G-proteins, since a pretreatment (18 hr) of these cells with pertussis toxin (PTX; 0.1 μg/ml) selectively suppressed the en-

Table 1. Release of ^{3}H-arachidonic acid from striatal astrocytes upon the combined application of 2-chloroadenosine and methoxamine

	^{3}H-arachidonic acid release (% above basal)
Methoxamine	5 ± 4
2-Chloroadenosine	7 ± 5
Methoxamine + 2-chloroadenosine	44 ± 7*

Striatal astrocytes were preincubated for 18 hr with ^{3}H-arachidonic acid as described in Materials and Methods. The combined addition of 2-chloroadenosine (10 μM) and methoxamine (25 μM) for 20 min significantly (*, $p < 0.05$) increased the release of ^{3}H-arachidonic acid as compared to the basal level measured in the absence of effectors. Alone, 2-chloroadenosine and methoxamine were without effect. The basal release of ^{3}H-arachidonic acid represented 2% of the incorporated radioactivity (approximately 85–90% of the radioactivity added to the cells). HPLC analysis indicated that more than 99% of the released radioactivity had a retention time strictly identical to that of authentic arachidonic acid. Results, expressed as percentage above basal amount of ^{3}H-arachidonic acid released in the absence of any effector (39.250 ± 450 dpm/well) are the means ± SEM of three determinations obtained in a typical experiment. Three other independent experiments yielded similar results.

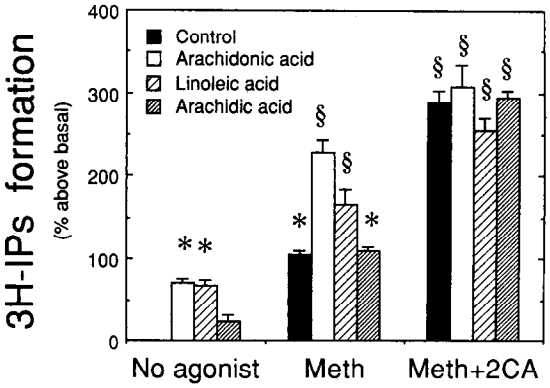

Figure 3. Effects of fatty acids on the formation of ^{3}H-inositol phosphates (*IPs*). Arachidonic, linoleic, or arachidic acid (10 μM) was added into the incubation medium in the absence or the presence of either methoxamine alone (*Meth*; 25 μM) or both methoxamine (25 μM) and 2-chloroadenosine (10 μM) (*Meth+2CA*). Results, expressed as in Figure 1, are the means ± SEM of values obtained in a typical experiment performed in triplicate. *, significantly different ($p < 0.01$, Student's *t* test) from basal ^{3}H-inositol phosphate production; §, significantly different ($p < 0.01$) from ^{3}H-inositol phosphate production induced by methoxamine alone. Two other independent experiments yielded similar results.

hancing effect of 2-chloroadenosine on the methoxamine-evoked production of ^{3}H-inositol phosphates but did not affect the response of the α_1-agonist alone (Fig. 1).

Involvement of arachidonic acid in the potentiating effect of 2-chloroadenosine

Several observations strongly suggest that the potentiating effect of 2-chloroadenosine on the methoxamine-evoked response involves arachidonic acid formed through the activation of phospholipase A2.

(1) Nonspecific blockers of phospholipase A2 such as mepacrine (100 μM) and 4-*p*-bromophenacyl bromide (10 μM), which slightly inhibited the accumulation of ^{3}H-inositol phosphates induced by methoxamine alone, completely suppressed the potentiating effect of 2-chloroadenosine. Moreover, cyclosporine A (8 μM), which has also been shown to inhibit phospholipase A2 (Fan and Lewis, 1985; Yukie et al., 1986), suppressed the enhancing effect of 2-chloroadenosine on the methoxamine-evoked response without altering the methoxamine-evoked response (Fig. 2).

(2) Similar results were obtained when astrocytes were pretreated for 48 hr with corticosterone (0.1 μM), the natural glucocorticoid in the mouse (Fig. 2).

(3) The involvement of arachidonic acid in the potentiating effect of 2-chloroadenosine was further confirmed by the stimulatory actions of the unsaturated fatty acids and arachidonic and linoleic acids (10 μM) (but not of the saturated fatty acid arachidic acid) on phospholipase C activity, which were additive with that evoked by methoxamine (Fig. 3).

