Role of Receptors in Metabolic Interaction of Histamine with Human Vascular Endothelial Cells and Skin Fibroblasts

AN ORDERED SEQUENCE OF ENZYME ACTION*

(Received for publication, December 29, 1986)

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The interaction of histamine with an H1 receptor on human endothelial cells evokes production of the lipid mediator prostaglandin I2 (PGI2) and is accompanied by tachyphylaxis of this H1 receptor response (Baenziger, N. L., Fogerty, F. J., Mertz, L. F., and Chernuta, L. F. (1981) Cell 24, 915–923). We have explored the affected cells' capability for subsequent metabolic degradation of histamine molecules. Human vascular endothelial cells and skin fibroblasts exhibit a two-stage histamine degradation sequence whose participants are an enzyme native to the cells themselves and one provided from an extracellular source. Initially, the cells' endogenous histamine N-methyltransferase activity mediates conversion of cell-associated [3H]histamine to tele-methylhistamine with retention of this intermediate metabolite. Subsequently, in the presence of exogenous diamine oxidase derived from fetal calf serum or human placenta, cell-associated tele-methylhistamine is further converted to the end product methylimidazoleacetic acid. After an initial lag phase lasting 3–6 min, the cell-associated radioactivity accumulates as methylimidazoleacetic acid at a linear rate substantially enhanced over that without diamine oxidase. The entire sequence is blocked by the histamine methyltransferase inhibitor homomaprit. Accumulation of [3H]histamine metabolites by endothelial cells is saturable both with respect to exogenous diamine oxidase and to histamine. Thus this metabolic pathway is carried out at the level of the individual cell by means of binding sites or receptors for the substrate and for the distal degradative enzyme, diamine oxidase.

Histamine (β-imidazolylyethamaine) is a mast cell-derived mediator which affects organ functions such as vascular tone, vascular permeability, and gastric acid secretion by interaction with cell surface receptors. Ash and Schild (1966) and Black et al. (1972) defined the properties of distinct H1 and H2 histamine receptor subtypes on the basis of specific antagonist and agonist structure/activity relationships. Cellular pathways activated by H1 and H2 receptors include those for synthesis of lipid mediators and cyclic nucleotides. We and others have demonstrated a histamine H1 receptor stimulating production of lipid mediators such as prostacyclin in cultured human vascular endothelial cells (Baenziger et al., 1980, 1981; McIntyre et al., 1985; Revtyak et al., 1985) so that H1-mediated vascular events may reflect the action of one or more of these lipid secondary mediators. Cyclic GMP and cyclic AMP formation in neural cells is linked to H1 and H2 receptors, respectively (Hill et al., 1977; Study and Greengard, 1978), and gastric acid secretion is an H2 receptor-mediated activity. These receptors initially outlined on the basis of biologic activities have also been explored via labeled antagonist or agonist binding (Blank and Hollis, 1986; Heltianu et al., 1982; Richelson, 1978; Toll and Snyder, 1982; Tran et al., 1981). Recent investigations are continuing to uncover new biologic activities and binding sites such as a reported H3 subtype; thus, other receptor interactions are available for histamine in addition to the classical H1 and H2 species (Arrang et al., 1983; Barbin et al., 1980; Palacios et al., 1978; Trzeciakowski and Cox, 1986).

Our previous studies of histamine action on endothelial cells have indicated that this ligand-target cell interaction results in modulation of H1 receptor biologic activity. We found that stimulation of the human endothelial cell H1 receptor to cause PGI2 production establishes a period of refractoriness or tachyphylaxis to histamine lasting several hours, a time frame during which the cells are still capable of responding to another agonist (thrombin) interacting at a separate receptor. Recovery from tachyphylaxis to histamine is dependent on the presence of fetal calf serum in a time- and concentration-dependent manner and is blocked in the continued presence of histamine (Baenziger et al., 1981; Fogerty et al., 1981). We have subsequently identified and characterized a histamine-metabolizing activity present in fetal calf serum and sought to uncover its relationship to our observed recovery from histamine tachyphylaxis (Fogerty and Baenziger, 1983, 1984; Haddock and Baenziger, 1985). In an analogous system Roscher et al. (1984) have shown that human skin fibroblast bradykinin receptor activity which mediates prostacyclin formation is regulated through degradation of cell-associated ligand; they have suggested that a similar regulatory mechanism may apply to other receptor systems where ligands are rapidly degraded at their site of action. The degradation of a ligand while in a state of association with the target cell poses interesting questions about the cellular mechanisms involved. What is the source of the degradative activity? Is ligand degraded while bound to a

* This work was supported by United States Public Health Service Grant HL 33699 (to N. B.) and National Research Service Award 2T32 AM07296 (to R. C. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PGI2, prostaglandin I2; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

2 N. L. Baenziger, L. F. Mertz, and F. J. Fogerty, manuscript in preparation.

3 F. J. Fogerty, P. Mack, and N. L. Baenziger, manuscript in preparation.
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Histamine is known to be degraded rapidly in vivo, and its degradation at the whole organ and organism level has been identified in mammals in terms of two alternative pathways: (a) direct oxidation of the aminothiol side chain by diamine oxidase, yielding imidazoleacetic acid and (b) methylation at the γ-nitrogen of the imidazole ring by histamine methyltransferase (EC 2.1.1.8), yielding tele-methylhistamine, followed by side chain oxidation by diamine oxidase (EC 1.4.3.6) or type B monoamine oxidase to give as the final product methylimidazoleacetic acid (Reilly and Schayer, 1971; Rothscchild and Schayer, 1958; Schayer, 1974; Schayer and Reilly, 1975; Suzuki et al., 1979). However, the detailed mechanisms by which these degradative steps are accomplished at the cellular level have remained to be completely defined. We have therefore explored in depth the degradation of [3H]histamine by intact cultured human vascular endothelial cells and fibroblasts in terms of the enzymatic steps involved and the products generated and accumulated by these cells.

