Biochimica et Biophysica Acta, 778 (1984) 503–510
Elsevier

BBA 72332

NICOTINE-INDUCED MEMBRANE PERTURBATION OF INTACT HUMAN GRANULOCYTES SPIN-LABELED WITH 5-DOXYLSTEARIC ACID

CORRELATION WITH CHEMOTAXIS

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(Received May 25th, 1984)

Key words: Nicotine-membrane interaction; Granulocyte membrane; Chemotaxis; Spin label; ESR

The effects of nicotine on intact human granulocytes were examined, using 5-doxylstearic acid as a spin probe. At micromolar concentrations, (−)-nitocine produces a membrane perturbation in granulocytes not observable with oriented lipid bilayers. The effect, which is stereoselective for the (−)-isomer, occurs at concentrations of nicotine that bind to noncholinergic nicotine receptors on granulocytes and which are present in the blood after smoking. At comparable concentrations, (−)-nicotine modulates granulocyte chemotaxis towards a chemotactic peptide in a stereospecific and dose-dependent manner. Cotinine, the major metabolite of nicotine, does not bind to the receptor, does not produce the membrane perturbation observed with nicotine, and has no effect on chemotaxis. These results suggest that (−)-nicotine present in the blood after smoking binds to a receptor on granulocytes, perturbs granulocyte membranes and modulates chemotaxis.

Introduction

It has been known for several years that tobacco smoking increases leukocyte number [1,2] and impairs granulocyte chemotaxis [2]. More recently, it was shown that peripheral blood leukocytes have a specific receptor for nicotine [3] which is the principal alkaloid of the tobacco plant. The receptor, which is noncholinergic, binds nicotine at concentrations found in the blood after smoking [4]. Although these studies demonstrate specific interactions of nicotine with granulocytes, the effects of nicotine on leukocyte membranes have not been investigated.

Electron spin resonance has been used widely for showing effects of various drugs on membranes [5], enzyme-substrate binding [6] and antigen-antibody interactions [7]. In the present study, 5-doxylstearic acid was used as a spin probe to examine the effects of nicotine on granulocyte membranes, comparing the results obtained with lipid bilayers. In addition, these data were correlated with the effects of nicotine on granulocyte chemotaxis. The results show that low concentrations of nicotine produce a stereoselective alteration in the membrane structure of intact granulocytes not observable with lipid bilayers, as well as a stereospecific inhibition of granulocyte chemotaxis.

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Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DPPS, dipalmitoylphosphatidylserine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Materials

Materials for the preparation of bilayers were purchased from Sigma Chemical Co. 5-Doxylstearic acid and 3-doxylcholestane were obtained from Molecular Probes, Inc., (−)-nicotine, Ficoll and sodium diatrizoate were purchased from Sigma Chemical Co. and (+)− nicotine and cotinine were gifts from Leo G. Abood. The nicotines and cotinine were pure as determined by HPLC as described in the Experimental Methods section. For the chemotaxis experiments, Eagle's minimum essential medium (10×) and penicillin/streptomycin (5000 units each/ml) were obtained from Gibco, agarose (SeaKem LE) from the Biochemical Division of Marine Colloids and plastic culture dishes (60 × 15 mm) from Falcon Plastics.

Experimental Methods

ESR measurements. Electron spin resonance spectra were recorded with an IBM-Bruker model ER-200D ESR spectrometer interfaced with a computer system as described elsewhere [8]. The percent increase in mobile spin label was calculated from the following formula: $100(1-[(B/F)_{exp}/(B/F)_{control}])$. The ratio of bound label to free label (B/F) was measured as shown in Figs. 1 and 3. The experimental samples contained various concentrations of nicotine or cotinine and the control contained 10 μl of water.

Preparation of dipalmitoylphosphatidylcholine (DPPC) oriented bilayers. For preparation of the bilayers, 7.5 μl of 0.123 M DPPC, (0.922 μmol), 3.75 μl of 0.10 M cholesterol (0.375 μmol) and 0.5 μl of spin probe (either 3-doxycholestane, 5-doxylstearic acid (0.006 μmol) were mixed with 11.25 μl of chloroform in a glass vial and shaken for 1 min and then sonicated for 2 s at room temperature. The molar percentage ratio of DPPC/cholesterol/spin probe was 70.93 : 28.83 : 0.23 and the molar ratio of DPPC/spin label was 308 : 1.

A 10 μl portion of the lipid mixture described above was applied uniformly to a quartz plate over an area of 20 × 8 mm. After the chloroform was evaporated using a gentle stream of moist N_2, the plates were subjected to a vacuum of 0.3–0.15 mm of Hg for 3 h in a desiccator containing dryrite. The bilayers were hydrated by storing plates in a chamber maintained at 100% humidity with 1% aqueous sulfuric acid for 30 min. Alternatively, the bilayers could be hydrated by the addition of 10 μl of 1 mM sodium phosphate buffer at pH 7.0 containing 0.9% NaCl.

Preparation of mixed DPPC/DPPS bilayers. The mixed DPPC/DPPS bilayers were prepared in a manner similar to that described above for DPPC bilayers. The following amounts of reagents were used: 11.25 μl of $CHCl_3$, 3.75 μl of 0.123 M DPPC (0.461 μmol), 3.75 μl of 0.0615 M DPPS (0.231 μmol), 3.75 μl of 0.10 M cholesterol (0.375 μmol) and 0.5 μl of spin probe, 3-doxylcholestane or 5-doxylstearic acid (0.0030 μmol). The molar percentage ratio of DPPC/DPPS/cholesterol/spin probe was 43.12 : 21.55 : 35.05 : 0.28 and molar ratio of lipids/label was 230 : 1.