(4) Moreover, as expected, the accumulation of ^{3}H-inositol phosphates evoked by the combined application of methoxamine and 2-chloroadenosine was increased neither by arachidonic acid nor by linoleic acid (Fig. 3). Arachidonic acid seems to act directly since nordihydroguaiaretic acid (10 μM) or indomethacin (10 μM), which inhibit lipoxygenases and cyclooxygenase activities, respectively, did not impair the enhancing effect of 2-chloroadenosine on the methoxamine-evoked response (data not shown). Nevertheless, the nonmetabolizable analog of arachidonic acid 5,8,11,14 eicosa-tetrayonic acid (ETYA; 20 μM), which partially inhibited the methoxamine-induced activation of phospholipase C, completely suppressed the potentiation evoked by 2-chloroadenosine. This latter observation could suggest either an involvement of arachidonic acid metabolites of the epoxygenase pathway or a direct inhibitory effect of ETYA on some targets of arachidonic acid since, for instance, ETYA has already been shown to block the arachidonic acid–induced activation of guanylate cyclase (Gerzer et al., 1986).

(5) When striatal astrocytes were preincubated for 18 hr with ^{3}H-arachidonic acid, neither methoxamine (25 μM) nor 2-chloroadenosine (10 μM) alone affected the release of the labeled unsaturated fatty acid. However, the release of ^{3}H-arachidonic acid was significantly increased (Table 1) under the combined application of the two agonists. HPLC analysis (see Materials and Methods) indicated that 99% of the released radioactive material was recovered in a peak having a retention time identical to that of authentic arachidonic acid.

(6) Finally, the addition, during the incubation period, of BSA (0.5 mg/ml), which is known to adsorb arachidonic acid (Réf), decreased the potentiating effect of 2-chloroadenosine on the methoxamine-induced formation of ^{3}H-inositol phosphates (Table 2).

Involvement of glutamate in the potentiating effect of 2-chloroadenosine

It has been reported that arachidonic acid inhibits the uptake of glutamate into Müller cells (astrocytes) of the salamander retina (Barbour et al., 1989). Therefore, through arachidonic acid, 2-chloroadenosine in the presence of methoxamine could indirectly induce an accumulation of glutamate into the extracellular medium. In turn, glutamate could stimulate phospholipase C activity, and this could account for the potentiating effect of 2-chloroadenosine. In fact, in our experimental conditions, glutamate (100 μM) stimulated the production of ^{3}H-inositol phosphates (Fig. 4), confirming that astrocytes possess glutamate receptors coupled to phospholipase C (Pearce et al., 1986).

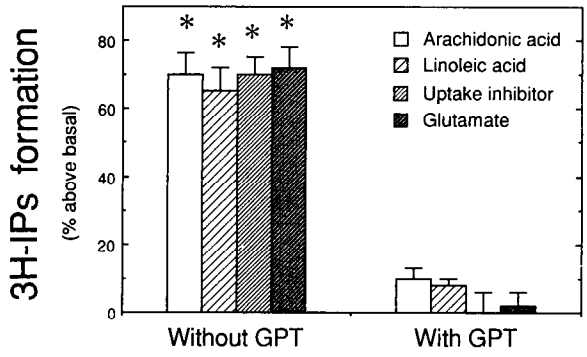

Figure 4. Removal of external glutamate by GPT suppresses the effects of unsaturated fatty acids and glutamate or of a glutamate uptake inhibitor on the formation of ^{3}H-inositol phosphates (*IPs*). Arachidonic and linoleic acids (both 10 μM), the glutamate uptake inhibitor β-methyl-DL-aspartic acid (*Uptake inhibitor*; 1 mM), and glutamate (100 μM) were added to the incubation medium in the presence of pyruvate (1 mM) either in the absence or presence of GPT. The basal formation of ^{3}H-inositol phosphates is not altered by the presence of GPT and pyruvate, suggesting that in the absence of agonist, the external glutamate concentration is too low to stimulate phospholipase C. Results, expressed as in Figure 1, are the means ± SEM of values obtained in a typical experiment performed in triplicate. *, significantly different ($p < 0.01$, Student's t test) from the basal production of ^{3}H-inositol phosphates. Two other independent experiments yielded similar results.