EXPERIMENTAL PROCEDURES

Materials—1,25-3H]Histamine dihydrochloride was obtained from Amersham Corp., fetal calf serum from Kansas City Biological, Lenera, KS, and human serum albumin from Cutter Biological, Berkeley, CA. Unlabeled histamine dihydrochloride was from Sigma. Human umbilical cords were provided by the National Diabetes Research Interchange, Philadelphia and by our cooperating local hospitals as were samples of human tissues used as sources of primary fibroblasts. Additional fibroblasts were purchased from the National Institute of General Medical Sciences Institute for Medical Research, Camden, NJ. Tele-methylhistamine, imidazoleacetic acid, and methylimidazoleacetic acid standards were from Behring Diagnostics, and homodimaprit (SKF91488) was a gift from Smith Kline & French Laboratories.

Cell Culture—Human umbilical vein endothelial cells were isolated by a modification of the method of Jaffe et al. (1973) and cultured in medium 199 with either 20% fetal calf or human serum or 20% human plasma and 0.06-0.15 A280 units/mL of bovine hypothalamic extract (Maciag et al., 1979). They were used as confluent monolayers at either primary level or passage 3-10, generally of a purity of 0.5-0.8 X 10^6 cells/25-cm Costar well except where noted. Human skin fibroblasts were isolated from surgically removed dermis and cultured in α-minimum Eagle’s medium with 10% fetal calf serum or 10% human serum. Cells were used at passage 3-15 as confluent monolayers generally containing 0.3-1.2 X 10^5 cells/8-mm well.

3H]Histamine Uptake Experiments—[3H]Histamine uptake was measured as a function of incubation time or of varying radioligand concentration at 10- and 30-min time points. Cells were incubated at 37°C with [3H]histamine in either medium 199, a pH 7.4 HEPES-buffered saline described previously (Baenziger et al., 1981), or Hank’s balanced salt solution, 15 mM HEPES, pH 7.2-7.5. These incubation media contained either (a) 10 mg/mL human serum albumin (denoted "serum-free medium"), (b) 20-50% v/v fetal calf serum, or (c) 10 mg/mL human serum albumin and aliquots of diamine oxidase activity derived from fetal calf serum or human placenta. Incubations were terminated by chilling on ice and washing three times with cold Dulbecco’s phosphate-buffered saline, and the cellular content of accumulated radiolabel was determined by removing the cells from wells with 0.25% trypsin in Hanks’ balanced salt solution and lysing with Cetrime detergent solution (82.3 mM hexadecyltrimethylammonium bromide, 1.2 mM disodium EDTA, and 141.5 mM sodium chloride) to release nuclei. Duplicate aliquots of lysate were counted in a liquid scintillation spectrometer for assessment of [3H]histamine uptake and a Model ZF Coulter Counter for enumeration of cell nuclei. Some experiments utilized 16-mm Costar wells containing 0.8-2.5 X 10^5 cells with final solubilization in 0.1 N NaOH for determination of total cell-associated radioactivity or trypsinization for a representative cell count determination by hemacytometer.

Specific [3H]histamine uptake was calculated as total uptake minus the nonspecific uptake occurring in the presence of 44-100 µM unlabeled histamine. The endothelial cell rate of uptake by fluid phase pinocytosis was assessed by similar experiments measuring uptake of 3.5 µM [3H]fibrinogen.

Analysis of Cell-associated Products—All product analysis experiments were performed with [3H]histamine repurified by the miniature thin-layer chromatographic method described below. Those endothelial cells and fibroblasts which had been cultured in fetal calf serum were washed and transferred into medium containing 20% human serum at least 12 h before the experiments. Cells in 35-mm wells were incubated 10-30 min at 37°C with 1 µM repurified [3H]histamine in 800 µL of medium without or with 20% fetal calf serum. Cells were scraped with 800 pl of medium without or with ice-cold phosphate-buffered saline. The cell-associated radioactivity was extracted at 4°C by scraping with 800 µL of 1.7 M acetic acid, and the contents were transferred to 12 X 75-mm polypropylene tubes by rinsing with 3.2 ml of 100% ethanol. Extraction mixtures were vortexed and centrifuged at 1000 x g for 15 min. The supernatant was removed to a new tube, dried under a stream of nitrogen, and then dissolved in 30 µL of ethanol, and its components were separated by thin-layer chromatography.

A miniature thin-layer chromatographic procedure for separation of histamine metabolites was developed via modification of larger scale methods. Lown and Wragg’s solvent systems (Lown and Wragg, 1968; Schwartzman, 1973; White, 1966). Samples with 25-50 µg of histamine, tele-methylhistamine, and/or methylimidazoleacetic acid standards added were spotted on a 1-cm-wide line 1 cm from the bottom of a 8 x 2.5-cm silica gel chromatography plate (Eastman Chromagram 250 µm thickness without fluorescent indicator). For development, standards of [3H]histamine, standards of histamine, telemethylhistamine, and/or methylimidazoleacetic acid were spotted simultaneously on separate parallel plates. The thin-layer plates were developed with solvent system I, ethanol-chloroform-ammonia (8:2:16:1.6), modified from Navert et al. (1969), poured to a depth of 0.5 cm in a 4 X 9 X 9-cm chromatography tank (Eastman Kodak). Alternating chromatographic solvent systems also used were: solvent system II, benzene:ethanol:ammonia (80:18:2) (White, 1966) and solvent system III, butanol:acetic acid:water (60:15:15) (Schwartzman, 1973). When the solvent front reached the top in approximately 30 min, plates were dried and stained with iodine vapor, and the standards were circled with pencil. Metabolites of metabolites in all systems were verified by chromatographing each standard alone. In addition, the position of methylmethanol acetic acid in these solvent systems was confirmed by visualizing the standard on the developed plate with 0.02% bromocresol green in acetone (Schippert et al., 1979). The plates were coated with ENHiCARE, Du Pont-New Englad Nuclear, mounted against x-ray film (Kodak XAR-5), and exposed for 3-10 weeks at ~80°C.

