A Neuroglial Cooperativity Is Required for the Potentiation by 2-Chloroadenosine of the Muscarinic-Sensitive Phospholipase C in the Striatum

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In rat striatal slices, 2-chloroadenosine, which had no direct effect on inositol phosphate formation, potentiated in a dose-dependent manner the accumulation of inositol phosphates induced either by carbamylcholine (10^{-3} M) or by noradrenaline (10^{-6} M). Experiments made on pure populations of striatal neurons or striatal glial cells in primary culture from mouse embryos indicated that 2-chloroadenosine potentiated the noradrenaline-elicited phosphoinositide breakdown in striatal glial cultures but did not modify the responses evoked either by noradrenaline or by carbamylcholine in striatal neuronal cultures. However, 2-chloroadenosine enhanced both the carbamylcholine and the noradrenaline-induced accumulation of inositol phosphates in neuroglial cocultures just as it did in rat striatal slices. The potentiation by 2-chloroadenosine of the carbamylcholine response, which is neuron specific, involved a cooperative effect between neurons and glial cells and, as shown by additional experiments, required a brief contact only between the 2 types of cells.

The potentiating effect of 2-chloroadenosine was blocked completely by a nonselective A1, A2 adenosine antagonist isobutylmethylxanthine either on rat striatal slices or on mouse embryonic cocultures (noradrenaline and carbamylcholine responses) or on mouse embryonic glial cultures (noradrenaline response).

These data indicate the involvement of an extracellular membrane-bound adenosine receptor, possibly of the A1 subtype since *N*⁶-cyclohexyladenosine, an A1 adenosine receptor agonist, was more efficient than 5'-*N*-ethylcarboxamideadenosine, a rather selective A2 adenosine receptor agonist.

We propose that 2-chloroadenosine acts through an adenosine receptor located on glial cells and induces the synthesis of a substance that improves the coupling between carbamylcholine or noradrenaline and phospholipase C located in glial cells or neurons.

Adenosine and its stable analogs appear to be potent psychotropic agents, inducing sedative and anticonvulsant effects in mammals following their systemic or central administration (Snyder et al., 1981; Dunwiddie and Worth, 1982; Dunwiddie, 1985). Adenosine has been shown to inhibit both spontaneous and evoked neuronal firing after local iontophoretic application either *in vivo* or *in vitro* (Phillis and Wu, 1981). Both the behavioral and electrophysiological actions of adenosine have been ascribed to its effect on transmitter release (Phillis and Barraco, 1985). The nucleoside has indeed been shown to prevent the release of several neurotransmitters, including that of noradrenaline or ACh (Fredholm and Hedquist, 1980). Adenosine may have not only pre- but also postsynaptic actions related to calcium flux (Snyder, 1985; Williams, 1987). In addition, adenosine was first demonstrated to increase cAMP content in brain slices (Sattin and Rall, 1970) and later to alter adenylate cyclase activity through A1 and A2 receptors that are coupled negatively and positively, respectively, to adenylate cyclase (Daly, 1977; Londos et al., 1980).

However, changes in cAMP accumulation cannot always account for events modulated by adenosine. The nucleoside probably influences cellular functions by acting also on other second-messenger systems such as inositol phosphates (IPs), as recently reported by Hollingsworth et al. (1986). Indeed, these authors have observed that 2-chloroadenosine (an agonist of A1 and A2 receptors), which had no effect alone on guinea pig cortical slices, was able to potentiate the histamine-induced accumulation of IPs. The latter results led us to look for a possible modulatory action of adenosine on transmitter responses mediated by phospholipase C in the striatum. This structure was selected for several reasons. (1) A1 and A2 receptors are present in the adult striatum. (2) In addition, these receptors have been identified on striatal neurons from the mouse in primary culture (Ebersolt et al., 1983; Premont et al., 1983), and there is some evidence that these receptors exist as well on striatal astrocytes (Daly, 1985). (3) By acting respectively on α_1 -adrenergic and muscarinic receptors, noradrenaline and carbamylcholine have been shown to stimulate markedly phosphatidylinositol (PI) turnover in adult striatum (Gonzales and Crews, 1984), and these effects were also demonstrated on embryonic striatal neurons from the mouse in primary culture (Weiss et al., 1986). Therefore, the effects of 2-chloroadenosine on α_1 -noradrenergic and muscarinic-sensitive phospholipase C were examined first on striatal slices of the rat and then either on pure neuronal or glial primary cultures from the striatum of embryonic mouse or on cocultures of these 2 cell types. In particular, it will be shown that

Received June 14, 1988; revised Sept. 7, 1988; accepted Sept. 23, 1988.