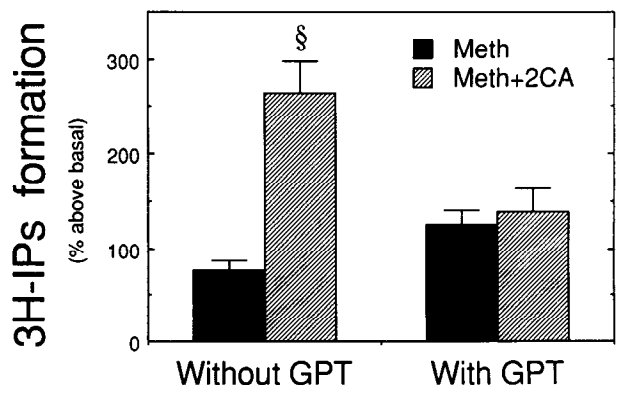

Figure 5. Enzymatic removal of external glutamate suppresses the enhancing effect of 2-chloroadenosine on the methoxamine-evoked formation of ^{3}H-inositol phosphates (*IPs*). GPT was added or not, as in Figure 4, to the incubation medium in the absence or presence of either methoxamine alone (*Meth*; 25 μM) or both methoxamine (25 μM) and 2-chloroadenosine (10 μM) (*Meth+2CA*). Results, expressed as in Figure 1, are the means ± SEM of values obtained in a typical experiment performed in triplicate. §, significantly different ($p < 0.01$) from ^{3}H-inositol phosphate production induced by methoxamine alone. Three other independent experiments yielded similar results.

The involvement of glutamate in the stimulating effect of arachidonic acid on phospholipase C activity was supported by several observations.

(1) The 2-chloroadenosine–induced production of ^{3}H-inositol phosphates observed in the presence of methoxamine was drastically reduced when external glutamate was enzymatically removed by incubating the astrocytes with GPT (Fig. 5).

(2) Conversely, the addition of a high-affinity and Na^+-dependent glutamate uptake inhibitor, β-methyl-DL-aspartic acid (1 mM), resulted in an increased production of ^{3}H-inositol phosphates, which was of amplitude similar to that obtained with glutamate (Fig. 4) and which disappeared in the presence of GPT (Fig. 4). Similar responses were obtained with other glutamate uptake inhibitors such as DL-threo-β-hydroxyaspartic acid (1 mM) or DL-aspartic acid β-hydroxamate (1 mM) (Kimelberg et al., 1989) (data not shown).

Table 2. Reduction by BSA of the 2-chloroadenosine-induced potentiation of the formation of ^{3}H-inositol phosphates evoked by methoxamine

	^{3}H inositol phosphate formation (% above basal)		
	Meth	2CA	Meth + 2CA
Without BSA	101 ± 9	39 ± 5	285 ± 27*
With BSA	95 ± 10	15 ± 7	182 ± 14*†

Fatty acid–free BSA (0.5 mg/ml) was added to the incubation medium in the absence or the presence of 2-chloroadenosine (2CA; 10 μM), methoxamine (Meth; 25 μM), or both agonists (Meth+2CA). The addition of fatty acid–free BSA did not modify the basal production of ^{3}H-inositol phosphates. Results, expressed as percentage above the basal formation of ^{3}H-inositol phosphates (6550 ± 260 dpm/well) measured in the absence of any agonist, are the means ± SEM of values obtained in a typical experiment performed in triplicate. *, significantly different ($p < 0.01$, Student's t test) from ^{3}H-inositol phosphate production induced by methoxamine alone; †, significantly different ($p < 0.01$, Student's t test) from ^{3}H-inositol phosphate production measured in the presence of both methoxamine and 2-chloroadenosine, but in the absence of fatty acid–free BSA. Two other independent experiments yielded similar results.

(3) As expected, the inhibitor of glutamate uptake β-methyl-DL-aspartic acid (1 mM) did not further increase the production of ^{3}H-inositol phosphates when it was simultaneously added with both methoxamine and 2-chloroadenosine (Fig. 6). In addition, it partially reproduced the enhancing effect of 2-chloroadenosine on the methoxamine-evoked response (Fig. 6).

