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CHARACTERIZATION OF NONCHOLINERGIC NICOTINE RECEPTORS ON HUMAN GRANULOCYTES

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Abstract—The noncholinergic nicotine receptor on leukocytes identified earlier [Davies *et al.*, *Molec. cell. Biochem.* **44**, 23 (1982)] was further characterized. Structure–activity relationships showed that a pyrrolidine ring containing a basic N atom is an important structural feature for ligands that bind to the receptor. Accordingly, the carcinogenic component of tobacco smoke, *N*-nitrosonornicotine, does not bind to the receptor. The stereoselectivity for the *d*-isomer, which was confirmed using [^{3}H]*d*-nicotine as a ligand, together with the absolute configurational relationship between *d*-nicotine and L-proline, suggested that basic peptides containing proline as the N-terminal amino acid would bind to the receptor. The finding that Pro-Lys-Pro-Arg, which has been reported to inhibit granulocyte phagocytosis, bound to the receptor with an IC_{50} value of 3.5 μM is compatible with this idea. An increase in receptor binding, which was observed in the presence of plasma, could be ascribed to bicarbonate. The presence of bicarbonate in the binding assay, even when the pH of the buffer was carefully controlled, resulted in an increase (approximately 2-fold) in the apparent number of receptors without affecting the K_d value significantly. Increasing the pH of the buffer in the absence of bicarbonate also increased receptor binding, suggesting that bicarbonate may increase receptor binding by its known ability to increase intracellular pH at constant extracellular pH. Preincubation of cells with *d*-nicotine under certain conditions reduced the subsequent binding of [^{3}H]*d*-nicotine to the receptor.

Tobacco smoking has a number of effects on leukocytes, including an increase in leukocyte number [1, 2], impairment of granulocyte chemotaxis [2], random migration [3, 4], and increased release of elastase [5] and superoxide [6] from lung macrophages. In addition, smoking has been claimed to increase thrombus formation [7], increase degranulation of basophils [8], and increase chromosomal aberration in lymphocytes [9, 10]. Nicotine, which is the principal alkaloid of the tobacco plant, is apparently responsible for the vasoconstriction and increased heart rate that occurs as a result of smoking [11, 12]. Nicotine also affects prostaglandin metabolism in various tissues including aorta [13], increases collagen synthesis in cultured aortic endothelial cells [14], and potentiates platelet thrombus formation [7].

Recently, using tritiated *d,l*-nicotine, we demonstrated a non-cholinergic receptor for nicotine on human leukocytes [15] and platelets‡. Although the receptor is stereoselective for the *d*-isomer, *l*-nicotine (the naturally-occurring isomer) binds at concentrations present in the blood after smoking [16]. Further work using 5-doxylstearic acid as a spin probe provided evidence that *l*-nicotine produced a specific and receptor-mediated perturbation in granulocyte membrane as well as a modulation of chemotaxis [17].

The present paper further characterizes nicotine receptors on granulocytes, showing the importance of the pyrrolidine ring containing a basic N atom for receptor binding and the stereochemical relationship between *d*-nicotine and N-terminal proline peptides. We also demonstrate that the tobacco-specific nitrosamines do not interact at this site, that bicarbonate ion is the factor responsible for the increased binding of nicotine to granulocytes in the presence of blood plasma, and that prior exposure to *d*-nicotine reduces the specific binding of [^{3}H]*d*-nicotine to granulocytes.

MATERIALS AND METHODS

Materials. Tritiated *d,l*- and *d*- and *l*-nicotines were obtained from the New England Nuclear Corp. at specific activities ranging from 30 to 70 Ci/mmole. Thin-layer chromatography on silica gel using ethanol–acetone–diethanolamine (60:60:1.5) as the solvent showed that all labeled compounds were >95% pure. This system separates nicotine, cotinine, nornicotine and nicotine *N*-oxide. Unlabeled *d*- and *l*-nicotine bitartrate salts, which were gifts from Prof. L. G. Abood, were pure by HPLC and TLC criteria. Ficoll and sodium diatrizoate used for the density gradient were purchased from the Sigma Chemical Co. and phosphate-buffered saline (PBS) and Hanks' balanced salt solution (without calcium, magnesium, phenol red and sodium bicarbonate) from M.A. Bioproducts and GIBCO. Other chemicals and supplies were high quality materials obtained from commercial sources.