We would like to thank Dr. A. Prochiantz and B. Chamaek for their judicious help in cell characterization experiments, and L. Behehradek for her assistance in preparing the manuscript. This work has been supported by grants from INSERM, DRET 87/201 and Rhône Poulenc Santé.

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Figure 1. Immunostaining of embryonic striatal cells cultured in 3 different conditions and labeled with anti-neurofilament-triplet or anti-GFAP antibodies. The presence of neurofilament-triplet (*A*, *C*, *E*) or GFAP (*B*, *D*, *F*) was detected by indirect immunofluorescence (see Materials and Methods). *A* and *B*, Striatal cells were cultured for 5 d in a MEM/F12 medium supplemented with a mixture of hormones and salt as described in Materials and Methods. Note the absence of immunostaining with anti-GFAP antibodies (*B*); conversely, all cells were labeled with anti-neurofilament-triplet antibodies, indicating the neuronal nature of the cells (*A*). *C* and *D*, Striatal cells were cultured for 4 weeks in a MEM/F12 medium supplemented with 10% NU-Serum, as described in Materials and Methods. Note the absence of immunostaining with anti-neurofilament-triplet antibodies (*C*). Conversely, all cells were labeled with the anti-GFAP antibody, indicating the glial nature of the cells (*D*). *E* and *F*, Striatal cells were cultured for 12 d in a MEM/F12 medium supplemented with 10% NU-Serum, as described in Materials and Methods. Note that the cells were immunostained with both antibodies, indicating the presence of glial cells (*F*) and neurons (*E*). $\times 1600$.

2-chloroadenosine, which has no effect alone, potentiates the α_1 -noradrenergic-sensitive phospholipase C on striatal glial cells but not on pure striatal neuronal cultures, whereas the carbamylcholine-sensitive phospholipase C, specifically located on neurons, requires the presence of glial cells to be potentiated by 2-chloroadenosine.

Materials and Methods

Striatal slice preparation

Sprague-Dawley male rats (300 gm) were decapitated, their brains rapidly removed, and their striata dissected. Striata were cut twice each perpendicularly at 0.3 mm intervals, using a McIlwain tissue chopper.

The minced tissue was resuspended in a gassed (5:95 CO₂/O₂) modified Krebs bicarbonate buffer [KBB (in mM): NaCl, 90; KCl, 4.7; NaHCO₃, 10; Na₂HPO₄, 10; KH₂PO₄, 1.2; MgSO₄, 1.2; CaCl₂, 1; glucose, 11.7; pH 7.4].

Cell cultures

Neuronal primary cultures. Striata were removed from 16 d-old Swiss mouse embryos (Iffa Credo, Lyon, France) and mechanically dissociated with a narrowed Pasteur pipette in serum-free medium, according to Weiss et al. (1986). Cells were plated (10⁶ cells/ml) in 24 well (0.5 ml/well) NUNC culture dishes previously coated successively with 1.5 µg/ml poly(L-ornithine) (M, 40,000; Sigma) and culture medium containing 10% fetal calf serum. After withdrawing the last coating solution, cells were seeded in a serum-free medium composed of a 1:1 mixture of Minimal Essential Medium (MEM) and F-12 nutrient (GIBCO Europe, Paris) and supplemented with glucose (33 mM), glutamine (2 mM), NaHCO₃ (3 mM), *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid (HEPES; 5 mM) and a mixture of hormones and salt: insulin (25 µg/ml), transferrin (100 µg/ml), putrescine (60 µM), progesterone (20 nM), and selenium salt (NaSeO₃, 30 nM).

Immunostaining of cultured cells was performed by indirect immunofluorescence according to the procedure described by Chamak et al. (1987). Using this technique, these cultures were immunocytochemically defined [with antibodies to neurofilament-triplet-proteins (kindly provided by Dr. R. K. Liem, Columbia University) and glial fibrillary acidic proteins] as purified neurons (Fig. 1A) devoid of detectable glial elements at five days in culture (Fig. 1B).