(4) The stimulatory action of the two unsaturated fatty acids arachidonic and linoleic acid (but not arachidic acid) on phospholipase C activity was no longer observed in the presence of GPT (Fig. 4).

(5) The enhancing effect of 2-chloroadenosine on the methoxamine-evoked production of ^{3}H-inositol phosphates was only observed when the incubation period was of long duration, that is, 20 min (Fig. 7). This delayed response likely corresponds to the time required to reach an extracellular concentration of glutamate sufficient for stimulating the activity of phospholipase C through glutamate receptors present on astrocytes.

Requirement of protein kinase C activation for the stimulatory effect of 2-chloroadenosine on phospholipase A2 activity

The stimulation of α_1-adrenoreceptors seems to be necessary for the activating effect of 2-chloroadenosine on phospholipase A2 activity since in the absence of methoxamine, 2-chloroadenosine was nearly ineffective (Table 1). This permissive effect of methoxamine seems to be mediated by the activation of protein kinase C resulting from its stimulatory effect on phospholipase C. Supporting this statement, 2-chloroadenosine alone markedly increased the production of ^{3}H-inositol phosphates when cells were simultaneously incubated with an activator of protein kinase C, the diacylglycerol analog 1-oleyl-2-acetyl-*rac*-glycerol (OAG; 25 μg/ml) (Table 3).

Discussion

We have previously shown that 2-chloroadenosine increases the noradrenaline-evoked production of inositol phosphates in cultured striatal astrocytes from mouse embryos (El-Etr et al., 1989). The present study was undertaken to investigate further the mechanism involved in this phenomenon.

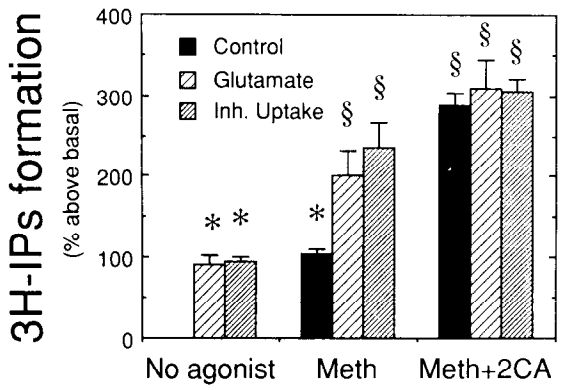

Figure 6. Effects of glutamate and of an inhibitor of glutamate uptake on the formation of ^{3}H-inositol phosphates (*IPs*). Glutamate (100 μM) or β-methyl-DL-aspartic acid (*Inh Uptake*; 1 mM), an inhibitor of the high-affinity and Na^+-dependent glutamate uptake process, was added into the incubation medium in the absence or presence of either methoxamine alone (*Meth*; 25 μM) or both methoxamine (25 μM) and 2-chloroadenosine (10 μM) (*Meth+2CA*). Results, expressed as in Figure 1, are the means ± SEM of values obtained in a typical experiment performed in triplicate. *, significantly different ($p < 0.01$, Student's t test) from the basal production of ^{3}H-inositol phosphates; §, significantly different ($p < 0.01$) from ^{3}H-inositol phosphate production induced by methoxamine alone. Two other independent experiments yielded similar results.

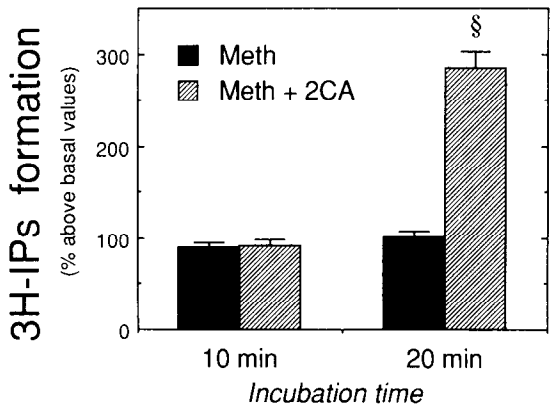

Figure 7. Delayed effect of 2-chloroadenosine on the methoxamine-induced formation of ^{3}H-inositol phosphates (*IPs*). The accumulation of ^{3}H-inositol phosphates was measured 10 or 20 min after the addition of either methoxamine alone (*Meth*; 25 μM) or both methoxamine (25 μM) and 2-chloroadenosine (10 μM) (*Meth+2CA*). Results, expressed as in Figure 1, are the means ± SEM of values obtained in a typical experiment performed in triplicate. §, significantly different ($p < 0.01$) from ^{3}H-inositol phosphate production induced by methoxamine alone. Two other independent experiments yielded similar results.