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Cell preparation. Most of the studies reported here used granulocytes prepared essentially by the method of Aguado *et al.* [18], as reported previously [15]. Briefly, buffy coat (60 ml) from a unit of blood drawn into citrate-phosphate-dextrose anticoagulant was diluted with 1 vol. of phosphate-buffered saline. Mononuclear and polymorphonuclear cells were separated on discontinuous density gradients comprised of two layers of Ficoll and sodium diatrizoate by centrifugation at 400 *g* for 40 min at 22°. The lower density solution (1.075 g/ml; 264 mosM) consisted of 10 vol. of a 34% solution of sodium diatrizoate (Sigma Chemical Co.) and 24 vol. of a 9% Ficoll solution. The higher density (1.090 g/ml) contained 10 vol. of a 34% solution of sodium diatrizoate and 24 vol. of 15% Ficoll solution.

After centrifugation, the mononuclear cells were collected from the upper interface and granulocytes from the lower one using a spinal syringe needle which had been bent 90° approximately 10 mm from its tip. Cell suspensions were diluted with an equal volume of phosphate-buffered saline and were collected by centrifugation at 400 *g* for 10 min. The resultant cell pellets were washed four times with 20 ml of phosphate-buffered saline and finally resuspended in 10 ml of phosphate-buffered saline.

Cell counting. Cell suspensions were diluted 1 to 20 or 1 to 50 from concentrated suspensions in PBS with gentian violet stain in 2% acetic acid and counted with the aid of an improved Neubauer chamber obtained from the American Optical Co.

Cell viability. The viability of the cells was determined by the fluorescein diacetate–ethidium bromide test as described by Dankberg and Persidsky [19]. Cell viability measured in this manner was routinely 95%.

Binding assays. Tritiated *d,l-* or *d*-nicotine was used as the ligand for measuring binding to nicotine receptors, employing a centrifugation assay as described previously [15]. Binding assays were generally performed in Hanks' buffer (without calcium, magnesium, phenol red and sodium bicarbonate) at 0–4°, pH 7.5, using 3 million cells in 1 ml. Nonspecific binding was evaluated by adding excess unlabeled *d*-nicotine to one-half of the samples. Values for K_d and B_{max} were obtained from Scatchard or double-reciprocal plots of the specific binding. Binding of unlabeled ligands to the receptor was evaluated by competition experiments in which the reduction in the specific [^{3}H]nicotine binding was used to measure the IC_{50} value of the unlabeled ligand.

Preincubation with d*-nicotine.* Cells were incubated batchwise in Hanks' buffer at 37°, pH 7.5, in the presence of 400 μg/ml of bacitracin and 50 units/ml each of penicillin and streptomycin. Subsequently, the cells were washed three times and resuspended in cold buffer. The effectiveness of the washing procedure was determined by including [^{3}H]*d*-nicotine in some samples. Binding of [^{3}H]*d*-nicotine was then determined in the usual manner.

RESULTS

Compounds with structures related to nicotine. The tobacco-specific nitrosamines were of interest because of the possible relation of these compounds to carcinoma [20]. As shown in Fig. 1, there was virtually no effect with *N*-nitrosonornicotine and only a slight but concentration-independent effect of 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone. The compound metyrapone, which was tested because it prevents the accumulation of radiolabeled nicotine in animal tissues *in vivo* [21], also bears some structural similarity to nicotine, having two pyridine rings (Fig. 2). We found that metyrapone was a very weak inhibitor, causing a maximum of 33% inhibition at 10^{-4} M. The results with *N*-benzylnornicotine are shown for comparison (Fig. 1).

