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Histamine Stimulates Glycogen Breakdown and Increases $^{45}Ca^{2+}$ Permeability in Rat Astrocytes in Primary Culture

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SUMMARY

In astrocyte-enriched cultures from rat brain hemispheres prelabeled with [^{3}H]glucose, histamine stimulates [^{3}H]glycogen breakdown in a concentration-dependent manner, with an EC_{50} of 0.6 μM. This effect can be induced by activation of both H_1 and H_2 receptors independently. Thus, neither 1 μM promethazine, an H_1 antagonist, or 100 μM metiamide, an H_2 antagonist, inhibited the glycogenolytic response to histamine unless they were present together. In addition, the maximal effect of histamine (55% decrease in [^{3}H]glycogen) was also elicited by 300 μM 2-thiazolylethylamine, an H_1 agonist, and by 1 mM dimaprit, an H_2 agonist. These agonist effects were inhibited by promethazine and metiamide, respectively, and were not additive, indicating that the same glycogen pool was affected. Histamine was more potent in eliciting glycogenolysis through H_1 (EC_{50} of 0.4 μM in the presence of 100 μM metiamide) than through H_2 (EC_{50} of 3.3 μM in the presence of 1 μM promethazine) receptors, as also shown previously for the H_1-mediated phosphoinositide hydrolysis compared with the H_2-mediated cAMP formation in the same cells. Both dibutyryl cyclic AMP and the Ca^{2+} ionophore A23187 could independently mimic the glycogenolytic effect of histamine, whereas the absence of extracellular Ca^{2+} abolished the H_1 component of the response. Histamine also stimulated rapid transmembrane $^{45}Ca^{2+}$ influx (maximum, 48% of basal at 15 sec) and efflux (maximum, 25% of basal at 1 min) in astrocytes by activation of H_1 receptors. This histamine-increased $^{45}Ca^{2+}$ entry was abolished by the nonspecific Ca^{2+} channel blocker lanthanum but not by the voltage-operated Ca^{2+} channel inhibitor nifedipine. The enhanced $^{45}Ca^{2+}$ release was more a consequence of the histamine-increased Ca^{2+} permeability than intracellular Ca^{2+} mobilization, because it was largely diminished when Ca^{2+} entry was prevented and was little affected by pretreatment of the cells with 12-*O*-tetradecanoyl-phorbol-13-acetate. Thus, the histamine-induced glycogen breakdown in astrocytes may involve increases in cAMP formation and in intracellular Ca^{2+} levels, this latter resulting mainly from H_1-mediated extracellular Ca^{2+} uptake.

Substantial evidence has now accumulated to assign HA a neuromodulator role in the central nervous system (for reviews, see Refs. 1–3). The numerous functions in which the amine has been implicated are mediated by three types of receptors, termed H_1, H_2, and H_3 (1, 2). We have recently demonstrated the presence of H_1 and H_2 receptors in rat brain astrocytes in primary culture (4, 5).[1] In these cells, as previously shown in different brain preparations (1, 2), activation of H_1 receptors induces phosphoinositide breakdown, whereas H_2 receptors mediate stimulation of cAMP formation. However, the physiological role of HA and its receptors in brain astrocytes is unknown.

In early studies, HA was shown to stimulate glycogen breakdown in rat astrocytoma cells (6) and chick brain hemispheres (7) and, because most of the brain glycogen appeared to be associated with astrocytes (8), the presence of HA receptors in these cells regulating brain glial glycogen stores was suggested (7). In astrocytoma cells HA did not activate adenylate cyclase and its glycogenolytic effect was, thus, independent of cAMP formation (6), whereas in chick brain HA induced a marked H_2-mediated increase in cAMP (9), but the glycogenolytic effect was only partially inhibited by H_2 antagonists (7). More recently, Quach *et al.* (10) demonstrated that in mouse brain cortical slices HA elicits glycogen breakdown through interaction with H_1 receptors in a process that depends on extracellular Ca^{2+}. A requirement for the presence of extracellular Ca^{2+} is common to other H_1-mediated effects described in intact cell preparations from nervous tissue, such as increase in cGMP (11, 12) and potentiation of cAMP accumulation induced by directly acting agonists (13, 14). Even the HA-stimulated phosphoinositide hydrolysis shows a strong extracellular Ca^{2+} dependency, in contrast to what occurs with other agonists (4, 15). This suggests that HA might cause increases in the intracellular Ca^{2+} concentration not only through the inositol tris-

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ABBREVIATIONS: HA, histamine; DMEM, Dulbecco's modified Eagle's medium; EMEM, minimal Eagle essential medium; FBS, fetal bovine serum; dBcAMP, N^6,2′-*O*-dibutyryl 3′:5′-cyclic monophosphate; TPA, 12-*O*-tetradecanoyl-phorbol-13 acetate; TEA, 2-thiazolylethylamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

phosphate-induced release from intracellular stores (16) demonstrated in astrocytoma cells (17, 18) but also through Ca^{2+} entry, as appears to occur in neuroblastoma cells (19, 20). Both intracellular Ca^{2+} mobilization and Ca^{2+} entry mediated by H_1 receptors have been described in muscle cells (21).