Involvement of two G-proteins. As already suggested, the noradrenaline- and 2-chloroadenosine–evoked responses seem to be mediated by α_1-adrenergic receptors and purinergic receptors of the A_1 subtype, respectively. Indeed, methoxamine, the α_1-adrenergic agonist, reproduced the effect of noradrenaline on the formation of ^{3}H-inositol phosphates, and both responses were blocked by prasozin, a selective α_1-noradrenergic antagonist. An activation of A_1 adenosine receptors is probably involved in the potentiating effect of 2-chloroadenosine as indicated by the relative potencies of the adenosine agonists 5'-*N*-ethylcarboxamide adenosine and N_6-cyclohexyladenosine and by the potent antagonistic activity of PACPX (El-Etr et al., 1989). The involvement of A_1 adenosine receptors was further confirmed by the present results. In agreement with numerous studies indicating that A_1 receptors are coupled to G-proteins sensitive to PTX (Dolphin and Pestwich, 1985; Stiles, 1986; Cooper et al., 1989), the pretreatment of striatal astrocytes with the toxin was shown indeed to abolish selectively the potentiating effect of 2-chloroadenosine while it did not alter the α_1-adrenergic–evoked production of ^{3}H-inositol phosphates. This suggests that both types of receptors are coupled to different transduction systems (G-proteins) and effectors, the α_1-adrenoceptors probably being linked to phospholipase C via a G-protein insensitive to PTX while the adenosine receptors are coupled to another effector regulated by a G-protein sensitive to this toxin (Fig. 8).

Involvement of arachidonic acid. Several observations strongly suggest that arachidonic acid is involved in the potentiating effect of 2-chloroadenosine and are thus in favor of a coupling of A_1 adenosine receptors to phospholipase A2. (1) Three different compounds known to inhibit the activity of phospholipase A2, mepacrine, 4-*p*-bromophenacyl bromide, and cyclosporine A, suppressed the potentiating effect of 2-chloroadenosine on the methoxamine-induced formation of ^{3}H-inositol phosphates. (2) In agreement with the well-known indirect inhibitory action of corticosterone on phospholipase A2 activity observed in numerous cell types and the presence of glucocorticoid receptors on astrocytes (De Georges et al., 1987), 2-chloroadenosine was without effect when astrocytes had been pretreated for two days with corticosterone (100 nM), the natural glucocorticoid in the mouse. (3) Arachidonic acid alone (or linoleic acid) but not the C_{20}-saturated fatty acid arachidic acid increased the formation of ^{3}H-inositol phosphates. (4) In the presence of both methoxamine and 2-chloroadenosine, arachidonic and linoleic acids did not further increase the formation of ^{3}H-inositol phosphates. (5) The nonmetabolizable analog of arachidonic acid, ETYA (Hawkins et al., 1985; Clark and Linden, 1986; Gerzer et al., 1986), suppressed the potentiating effect of 2-chloroadenosine. (6) The adsorption of arachidonic acid by the addition of bovine serum albumin to the incubation medium led to a significant decrease of the 2-chloroadenosine–induced potentiating effect. (7) Finally, 2-chloroadenosine induced a release of ^{3}H-arachidonic acid only in the presence of methoxamine.

As expected for phospholipase A2, an enzyme highly sensitive

Table 3. Permissive effect of the activation of protein kinase C upon 2-chloroadenosine–induced formation of ^{3}H-inositol phosphates

	^{3}H-inositol phosphates (% above basal)
OAG	−9 ± 5
2-Chloroadenosine	32 ± 4
OAG + 2-chloroadenosine	112 ± 10*†

An activator of protein kinase C, the diacylglycerol analog OAG (25 μg/ml), was added into the incubation medium in either the absence or presence of 2-chloroadenosine (10 μM). In the same experiment, the effect of 2-chloroadenosine alone was compared. Results, expressed as percentage above the basal formation of ^{3}H-inositol phosphates measured in the absence of any agonist, are the means ± SEM of values obtained in a typical experiment performed in triplicate. *, significantly different ($p < 0.05$, Student's t test) from the basal ^{3}H-inositol phosphate production measured in the absence of any effectors; †, significantly different ($p < 0.01$) from the ^{3}H-inositol phosphate production induced by 2-chloroadenosine alone. Two other independent experiments yielded similar results.