Glial primary cultures. Dissociated cells were plated (7 × 10⁵ cells/ml) in 24 well (0.5 ml/well) NUNC culture dishes previously coated with 1.5 µg/ml poly(L-ornithine). The culture medium included 10% NU-Serum (Collaborative Research) instead of the mixture of hormones and salt and was changed every 3 d during 4 weeks until glial elements had formed a confluent monolayer, devoid of neuronal cells. More than 95% of the cells were stained significantly by the immunofluorescence technique using a rabbit antibody against glial fibrillary acidic protein (GFAP) (Fig. 1D). The absence of surviving neurons was checked by immunofluorescence using antibodies against neurofilament-triplet-proteins (Fig. 1C). A monoclonal rat galactocerebroside antibody (gift from Dr. Goridis) and a rabbit antiserum against fibronectin were used to identify the eventual presence in these cultures of oligodendrocytes and fibroblasts, respectively. No staining could be observed with other antibodies.

Neuronal and glial cell cocultures: 12-d-old cultures. When striatal cells were cultured for 12 d in the presence of 10% NU-Serum, both neurons and glial cells were present, as demonstrated immunocytochemically using antibodies specific either for neurons or for glial cells, as already described (Fig. 1, E, F).

Neuronal (2-d-old) and glial cell (4-week-old) cocultures. Dissociated embryonic striatal cells (10⁶ cells/ml; 0.5 ml/well) were layered on confluent glial cells cultured during 26 d in 24 well culture dishes (0.5 ml/well). After 2 d in the presence of 10% NU-Serum, these cultures contained both neurons (2 d old) and glial cells (mainly, 4 week old).

Neuronal (5-d-old) and replated glial cells (4-week-old) cocultures. Another type of coculture consisted of primary neuronal cultures (5 d old), obtained as previously described, and of glial cells, which had been allowed to grow during 4 weeks before they were layered on neuronal cells. For this purpose, striatal cells were cultured in the presence of 10% NU-Serum in 100-mm-diameter culture dishes coated with poly(L-ornithine), at the density of 8 × 10⁶ cells/dish. After 26 d of culture, glial cells were scraped in serum-free medium, centrifuged at 300 × *g* at room temperature, and resuspended in 24 ml of culture medium supplemented with 10% NU-Serum. Five hundred microliters of this cell suspension were seeded on the neuronal primary cultures after withdrawing of their defined hormones and salt mixture supplemented culture medium.

³H-IP assay

Striatal slices. The method of Berridge et al. (1982) was used with minor modifications. Slices (10 mg wet weight tissue/ml) were preincubated for 60 min at 37°C with gassed KBB containing ³H-myoinositol (8 µCi/ml, 14 Ci/mM, New England Nuclear) and then washed 4 times with fresh oxygenated KBB and preincubated for further 15 min in KBB supplemented with 10 mM lithium chloride at 37°C in the same volume. Finally, 200 µl aliquots of labeled slices (approximately 150 µg proteins)

were added to 100 µl of the buffer containing 10 mM LiCl, 0.3 IU adenosine deaminase (Boehringer, Mannheim), and appropriate agonists (final volume, 300 µl). Incubation was terminated after 30 min at 37°C by addition of 1 ml chloroform-methanol (1:2, vol/vol), 300 µl of chloroform, and 300 µl of water. Lower organic and upper aqueous phases were separated by 10 min centrifugation at 1000 × *g*. A 1 ml aliquot of the upper aqueous phase was diluted to 5 ml with cold 5 mM myoinositol and loaded onto columns (Bio-Rad) of Dowex AG1X8 (formate form, 1.6 ml/column, 1:2 wt/vol). Columns were then washed with 10 ml of 0.1 M formic acid. Total labeled IPs, containing mainly ³H-inositol monophosphate (more than 90% of total IPs) were eluted with 5 ml of 1 M ammonium formate/0.1 M formic acid. Aquasol 2 scintillation fluid (DuPont), 10 ml, was added to the eluates and the radioactivity measured.

Cell cultures. ³H-myoinositol was added to the culture medium (1 µCi/well containing 500 µl medium), 24 hr before the experiment. The assay began with 4 washes with Krebs phosphate buffer (in mM: NaCl, 120; NaH₂PO₄, 15.6; KCl, 4.8; MgSO₄, 1.2; CaCl₂, 1.2; glucose, 33.4; pH 7.4). Cells were preincubated for 15 min with this buffer supplemented with 10 mM LiCl at 37°C. The medium was then removed and replaced with 500 µl fresh buffer supplemented with 10 mM LiCl, 0.5 IU adenosine deaminase and the agonists to be tested. The incubation was stopped 20 min later by successively adding 200 µl of 0.1% Triton /0.1N NaOH, 200 µl of 0.1% Triton/0.1 N HCl, and 500 µl of water to the incubation medium. The cells were then scraped and the contents of the wells transferred to glass tubes with an additional 1.5 ml chloroform-methanol (1:2, vol/vol) wash. Chloroform, 500 µl, was then added to the tubes, which were centrifuged at 1000 × *g* for 10 min. The rest of the assay was the same as described for the slices.