Astrocyte primary cultures are able to accumulate glycogen (22, 23) and regulate its metabolism by mechanisms similar to those operating in brain (24). Physiological increases in K^+ concentration and neurotransmitters that stimulate cAMP formation and/or Ca^{2+} mobilization, such as norepinephrine, vasoactive intestinal peptide, adenosine, and serotonin, produce significant changes in the glycogen levels of those cultures (23, 25, 26), suggesting that neuronal activity may control mobilization of this important metabolic energy reserve.

In this work, we have used astrocyte-enriched cultures from rat brain to examine the effect of HA on [^{3}H]glycogen levels in [^{3}H]glucose-prelabeled cells. We show that HA is able to produce breakdown of the same pool of glycogen by stimulation of both H_1 and H_2 receptors, through independent mechanisms that seem to involve Ca^{2+} and cAMP, respectively. We also show by measuring unidirectional $^{45}Ca^{2+}$ fluxes that in these cells HA increases Ca^{2+} permeability. Preliminary accounts of this work have been published in abstract form (27, 28).

Materials and Methods

Chemicals. DMEM, EMEM, and FBS were obtained from Flow, D-[6-^{3}H(N)]glucose (30.2 Ci/mmol) from New England Nuclear, $^{45}CaCl_2$ (10–40 mCi/mg) from Amersham, penicillin G, streptomycin sulfate, histamine dihydrochloride, norepinephrine, promethazine, mepyramine, nifedipine, calcium ionophore A23187, dBcAMP, and TPA from Sigma, and amylo-1-6-glucosidase (from *Aspergillus nigrum*) from Boehringer Mannheim. Dimaprit, metiamide, and TEA were generous gifts from Smith, Kline and French.

Cell cultures. Astrocyte-enriched primary cultures were obtained from newborn rat brain hemispheres as previously described (4). Dissociated cells were seeded onto 30-mm diameter plastic Petri dishes, grown in a culture medium of 90% DMEM (25 mM glucose), 10% FBS, 20 units/ml penicillin, and 20 μg/ml streptomycin at 37° in a humidified atmosphere of 90% air/10% CO_2, and used at confluence after 16–23 days in culture.

Indirect immunocytochemical studies indicated that more than 90% of the cells present in the cultures were glial fibrillary acidic protein positive and less than 5% neurone-specific enolase positive.

[^{3}H]Glycogen assay. Confluent cultures grown as described above were incubated in 2 ml of a low glucose medium, consisting of 90% EMEM (5.5 mM glucose), 10% FBS, 20 units/ml penicillin, and 20 μg/ml streptomycin, for 48 hr before the cells were labeled in 1 ml of EMEM, containing 4–5 μCi of [^{3}H]glucose, for 140 min. Afterwards, the medium was removed and the cells were incubated for 10 min in 950 μl of HEPES-buffered saline (in mM: 70 NaCl, 5 KCl, 2.6 $CaCl_2$, 0.67 $MgSO_4$, 1.2 KH_2PO_4, 3 glucose, 27.5 $NaHCO_3$, 50 HEPES, pH 7.4) and antagonist drugs when present. Agonists were then added in a volume of 50 μl and cultures were incubated for an additional 20 min (unless indicated). To terminate the incubations, the media were aspirated and the cells were washed with 2 ml of ice-cold HEPES-saline. Monolayers were scraped from the dishes with a rubber spatula in 0.5 ml of the same buffer, transferred to plastic tubes, and sonicated. Samples were then placed in a water bath at 95° for 10 min and centrifuged (3000 × *g*, 10 min). Determination of [^{3}H]glycogen in the deproteinized samples was performed by the ethanol precipitation technique of Sølling and Esmann (29). Briefly, aliquots (150 μl) of the supernatants were pipetted on squares (2 × 2 cm) of Whatman filter paper (31 ET) and immersed in ice-cold 66% ethanol (10 ml/filter) for 20 min. Filters were washed twice with the same volume of 66% ethanol at room temperature for 1 more hr and afterwards were soaked for 5 min in acetone, dried, and placed in scintillation vials; glycogen was extracted by addition of 2 ml of boiling water. Fifteen milliliters of a toluene/Triton X-100 (2:1, v/v) scintillation fluid were added and radioactivity was counted with an efficiency of 50%. The ethanol washings completely removed [^{3}H]glucose from the filters, as demonstrated on filters where an aliquot of [^{3}H]glucose solution had been pipetted.

To verify that the ^{3}H-containing compound retained by the filters was [^{3}H]glycogen, 0.4 ml of the deproteinized supernatants from cells that had been collected in acetate buffer (0.1 M, pH 4.8) was incubated at 37° for 30 min in the presence or absence of 4 units of amylo-1-6-glucosidase. Tritium remaining on washed filters from samples incubated with the enzyme was 914 ± 68 dpm, compared with 13,926 ± 96 dpm (three experiments) in control samples.