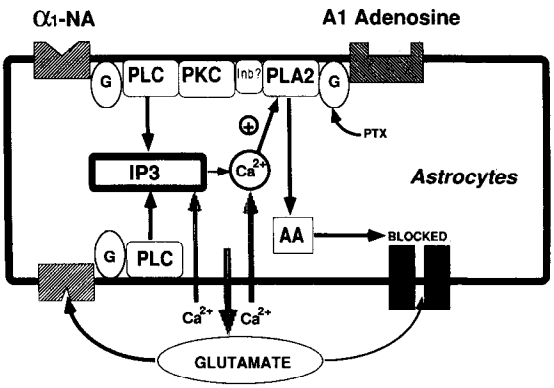

Figure 8. Proposed mechanism for the regulation by 2-chloroadenosine of the formation of inositol phosphates in striatal astrocytes. Following the binding of methoxamine and 2-chloroadenosine on α_1-adrenergic (α_1-NA) and A_1 adenosine receptors, respectively, the activity of phospholipase C (*PLC*) is first stimulated. The 1,4,5-inositol triphosphate (*IP3*) formed opens Ca^{2+} channels located on the endoplasmic reticulum (the inositol triphosphate–sensitive calcium stores). Diacylglycerol simultaneously formed activates protein kinase C (*PKC*), which by phosphorylation of a putative inhibitor (*Inb*) allows a lipase, likely phospholipase A2 (*PLA2*), to be stimulated by 2-chloradenosine via a *PTX*-sensitive G-protein (*G*). The released arachidonic acid (*AA*) induces an hyperpolarization, which by increasing the driving force for Ca^{2+} improves the refilling of intracellular Ca^{2+} stores and/or potentiates the net influx of Ca^{2+} leading to a sustained elevation of cytosolic Ca^{2+} (Delumeau et al., 1991), which further increases phospholipase A2 activity. Due to the presence of external glutamate receptors coupled to phospholipase C on striatal astrocytes, the accumulation of external glutamate resulting from the blocking effect of arachidonic acid on glutamate uptake is responsible for the enhanced formation of inositol phosphates.

to calcium, the absence of external calcium impaired the potentiating effect of 2-chloroadenosine on the methoxamine-evoked production of ^{3}H-inositol phosphates. In fact, complementary studies, in which variations in the cytosolic concentration of calcium were estimated in single cultured striatal astrocytes using the microfluorimetric INDO-1 method provided additional evidence for the involvement of external calcium. Indeed, although 2-chloroadenosine and methoxamine alone induced only transient elevations in the cytosolic concentration of calcium, their combined application resulted in a prolonged influx of external calcium (>30 min) and this effect was immediately interrupted when calcium was removed from the extracellular medium (Delumeau et al., 1991).

Involvement of glutamate. It has recently been shown that arachidonic acid inhibits glutamate uptake into Müller cells (astrocytes) of the salamander retina (Barbour et al., 1989) and that glutamate is spontaneously released from cultured astrocytes (Kimelberg et al., 1990). Therefore, it can be suggested that in the presence of methoxamine, 2-chloroadenosine through the release of arachidonic acid inhibits the reuptake of glutamate and that the resulting accumulation of the amino acid in the external medium is responsible for the enhanced phospholipase C activity (Fig. 8). This hypothesis is supported by several observations. (1) The enzymatic elimination of glutamate by the addition of GPT prevented both the enhancement by 2-chloroadenosine of the methoxamine-evoked response and the increased formation of ^{3}H-inositol phosphates induced by the unsaturated fatty acids arachidonic acid and lineolic acid. (2) As expected, since astrocytes are known to possess glutamate receptors coupled to phospholipase C (Pearce et al., 1986), glutamate stimulated the production of ^{3}H-inositol phosphates with an amplitude similar to that observed with arachidonic acid, and its effect was additive with that of methoxamine. (3) The stimulatory effect of glutamate on phospholipase C activity could not be seen under the combined presence of methoxamine and 2-chloroadenosine. (4) Finally, the inhibition by specific inhibitors of the high-affinity and Na^+-dependent glutamate uptake process (Kimelberg et al., 1989) resulted in the formation of ^{3}H-inositol phosphates with an amplitude similar to that observed with arachidonic acid, in both the absence and the presence of methoxamine. Moreover, in these conditions, the α_1-adrenergic response was no longer enhanced by the combined application of 2-chloroadenosine.