Results

Effects of adenosine deaminase and 2-chloroadenosine on carbamylcholine- and noradrenaline-evoked accumulation of ³H-IPs in striatal slices of adult rats

Maximally effective concentrations of carbamylcholine (10⁻³ M) and noradrenaline (10⁻⁴ M) stimulated PI turnover in rat striatal slices labeled with ³H-myoinositol by 502 ± 40% and 363 ± 54% (*n* = 11), respectively. The stimulatory effect of carbamylcholine was completely blocked by the muscarinic antagonist atropine (10⁻⁶ M), while that of noradrenaline was suppressed by the specific α₁-adrenergic antagonist prazosin (10⁻⁶ M) (data not shown), as already described (Brown et al., 1984).

When adenosine deaminase (1 IU/ml) was present during the incubation, the increase in ³H-IPs accumulation induced either by carbamylcholine (10⁻³ M) or by noradrenaline (10⁻⁴ M) was slightly reduced, while basal accumulation did not change (Fig. 2). In the presence of adenosine deaminase, the resistant adenosine analog, 2-chloroadenosine had no effect when added alone, but it potentiated both the carbamylcholine- and the noradrenaline-induced accumulation of ³H-IPs in a dose-dependent manner between 10⁻⁶ and 10⁻⁴ M. In addition, the potentiating effect of 2-chloroadenosine was much more pronounced on the carbamylcholine-induced than on the noradrenaline-induced response (Fig. 2).

2-Chloroadenosine potentiates the noradrenaline-induced accumulation of ³H-IPs in striatal glial cultures but not the noradrenaline- or carbamylcholine-evoked responses in striatal neuronal cultures

Membrane-bound adenosine receptors (A1 and A2) are known to be present either on neurons or on glial cells in the striatum (Daly, 1985). Therefore, primary cultures of immunocytochemically defined neurons or glial cells (see Materials and Methods; refer also to Figs. 1, A, B and 1, C, D, respectively) originating from mouse embryonic striata were used to determine the cell type(s) on which 2-chloroadenosine was acting in order to po-

Figure 2. Effects of adenosine deaminase and 2-chloroadenosine on noradrenaline- and carbamylcholine-elicited accumulation of ^3H -IPs in striatal slices of adult rat. Effects of carbamylcholine (CARB) and noradrenaline (NA) in maximally effective concentrations were first examined in the absence (–ADA) or presence (+ADA) of adenosine deaminase (1 IU/ml). The effects of various concentrations of 2-chloroadenosine in the absence (CONTROL) or presence of agonists were always examined in the presence of ADA. Results are means $\pm$ SEM of values obtained in a typical experiment performed in triplicate and are expressed as a percentage of the control values obtained in the presence of ADA and the absence of 2-chloroadenosine (dpm/assay: 140 $\pm$ 30; column with filled circle). Asterisks denote a significant difference ($p < 0.05$) compared with results obtained in the presence of respective agonists and with ADA and in the absence of 2-chloroadenosine. Four other independent experiments provided similar results.

tentiate the carbamylcholine- or noradrenaline-evoked responses on ^3H -IP accumulation.

Surprisingly, 2-chloroadenosine was without effect on the accumulation of ^3H -IPs induced either by carbamylcholine (10^{-3} M) or by noradrenaline (10^{-4} M) in labeled striatal neurons. Carbamylcholine (10^{-3} M) had no significant effect on striatal glial cells either in the absence or in the presence of the nucleoside analog. However, 2-chloroadenosine markedly potentiated the noradrenaline (10^{-4} M)-mediated response present in glial cells, whereas it had no effect when added alone (Fig. 3).

Potentiating effect of 2-chloroadenosine both on noradrenaline- and carbamylcholine-induced accumulation of ^3H -IPs in neuroglial cocultures

Striatal cells cultured for 12 d in the presence of serum contain both neurons and glial cells, as immunohistochemically demonstrated in Figure 1, E, F. As observed with rat striatal slices, when labeled cocultures of cells from the mouse striatum were used, 2-chloroadenosine significantly enhanced both the noradrenaline- and carbamylcholine-induced accumulation of ^3H -IPs in a dose-dependent manner, with the latter effect being more pronounced than the former (Fig. 4). When added alone, increasing concentrations of 2-chloroadenosine had no effect.