Preparation of Exogenous Amine Oxidase—The purification, properties, and histamine assay developed for fetal calf serum diamine oxidase are described in detail elsewhere (Fogerty and Baenziger, 1983, 1984). Briefly, the enzyme from whole fetal calf serum at an initial specific activity ranging from 0.15 to 0.25 units/mg has been purified by two alternative procedures, either (a) sequential affinity chromatography on concanavalin A-Sepharose, aminohexyl-Sepharose, and Blue Sepharose, yielding enzyme with a maximal specific activity of 187 units/mg, or (b) affinity chromatography on putrescine-Affi-Gel 10 followed by ion exchange chromatography on DEAE-Sepharose, yielding enzyme with a maximal specific activity of 200 units/mg. A cytosolic fraction of human placenta containing diamine oxidase activity was prepared by rinsing fresh human placenta in 0.02 M HEPES, pH 7.5, at 4°C and homogenizing in 0.02 M HEPES, pH 7.5, in an industrial Waring Blender at a low setting for 3 X 30-s cycles and at a medium setting for 5 X 30-s cycles. The homogenate was centrifuged at 48,000 X g for 30 min, and the supernatant containing 45 units/mg of diamine oxidase used directly as the source. Activity of both enzymes for these studies was measured by the method of Okuyama and Kobayashi (1961) utilizing 4.4 µM putrescine as the substrate. One unit is defined as 1 nmol of [14C]putrescine substrate degraded per minute under the particular assay conditions (Navert et al., 1969). Specific histamine degradation by both enzymes are very similar to those for putrescine degradation (Bardsey et al., 1974; Fogerty and Baenziger, 1983, 1984; Paolucci et al., 1971).

RESULTS

Accumulation of [3H]Histamine Radiolabel by Endothelial Cells—We have previously observed that interaction of his-
Histamine with endothelial cells at the H₁ receptor results in a burst of PGI₂ production which ceases within 4 min, concomitant with the development of the desensitized state (Baenziger et al., 1980, 1981). We therefore incubated cells with [³H]histamine in a serum-free medium at the concentrations used for evoking PGI₂ production and desensitization to ascertain the fate of this ligand. Fig. 1 indicates that the cells accumulated or took up radiolabel in a time-dependent manner. A brief initial phase of rapid uptake occurred within the first 10 min, yielding approximately 6-23 fmol/10⁶ cells. An extended phase of slower uptake then followed through the next 90 min. Cultured human skin fibroblasts in serum-free medium exhibited properties of [³H]histamine uptake quantitatively similar to those of endothelial cells. In both cell types this process was partially inhibited by a 100-fold excess of unlabeled histamine; the competitive or specific portion accounted for 30-50% and the noncompetitive component for 50-70% of the total accumulated radiolabel in each of the cell types over all of the experiments done. When this latter component was subtracted out, fibroblasts specifically took up [³H]histamine radiolabel in the range of 58-153 fmol/10⁶ cells (n = 3) and endothelial cells 35-115 fmol/10⁶ cells (n = 6) in 30 min of incubation.

[³H]Histamine Metabolites in Whole Cells—The observation that these cells accumulated radiolabeled ligand within the time course of known endothelial cell H₁ receptor activation, and desensitization events raised the question of whether this cell-associated radioligand was in its native or an altered form. The biochemical nature of the radioactivity taken up by endothelial cells was therefore determined by thin-layer chromatography of acetic acid:ethanol total cell extracts. Endothelial cells incubated at 37°C for 4.5 h with 1 μM [³H]histamine in serum-free medium retained [³H]histamine to the extent of 18-30 fmol/10⁶ cells, but no metabolic conversion of the histamine took place (Fig. 2a). However, when cells were incubated with [³H]histamine at 37°C for 10 min, they accumulated it in a metabolized form identified as tele-methylhistamine rather than the native compound (Fig. 2b). Both primary and passage 2-5 endothelial cells carried out the conversion to tele-methylhistamine. Human skin fibroblasts at 37°C also converted [³H]histamine to tele-methylhistamine. A number of strains of human skin fibroblasts obtained from cell culture banks or established as primary strains in our laboratory all generated the methylated derivative as their cell-associated product (Fig. 2, c-f). The cell strains AG1518 and GM2987 from the National Institute of General Medical Sciences bank (Fig. 2, c and d) were tested at a mid- to late-passage level, and our own strain LF (Fig. 2e) was tested at passage 1. Conversion of [³H]histamine to tele-methylhistamine was quantitative at the earliest time point measurable, 3 min at 37°C (Fig. 2f), and this methylated derivative was the radiolabeled product accumulated by the cells for up to 2 h (data not shown). These findings indicated that both endothelial cells and fibroblasts possess endogenous histamine methyltransferase and retain it throughout their lifespan in culture. Its activity results in conversion of cell-associated [³H]histamine to a product which is an intermediate rather than a final product in a degradative pathway, occurring within the time frame of early postreceptor events and for a prolonged period afterward.