Unidirectional $^{45}Ca^{2+}$ flux assays. For the unidirectional $^{45}Ca^{2+}$ efflux assay, cells were labeled by incubation in 1 ml of culture medium (90% DMEM, 10% FBS) with 2.5–5 μCi of $^{45}Ca^{2+}$ for 12–18 hr. Before the assay, media were aspirated and cells were rapidly washed two times with 2 ml of HEPES-buffered saline (in mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 10 glucose, 20 HEPES, pH 7.4) at 4°. Then cells were incubated at 37° in 2 ml of the same buffer (unless indicated), in the presence or absence of HA, for the indicated times. When antagonists were present, they were added in 10 μl 10 min before the end of the labeling period and were present throughout the washings and stimulation. For TPA pretreatment, the compound (0.2 μM final concentration) was present only during the last 15 min of the labeling period. Incubations were terminated by aspiration of the media and washing of the monolayers three times with 2 ml of a modified buffer containing lanthanum (in mM: 118 NaCl, 4.7 KCl, 3 $CaCl_2$, 0.5 EDTA, 5 $LaCl_3$, 20 HEPES, pH 7.4) at 4°. Aspiration of the medium and successive washings were completed in less than 8 sec/sample. Cells were left for 2 hr at room temperature in 0.5 ml of a solution containing 10 mM EGTA, 3% (w/v) Trition X-100, and 10 mM HEPES, pH 7.4, and the solubilized cells together with 0.5 ml of the same solution used to rinse the dishes were transferred to scintillation vials. Four milliliters of scintillation fluid were added and radioactivity was measured.

To measure unidirectional $^{45}Ca^{2+}$ influx, monolayers were washed and preincubated at 37° for 5 min in 2 ml of the HEPES-buffered saline. Afterwards, cells were incubated for 2 min in 0.9 ml of the same buffer with antagonists when used. Stimulations were started by addition of 2.5–5 μCi of $^{45}Ca^{2+}$ in 0.1 ml of buffer, with or without HA, at the indicated times. Incubations were terminated and cellular $^{45}Ca^{2+}$ was measured as described above for the $^{45}Ca^{2+}$ efflux studies.

Analysis of data. To calculate the average parameters (EC_{50}, n, and E_{max}) for HA-induced glycogenolysis, data obtained from different concentration-effect curves performed under the same conditions and expressed as percentage of the maximum effect were pooled and fitted to a single hyperbola, using the BMDP-AR nonlinear regression iterative program implemented on a VAX 11/785 system. Each point was weighted by the reciprocal of the variance associated with it. The equation fitted was $E = E_{max} \times HA^n/(EC_{50} + HA^n)$, where E is the percentage of [^{3}H]glycogen breakdown induced by a given HA concentration, EC_{50} is the HA concentration that elicits half maximal effect (E_{max}), and n is the Hill coefficient. The EC_{50} values for HA-stimulated $^{45}Ca^{2+}$ fluxes were averaged from those calculated from linear Hill transformations of concentration-effect curves. All experiments were performed at least in triplicate using Petri dishes from the same culture batch. Student's t test was used for statistical evaluation.

Results

Preliminary experiments designed to optimize [^{3}H]glucose incorporation into cellular glycogen showed that the incorporation was higher when the cultures were maintained for the last 48 hr in a lower glucose (5.5 mM) medium before they were incubated with [^{3}H]glucose (not shown). Under these condi-

tions, the incorporation of label into [^{3}H]glycogen increased linearly for the first 2 hr, reaching a steady state that was maintained for at least 1 more hr (Fig. 1). After 140 min, the time chosen for routine experiments, the incorporation of tritium into the cells was approximately 1% of that present in the medium. The rate of [^{3}H]glucose incorporation into glycogen, calculated from the linear part of the curve (Fig. 1), was 5.6 ± 2.1 nmol of glucose/mg of protein/hr (four experiments).

When prelabeled astrocyte cultures were incubated with HA (100 μM), there was a rapid and significant decrease in [^{3}H]-glycogen that reached a plateau (50–60% of basal levels) by 10 min (Fig. 2), whereas no change in the amount of [^{3}H]glycogen was observed in control cultures for at least 20 min. The glycogenolytic effect of HA was concentration dependent (Fig. 3), and maximal stimulation was attained at a HA concentration of 10 μM. Nonlinear Hill transformation of the data from four independent experiments allowed the calculation of an EC_{50} for HA of 0.57 ± 0.04 μM and a Hill coefficient (n) significantly lower than 1 (0.74 ± 0.03) (Table 1), which suggested the involvement of more than one component in the response to HA. Confirmation of this suggestion came from the study of the effect of HA (10 μM) in the presence of specific H_1 and H_2 antagonists at concentrations high enough to block their respective receptors. As shown in Table 2, the glycogenolytic effect of HA was not inhibited when either the H_1 antagonist promethazine (1 μM) or the H_2 antagonist metiamide (100 μM) were present separately but was completely abolished when both compounds were added together. Furthermore, both the more selective H_1 agonist TEA (300 μM) and the specific H_2 agonist dimaprit (1 mM) were as efficacious as HA in eliciting the response and their effects were not additive. The response to TEA was inhibited by promethazine but not by metiamide, whereas the opposite was true for the response to dimaprit (Table 2). Thus, it appears that in rat astrocytes HA is able to induce breakdown of the same pool of glycogen by stimulating either H_1 or H_2 receptors independently. In order