Requirement of a brief contact between glial cells and neurons for the demonstration of the 2-chloroadenosine potentiating activity

The 2-chloroadenosine potentiating activity on the carbamylcholine- and noradrenaline-evoked accumulation of ^3H -IPs seen

Figure 3. Effects of 2-chloroadenosine on agonist-induced accumulation of ^3H -IPs in mouse striatal neurons or glial cells in primary culture. Striatal neurons (5 d old) or glial cells (4 week old) from embryonic mouse were cultured as described in Materials and Methods (see Figs. 1, A, B and 1, C, D, respectively). In all cases, experiments were performed in the presence of adenosine deaminase (1 IU/ml) in the absence (CONT) or presence either of carbamylcholine (CARB) or noradrenaline (NA). Results are means $\pm$ SEM of values obtained in a typical experiment performed in triplicate and are expressed as a percentage of respective controls (columns with filled circle; dpm/well: 230 $\pm$ 12 and 923 $\pm$ 53 for striatal neurons and glial cells, respectively). Asterisks indicate significant difference ($p < 0.01$) compared with results obtained in the presence of maximally effective concentrations of NA. Four other independent experiments provided similar results.

in striatal cocultures (neurons plus glial cells) was largely prevented when glial cell growth was reduced by a cytosine arabinoside treatment (which had no effect on neuronal survival) (Table 1).

For the carbamylcholine response particularly, which is neuron-specific, the results described above seem to indicate that glial cells are required for the 2-chloroadenosine potentiating activity or that 12-d-old neurons, grown in presence of glial cells, have developed a direct sensitivity to 2-chloroadenosine. Supporting the former hypothesis (requirement of glial cells), 2-chloroadenosine still potentiated the carbamylcholine (10^{-3} M)-evoked response when dissociated embryonic striatal cells (known to contain mainly neurons) were grown for 2 d only with 4-week-old striatal glial cells (Fig. 5).

Additional experiments indicated that only a brief membrane contact between glial and neuronal cells was required for the occurrence of the 2-chloroadenosine-potentiating activity on the carbamylcholine neuronal response. Indeed, 2-chloroadenosine (10^{-4} M) potentiated the carbamylcholine-induced (10^{-3} M) accumulation of ^3H -IPs when added only 30 min (time necessary for glial attachment) after the layering of dissociated unlabeled glial cells (grown for 4 weeks *in vitro*) on 5-d-old striatal neurons previously labeled with ^3H -myo-inositol (Table 2). In parallel experiments, it was confirmed that labeled replated glial cells remained sensitive to noradrenaline (10^{-4} M) and that this response was still amplified by 2-chloroadenosine (data not shown).

Selectivity of the adenosine receptor subtype involved in the potentiating effect of 2-chloroadenosine

Experiments made first on rat striatal slices indicated that the potentiating effect of 2-chloroadenosine (up to 10^{-4} M) on nor-

Figure 4. Potentiating effect of 2-chloroadenosine on agonist elicited accumulation of ^3H -IPs in striatal neuroglial cocultures (12 d old). Striatal cells were cultured during 12 d in a MEM/F12 medium supplemented with 10% NU-Serum (see Materials and Methods). Both neurons (N) and glial (G) cells were present under these culture conditions (see Fig. 1, E, F). Experiments were carried out as described in Figure 2, adenosine deaminase (1 IU/ml) being present in all cases. Results are means $\pm$ SEM of values obtained in a typical experiment performed in triplicate and are expressed as a percentage of the mean control value obtained in absence of 2-chloroadenosine (columns with filled circle; dpm/well: 640 ± 40). Asterisks indicate significant difference ($p < 0.05$) compared with results obtained in the presence of maximally effective concentrations of carbamylcholine (CARB) or noradrenaline (NA) and in the absence of 2-chloroadenosine. Four other independent experiments yielded similar results.

adrenaline- and carbamylcholine-mediated increase in IP accumulation was blocked completely by 10^{-4} M isobutylmethylxanthine (IBMX), a nonselective adenosine antagonist of A1, A2 receptors. Papaverine (10^{-4} M), an adenosine uptake blocker, had no effect. Similar results were found both with IBMX and papaverine on mouse embryonic glial cultures (noradrenaline response) or cocultures (noradrenaline and carbamylcholine responses) from the striatum (data not shown).

Since 2-chloroadenosine exerts its effects by acting on glial cells, preliminary attempts were then made to determine whether A1 or A2 receptors were involved in the potentiation of the glial noradrenaline-sensitive phospholipase C. The selective agonist of the A1 adenosine receptor subtype N^6 -cyclohexyladenosine (CHA) was more potent in increasing the noradrenaline-induced accumulation of ^3H -IPs in glial cells than 5'-N-ethylcarboxamide-adenosine (NECA), which is believed to have a slightly higher affinity for the A2 than the A1 adenosine receptor subtype (Londos et al., 1980) (Table 3).

