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NICOTINE AND PYRROLIDINE-INDUCED RELEASE OF 5-HYDROXYTRYPTAMINE AND HISTAMINE FROM NEOPLASTIC MAST CELLS*

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Abstract—Nicotine and other pyrrolidines induce the release of the components of the multivesicular granules of mouse neoplastic mast cells, including 5-HT (serotonin), histamine and heparin. The elevation of the pH required for the compounds to show activity makes a mast cell site available for the chemical initiation and the maintenance of the release process. At the optimum pH, only the pyrrolidine ring of the nicotine molecule is needed for activity.

NICOTINE stimulates the secretion of biogenic amine-containing granules from the adrenal medulla,[1–6] the gastrointestinal tract,[7–9] from blood platelets,[10,11] and from rat peritoneal mast cells.[12,13] In addition, Bond and Schwartz[14] have reported the nicotine-induced release of pinolysosomes from macrophages. Nicotine may function as an activator of the release process[15] in most or all of these systems, to produce the liberation of the contents of granules and vesicles from cells.

Normal mouse mast cells are unusually resistant to the liberation of their vesicular granules, at physiological pH, by a classical releasing agent such as compound 48/80.[16–18] We have found that nicotine triggers the amine–heparin release process in mouse neoplastic mast cells, at pH levels more alkaline than 7·4.[19]

This paper describes the conditions under which the nicotine-induced release of biogenic amines from the neoplastic mast cells can occur; it suggests the reason for the occurrence of release under these conditions; and it characterizes the structural subunit of the nicotine molecule that triggers and maintains the release process.

MATERIALS AND METHODS

Neoplastic mast cells. The origin and the methods of propagation of the HC and P-815Y lines of mouse neoplastic mast cells have been described.[20] The HC line was routinely carried in LAF_1 female mice and the P-815Y line was carried in AKD_2F_1 female mice that were obtained from Jackson Labs, Bar Harbour, Me. For these experiments, both cell types were grown in the LAF_1 female mice and they were harvested 5–8 days after the injection of the tumor. Each P-815Y bearing animal received an intraperitoneal injection of 0·2 ml of a solution of 0·83 mg histamine dihydrochloride plus 1·15 mg 5-hydroxytryptamine (5-HT) creatinine sulfate/ml, 3 hr

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prior to removal of the cells. This was done only for the P-815Y cells, to increase the intracellular amine content in order to raise their amine level for ease of assay. HC cells contained 0·57 ± 0·15 and 0·27 ± 0·02 μg 5-HT and histamine, respectively, per 10^6 cells. P-815Y cells contained 0·67 ± 0·32 and 14·56 ± 0·47 ng 5-HT and histamine per 10^6 cells prior to the histamine–5-HT injection and 14·56 ± 0·47 and 13·11 ± 0·60 ng 5-HT and histamine per 10^6 cells at the time of harvest (3 hr post-injection). The mice were sacrificed and the cells were obtained from their peritoneal cavities and centrifuged at 3000 *g* for 2 min in a Sorvall RC-2B, at 4°. The cells were washed twice with normal saline and centrifuged as above. They were resuspended in modified Tyrode–phosphate–albumin buffer at a concentration of 0·2 g wet wt (~2×10^8 cells)/ml; 1 ml of the cell suspension was used per incubation vessel containing a final volume of 5 ml.

Chemical assays. The intracellular and extracellular amine levels were determined spectrophotofluorometrically with the Hitachi–Perkin Elmer MPF-2A, using the method of Shore *et al.*[21] for histamine, and the modified method of Bogdanski *et al.*,[22] as described by Carlini *et al.*,[23] for 5-HT. 5-HT creatinine sulfate (Calbiochem) and histamine dihydrochloride (Eastman Organic Chem.) were used as standards. Heparin was assayed in un-incubated cells, control cells and nicotine-treated cells, as described under Incubation techniques. Release was determined by difference. The heparin levels were determined by the method of Glick *et al.*,[24] with a Gilford-2400 spectrophotometer, using a 10-fold increase in the volume of each reagent in the procedure. Sodium heparin (Sigma Chemical Co.) was used as a standard. Protein was determined by the biuret method.[25]