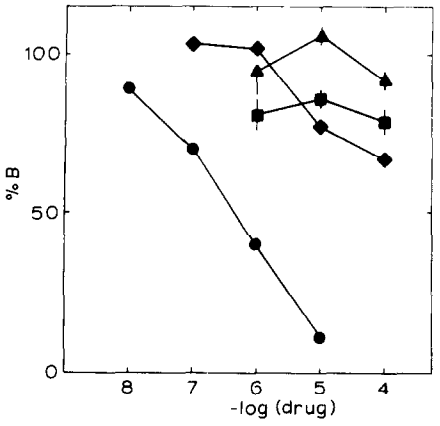

Fig. 1. Inhibition of the specific binding of 10^{-8} M [^{3}H]-*d,l*-nicotine to granulocytes by *N*-nitrosonornicotine (▲), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (■) and metyrapone (◆). The inhibition by *N*-benzylnornicotine (●) is also shown for comparison.

Peptides. The presumed native ligand for the nicotine receptor of polymorphonucleocytes (PMNs) is unknown. The only peptides, however, that would contain a pyrrolidine ring with a basic N are those having proline as the N-terminal amino acid. The inhibition of specific nicotine binding by two such peptides—Pro-Lys-Pro-Arg and Pro-Leu-Gly-NH_2—is shown in Fig. 3. Whereas the IC_{50} value for Pro-Leu-Gly-NH_2 was 79 μM, similar to that for *N*-f-Met-Leu-Phe, the value for Pro-Lys-Pro-Arg was 3.5 μM. It should be noted that the inhibition of specific binding by the peptides was only partial even at high concentrations ($\geq 10^{-5}$ M). Peptides that showed no effects at 10^{-5} M included β-endorphin, Thr-Lys-Pro-Arg (tuftsin), and Arg-Pro-Lys-Pro (SP

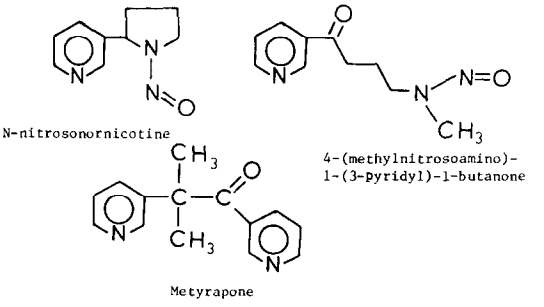

Fig. 2. Structures of the tobacco-specific nitrosamines and metyrapone.

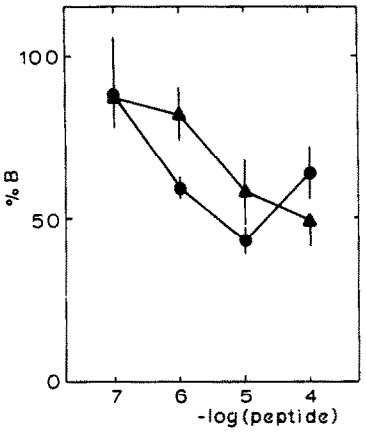

Fig. 3. Inhibition of the specific binding of 10^{-8} M [^{3}H]-*d,l*-nicotine to granulocytes by the peptides Pro-Lys-Pro-Arg (●) and Pro-Leu-Gly-NH_2 (▲). The error bars represent the S.E.M. of three independent experiments each performed in triplicate.

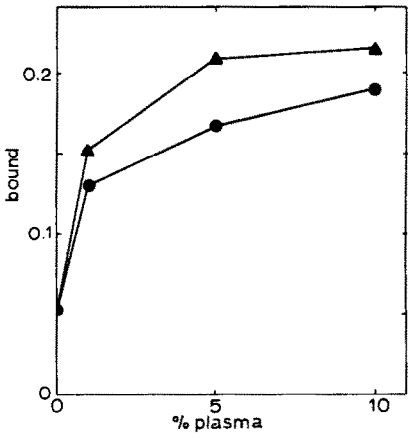

Fig. 5. Comparison between the enhancement of the specific binding of 10^{-8} M [^{3}H]-*d,l*-nicotine to granulocytes by sodium bicarbonate (▲) and plasma (●). Separate solutions were prepared for each concentration of sodium bicarbonate in phosphate-buffered saline, and the pH of each was adjusted to a value of 7.4. The concentrations of bicarbonate were matched to their percent plasma values, for example, 2.5 mM sodium bicarbonate is equivalent to 10% of the plasma value. The ordinate represents specific binding in units of pmoles/3 × 10^6 cells.