Discussion

In the present study, using rat striatal slices, we have demonstrated, first, that 2-chloroadenosine, an adenosine deaminase-resistant analog, which had no effect alone, potentiated not only the noradrenaline- but also the carbamylcholine-induced stimulation of PI turnover. Similar results were obtained in 12-d-old cocultures of striatal glial cells and neurons from the mouse embryo, indicating that the modulatory action of 2-chloroadenosine can be seen at an early stage of ontogenesis. Experiments made on pure striatal neuronal populations in primary culture revealed that 2-chloroadenosine potentiated neither the nora-

Figure 5. Potentiating effect of 2-chloroadenosine on carbamylcholine-induced accumulation of ^3H -IPs in 2-d-old cocultures containing neurons (2 d old) and glial cells (4 week old). Effects of carbamylcholine (CARB) in the absence or presence of 2-chloroadenosine were examined either in 4-week-old glial cells or in 2-d-old neurons or in 2-d-old cocultures of both cell populations as described in Materials and Methods. In all cases, experiments were performed in the presence of adenosine deaminase (1 IU/ml). Results are means $\pm$ SEM of values obtained from a typical experiment performed in triplicate and are expressed as a percentage of respective control values. dpm/well: 1295 ± 200 , 103 ± 7 , and 1748 ± 80 for glial cells, neurons, and cocultures, respectively. It should be noted that the control value in cocultures corresponds to the accumulation of ^3H -IPs in both cell types and that neurons only respond to carbamylcholine. This explains the lower stimulation induced by carbamylcholine in cocultures. Asterisks indicate significant difference ($p < 0.05$) compared with control values; filled circles indicate significant differences ($p < 0.05$) compared with results obtained in the presence of carbamylcholine and in the absence of 2-chloroadenosine. Three other independent experiments provided similar results.

Table 1. Effects of cytosine arabinoside treatment of striatal neuroglial cocultures (12 d old) on the potentiating effect of 2-chloroadenosine on the carbamylcholine-induced accumulation of ^3H -IPs

	Increase in ^3H -IP accumulation above control values (dpm/well)	
	Untreated cells	ARA-C-treated cells
Carbamylcholine	1250 ± 220 (100)	1810 ± 300 (100)
Carbamylcholine + 2-chloroadenosine	4250 ± 320 (340)	3100 ± 110 (171) ^a

Striatal cells were cultured in a MEM/F12 medium supplemented with 10% NU-Serum for 12 d (corresponding to Fig. 1, E, F). When cells were treated with cytosine arabinoside (ARA-C) to reduce glial cell division, ARA-C (2.5×10^{-6} M) was present in the culture medium between days 5 and 7 of the culture. The culture medium was changed at the same time for control and ARA-C-treated cells. Experiments were performed in the presence of adenosine deaminase (1 IU/ml). Carbamylcholine (CARB) alone (10^{-3} M) increased the accumulation of ^3H -IPs by 68 ± 8 and $450 \pm 36\%$ in untreated cells and ARA-C-treated cells, respectively, compared with control values. Results expressed in dpm/well of ^3H -IP accumulation above control values are means $\pm$ SEM of data obtained in a typical experiment performed in triplicate. The numbers in brackets indicate the increase in ^3H -IP accumulation due to 2-chloroadenosine (10^{-4} M) expressed as a percentage.

^aSignificantly different ($p < 0.05$) compared with results obtained in untreated cells similarly incubated in the presence of carbamylcholine and 2-chloroadenosine. Two other independent experiments provided similar results.

Figure 6. Proposed model for the involvement of glial cells in the potentiating effect of 2-chloroadenosine on agonist-induced activation of phospholipase C in striatal cell cultures. 2-Chloroadenosine first acts on an adenosine receptor located on the surface of glial cells (A) and induces the synthesis of a putative second messenger or a "second agonist" which originates from glial cells. This putative substance could then trigger the potentiation of agonist-induced phospholipase C (PLC) stimulation of each cell type. Two situations could be distinguished: (1) homologous cell interaction in which 2-chloroadenosine might amplify the α_1 -noradrenergic-sensitive PLC located on the same glial cell (1) or on surrounding ones (2); and (2) heterologous cell interaction in which 2-chloroadenosine first acts on glial cells which send a signal to surrounding neurons, allowing enhancement of their response to carbamylcholine through muscarinic receptors (M; 4). We have, as yet, no information concerning the eventual potentiation by the nucleoside, of the α_1 -noradrenergic response in neurons (3).