Enhancement of [³H]Histamine Uptake by Fetal Calf Serum—Because we had shown that fetal calf serum component(s) both increased histamine receptor desensitization and metabolized native histamine (Fogerty et al., 1981; Fogerty and Baenziger, 1983, 1984), we examined the effect of fetal calf serum on the interaction of [³H]histamine with endothelial cells. The presence of 20% v/v fetal calf serum in the incubation medium enhanced the rate of [³H]histamine uptake by human vascular endothelial cells 10-fold (Fig. 3A), yielding 558 fmol/10⁶ cells in 30 min as opposed to 81 fmol/10⁶ cells in the absence of fetal calf serum. A similar serum-dependent 6-fold enhancement of the uptake rate occurred in human skin fibroblasts, giving 909 fmol/10⁶ cells compared with 143 fmol/10⁶ cells in the absence of fetal calf serum (Fig. 3B). The serum-enhanced [³H]histamine uptake process consistently demonstrated an initial lag in rate lasting 3 to 6 min, as is apparent in Fig. 3, A and B. Following this initial lag phase, the uptake of [³H]histamine was linear for up to 120 min (Fig. 3C). In the presence of 20% v/v fetal calf serum, for endothelial cells this enhanced linear rate ranged from 9.3 to 34.8 fmol/10⁶ cells/min (n = 7) and for fibroblasts from 7.3 to 37.9 fmol/10⁶ cells/min (n = 5). Such a rate was considerably greater than that observed for both endothelial cells and fibroblasts accumulating radiolabel as tele-methylhistamine.
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The enhanced uptake of \(^{3} \text{H}\)histamine by endothelial cells was a function of the fetal calf serum concentration in the incubation medium as indicated in Fig. 3C. In these experiments conducted at 37 °C, the total uptake of \(^{3} \text{H}\)histamine in the presence of 20% v/v fetal calf serum was 15 fmol/min/10^6 cells. Comparable values for uptake in 50% v/v fetal calf serum were 34 fmol/min/10^6 cells. Three additional experiments at 37 °C measuring uptake at a lower \(^{3} \text{H}\)histamine concentration, 0.5 \(\mu\)M rather than 1 \(\mu\)M in 50% fetal calf serum, yielded an uptake rate of 11.6 fmol/min/10^6 cells, which was similar to the 15 fmol/10^6 cells above. The rate of enhanced uptake was also temperature-dependent; cells in 50% fetal calf serum at 25 °C exhibited a slower uptake rate of 4.8–7.8 fmol/min/10^6 cells (n = 3).

The serum-enhanced uptake process was inhibited by a 100-fold excess of unlabeled histamine to a significantly greater degree than the uptake in serum-free medium accompanying the tele-methylation step. The noncompetible component of uptake was somewhat higher at shorter incubation times; it ranged from 27 to 42% of the total uptake at 10 min, where the transition from the lag phase to the linear phase had just occurred, to 18–30% of total uptake at 30 min. This noncompetible component decreased even further at the 60–120-min time points where it accounted for 12% of the total uptake.

Endothelial Cells in the Presence of Fetal Calf Serum Accumulate an Oxidized \(^{3} \text{H}\)Histamine Metabolite—Based on the fetal calf serum enhancement of uptake and our prior identification of its histamine degrading activity in solution (Fogerty and Baenziger, 1983, 1984), we examined the nature of the endothelial cell-associated products generated in the presence of fetal calf serum. The enhanced uptake of \(^{3} \text{H}\)histamine under these conditions resulted in accumulation of a cell-associated histamine metabolite which was neither tele-methylhistamine nor imidazoleacetic acid, which would be the product of direct amine oxidation at the aminoethyl side chain. Fig. 4, a–d, demonstrates the identification of this endothelial cell product as methylimidazoleacetic acid by virtue of its chromatographic position with the appropriate standard in multiple solvent systems. In a butanol:acetic acid:water solvent system initially employed, the cell-associated activity moved with the retention factor value of both imidazoleacetic acid and methylimidazoleacetic acid standards, and these two compounds failed to resolve from one another (data not shown).

Experimental Procedures." A, specific uptake of \(^{3} \text{H}\)histamine by endothelial cells (○) in serum-free medium and (□) in medium containing 20% fetal calf serum. B, specific uptake of \(^{3} \text{H}\)histamine by skin fibroblasts (●) in serum-free medium and (□) in medium containing 20% fetal calf serum. Fig. 3, A and B, shows representative experiments from a series of three each for endothelial cells and fibroblasts. Specific uptake shown for each time point was determined by subtracting the noncompetible uptake in the presence of 100 \(\mu\)M unlabeled histamine from the value for total uptake. The noncompetible component for endothelial cells represented 55–62% of the total uptake in serum-free medium. In medium containing fetal calf serum, the noncompetible component was 54–60% of the total during the first 6 min; it decreased at the 10- and 30-min points to 26 and 13% of the total, respectively. C, total uptake of 1 \(\mu\)M \(^{3} \text{H}\)histamine by endothelial cells over a 2-h period in the presence of 20% (○) or 50% (□) fetal calf serum at 37 °C and 50% fetal calf serum at 37 °C (△). Representative wells counted had 1×1.5×10^6 cells over the three experiments shown; data represents mean ± S.E. Although the non-specific or noncompetible component has not been subtracted out in the data shown, in other experiments it was found to be similar to that in A and B regardless of the temperature, \(^{3} \text{H}\)histamine concentration, or fetal calf serum concentration.