1–4), Met-enkephalin, and substance P; none of these peptides contain proline as the N-terminal amino acid.

Effects of plasma components. A robust activation of specific binding was noted in the presence of plasma with a maximum effect occurring between 5 and 10% plasma in the incubation medium (Fig. 4). Both serum from freshly clotted blood and heat-treated plasma were also active. Fractionation of heat-treated plasma by column chromatography on Sepharose CL-6B failed to yield a protein fraction containing activity. In addition, the following proteins, which were tested independently, were without effect: bovine serum albumin, 2 and 5 mg/ml; human globulin (Cohn Fraction IV), 2 mg/ml; human γ-globulin (Cohn Fraction II), 2 mg/ml; aggregated γ-globulin, 0.75 mg/ml; and human fibrinogen, 2 mg/ml. Additional inactive components included glucose (1 mg/ml), urea (5 mM) and NaH_2PO_4 (2 mM).

The only active component identified was bicarbonate. The data in Fig. 5 suggest that bicarbonate ion accounts for the activation by plasma since the maximal effect in this and three additional experiments occurred with 2.5 mM HCO_3^-, which is equivalent to 10% of the plasma value (25 mM). The magnitude of the activation by bicarbonate ion was variable depending on the individual preparation of PMNs. The mean percent of the control value for specific binding of 10^{-8} M [^{3}H]*d,l*-nicotine was 213 ± 56 (S.E.M. of six determinations each performed in triplicate) in the presence of 5 mM HCO_3^-. A trend toward greater increases in preparations with lower initial values for specific binding was also noted.

Since HCO_3^- (25 mM in blood) has a strong capacity for buffering blood, the effect of pH on specific binding was investigated. As shown in Fig. 6, increasing pH also dramatically increased specific binding to nicotine receptors. Figure 6 also shows that the enhancing effect of added HCO_3^- was much less at pH 8.5 compared with pH 7.5.

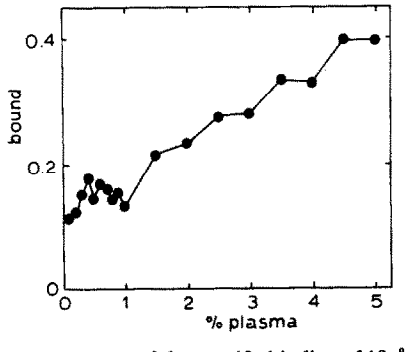

Fig. 4. Enhancement of the specific binding of 10^{-8} M [^{3}H]-*d,l*-nicotine by plasma. The ordinate represents specific binding in units of pmoles/3 × 10^6 cells.

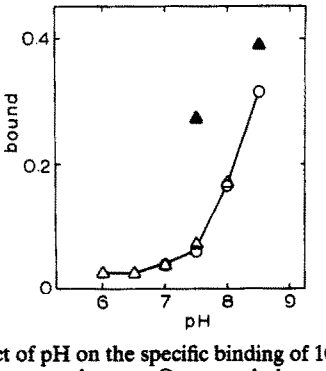

Fig. 6. Effect of pH on the specific binding of 10^{-8} M [^{3}H]-*d,l*-nicotine to granulocytes. Open symbols represent data from two independent experiments each performed in triplicate. The maximal stimulation with bicarbonate is shown for comparison (▲).