Materials. L(−)Nicotine was purchased from Matheson, Coleman & Bell and redistilled at 84° under a vacuum of 2·6 mm Hg. Aliquots were stored in amber bottles at 4°. Pyrrolidine and 1-methyl-2-pyrrolidinone were purchased from Aldrich Chemical Co. (Milwaukee, Wis.) and used without further purification as was the 1-methylpyrrolidine purchased from K & K Laboratories (Plainview, N.Y.) and the bovine serum albumin from Sigma Chemical Co.

Incubation techniques. The incubation buffer consisted of: NaCl, $1{\cdot}4 \times 10^{-1}$ M; KCl, $5{\cdot}4 \times 10^{-3}$ M; $CaCl_2$, $1{\cdot}8 \times 10^{-3}$ M; $MgCl_2$–$6\,H_2O$, $1{\cdot}1 \times 10^{-3}$ M; $NaHCO_3$, $1{\cdot}2 \times 10^{-2}$ M; D-glucose, $5{\cdot}6 \times 10^{-3}$ M; NaH_2PO_4, $4{\cdot}2 \times 10^{-4}$ M; bovine serum albumin, 1·0 g/l. Nicotine was used at a concentration of $1{\cdot}3 \times 10^{-2}$ M, unless otherwise stated. The pH of the buffer was adjusted with NaOH or HCl as required. The cells were resuspended in buffer at a concentration of 0·2 g wet wt/ml. Incubations were carried out in 10-ml beakers, at 37° unless otherwise stated. The vessels were gently agitated in a metabolic shaker. A final volume of 5 cm^3/incubation beaker was used, except where specifically indicated. The incubations were terminated by centrifugation at 3000 *g* for 2 min, at 4°. The supernatant was divided into two equal parts. One part was added to 0·75 N $HClO_4$ and the other part was added to the 0·1 N HCl for the histamine and 5-HT assays respectively. Control release values were subtracted in all cases. Total amine concentrations per 0·2 g wet wt of cells were determined prior to incubation for each experiment.

RESULTS

In contrast to rat peritoneal mast cells, the mouse neoplastic mast cells respond feebly to nicotine at physiological pH. As the pH was raised, the per cent amine

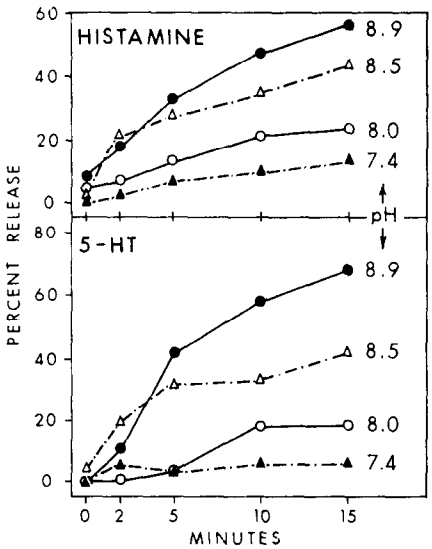


FIG. 1. Effect of pH on nicotine-induced histamine and 5-HT release. Incubation techniques for all figures are given under Methods. P-815Y cells were used except where indicated.

released increased up to values of 60–70 per cent (Fig. 1). The controls (Fig. 2) show practically no release in the absence of nicotine. Nicotine produces measurable amine release at about 0·5 to 2·5 mM (Fig. 3) with a maximum level achieved at 12–13 mM. The curves for histamine and 5-HT release are very similar and no marked qualitative difference in the release of one amine as compared with the other was observed.