Fig. 3. Uptake of \(^{3} \text{H}\)histamine shows a biphasic time course and enhancement in the presence of fetal calf serum. Cells were incubated with 1 \(\mu\)M \(^{3} \text{H}\)histamine for the times indicated and cell-associated radioactivity determined as described under “Ex-

during incubation in serum-free medium (Fig. 1); the latter was 3.4 fmol/10^6 cells/min and 1.3–5.5 fmol/10^6 cells/min, respectively.
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not shown). Other histamine metabolite reference compounds (native, tele-methyl, and N-acetylhistamine) moved out from the origin in all solvent systems. Imidazole acetic acid was at the origin in system I shown here, but it had an \( R_f \) value of 0.62 in system II, which did not match the position of the major band of cell-associated radioactivity generated in the presence of fetal calf serum. The observed endothelial cell-associated product indicated the existence of a diamine oxidase activity in fetal calf serum acting on histamine previously methylated by the cells themselves, outlining a two-stage enzymatic sequence. The converse order of enzymatic steps did not occur. Only traces of radioactivity, when they were present at all, appeared in a faint band with the mobility of imidazolacetic acid so that any solution-phase direct amine oxidation of histamine was not a major participant in interactions with the cells. Furthermore, imidazolacetic acid is not a substrate for histamine methyltransferase (Rothschild and Schayer, 1958), so the product observed here would not have arisen from endothelial cell methylation of imidazolacetic acid if it had been generated first by the diamine oxidase. In contrast, tele-methylhistamine is known to serve as a substrate for other amine oxidase activities (Bardsley et al., 1974; Hough and Domino, 1979; Kusche, 1982; Suzuki, 1979; Zeiger et al., 1976). Here generation of methylimidazolacetic acid was a function of the fetal calf serum concentration, providing further evidence for enzymatic activity. Endothelial cells incubated with 2% v/v fetal calf serum accumulated tele-methylhistamine without detectable quantities of methylimidazolacetic acid, whereas in the presence of 50% v/v fetal calf serum the latter was virtually the sole product (Fig. 4, a-d).

Further evidence for the stepwise nature of this enzymatic process in human endothelial cells was provided by the histamine methyltransferase inhibitor SKF91488 (homodimaprit). In three experiments this compound inhibited serum-enhanced endothelial cell uptake of \([^{3}H] \)histamine in a dose-dependent fashion; uptake was decreased >50% by 4–10 \( \mu \) M SKF91488, and a 50 \( \mu \) M concentration caused maximal inhibition, down to the same 30% background of noncompetible uptake as that observed with excess unlabeled histamine. This dose-response profile is similar to that for SKF91488 inhibition of histamine methyltransferase activity (\( ED_{50} = 1 \mu \) M); although SKF91488 can inhibit diamine oxidase as well, this latter inhibition requires much higher levels of inhibitor (\( ED_{50} = 70 \mu \) M) (Beaven and Schaff, 1979a, 1979b). Fig. 4e shows the product analysis of fetal calf serum-enhanced \([^{3}H] \)histamine uptake in this series of experiments, where cell-associated radioactivity consisted of small amounts of native and tele-methylhistamine and a large amount of label in the chromatographic position of methylimidazolacetic acid. Preincubation with SKF91488 (Fig. 4f) eliminated accumulation of the tele-methylhistamine intermediate and greatly decreased the amount of observable methylimidazolacetic acid formed as well, leaving native histamine and a small amount of material migrating at the origin as the only cell-associated radioactivity.

Enhancement of \([^{3}H] \)Histamine Uptake Reflects Diamine Oxidase Activity—We have characterized a diamine oxidase activity in fetal calf serum that preferentially utilizes histamine and putrescine as substrates (Fogerty and Baenziger, 1983, 1984). This enzyme is distinct and physically separable from the spermidine oxidase of fetal calf serum (Gahl and Pitot, 1975; Gahl et al., 1980), which oxidizes neither putrescine nor histamine. The activity is present in whole fetal calf serum at a concentration of 2–6 units/ml in different batches so that in \([^{3}H] \)histamine uptake studies employing whole fetal serum the level of enzyme present ranged from 0.3 to 3 units/ml. We tested the activity of this enzyme in purified preparations with regard to its enhancement of \([^{3}H] \)histamine uptake by cells. Fig. 5 compares uptake of \([^{3}H] \)histamine by human vascular endothelial cells in the presence of 1.48 units/ml diamine oxidase activity purified from fetal calf serum with uptake in 20% v/v fetal calf serum having an enzyme level of 0.68 units/ml. Enhanced histamine uptake by endothelial cells was proportional to diamine oxidase activity regardless of whether whole fetal calf serum or purified enzyme was used. Diamine oxidase and enhanced uptake activity tested in six experiments copurified through procedures A and B as described under "Experimental Procedures." These
purifications involved totally different affinity and ion exchange media and afforded complete separation from the spermdermin oxidase (Fogerty and Baenziiger, 1983, 1984). Fig. 5 (right) shows that the copurified diamine oxidase enzyme activity and uptake enhancing activity also caused generation of endothelial cell-associated methylimidazoleacetic acid from [3H]histamine. These experiments with purified preparations of enzyme further confirm its action on tele-methylhistamine generated by the cells as its substrate as well as native histamine in solution. In other experiments both the enhanced uptake activity and methylimidazoleacetic acid generation were similarly sensitive to heat denaturation and to inhibition of diamine oxidase activity by phenylhydrazine and aminoguanidine. In addition, endothelial cell uptake of 1 μM [3H]histamine was blocked by unlabeled putrescine, the substrate for this enzyme, but not by unlabeled spermidine which is the substrate for the spermidine oxidase purified away from diamine oxidase. These results indicated that enhanced [3H]histamine uptake and cellular accumulation of an oxidized metabolite specifically reflected the action of the putrescine and histamine-metabolizing diamine oxidase in fetal calf serum.