Table 2. Requirement of a brief contact between striatal glial cells and neurons for the potentiating effect of 2-chloroadenosine on the neuronal response to carbamylcholine

	Increase in ^3H -IP accumulation above control values (dpm/well)	
	Labeled neurons	Labeled neurons + unlabeled replated glial cells
Carbamylcholine	1314 $\pm$ 87 (100)	984 $\pm$ 68 (100)
Carbamylcholine + 2-chloroadenosine	1048 $\pm$ 65 (80)	2007 $\pm$ 42 (204) ^a

Neuronal cells were cultured for 5 d and glial cells for 4 weeks in appropriate culture media as described in Materials and Methods (see also Fig. 1, A, B and C, D for neurons and glial cells, respectively). Unlabeled glial cells were scraped and layered on neurons that had been previously exposed to ^3H -myo-inositol (after withdrawing the medium containing ^3H -myo-inositol). The incubation with carbamylcholine (10^{-5} M) with or without 2-chloroadenosine (10^{-4} M) was made after a 30 min period of contact between the 2 cell populations. All experiments were performed in the presence of adenosine deaminase (1 IU/ml). Carbamylcholine alone (10^{-5} M) increased the accumulation of ^3H -IPs above control values by 448 $\pm$ 29 and 300 $\pm$ 21% for labeled neurons alone and labeled neurons plus unlabeled replated glial cells, respectively. Results expressed in dpm/well of ^3H -IP accumulation above control values are means $\pm$ SEM of data obtained in a typical experiment performed in triplicate. The values in brackets indicate the relative change in ^3H -IP accumulation due to 2-chloroadenosine (10^{-4} M) expressed as a percentage.

^aSignificantly different ($p < 0.05$) compared with results obtained with labeled neurons alone. In these experiments, it was verified that 2-chloroadenosine (10^{-4} M) was still able to potentiate the noradrenaline (10^{-4} M)-evoked response in replated glial cells.

Table 3. Effects of 2 adenosine analogs on noradrenaline-induced ^3H -IP accumulation in striatal glial cells

Potentiation of noradrenaline-induced ^3H -IP accumulation by adenosine analogs (in %)

Concentrations (M)	CHA	NECA
0	100 $\pm$ 4	100 $\pm$ 4
10^{-7}	225 $\pm$ 10 ^a	125 $\pm$ 5
10^{-6}	275 $\pm$ 4 ^a	187 $\pm$ 7 ^a
10^{-5}	287 $\pm$ 3 ^a	212 $\pm$ 2 ^a
10^{-4}	300 $\pm$ 6 ^a	250 $\pm$ 7 ^a

Experiments were performed on 4-week-old striatal glial cells (see Materials and Methods, Fig. 1, C, D). Incubations were performed in the presence of noradrenaline (10^{-4} M) and various concentrations of either *N*-cyclohexyladenosine (CHA) or 5'-*N*-ethylcarboxamideadenosine (NECA). In all cases, adenosine deaminase (1 IU/ml) was present in the incubating medium. Results are expressed as a percentage of data obtained in the presence of noradrenaline alone. They are means $\pm$ SEM of data obtained in triplicate.

^a $p < 0.05$ compared with results obtained with noradrenaline alone. Two other independent experiments provided similar results.

drenaline- nor the carbamylcholine-induced accumulation of IPs. These results were surprising since we have demonstrated previously that both A1 and A2 adenosine receptors are present on striatal neurons from the mouse embryo in primary culture (Ebersolt et al., 1983). The lack of potentiating effect of 2-chloroadenosine on pure neuronal cultures suggested that glial cells were required for the expression of the modulatory action of the adenosine analog. This was particularly evident for the potentiation of the carbamylcholine-induced response since in contrast to the α_1 -adrenergic receptors, the muscarinic receptors coupled to phospholipase C were not found on glial cells.