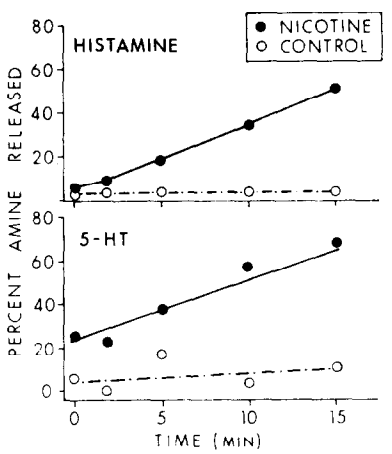


FIG. 2. Mast cell amine release. HC cells (0·2 g wet wt/ml) were incubated at 37°, pH 8·9, in Tyrode–phosphate–albumin buffer, final volume, 5 ml. Incubations were terminated by centrifugation at 4°, 3000 *g* for 2 min and the supernatants were divided into two parts for histamine and 5-HT assays. Controls (without nicotine) were run simultaneously with each experiment. See Incubation techniques and Chemical assays for detailed methods.

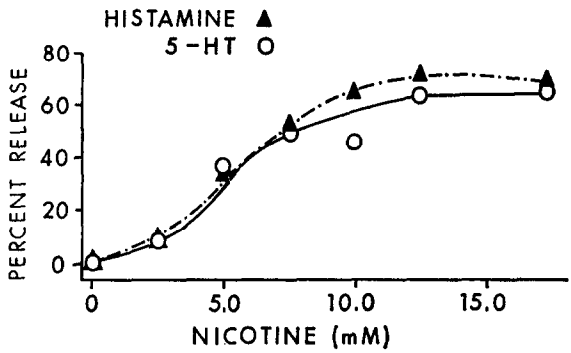


FIG. 3. Maximum amine release. HC cells (0·2 g wet wt/ml) were incubated at 37° for 15 min, pH 8·9, in Tyrode–phosphate–albumin buffer, final volume, 5 ml. Incubations were terminated by centrifugation at 4°, 3000 *g* for 2 min and the supernatants were divided into two parts for histamine and 5-HT assays. Incubations without nicotine were run simultaneously with each experiment and the per cent release values reported were corrected for the small amounts of release in these controls. See Incubation techniques and Chemical assays for detailed methods.

Rat peritoneal mast cells release their granule contents, in response to chemical stimuli, in what has been termed an "explosive" process[26] that is complete in about 15 sec. The nicotine-induced release from the mouse tumor mast cells does not plateau for about 15 min (Fig. 4), 60 times as long as that required for the rat mast cell amine release. The reason for the prolonged release has not been determined. The release process is markedly temperature sensitive (Fig. 5), suggesting enzymatic participation. Note that there is no measurable amine release below 20°. This is similar to an observation reported by Johnson *et al.*[27] for rat mast cells.

The quantities of the substances released from the neoplastic mast cells by nicotine treatment (Table 1) are similar to those reported for rat peritoneal mast cells treated with compound 48/80 by Uvnäs and Thon,[28] Fillion *et al.*,[29] Nosal *et al.*,[30] Slorach,[31] and Thon and Uvnäs.[32] The ratio of the per cent histamine released to the per cent

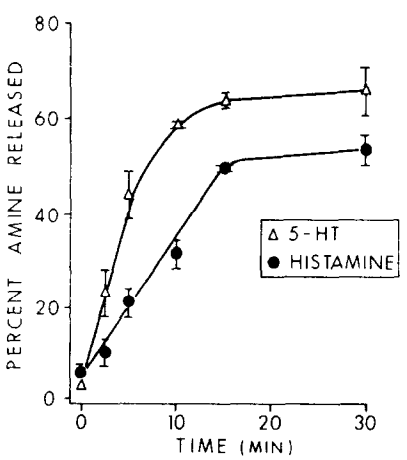


FIG. 4. Amine release by nicotine with time. HC cells were used. All values represent the mean of three or more experiments (± S.E. of the mean).