**Uptake Is Saturable with Respect to [3H]Histamine**—Having defined a process of [3H]histamine uptake or accumulation linked to the metabolism of this ligand, we sought to further define the mechanism by which the endothelial cells participated. Uptake of [3H]histamine by vascular endothelial cells was a concentration-dependent and saturable process with respect to [3H]histamine concentration whether or not diamine oxidase activity was present. As indicated in Fig. 6, the rate of uptake in the absence of diamine oxidase, accompanying only the histamine to tele-methylhistamine conversion step, increased over the concentration range of 0.5-5 μM [3H]histamine. A plateau in specific uptake occurred at histamine concentrations above 5 μM. The concentration for half-maximal uptake in the absence of diamine oxidase was 2.5 μM. Maximum specific uptake in the absence of diamine oxidase ranged from 626 to 2533 fmol/10^6 cells/30 min at the highest input level of [3H]histamine, 11 μM. These values represented an increment over a background not competitible by unlabeled histamine, averaging 69% of the total uptake. The measured fluid phase pinocytosis uptake rate was 126 fmol of [3H]insulin/10^6 cells/30 min at an input concentration of 3.5 μM so that the rate of [3H]histamine uptake was severalfold elevated above this pinocytic rate. This [3H]histamine dose response with its defined region of saturability was consistently observed over the concentration range of 0.5-11 μM.

The enhanced uptake of [3H]histamine by primary cultures of endothelial cells in the presence of the diamine oxidase of fetal calf serum, accompanying production of methylimidazoleacetic acid, also represented a concentration-dependent and saturable process; it had virtually the same dose-response curve for [3H]histamine over a range of 0.05-13 μM (Fig. 7) as that observed for the serum-free uptake described above which is linked to tele-methylhistamine generation. In six experiments uptake increased between 0.3 and 4.5 μM [3H]histamine and was saturated at 4.5-13 μM with a concentration for half-maximal uptake of 1.75 μM. In other experiments (n = 9) the uptake rate displayed saturability at slightly lower histamine concentrations, in the range of 1-2 μM with a half-maximal concentration of 0.7 μM. In an additional series of experiments (n = 3) where the rate of fluid phase pinocytosis measured by [3H]insulin uptake was compared with uptake of 1 μM [3H]histamine in the presence of human placental diamine oxidase (1.8 units/ml), insulin uptake was 116 fmol/10^6 cells/30 min, and [3H]histamine uptake was 4,800-10,700 fmol/10^6 cells/30 min. Fluid phase pinocytosis therefore was not increased during the strong enhancement of [3H]histamine uptake mediated by the diamine oxidase (Gibbs et al., 1986; Sullivan and Zigmond, 1986; Swanson et al., 1985).

The similarity of dose-response curves for [3H]histamine uptake in both the absence and presence of exogenous diamine oxidase activity was consistently observed. Thus the uptake of [3H]histamine reflects the specific contribution of a finite number of sites with a similar affinity for the interaction of this ligand with endothelial cells regardless of whether the uptake is occurring with only the methylation step or in the diamine oxidase-enhanced state yielding the final methylimidazoleacetic acid product. These properties indicate a binding site or receptor for histamine on endothelial cells and fibroblasts as an integral part of this metabolic process. Its properties will require further definition. In additional experiments, no inhibition of uptake at either the methylation step

**FIG. 7.** Diamine oxidase-enhanced uptake has the same [3H]histamine concentration dependence as the methylation step. Primary endothelial cells were incubated with [3H]histamine for 10-30 min in the presence of 50% fetal calf serum (2.5 units/ml diamine oxidase activity), washed with chilled phosphate-buffered saline, solubilized in 0.1 N NaOH, and cell-associated radioactivity quantitated. Values for maximal specific uptake in the presence of fetal calf serum at saturating [3H]histamine concentrations ranged from 316 to 860 fmol/approximately 1.7×10^6 cells min at 10 and 30 min among the experiments. Comparable values for specific [3H]histamine uptake in the absence of fetal calf serum for these experiments were 81-113 fmol/10^6 cells. Noncompetitive uptake in the presence of 44-100 μM unlabeled histamine has been subtracted from all points; equivalent values for competition of [3H]histamine uptake at levels beyond 4.5 μM were obtained with 100 and 250 μM unlabeled histamine. The figure represents data from six experiments. Values are mean ± S.E.
or amine oxidation step occurred in the presence of the H₁ receptor antagonist pyrilamine or the H₂ receptor antagonist cimetidine at levels of 5 nM–50 μM so that this binding site cannot be directly identified with known H₁ or H₂ receptor subtypes.

Enhanced Uptake Is Saturable with Respect to Exogenous Diamine Oxidase Activity—We also explored the concentration dependence for diamine oxidase in enhancing the uptake of [³H]histamine radiolabel. Endothelial cells incubated with increasing concentrations of exogenous diamine oxidase isolated from fetal calf serum showed an increasing rate of [³H]histamine uptake, culminating in a plateau at an input diamine oxidase level of 2.0 units/ml. The half-maximal response for enhanced uptake was observed at 1.0 units/ml of diamine oxidase activity (Fig. 8A). Similarly, diamine oxidase derived from human placenta enhanced endothelial cell [³H]histamine uptake in a saturable manner, showing a half-maximal rate enhancement at 0.26 units/ml and a plateau at 1.0 units/ml of diamine oxidase activity (Fig. 8B). These experiments were carried out at 5 μM [³H]histamine, which is a saturating substrate concentration for [³H]histamine uptake by endothelial cells (see above, Fig. 7), as well as for both the fetal calf serum and human placental diamine oxidases in solution (Paolucci et al., 1971; Zeller et al., 1956). Thus the plateau in rate enhancement observed for the diamine oxidase from both sources reflects interaction with a limited number of endothelial cell binding sites, indicating the presence of a receptor on endothelial cells for diamine oxidase which mediates this enzyme’s participation in endothelial cell histamine metabolism.