Experiments made to understand the process involved in the potentiating effect of 2-chloroadenosine on the carbamylcholine-induced PI breakdown demonstrated a cooperative effect between glial cells and neurons. This is supported by 4 observations: (1) As already mentioned, when striatal cells were cultured during 12 d in the presence of serum, the culture contained both neurons and glial cells. In this condition, 2-chloroadenosine potentiated the effect of carbamylcholine as it did in slices in which neurons are surrounded by glial cells but not when pure populations of neurons were used. (2) When the growth of glial cells in 12-d-old cocultures made in serum was slowed by treatment with cytosine arabinoside, the potentiating effect of 2-chloroadenosine decreased. (3) When pure populations of glial cells were mixed with pure populations of neurons and cocultured during 2 d, the 2-chloroadenosine-potentiating effect of the carbamylcholine-induced accumulation of IPs (a neuronal response) could be seen. (4) The nucleoside effect did not seem to be related to a neuronal maturation due to the presence of glial cells allowing neurons to be responsive to 2-chloroadenosine since a 30 min contact between these 2 different cell types was sufficient to obtain the potentiation. Cell contact between the 2 types of cells seems to be required since no potentiation was observed in preliminary experiments in which a conditioned medium of glial cells exposed to adenosine was added to neurons.

We have shown that a neuroglial cooperativity is a prerequisite for the potentiation by 2-chloroadenosine of the carbamylcholine-induced accumulation of IPs in neurons, but we do not know yet whether the nucleoside exerts a similar effect and

by a similar process on the neuronal α_1 -adrenergic receptors coupled to phospholipase C. Noradrenaline indeed stimulated the turnover of PI in 4-week-old cultures of pure striatal glial cells, and this effect was even more pronounced than that observed on neurons. In addition, 2-chloroadenosine, which was without effect alone, potentiated the noradrenaline-evoked response, indicating a functional heteroregulation between the 2 populations of receptors involved. Moreover it cannot be excluded that a cooperative effect also exists between glial cells. To our knowledge, this is the first demonstration of a modulatory action of adenosine on receptors coupled to phospholipase C on glial cells.

Therefore, the 2-chloroadenosine modulation of noradrenaline- and carbamylcholine-evoked responses seems to be mediated by an extracellular adenosine membrane-bound receptor located on glial cells. This modulation was blocked by the non-specific A1, A2 extracellular adenosine receptor antagonist IBMX. 2-Chloroadenosine does not need to penetrate into the cells to be effective, since papaverine, an adenosine-uptake blocker, did not suppress its potentiating effect. CHA, a selective A1 adenosine receptor agonist was more efficient than NECA, a compound that acts both on A1 and A2 adenosine receptors but that has been described to have a slightly higher affinity for A2 adenosine receptors. Although this characterization of the adenosine receptor subtype involved is far from being complete, our preliminary results favor the implication of an A1 receptor subtype. Indeed, this conclusion is not definitive and, despite their particular liposolubility, has to be confirmed with specific A1 antagonists. However, A1 receptors coupled negatively to adenylate cyclase are present on neurons but 2-chloroadenosine had no potentiating effect on pure neuronal populations. Therefore, it remains to be determined whether the glial adenosine receptors involved in the modulatory action of 2-chloroadenosine represent a new subtype of adenosine receptors not coupled to adenylate cyclase but to another second-messenger system.

We propose that 2-chloroadenosine acts through adenosine receptors located on glial cells and induces the synthesis of a substance that, directly or indirectly, is able to improve the coupling between the agonist receptor and the phospholipase C located on either glial cells or neurons. This implies that this putative substance originating from glial cells could act on neighboring cells (Fig. 6).

Our results may explain the potentiating effect of adenosine on the noradrenaline-induced accumulation of intracellular cAMP in rat cortical slices (Schwabe and Daly, 1977). This effect depended in fact, on the presence of extracellular calcium, suggesting the involvement of α_1 -adrenergic receptors, which, by the coupling to phospholipase C, leads to an increase in intracellular levels of calcium. Hollingsworth et al. (1986) have reported that 2-chloroadenosine amplifies histamine-elicited PI breakdown on guinea pig cortical slices, but these authors were unable to demonstrate a similar potentiation on the carbamylcholine- or noradrenaline-induced accumulation of IPs in this preparation. This could be due to a difference either of species or of brain region. Preliminary results support the latter hypothesis since 2-chloroadenosine did not potentiate the noradrenaline-induced accumulation of IPs on mouse embryonic glial cells from the cerebral cortex in primary cultures (M. El-Etr and J. Cordier, unpublished observations). This provides further evidence for a heterogeneity in the properties of glial cells, depending on their brain location, especially between the

cerebral cortex and the striatum, as already observed in our laboratory (Chneiweiss et al., 1985a, b).

We are now investigating the nature of the putative substance from striatal glial cells that, in response to 2-chloroadenosine, seems to play a critical role in the neuroglial cooperativity for the modulatory action of 2-chloroadenosine.

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