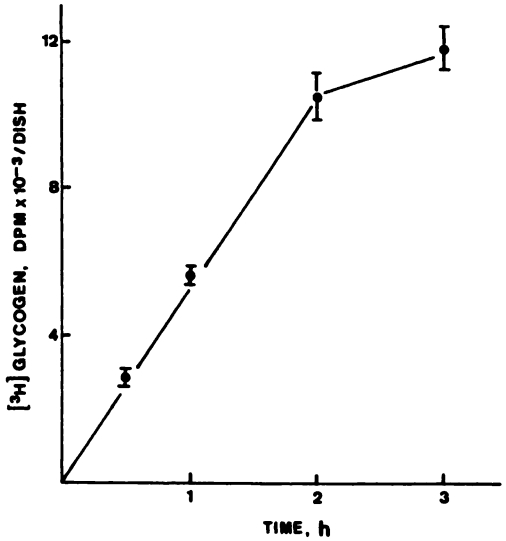

Fig. 1. Time course of [^{3}H]glucose incorporation into [^{3}H]glycogen. After 48 hr of incubation in a low glucose medium (90% EMEM, 10% FBS), astrocyte cultures were incubated with [^{3}H]glucose (5 μCi/dish, 5.5 mM final concentration) for the indicated times and [^{3}H]glycogen was determined as indicated in Materials and Methods. Results are means ± standard errors of triplicate determinations from a representative experiment, which was repeated three times with similar results.

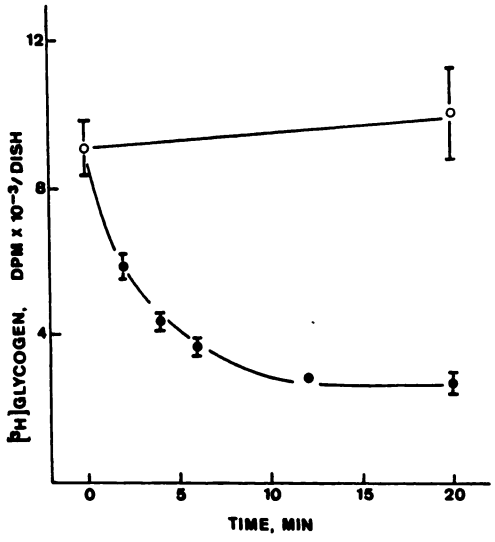

Fig. 2. Time course of HA-stimulated [^{3}H]glycogen breakdown. Astrocyte cultures prelabeled with [^{3}H]glucose (5 μCi/dish) for 140 min were incubated in the presence (●) or absence (○) of 100 μM HA for the indicated times. Results are means ± standard errors of triplicate determinations from a single experiment, which was replicated once.

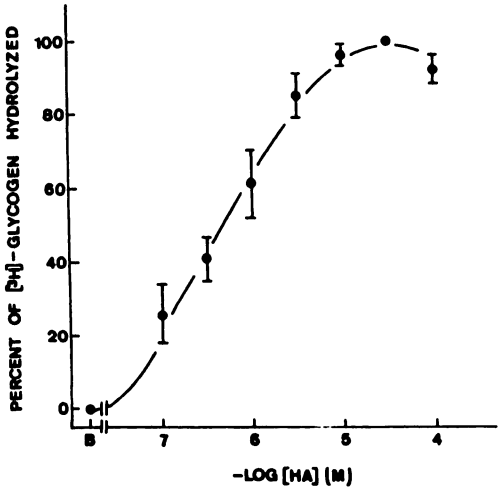

Fig. 3. Concentration-effect relationship for HA-induced [^{3}H]glycogen breakdown. Astrocyte cultures prelabeled with [^{3}H]glucose were incubated for 20 min with increasing concentrations of HA. Results, expressed as percentages of the maximal stimulation induced by HA, are means ± standard errors of four independent experiments. [^{3}H]Glycogen levels in controls (B) and after HA maximal stimulation were 6310 ± 782 and 2866 ± 386 dpm/dish, respectively.

TABLE 1

Concentration-response parameters for HA stimulation of glycogenolysis in rat astrocytes

Concentration response curves for HA were generated in the absence or presence of 1 μM promethazine or 100 μM metiamide. Maximal effects (E_{max}), potencies (EC_{50}), and Hill coefficients (n_H) were estimated by fitting the data from three or four curves, in each case, to a Hill equation, using the BMDP-AR nonlinear regression iterative program, as described in Materials and Methods.

Compound	E_{max}	EC_{50}	n_H
	% of glycogen hydrolyzed	μM	
HA	55.0 ± 4.2	0.57 ± 0.04	0.74 ± 0.03
HA + promethazine (1 μM)	54.3 ± 0.8	3.30 ± 0.16	0.90 ± 0.02
HA + metiamide (100 μM)	52.7 ± 1.4	0.40 ± 0.04	0.86 ± 0.06

TABLE 2

Glycogenolytic effect of histaminergic agonists and antagonists in rat astrocyte primary cultures

Astrocyte cultures prelabeled with [^{3}H]glucose were incubated for 20 min with agonists. Antagonists, when present, were added 10 min before. Results are expressed as percentage of the effect induced by 10 μM HA and are means ± standard errors of *n* independent experiments. [^{3}H]Glycogen levels of controls and HA-stimulated cells were 7770 ± 1132 and 3820 ± 760 dpm/dish, respectively.