**DISCUSSION**

Human vascular endothelial cells represent a significant target for the biologic action of histamine, possessing defined subtypes of receptors linked to pathways for production of secondary mediators. Conversely, these target cells can affect histamine molecules interacting with cell surface receptors by virtue of their enzymatic capability for metabolizing histamine. The type and efficiency or relative activity of histamine metabolizing enzymes, as well as their cellular location relative to the ligand, may exert a profound influence on both ligand-receptor interactions and the efficacy of ligand clearance from the immediate cellular microenvironment.

The sequence of steps which takes place in association with an individual endothelial cell or fibroblast reflects that defined by Schayer and co-workers (1968, 1974, 1975) and Schippert et al., (1979) for histamine metabolism at the level of whole tissue and whole organism. Our studies are the first to demonstrate expression of this sequential pathway at the level of an individual cell via a mechanism which reconciles the necessary enzymological representation of these enzymes: a ubiquitous distribution of diamine methyltransferase in most human tissues except plasma (Beaven, 1982; Gustafsson and Fersl-II), 1964; Lindell and Westling, 1966; Snyder et al., 1966; Thitilapanda and Cohen, 1978) versus a highly restricted prevalence of diamine oxidase to specific organs, primarily kidney and intestine (Almeida et al., 1980; Beaven, 1982; Gustafsson and Forsell, 1964; Schayer and Reilly, 1974; Shaff and Beaven, 1976; Shakir et al., 1977). Participation of the latter enzyme in the sequence by means of a specific receptor makes it, in a sense, an "optional" step whose deployment may offer possibilities for additional levels of regulation.

The metabolic sequence which we have characterized here may have a bearing on the mechanism for the H₁ histamine receptor tachyphylaxis phenomenon and recovery properties we observed previously (Baenziger et al., 1981; Fogerty et al., 1981) if histamine during its interaction with the H₁ receptor is accessible to this degradation pathway. Endothelial cell conversion of histamine to tele-methylhistamine, initiated immediately upon contact, is a biochemical modification which would abrogate histamine’s biologic activity at the H₁ receptor (Lee and Jones, 1949). After a few minutes of incubation, most of the cell-associated product accumulated is the tele-methyl derivative rather than native histamine. In the absence of any additional metabolizing activity provided from an outside source, both endothelial cells and fibroblasts retain and accumulate the methylated metabolite. This system is essentially trapped at an intermediate state of degradation until the next enzyme, diamine oxidase, is made available from an exogenous source.

Uptake of [³H]histamine by endothelial cells and fibroblasts is thus directly coupled to the metabolism of this compound. The observed saturable concentration dependence for both histamine and for exogenous diamine oxidase activity is consistent with the hypothesis that this uptake and metabolism is a receptor-mediated process. The similar half-maximal point and plateau near 4 μM in the concentration curve for [³H]histamine, regardless of whether or not diamine oxidase is present, indicate that a distinctive binding site or receptor is involved in the histamine-endothelial cell interaction which directs the ligand into this metabolic pathway. The saturable

| FIG. 8 | Enhance uptakes is saturable with respect to diamine oxidase activity from human and bovine sources. Human vascular endothelial cells at passage 2–3 were incubated for 30 min with 5 μM [³H]histamine at the indicated diamine oxidase concentration, expressed in units/ml, from (A) fetal calf serum or (B) human placenta. Cell-associated radioactivity was quantitated; the nonspecific or noncompetible component of uptake has been subtracted. A, several diamine oxidase preparations were used which were purified from 61- to 324-fold by procedure B (see "Experimental Procedures") from fetal calf serum. Maximal specific uptake ranged from 17,000 to 33,000 fmol/10⁵ cells/30 min (n = 3). Cell density was 0.11–0.15 × 10⁶/8-mm-well. B, human placental diamine oxidase was prepared as a 48,000 × g supernatant containing 46 units/ml of enzyme activity. Maximal specific uptake at a cell density of 0.05–0.07 × 10⁶/8-mm well was 67,000–123,000 fmol/10⁵ cells/30 min (n = 3). Three additional experiments (data not shown) utilizing 0.29–0.39 × 10⁶ cells/8-mm well yielded maximal specific uptake of 20,950–36,725 fmol/10⁵ cells/30 min with the same concentration dependence as seen here. |
Histamine Uptake: a Two-step Ordered Sequence

binding site in the metabolic pathway is clearly distinct from the classical H1 and H2 receptor species, since uptake is not inhibited by their known antagonists. It is intriguing to note, however, that the observed saturable [3H]histamine concentration curve parallels that for the H1 receptor activation which evokes synthesis of lipid mediators (Baenziger et al., 1980; McIntyre et al., 1985). Histamine may bind to some common site, a type of receptor previously undescribed, which interfaces with both the H1 apparatus and that for histamine metabolism. Alternatively, histamine methyltransferase itself may well be the saturable binding site mediating uptake, since inhibition of this enzyme by homodimaprit (SKF91488) totally blocks specific uptake and formation of the distal oxidized product. The micromolar histamine concentrations operative here are in fact ones which can be generated in local vascular beds in humans when mast cell release occurs by natural stimuli (Kaplan et al., 1975) so that the observed uptake/metabolism process in endothelial cells reflects a physiologically relevant context.