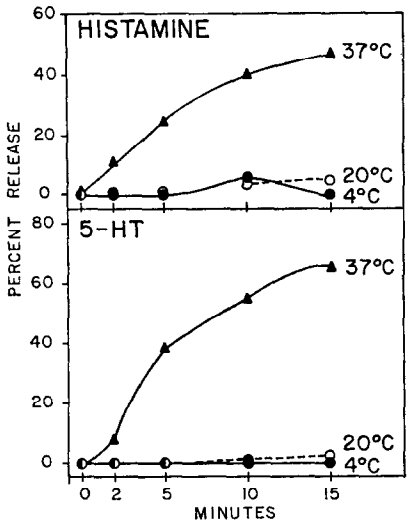


FIG. 5. Effect of temperature on nicotine-induced histamine and 5-HT release.

heparin released is 1·3 for the mouse tumor cells, therefore, within the range of reported values of 1·1 to 2·0 for the rat cells.[29–31] The protein release (Table 1) is close to the value of 21 per cent that has been reported for rat mast cells.[29]

TABLE 1. NICOTINE-INDUCED RELEASE OF MAST CELL COMPONENTS*

Substance released	Per cent release†	Substance released	Per cent release†
Histamine	48·50 ± 0·53	Heparin	37·43 ± 3·27
5-HT	61·48 ± 1·92	Protein	23·02 ± 2·32

* See incubation techniques and chemical assays under Methods. Nicotine was used at a concentration of $1{\cdot}3 \times 10^{-2}$ M.

† All values represent the mean of six or more experiments (± S.E. of the mean).

In order to establish the structures in the nicotine molecule that are essential for the releasing activity, the molecule was divided into pyridine and 1-methylpyrrolidine portions. The activity of each of these subunits and of the combination of the two subunits was compared with that of the intact nicotine molecule, under optimum release conditions. The results (Fig. 6) show that all of the releasing activity is retained by the 1-methylpyrrolidine subunit, and that the pyridine subunit has no activity alone or in combination with the methylated pyrrolidine. As shown in Fig. 6, 1-methylpyrrolidine causes even greater amine release than does nicotine. The methyl group on the pyrrolidine ring is not required for releasing activity (Fig. 7), but the introduction of a carbonyl group adjacent to the pyrrolidine ring nitrogen (1-methyl-2-pyrrolidinone) destroys all activity. Note that the dose–response curves are similar, with detectable release occurring at about 5×10^{-4} M in each case and optimum release at about $1{\cdot}2$ to $1{\cdot}3 \times 10^{-2}$ M. Low concentrations of the releasing agents gave variable

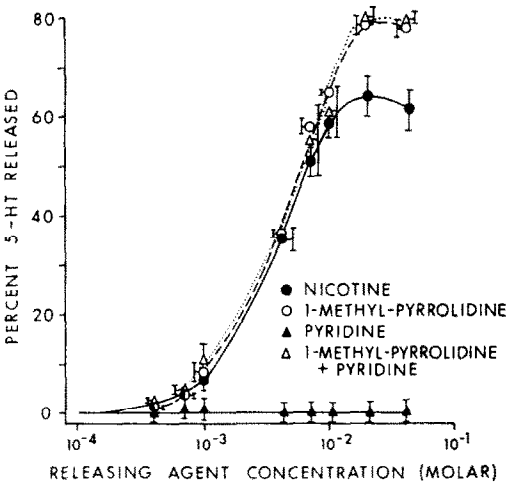


FIG. 6. Structural requirements for amine release. All values represent the mean of three or more experiments (± S.E. of the mean).

results, as can be seen by comparing Figs. 6 and 7. By measuring the effect of pH on amine release (Fig. 8), it was observed that nicotine's releasing ability was greater than that of the pyrrolidines at the intermediate pH range of approximately 7·75 to 8·75. Further experiments have shown that the addition of an equimolar amount of pyridine to either of the pyrrolidine compounds, over the intermediate pH range, produced no enhancement of their releasing activity. The results suggest that the pyridine and pyrrolidine rings must be covalently linked in the conformation of the nicotine molecule