Drug	Effect	*n*
	% of HA effect	
HA (10 μM) + promethazine (1 μM)	80 ± 5	4
HA (10 μM) + metiamide (100 μM)	96 ± 4	3
HA (10 μM) + promethazine (1 μM) + metiamide (100 μM)	2 ± 1	4
TEA (300 μM)	104 ± 5	4
Dimaprit (1 mM)	103 ± 2	4
TEA (300 μM) + dimaprit (1 mM)	102 ± 2	3
Dimaprit (1 mM) + metiamide (100 μM)	24 ± 9	4
Dimaprit (1 mM) + promethazine (1 μM)	86 ± 8	4
TEA (300 μM) + metiamide (100 μM)	80 ± 8	4
TEA (300 μM) + promethazine (1 μM)	17 ± 7	4

TABLE 3

Glycogenolytic effect of different compounds in rat astrocytes

Prelabeled astrocyte cultures were incubated for 20 min with the indicated compounds. Results, expressed as percentage of the [^{3}H]glycogen hydrolyzed, are means ± standard errors of *n* independent experiments.

Compound	Effect	*n*
	% of [^{3}H] glycogen hydrolyzed	
HA (100 μM)	52 ± 4	9
dBcAMP (1 mM)	59 ± 3	6
A23187 (10 μM)	60 ± 3	6
Norepinephrine (100 μM)	65 ± 4	2
HA (100 μM) + dBcAMP (1 mM)	64 ± 3	2
HA (100 μM) + A23187 (10 μM)	62 ± 3	2
dBcAMP (1 mM) + A23187 (10 μM)	69 ± 4	2
HA (100 μM) + norepinephrine (100 μM)	67 ± 3	2

to characterize the response further, we measured the concentration-effect relationships for HA in the presence of 1 μM promethazine or 100 μM metiamide. Results are summarized in Table 1 and show that 1) the maximal glycogenolytic response to HA is the same regardless of the blockade of H_1 or H_2 receptors by specific antagonists; 2) in the presence of metiamide, the potency of HA was higher (EC_{50} = 0.40 ± 0.04 μM) than in the presence of promethazine (EC_{50} = 3.30 ± 0.16 μM), in accord with the higher potency shown by HA acting at H_1 compared with H_2 receptors in these cells (4, 5)[1]; and 3) Hill coefficients of the curves in the presence of the antagonists were closer to unity, suggesting that under those conditions only one component of the response to HA was evident.

Because in astrocytes H_2 receptors mediate increases in cAMP and H_1 receptors mediate stimulation of phosphoinositide hydrolysis (4, 5),[1] both cAMP- and Ca^{2+}-dependent regulatory mechanisms may be involved in the HA modulation of glial glycogen turnover. As shown in Table 3, both the cAMP analog dBcAMP and the Ca^{2+} ionophore A23187 elicit glycogenolytic responses of a magnitude similar to those of HA, when present either separately or together, and their effects are not additive to that of HA. Also not additive is the effect of norepinephrine, another agonist that stimulates cAMP formation and phosphoinositide hydrolysis in astrocytes (30, 31).

All the H_1-linked responses described in nervous tissues show a strong dependency on the presence of extracellular Ca^{2+} (4, 10–14). Thus, we investigated whether the H_1 component of the glycogenolytic effect of HA also required extracellular Ca^{2+}. When we measured the response to HA in a Ca^{2+}-free medium containing 0.5 mM EGTA (Table 4), the percentage of decrease in [^{3}H]glycogen was lower than in medium containing Ca^{2+}, which may result from the reduction in basal [^{3}H]glycogen content observed in all experiments under those conditions, whereas the decrease induced by HA reached the same absolute levels. Nevertheless, the HA effect was completely blocked by 100 μM metiamide but not affected by 1 μM promethazine. Thus, the absence of extracellular Ca^{2+} eliminates the H_1 component of the response, suggesting a need for Ca^{2+} entry in order for it to be observed. In light of these results, we then investigated whether HA was actually causing Ca^{2+} entry and/or mobilization in astrocytes, by measuring stimulation of unidirectional $^{45}Ca^{2+}$ fluxes. As shown in Fig. 4, HA stimulates both $^{45}Ca^{2+}$ uptake into the cells and $^{45}Ca^{2+}$ efflux from preloaded cells. Maximal $^{45}Ca^{2+}$ influx was of greater magnitude and appeared to be attained more rapidly (48 ± 9% of basal by 15 sec; three experiments) than maximal $^{45}Ca^{2+}$ release (25 ± 1% 1 min; three experiments), indicating that HA-induced Ca^{2+} entry could be an early event and not a consequence of Ca^{2+} mobilization. Both effects were concentration dependent (Fig. 5) and showed similar EC_{50} values for HA (0.60 ± 0.31 μM and 0.40 ± 0.10 μM, three experiments, for $^{45}Ca^{2+}$ influx and efflux, respectively) but, whereas the concentration-effect curve for $^{45}Ca^{2+}$ influx presented a Hill coefficient not different from 1 (0.90 ± 0.24), that corresponding to $^{45}Ca^{2+}$ efflux was significantly lower (0.67 ± 0.04), indicating a more complex nature of this latter response. In both cases, the effect elicited by 5 μM HA was inhibited by 1 μM mepyramine but not by 100 μM metiamide (not shown), as expected for H_1 receptor mediation. The HA-stimulated $^{45}Ca^{2+}$ entry was abolished by the nonspecific Ca^{2+} channel blocker lanthanum (0.1 mM) and was not affected by the voltage-operated Ca^{2+} channel inhibitor nifedipine (10 μM) (Fig. 6A); thus, dihydropyridine-sensitive channels do not seem to be involved. On the other hand, the observed unidirectional $^{45}Ca^{2+}$ efflux seems to be due to a large extent to the HA-increased Ca^{2+} permeability, because, when $^{45}Ca^{2+}$ release was measured under conditions that prevent Ca^{2+} entry, i.e., in Ca^{2+}-free medium or in the presence of 0.1 mM lanthanum, the effect of HA decreased to around 30% of that elicited in the presence of Ca^{2+} (Fig. 6B). In agreement with this, preincubation of the cells for 15 min with 0.2 μM TPA, treatment that inhibits agonist-stimulated phosphoinositide