The α_1-adrenergic stimulation of phospholipase C exerts a permissive action on the 2-chloroadenosine–evoked response. Our results strongly suggest that in striatal astrocytes the stimulation of A_1 adenosine receptors coupled to phospholipase A2 leads indirectly to a modulation of phospholipase C activity. Nevertheless, the α_1-adrenergic stimulation of phospholipase C exerts a permissive action on the 2-chloroadenosine–evoked response. If the adenosine receptors involved in the observed phenomenon are coupled to phospholipase A2, this enzyme must be tonically inhibited since 2-chloroadenosine alone was ineffective on the release of arachidonic acid. We suggest that the α_1-adrenergic sitmulation of phospholipase C and the expected activation of protein kinase C allow the disinhibition of phospholipase A2 activity through a phosphorylation of inhibitory components that have yet to be identified (Fig. 8). Supporting this hypothesis, in the presence of OAG, an activator of protein kinase C, 2-chloroadenosine alone stimulated the production of ^{3}H-inositol phosphates.

In conclusion, the concentration of extracellular glutamate depends on the regulation of either the release of glutamate from neurons or its uptake into neurons and astrocytes. Arachidonic acid has indeed been shown to decrease glutamate uptake into both cell types (Chan et al., 1983; Barbour et al., 1989). It has also been reported that arachidonic acid can be released from striatal neurons in response to glutamate (Dumuis et al., 1988, 1990). The present results indicate that arachidonic acid can also be released from astrocytes under the combined activation of A_1 adenosine and α_1-adrenergic receptors. Similarly, we have recently shown that somatostatin receptors coupled to phospholipase A2 are present on striatal astrocytes and that the combined activation of somatostatin and α_1-adrenergic receptors results in the formation of arachidonic acid and the same cascade of events as that observed in the present study (Marin et al., 1991). If arachidonic acid is really involved in long-term potentiation, this phenomenon could be linked not only to neuroneuronal interactions but also to events occurring in a more complex cellular network including astrocytes.

References

Barbour B, Szatkowski M, Ingledew N, Attwell D (1989) Arachidonic acid induces a prolonged inhibition of glutamate uptake into glial cells. Nature 342:918–920.

Chan PH, Kerlan R, Fishman RA (1983) Reduction of γ-aminobutyric acid and glutamate uptake and (Na^+/K^+)ATPase activity in brain slices and synaptosomes by arachidonic acid. J Neurochem 40:309–316.

Clark DL, Linden T (1986) Modulation of guanylate cyclase by lipoxygenase inhibitors. Hypertension 8:947–950.

Cooper DMF, Caldwell KK, Boyajian CL, Petcoff DW, Schlegel W (1989) Adenosine A_1 receptors inhibit both adenylate cyclase activity