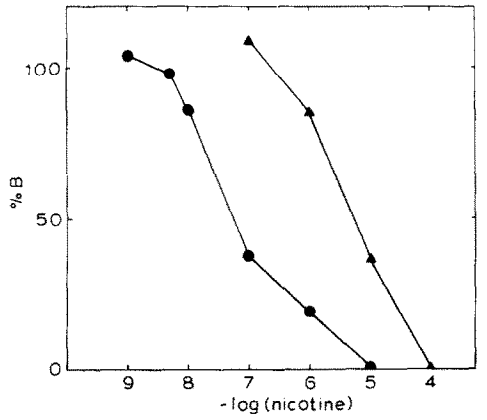

Fig. 7. Inhibition of the binding of 10^{-8} M [^{3}H]*d*-nicotine to granulocytes by *d*- (●) and *l*-nicotine (▲). The K_i values, which were calculated from the expression $K_i = IC_{50}/(1 + L/K_d)$ where L is the free ligand concentration, were 23 nM and 2.3 μM respectively. The Hill coefficients were 0.92 and 1.0 respectively.

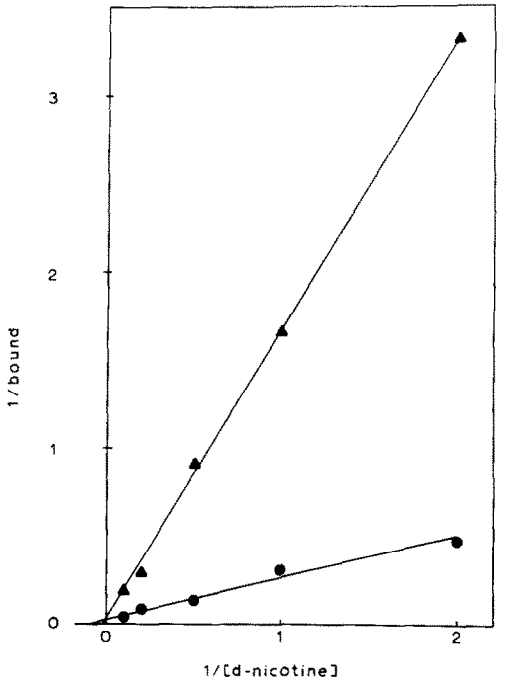

Fig. 8. Double-reciprocal plot of the specific binding of [^{3}H]*d*-nicotine to granulocytes in the presence (▲) and absence (●) of 10^{-5} M *l*-nicotine. The calculated values for K_d and B_{max} were 6.6 nM and 57,400 sites/cell respectively. The Hill coefficient was 1.3.

Double-reciprocal plots of the binding of [^{3}H]*d,l*-nicotine in the presence and absence of HCO_3^- demonstrated that the increase in binding is owing to an increase in the apparent number of receptors with relatively small changes in the K_d value. In three experiments each performed in triplicate, the mean value of B_{max} increased from 65,300 ± 17,700 to 145,000 ± 43,000 sites/cell (±S.E.M.) in the presence of 5 mM HCO_3^-, whereas the mean K_d values in the absence and presence of HCO_3^- were 16.8 ± 2.9 and 14.2 ± 3.7 nM (±S.E.M.) respectively.

Stereoselectivity. We had initially reported, using [^{3}H]*d,l*-nicotine, that the stereoselectivity of the *d*- over the *l*-isomer was approximately 30-fold, comparing IC_{50} values for inhibition of specific binding [15]. The recent availability of [^{3}H]*l*-nicotine and [^{3}H]*d*-nicotine made possible the confirmation of the stereoselectivity. Figure 7 shows the inhibition of the binding of [^{3}H]*d*-nicotine by both *d*- and *l*-nicotines. A robust stereoselectivity of approximately 100-fold is evident.

An example of a double-reciprocal plot of the binding of [^{3}H]*d*-nicotine using 10^{-5} M *d*-nicotine to define non-specific binding is shown in Fig. 8. The mean value of K_d was 7.73 ± 1.6 nM (mean ± S.D. of two experiments each performed in triplicate) and the number of sites/cell was 48,700. Figure 8 also shows that *l*-nicotine is apparently competitive for the binding site. As expected from the relatively low affinity of the *l*-isomer, we were unable to use it in tritiated form as a ligand for measuring specific binding to leukocytes.

Preincubation with d-*nicotine.* To determine whether exposure of granulocytes to a saturating concentration of *d*-nicotine would affect subsequent binding, cells were preincubated at 37° with 10^{-4} M *d*-nicotine, washed and then specific binding of [^{3}H]-*d*-nicotine was measured in the usual manner.