TABLE 4

Effect of Ca^{2+} on HA-induced glycogenolysis in rat astrocytes

Labeled astrocytes were preincubated and stimulated in HEPES-buffered saline containing 2.6 mM $CaCl_2$ or no $CaCl_2$ plus 0.5 mM EGTA. Results are means ± standard errors of three independent experiments.

Compound	[^{3}H]Glycogen	
	dpm/dish	*% hydrolyzed*
With Ca^{2+}		
Control	6924 ± 1820	
HA (10 μM)	2814 ± 588[a]	59 ± 3
Without Ca^{2+} + EGTA		
Control	4918 ± 1,022	
HA (10 μM)	2840 ± 528[b]	42 ± 5
HA (10 μM) + metiamide (100 μM)	4386 ± 626	9 ± 6
HA (10 μM) + promethazine (1 μM)	2714 ± 350[a]	44 ± 7

[a] $p < 0.01$, significantly different from controls in each experiment by Student's *t* test.
[b] $p < 0.05$.

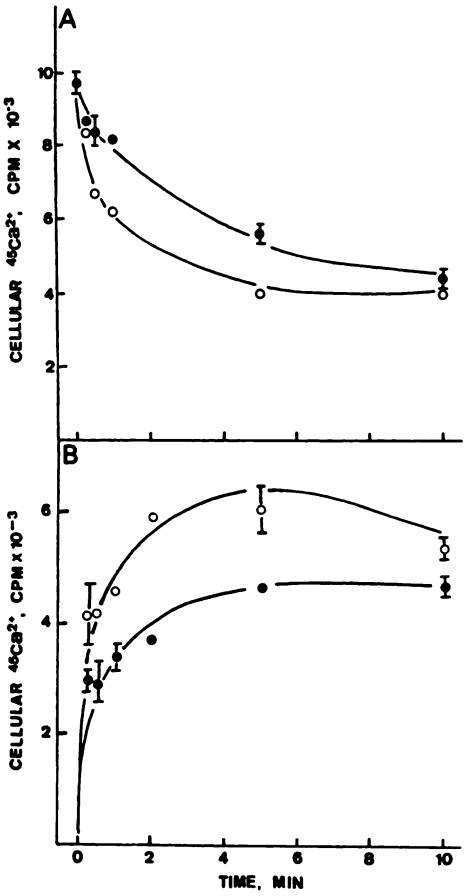

Fig. 4. Time courses of unidirectional $^{45}Ca^{2+}$ fluxes. Astrocyte cultures were incubated in 1 ml of HEPES-buffered saline with (○) or without (●) 1 mM HA for the indicated times. To measure unidirectional $^{45}Ca^{2+}$ efflux (A), cells prelabeled overnight with $^{45}Ca^{2+}$ (4.8 μCi/dish) were then incubated in a buffer without $^{45}Ca^{2+}$. To measure unidirectional Ca^{2+} influx (B), cells were incubated in the presence of 2.5 μCi of $^{45}Ca^{2+}$. Cellular $^{45}Ca^{2+}$ was measured as described in Materials and Methods. Results are means ± standard errors of triplicate determinations from representative experiments, which were repeated two times with similar results.

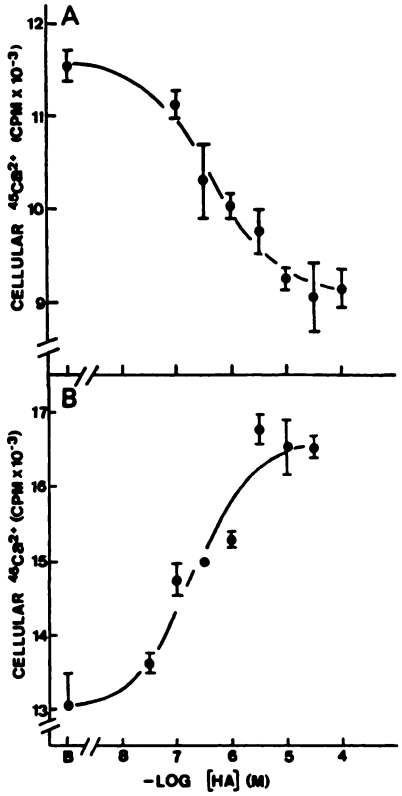

Fig. 5. Concentration-response curves for HA-induced unidirectional $^{45}Ca^{2+}$ fluxes. A, Astrocyte cultures prelabeled overnight with $^{45}Ca^{2+}$ (4.4 μCi/dish) were washed free of $^{45}Ca^{2+}$ and incubated for 1 min with increasing HA concentrations. B, Cells were incubated for 2.5 min with $^{45}Ca^{2+}$ (5.0 μCi/dish) and increasing concentrations of HA. Results are means ± standard errors of triplicate determinations from representative experiments, which were repeated two times with similar results.

hydrolysis and $^{45}Ca^{2+}$ mobilization in astrocytes (32), leaves 79 ± 9 (two experiments) of the HA-evoqued $^{45}Ca^{2+}$ efflux unaffected (Fig. 6C).