Saturability with respect to exogenous diamine oxidase activity indicates that a receptor for diamine oxidase is utilized in its interaction with cells which results in the uptake of [3H]histamine as metabolites as we have shown here. Both diamine oxidases exhibited a consistent value for the units/ml required to achieve saturation (1–2 units/ml), as would be predicted if the cells express receptors for this enzyme itself. The apparent affinity reflected in the concentration of human placental diamine oxidase needed to mediate half-maximal uptake by endothelial cells was 4-fold better than that for the fetal calf serum enzyme, suggesting a slightly greater preference for the human placental enzyme interacting with human cells in a completely homologous system. The studies of Robinson-White et al. (1985) have outlined saturable binding of placental diamine oxidase to freshly isolated rodent microvascular endothelial cells in suspension. Our observed value for half-maximal diamine oxidase-mediated enhancement of uptake in human endothelial cells is relatively close to the Km reported by these investigators (Robinson-White et al., 1985) for their binding of enzyme activity; when the units of enzyme activity in their study are compared to ours, expressed as nmol of putrescine substrate degraded per hour, their measured Kms would be 0.092 units/ml whereas we found the half-maximal value to be 0.26 units/ml.

The restriction of observable histamine metabolizing activity in human vascular endothelial cells and skin fibroblasts to the tele-methylation step alone and utilization of a receptor for the distal enzyme from an extracellular source should be considered in light of what is known about the types of amine oxidase activity found in or accessible to these cells. Classical diamine oxidase activity toward native histamine and aliphatic polyamines as exemplified by the two enzymes used in our studies occurs in human plasma only in the case of pregnant women and individuals with specific types of tumors. It has been found associated only with rodent microvessels (Robinson-White and Beanen, 1982). Other types of endothelial cells and fibroblasts in culture lack a diamine oxidase. They may possess type B monoamine oxidase, which is capable of utilizing tele-methylhistamine as a substrate in a solution phase assay, in strong preference over native histamine (Hough and Domino, 1979; Kusche et al., 1982; Roth et al., 1976; Roth and Venter, 1978; Small et al., 1976; Suzuki et al., 1979; Treveithick et al., 1981; Zeller et al., 1956). For example, human skin fibroblasts contain this enzyme, but it is located in mitochondria; our studies indicate that it is not acting at all on tele-methylhistamine which the cells have generated. Their failure to carry out the entire degradation sequence forming methylimidazolacetic acid by themselves suggests that the tele-methylhistamine substrate may accumulate in a cellular compartment relatively sequestered from the mitochondrial enzyme, precluding the distal step by the latter even when it is present in the cells. A somewhat similar situation has been observed for B-phenylethylamine, a naturally occurring structural analogue of histamine, which does not undergo a ring methylation step but is accumulated as the native amine in tissues known to possess mitochondrial type B monoamine oxidase activity (Garca et al., 1985).

We have shown that the enzymatic steps involved in degradation of histamine by human vascular endothelial cells and skin fibroblasts conform to an ordered sequence. Similarly, the histamine uptake process itself also appears to include multiple steps closely linked to the enzymatic sequence. The lag phase in diamine oxidase-enhanced uptake during the first 3–6 min of exposure to radioligand has certain properties, including its substantial noncompetitive component, which resemble the tele-methylation step alone. An early step may be rate-limiting, for example tele-methylation or cellular binding of diamine oxidase, until a sufficient amount of some product has accumulated to facilitate subsequent metabolism/uptake by a mass action effect or some other mechanism. The cell-associated two-stage metabolic sequence occurs preferentially over direct amine oxidation even though the diamine oxidase can and likely does carry out this latter reaction concurrently in the solution phase. This indicates that the endogenous histamine methyltransferase and exogenously provided diamine oxidase may be in close proximity to one another at the binding sites or receptors identified in the present study.

Metabolism of histamine by a target cell thus serves to terminate its biologic activity at or near its site of direct action on receptors such as the H1 type. The enzymatic sequence which we have described and the accompanying uptake process may also serve a more general function in the clearance of released histamine from the circulation and the interstitial spaces. This clearance role would be of particular interest in the case of fibroblasts, which do not have the H1-receptor triggered lipid mediator pathway present in endothelial cells (Baenziger et al., 1981) but do not metabolize histamine by the same mechanism. In addition to the effects of metabolism on histamine action at the microenvironment of the vascular or interstitial connective tissue cell, total histamine uptake by endothelial cells and fibroblasts is considerably enhanced by the action of the exogenous diamine oxidase. Further studies are in progress to identify an amine oxidase activity in humans which would fulfill in vivo the functions of human placental or fetal calf serum diamine oxidase delineated in our tissue culture model. The metabolic step carried out by diamine oxidase is not necessary for inactivation of histamine in this system since the prior methylation step performs that role. We postulate that an important function of the diamine oxidase may be to modulate the rate of histamine uptake by removing the intermediate tele-methylhistamine. This clearance mechanism in turn may serve to regulate the pharmacologic activities of histamine at the level of the local tissue or the whole organism.4

Acknowledgments—We acknowledge the skilled and enthusiastic technical assistance of Lisa Mertz and dedicate this publication to the memory of Matthew N. Mertz. We thank Dr. Elsa Bello-Resus for the gift of [3H]histamin, Dr. Gary Bass for gifts of human plasma, and the staff nurses and physicians at our participating hospitals for their support and cooperation in providing human tissues.

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