Table 1. Effect of preincubation on binding of [^{3}H]*d*-nicotine

Experimental conditions	Specific binding* (pmoles bound/3 million cells)	P‡
5 nM [^{3}H]*d*-Nicotine		
Control	0.065 ± 0.023	
Preincubated	0.035 ± 0.011	<0.01
1 nM [^{3}H]*d*-Nicotine		
Control	0.020 ± 0.0071	
Preincubated	0.0071 ± 0.0076	<0.02

Cells were preincubated with and without (control) 10^{-4} M *d*-nicotine for 30 min, 37°, washed, and then binding of [^{3}H]*d*-nicotine was subsequently measured.

* Mean ± S.D. of pooled raw data from two independent experiments each performed in triplicate.

‡ Student's *t*-test.

Preliminary experiments showed that the washing procedure completely removed unlabeled *d*-nicotine and that maintaining the cells at 37° without *d*-nicotine also reduced specific binding (approx. 50% in 30 min).

Table 1 shows that preincubation with 10^{-4} M *d*-nicotine for 30 min at 37° reduced the subsequent binding of [^{3}H]*d*-nicotine relative to the control, which was preincubated without *d*-nicotine. The nonspecific binding, however, was not affected by preincubation with *d*-nicotine. Although trends consistent with the data in Table 1 were noted with additional concentrations of *d*-nicotine and different incubation times, the data were not statistically significant. In one experiment, preincubation with 10^{-5} M Pro-Lys-Pro-Arg at 37° for 30 min reduced the specific binding of 1 nM [^{3}H]*d*-nicotine from 0.028 ± 0.0045 to 0.012 ± 0.0048 pmoles bound/3 million cells ($P < 0.02$).

DISCUSSION

Taken together, the structure–activity relationships, reported previously [15] and in the present work, suggest that a pyrrolidine ring containing a basic N atom is an important structural feature for binding to the nicotine receptor on granulocytes. The native ligand for this receptor, however, remains unknown. The possible activity of a basic peptide containing proline as the N-terminal amino acid was predicted on the basis of the structure–activity relationships as well as the absolute configurational relationship between L-proline (the natural form) and *d*-nicotine [22] (see Fig. 9). As expected, both L-proline and *d*-nicotine are dextrorotatory. The finding that one peptide, Pro-Lys-Pro-Arg, has an affinity for the receptor in the low micromolar range may shed light on the structure of the native ligand.

Radiolabeled nicotine accumulates in the bronchial and nasal epithelia regardless of the route of administration [21]. Metyrapone, which interacts with the cytochrome P-450 system in endoplasmic reticulum, completely prevents the accumulation of nicotine in these tissues [21]. *N*-Nitrosonornicotine also accumulates in the nasal and bronchial epithelia [23] where it has some carcinogenic activity [20]. Neither metyrapone, *N*-nitrosonornicotine nor the ring-opened butanone derivative, which is also present in tobacco smoke, bound to the nicotine receptor on granulocytes, showing that these compounds do not act by this mechanism. It is, therefore, unlikely that the nicotine receptor on granulocytes is involved in either the accumulation of nicotine by bronchial and nasal epithelia or the toxic effects of the tobacco-specific nitrosamines.

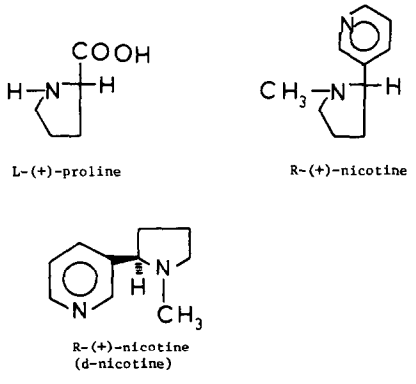

Fig. 9. The absolute configuration of *d*-nicotine (bottom) and its stereochemical relationship to L-proline (top).