Discussion

In this work, we show that, in astrocyte-enriched cultures from rat brain prelabeled with [^{3}H]glucose, HA induces a rapid and marked decrease in [^{3}H]glycogen. The glycogenolytic response to a maximally effective concentration of HA observed by us is higher than that obtained by Magistretti *et al.* (23), using the same technique but somewhat different astrocyte culture preparations, and similar to the responses the same authors found for norepinephrine (also in this work), adenosine, and vasoactive intestinal peptide.

In contrast to mouse cortex, where the glycogenolytic response to HA appears to be mediated exclusively by H_1 receptors (10), in rat astrocyte cultures the HA effect can be mediated by both H_1 and H_2 receptors through independent mechanisms acting on the same glycogen pool, as demonstrated by the observations that 1) neither an H_1 (promethazine) nor an H_2 (methiamide) antagonist inhibits the response to HA unless the antagonists are present simultaneously; 2) both an H_1 (TEA) and an H_2 (dimaprit) agonist are able to elicit separately the same response as HA; and 3) these agonist effects, which are specifically inhibited by H_1 and H_2 antagonists, respectively, are not additive. The participation of both H_1 and H_2 receptors in the glycogenolytic effect of HA was also suggested to occur in chick brain (7) and was demonstrated in rat hepatocytes (33), although in these cases the H_1- and H_2-mediated effects seem to be additive.

Concentration-response curves for HA generated under conditions of blockade of either H_1 or H_2 receptors with specific antagonists allowed the calculation of an EC_{50} for the amine acting at H_1 receptors of 0.4 μM, which is comparable to that we found earlier for the stimulation of phosphoinositide hydrolysis (1.7 μM) (4) and on the same order as those reported for the glycogenolytic response in mouse brain (10) and hepatocytes (33). However, the calculated EC_{50} at H_2 receptors (3.3 μM) was considerably lower than that obtained for the stimulation of cAMP accumulation (43 μM) mediated by H_2 receptors in the same cultures (5).[1] This higher potency of HA in glycogenolysis, compared with cAMP formation, together with the observation that dimaprit, which behaves as a partial agonist in the cAMP system (5),[1] is as efficacious as HA eliciting glycogen breakdown, suggests the existence of spare H_2 receptors in rat astrocytes for this response.

Meningeal fibroblasts are the major contaminating cell type in astrocyte cultures, and meningeal cultures have been shown

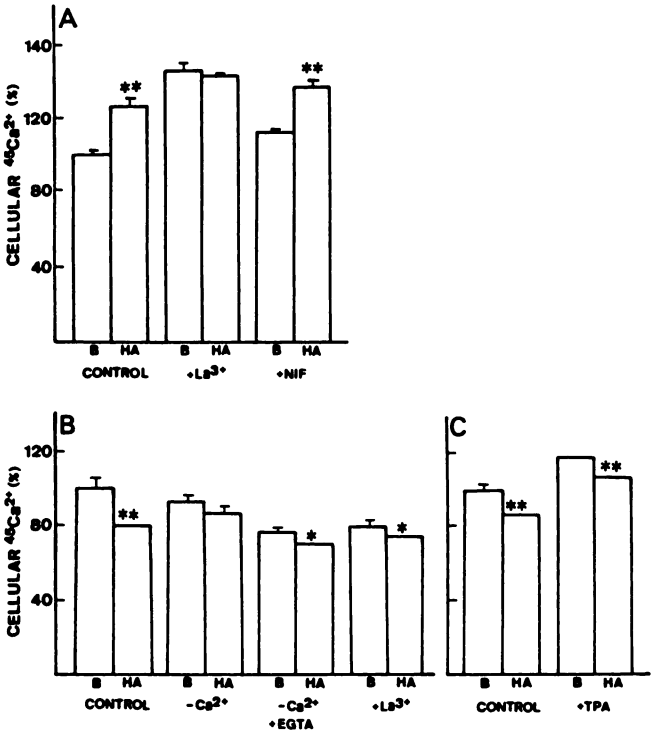

Fig. 6. Effect of different treatments on HA-induced unidirectional $^{45}Ca^{2+}$ fluxes. A, Astrocytes were incubated with $^{45}Ca^{2+}$ (3.2 μCi/dish) for 2.5 min in the absence (*B*) or presence of 10 μM HA under control conditions or with $LaCl_3$ (0.1 mM) or nifedipine (*NIF*) (10 μM). B, Cells prelabeled overnight with $^{45}Ca^{2+}$ (3.4 μCi/dish) were stimulated for 1 min with 100 μM HA under control conditions or in buffer containing no $CaCl_2$, no $CaCl_2$ plus EGTA (0.1 mM), or $LaCl_3$ (0.1 mM). C, Cells prelabeled overnight with $^{45}Ca^{2+}$ (5.5 μCi/dish) and incubated with or without TPA (0.2 μM) for 15 min, as indicated in Materials and Methods, were stimulated for 1 min with 10 μM HA. Results, expressed as percentages of basal $^{45}Ca^{2+}$ in controls (A, 5,611 ± 98 cpm/dish; B, 5,260 ± 322 cpm/dish; C, 10,015 ± 368 cpm/dish) are means ± standard errors of three or four determinations. Experiments A and C were repeated with similar results. $^{**}p < 0.01$; $^{*}p < 0.05$, significantly different from basal levels by Student's *t* test.