and TRH-activated Ca^{2+} channel by a pertussis toxin–sensitive mechanism in GH_3 cells. Cell Signal 1:85–97.
De Georges J, Ousley AH, McCarthy KD, Morell P, Lapetina EG (1987) Glucocorticoids inhibit the liberation of arachidonate but not the rapid production of phospholipase C–dependent metabolites in acetylcholine-stimulated C62B glioma cells. J Biol Chem 262:9979–9983.
Delumeau JC, Tencé M, Marin P, Cordier J, Glowinski J, Prémont J (1991) Synergistic regulation of cytosolic Ca^{2+} concentration by adenosine and α_1-adrenergic agonists in mouse striatal astrocytes. Eur J Neurosci 3:539–550.
Dolphin AC (1983) The adenosine agonist 2-chloroadenosine inhibits the induction of long term potentiation of the perforant path. Neurosci Lett 39:83–89.
Dolphin AC, Pestwich SA (1985) Pertussis toxin reverses adenosine inhibition of neuronal glutamate release. Nature 316:148–150.
Dumuis A, Sebben M, Haynes L, Pin JP, Bockaert J (1988) NMDA receptors activate the arachidonic acid cascade system in striatal neurons. Nature 336:68–70.
Dumuis A, Pin JP, Oomagari K, Sebben M, Bockaert J (1990) Arachidonic acid released from striatal neurons by joint stimulation of ionotropic and metabotropic quisqualate receptors. Nature 347:182–184.
Ebersolt C, Premont J, Prochiantz A, Perez M, Bockaert J (1983) Inhibition of brain adenylate cyclase by A_1 adenosine receptors: pharmacological characteristics and locations. Brain Res 267:123–129.
El-Etr M, Cordier J, Glowinski J, Prémont J (1989) A neuroglial cooperativity is required for the potentiation by 2-chloroadenosine of the muscarinic-sensitive phospholipase C in the striatum. J Neurosci 9:1473–1480.
Eng LF (1985) Glial fibrillary acid protein (GFAP): the major protein of glial intermediate filaments in differentiated astrocytes. J Neuroimmunol 8:203–214.
Fan T-PD, Lewis GP (1985) Mechanism of cyclosporin A–induced inhibition of prostacyclin synthesis by macrophages. Prostaglandins 30:735–747.
Gerzer R, Brash AR, Hardman JG (1986) Activation of soluble guanylate cyclase by arachidonic acid and 15-lipoxygenase products. Biochem Biophys Acta 886:383–386.
Harms HH, Wardeh G, Muller AH (1976) Adenosine modulates depolarization-induced release of ^{3}H-noradrenaline from slices of rat brain neocortex. Eur J Pharmacol 49:305–308.
Hawkins DJ, Ross AH, Gerzer R, Hardman JG (1985) Elevation of rabbit platelet cGMP by thrombin is not reduced by lipoxygenase/cyclooxygenase inhibition. Fed Proc 44:1817.
Kimelberg HK, Pang S, Treble DH (1989) Excitatory amino acid-stimulated uptake of $^{22}Na^+$ in primary astrocyte cultures. J Neurosci 9:1141–1149.
Kimelberg HK, Goderie SK, Higman S, Pang S, Waniewski RA (1990) Swelling-induced release of glutamate, aspartate, and taurine from astrocytes cultures. J Neurosci 10:1583–1591.
Marin P, Delumeau JC, Tencé M, Cordier J, Glowinski J, Prémont J (1991) Somatostatin potentiates the α_1-adrenergic activation of phospholipase C in striatal astrocytes through a mechanism involving arachidonic acid and glutamate. Proc Natl Acad Sci USA, 88:9016–9020.
O'Brien RJ, Fischbach GD (1986) Modulation of embryonic chick motoneuron glutamate sensitivity by interneurons and agonists. J Neurosci 6:3290–3296.
Okada Y, Ozawa S (1980) Inhibitory action of adenosine on synaptic transmission in the hippocampus of the guinea pig *in vitro*. Eur J Pharmacol 68:483–492.
Pearce BP, Albrecht J, Morrow C, Murphy S (1986) Astrocyte glutamate receptor activation promotes inositol phospholipid turnover and calcium flux. Neurosci Lett 72:335–340.
Siggins GR, Shubert P (1981) Adenosine depression of hippocampal neurones *in vitro*: an intracellular study of dose-dependent actions on synaptic and membrane potentials. Neurosci Lett 23:55–60.
Stiles GL (1986) Adenosine receptors: structure, function and regulation. Trends Pharmacol Sci 87:486–490.
Trussel LO, Jackson MB (1985) Adenosine-activated potassium conductance in cultured striatal neurones. Proc Natl Acad Sci USA 82: 4857–4861.
Vizie ES, Knoll J (1976) The inhibitory effect of adenosine and related nucleotides on the release of acetylcholine. Neuroscience 1:391–398.
Yukie N, Tadashi K, Shinkichi T, Yoshiki M, Tsuyoshi S (1986) Effects of cyclosporin A on the membrane associated events in human leukocytes with special reference to the similarity with dexamethasone. Biochem Pharmacol 35:947–951.