Although phagocytic leukocytes can be cytotoxic to tumor cells [24] and may also protect against malignancy [25], there is evidence that superoxide, which is a product of stimulated phagocytes, can itself be mutagenic [26]. Along these lines, Weitzman and Stosell [27] recently reported that the phagocytic leukocytes were mutagenic in the Ames test. Neither *d*- nor *l*-nicotine had any effect on the generation of superoxide or hydrogen peroxide by granulocytes or monocytes (data not shown).

The noncholinergic nature of the nicotine receptor on leukocytes and platelets [15] separates pharmacologically the effects of nicotine on these cells from the previously described agonist effects of nicotine at cholinergic synapses on skeletal muscle and in the central and peripheral nervous systems. In addition, the receptor is different from the nicotine receptor described by Abood *et al.* [28] in the brain on the basis of stereoselectivity. The neural receptor, the occupancy of which is linked behaviorally to a prostration-immobilization response [29], has a stereoselectivity for the *l*-isomer. Thus, there is an apparent chiral relationship between noncholinergic receptors for nicotine depending on whether the receptors are found in nervous tissue or on blood cells.

The effects of nicotine on phagocytosis remain to be evaluated. It has been reported that the peptide Pr-Lys-Pro-Arg is an inhibitor of granulocyte phagocytosis [30]. Neither Pro-Lys-Pro-Arg nor Pro-Leu-Gly-NH_2 stimulated chemotaxis measured by the method of Nelson *et al.* [31]. Pro-Lys-Pro-Arg, moreover, did not affect the chemotactic activity of N-f-Met-Leu-Phe (data not shown). In an earlier study, we had shown that *l*- but not *d*-nicotine modulates chemotaxis towards N-f-Met-Leu-Phe and that neither compound has chemotactic activity [17].

The observed increase in receptor binding in the presence of bicarbonate may be a consequence of intracellular pH changes. Many cells have mechanisms, for example, Na^+/H^+ and HCO_3^-/Cl^- exchange, for maintaining the value of intracellular pH in the range of 0.5 to 1 pH unit above that expected from the passive diffusion of H^+ across the plasma membrane [32]. In some systems, HCO_3^- is an essential component of the exchange process. In addition, there is evidence that HCO_3^- can increase the intracellular pH (approximately 0.5 pH unit) of leukocytes at constant extracellular pH [33]. This line of reasoning also implies that intracellular pH may regulate binding to the nicotine receptor on granulocytes.

Nicotine, with pK values of 6.16 and 10.96, would remain almost exclusively in the monoprotonated form over the pH range (7.5 to 8.5) where the binding is increasing. Therefore, the acid–base equilibrium of nicotine itself should not be a factor in the bicarbonate-induced increase in binding. Although unlikely, the possibility remains that bicarbonate

could have some specific interaction with the receptor and/or nicotine.

The experiments in which cells were preincubated with *d*-nicotine are complicated by the additional reduction in binding after exposure to 37° without *d*-nicotine and other factors such as cell aggregation. It appears, however, that at least under some conditions subsequent binding of [^{3}H]*d*-nicotine is reduced relative to the control values.

In conclusion, the structural requirement for a pyrrolidine ring containing a basic N atom for binding to nicotine receptors on granulocytes is shown by the relatively high affinities of nicotine, nornicotine, *N*-benzylnornicotine, and pyridylmethylpyrrolidine [15] compared with the virtual inactivity of cotinine and *N*-nitrosonornicotine. This finding, coupled with both the stereoselectivity for the *d*-isomer and its absolute configurational relationship to L-proline, led to the prediction that basic peptides containing *N*-terminal proline would bind to the receptor. This idea was confirmed by the binding inhibition of Pro-Lys-Pro-Arg, possibly providing a clue to the structure of the presumed native ligand for the receptor. The ability of raised pH of the buffer or of bicarbonate independent of the pH of the buffer to increase the apparent number of receptors suggests that the receptor is sensitive to the intracellular pH of granulocytes, unmasking additional sites at higher pH. The possibility of receptor desensitization is raised by the ability of exposure to *d*-nicotine to reduce subsequent binding to the receptor.

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