to contain an appreciable amount of glycogen (25). However, meningeal fibroblast contributions to the glycogenolytic effect of HA observed in our astrocyte cultures are unlikely, because we have not been able to detect functional H_1 or H_2 receptors in meningeal cell cultures (4).[1] On the other hand, some authors have reported that neurons are also able to accumulate glycogen (34). Contribution to the response from the very few neurons detectable by indirect immunocytochemical techniques in the astrocyte cultures was also ruled out, because, using neuronal cultures prepared from embryonic rat brain, we have observed that these cells can incorporate [^{3}H]glucose into [^{3}H]glycogen at a slow rate (two times slower than in astrocytes) and they do not show glycogenolytic response to HA.[2]

In brain, as occurs in muscle and liver (35, 36), glycogen breakdown can be induced through a cAMP-dependent activation of glycogen phosphorylase (37), as well as by Ca^{2+} activation of phosphorylase *b* kinase (38, 39). These two mechanisms seem to be operating also in astrocytes. Thus, various substances that increase cAMP levels in these cells as well as the synthetic analog dBcAMP and high K^+ concentrations, which would elevate intracellular Ca^{2+}, have been shown to induce glycogen breakdown (23, 25). In this work we show that dBcAMP and the Ca^{2+} ionophore A23187 are both able to promote glycogenolysis in the astrocyte cultures to the same extent and their effects are not additive, an indication that both operate on the same glycogen reservoir(s). The magnitude of the response to those compounds is similar to that to norepinephrine, an agonist that can increase both cAMP and intracellular Ca^{2+} through activation of β- and α_1-adrenergic receptors demonstrated to be present in astrocytes (30, 31). The slightly higher glycogenolytic response to these agents observed here, compared with HA, could be explained if, as has been suggested (26), they have an effect on the glycogen content of the contaminating meningeal fibroblasts whereas HA has not. In neither case, however, is their response additive to that of HA, which is most probably also acting through cAMP- and Ca^{2+}-dependent mechanisms, because, as already mentioned, in astrocytes the amine stimulates cAMP formation and phosphoinositide hydrolysis and in this latter case intracellular Ca^{2+} mobilization by the inositol trisphosphate product is expected. Also, Ca^{2+} entry into the cells seems to be involved, as judged by the extracellular Ca^{2+} dependency of the H_1-mediated glycogenolytic response, also reported in mouse brain (10) and rat hepatocytes (33). An increase in Ca^{2+} uptake induced by HA in astrocytes is shown here by a significant stimulation of $^{45}Ca^{2+}$ influx that is of greater magnitude than the HA-evoked $^{45}Ca^{2+}$ efflux from preloaded cells. Furthermore, a large portion of this latter effect seems to be due to the HA-increased Ca^{2+} permeability rather than to intracellular Ca^{2+} mobilization, as indicated by the greatly diminished effect of HA when Ca^{2+} entry is prevented and the observation that pretreatment of the cells with TPA, which abolishes agonist-stimulated phosphinositide turnover and subsequent Ca^{2+} mobilization (32), only affects to a small extent the HA-stimulated $^{45}Ca^{2+}$ release. This contrasts with the effect of other agonists that also promote phosphoinositide hydrolysis and Ca^{2+} mobilization in astroglial cells but induce little or no Ca^{2+} entry (40, 41). On the other hand, we showed previously that the HA-stimulated [^{3}H]inositol phosphate accumulation in astrocytes is absolutely dependent on the presence of extracellular Ca^{2+} (4), whereas only a partial dependency was reported for the response to other agonists such as carbachol and norepinephrine (31). Taking these observations together, it is not unreasonable to think that the H_1-mediated stimulation of phosphoinositide phospholipase C in astrocytes is largely due to the increase in intracellular Ca^{2+} provoked by the HA-stimulated extracellular Ca^{2+} uptake. Thus, H_1 receptors in these cells may be coupled to two different effector systems, i.e., GTP-binding protein-linked phospholipase C (42) and gating of extracellular Ca^{2+}, in a similar way as has been proposed for α_1-adrenergic receptors in muscle cells (43).

Although both H_1 and H_2 receptors can mediate mobilization of astrocyte glycogen, at low HA concentrations only stimulation of H_1 receptors would be physiologically relevant. However, involvement of the two types of receptors linked to different effector systems offers the amine more possibilities for homologous and heterologous regulation of glycogen metabolism. Glial glycogen stores constitute the largest energy reserve in brain, which may be vital for sustaining increased neural activity. Interestingly, low glucose concentrations enhance the K^+-induced HA release from mouse hypothalamus, in a process that seems to involve Na^+ channels (44). Thus, release of

[1] L. Arbonés, F. Picatoste, and A. Garcia, unpublished observations.

neuronal HA may be one of the signals from neurons to glial cells that contribute to ensure a continuous energy supply during normal and intense activity if glucose levels fall.

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