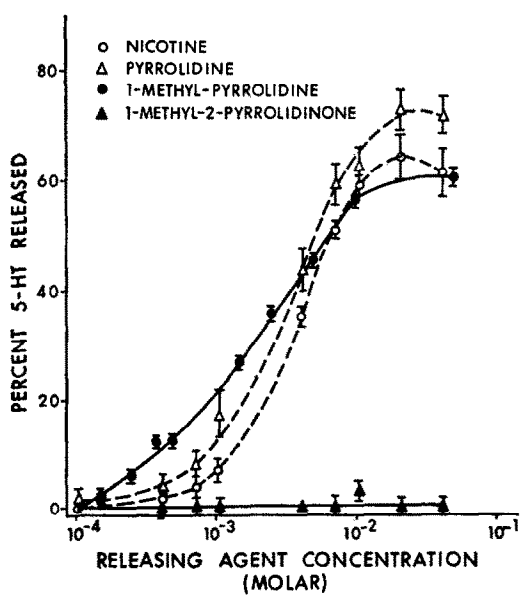
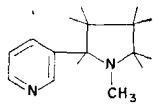
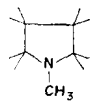
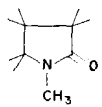


FIG. 7. Concentration of releasing agent vs amine release. All values represent the mean of three or more experiments (± S.E. of the mean).

TABLE 2. EFFECT OF pH ON MOLECULAR STRUCTURE AND AMINE RELEASE*

Releasing agent	Structure	Per cent†	pH 7·4	pH 8·0	pH 8·5	pH 8·9
Nicotine		Free base‡	30·58	63·69	84·72	93·30
		5-HT released	7·30 ± 1·25	24·72 ± 3·52	57·02 ± 4·87	59·17 ± 2·38
1-Methyl-pyrrolidine		Free base	0·22	0·87	2·70	6·53
		5-HT released	8·72 ± 0·36	12·86 ± 0·90	31·01 ± 0·72	55·02 ± 5·12
Pyrrolidine		Free base	0·02	0·10	0·31	0·78
		5-HT released	6·41 ± 0·80	11·28 ± 2·82	31·81 ± 3·08	62·48 ± 3·33
1-Methyl-2-pyrrolidinone		Free base	—	—	—	—
		5-HT released	1·84 ± 0·86	1·61 ± 0·79	2·00 ± 1·00	2·78 ± 2·78

* See Incubation techniques and Chemical assays under Methods. All compounds were used at a concentration of $1{\cdot}3 \times 10^{-2}$ M.
† All values represent the mean of six or more experiments (± standard error of the mean).
‡ Per cent free base calculated by the method of Hamilton.[33]

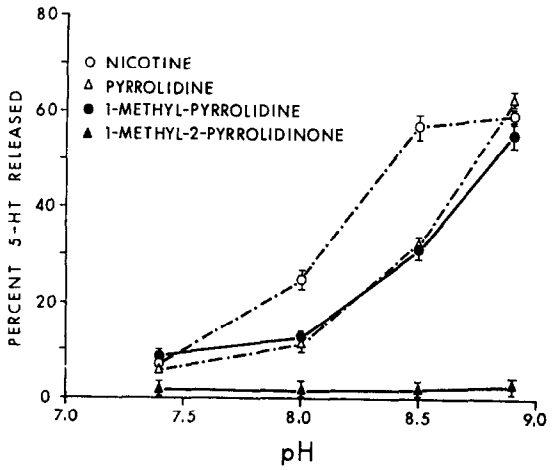


FIG. 8. Effect of pH on amine release. All compounds were used at a concentration of $1{\cdot}3 \times 10^{-2}$ M. All values represent the mean of three or more experiments (± S.E. of the mean).

in order to produce the increased release seen with nicotine below pH 8·9. The 1-methyl-2-pyrrolidinone (and 2-pyrrolidinone) again showed no activity. An examination of the relationship between pH, the molecular structure of the releasing agent, and the per cent amine release (Table 2) showed that an increase in pH produced an increase in amine release and a 3-fold increase in the per cent free base of nicotine present. In the case of 1-methylpyrrolidine and pyrrolidine, however, a rise in pH of the incubation mixture produced an increase in 5-HT released, and an increase of 30–40-fold in the per cent free base of these releasing compounds. Nicotine does not induce appreciable amine release at pH 7·4 even though more than 30 per cent of the drug molecules are present in the form of the free base. Increasing the drug (free base) concentration at this pH does not produce increased amine release. These results suggest that a change in conformation or availability of the trigger site, (or both of these factors that involve cell structure), as well as a transformation of the structure of some molecules of the releasing agents, occur as the pH rises. The 1-methyl-2-pyrrolidinone, which exists over the entire pH range tested as the free base, was inactive. The base is so weak that it requires nonaqueous titration for measurement.[34,35]

DISCUSSION

The rat peritoneal mast cell has been used as a model system for the study of amine release[36–42] and of substances that induce amine release,[43] but relatively few studies have used mouse mast cells. This appears to be due to their refractoriness to releasing agents.[16–18] We have found that the pH at which release can take place differs markedly in the two cell types. Nicotine triggers the liberation of amines from rat peritoneal mast cells at pH 7·4[13] and from neoplastic mast cells of the mouse at pH levels above 8·0.[19] In preliminary experiments, we have observed that compound 48/80 has the same alkaline pH-dependent releasing effect upon the tumor cells. The effect seems to be due to the availability in the mouse cell, only under alkaline conditions, of a site required to initiate or sustain amine release. The variability in the pH required for the action of the release-inducing agents in the mouse, as compared

with rat, may be ascribed to the malignant state of the mouse cells or, alternatively, to a species difference between the mast cells from the two animals.

In experiments with the mouse and the rat mast cell, the total cellular content of histamine and 5-HT is never released and often the level of 5-HT released exceeds that of histamine (Table 1, Fig. 4). This may reflect the distribution of the amines between the granular and the extragranular pools which are found in the tumor cells.[44–46] Amine release from rat peritoneal mast cells is incomplete,[26,29–31,42,47] even though an extragranular amine pool has been reported to be absent in these cells.[48,49] The presence of immature, non-releasable granules (progranules)[50,51] may account for the remaining intracellular amines and heparin in the rat mast cells.[31] This could also apply to the mouse tumor cells in which many immature multivesicular granules are present in young cells formed as a result of the rapid rate of cell division in the population.[20] The interpretation of the results of the measurement of heparin release is further complicated by the additional problems of adherence of the sulfated mucopolysaccharide to cells and cell fragments, and the aggregation of the polymer units alone or with protein to form precipitable clumps, all of which may contribute to low values.[28,29] Nevertheless, the results obtained for the liberation of mouse neoplastic mast cell granule components with nicotine (Table 1) are similar to those obtained for rat peritoneal mast cells by other laboratories using releasing agents other than nicotine.[29–31]

In addition to their action on mast cells, nicotine and other pyrrolidines produce stimulatory effects on nerve and muscle.[52,53] The pyrrolidinium ion of nicotine is the active form of the drug at the ganglionic and the neuromuscular junction sites.[33,54] This contrasts with the induction of amine release in mouse neoplastic mast cells where the free base appears to be the biologically active molecular species. This probably reflects a variability in the nicotine receptors of the different cell types. This view is also supported by the marked difference (100–1000 ×) in the concentration of nicotine that is required to produce the optimum stimulatory response of mast cells as compared with neurons and muscle cells.

Nicotine, chemically, is an alpha-substituted pyrrolidine. In the tumor mast cell system, we have shown that the pyrrolidine ring is the biologically active portion of the nicotine molecule that is required for the initiation of the amine release. The presence of a methyl group on the pyrrolidine ring nitrogen does not affect the activity of nicotine or the pyrrolidines in this system, probably because there is still a free electron pair on the ring nitrogen atom. The importance of this electron pair may be further illustrated by the lack of amine–heparin releasing activity shown by the pyrrolidinones. The pyrrolidinones are cyclic amides in which the partial polarization of the carbonyl group is reflected chemically by very weak basicity.[34,35] and it would be expected to make the electrons on the adjacent nitrogen atom less available for biochemical interaction with the mast cell, though the possible role of steric factors (due to the presence of the oxygen atom) has not been eliminated. A similar relationship between basicity and biological activity in the pyrrolidine series was observed by Craig in 1933.[55]

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