Effect of nicotine on the synthetic oriented bilayers. 10 μl of aqueous nicotine solution (prepared by serial dilution in doubly distilled, deionized water) was applied to the previously hydrated bilayer and allowed to stand at room temperature. After the excess water evaporated (about 20 min), the ESR spectra were recorded within the next 10 min.

Preparation of leukocytes. Granulocytes and a mononuclear fraction were prepared using a modification of the procedure of Aguado et al. [9]. Briefly, buffy coat (60 ml) from a unit of blood drawn into citrate-phosphate-dextrose anticoagulant was diluted with 1 vol. of phosphate-buffer saline. Mononuclear and polymorphonuclear cells were separated on discontinuous density gradients comprised of two layers Ficoll and sodium diatrizoate by centrifugation at 400 × *g* for 40 min at 22°C. The lower density solution (1.075 g/ml; 264 mosM) consists of 10 vol. of a 34% solution of sodium diatrizoate (Sigma Chemical Co.) and 24 vol. of a 9% Ficoll. The higher density (1.090 g/ml) contained 10 vol. of a 34% solution of sodium diatrizoate and 24 vol. of 15% Ficoll solution.

The gradient tubes were prepared as follows: 20 ml of the diluted buffy coat were added to plastic centrifuge tubes. The lower density Ficoll/sodium diatrizoate solution (15 ml) was gently added to the bottom of the tube, using an 18 gauge 75 mm spinal syringe needle, forcing the cell suspension

upward. Similarly, 15 ml of the higher density solution were added to the tube with a spinal syringe needle. After centrifugation, the mononuclear cells were collected from the upper interface and granulocytes from the lower one using another 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 $400 \times g$ 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. In order to remove residual red cells, the mononuclear fraction was then carefully layered over 20 ml of the lower density solution and the polymorphonuclear fraction was layered over 20 ml of the higher density solution. After centrifugation at $400 \times g$ for 20 min, the leukocytes were collected and washed as described above.

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

Cell viability. The viability of the preparation was routinely meaured by the method of Dankberg and Persidsky [10]. Cell suspensions were incubated with an equal volume of a fluorescein diacetate/ethidium bromide mixture and examined under a fluorescence microscope. The viable cells take up nonfluorescent fluorescein diacetate, which is cleaved by an esterase to yield fluorescein (green fluorescence). Damaged cells, having disrupted nuclear membranes, take up the ethidium bromide, which becomes intercalated into the DNA and fluoresces red. The percent viable is the ratio of the number of green cells to the total number of red plus green cells. All granulocyte preparations used in these experiments were greater than 95% viable.

Chemotaxis. Granulocyte chemotaxis was measuted by the method of Nelson et al. [11] in which cells migrate under a layer of agarose toward a chemoattractant. Briefly, 5 ml of a solution prepared by mixing equal volumes of 2% agarose and a culture medium comprised of 2 × Eagle's minimum essential medium (with glutamine), 100 units of each of streptomycin and penicillin/ml, 10 mg of gelatin/ml and buffered with 50 mM Hepes adjusted to pH 7.5 were poured into a 60 × 15 mm plastic culture dish. After cooling for at least 30 min in a refrigerator, six sets of three wells (2.8 mm diameter and 2.75 mm apart) were punched in the agarose with the aid of a plastic template and stainless steel punch. The innermost wells were filled with 10 μl of Eagle's minimum essential medium and the peripheral wells contained a chemoattractant in minimum essential medium. The central wells were filled with 10 μl of a suspension containing $1 \cdot 10^8$ cells/ml in phosphate-buffered saline. After incubation for 2 h at 37°C, the culture dishes were flooded with methanol for 30 min, 40% formaldehyde for 30 min and the agarose was removed intact. The cells were then stained with gentian violet in 2% aqueous acetic acid. The image of each individual well was then magnified 20-times and projected onto a piece of graph paper.

The chemotatic index is defined as the difference between the distance moved by the cells towards the chemoattractant (distance A) and away from the chemoattractant (distance B), representing random migration divided by the distance moved away from the chemoattractant. Therefore, the chemotactic index is $(A - B)/B$. For example, a value of 0 is no chemotaxis and a value of 1 means that the cells moved twice as far towards the chemoattractant compared with the value for random migration.

In order to find the optimal concentration for the chemotactic peptide *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe), we varied the concentration of fMet-Leu-Phe between 10^{-7} and 10^{-5} M; the maximum chematactic index was obtained with a concentration of 10^{-6} M. The effects of drugs were measured by preincubating them with the cells for 30 min at room temperature, using $5 \cdot 10^{-7}$ fMet-Leu-Phe as the chemoattractant.

Granulocyte ESR experiments. 6–8 μl of 0.006 M 5-doxylstearic acid in $CHCl_3$ were taken in a 2.5 ml polyethylene vial, and the $CHCl_3$ was evaporated using dry N_2 gas. To this vial, 2–2.5 ml of granulocyte preparation ($24 \cdot 10^6$ cells/ml in phosphate-buffered saline) were added. The vials were gently shaken periodically for the next 16–18 h at 4°C. If the cells aggregated, the entire pre-

paration was rejected.

Next, 200 μl of the above thoroughly mixed polymorphonulclear cell suspension, in which 5-doxylstearic acid was incorporated, were transferred to a polyethylene vial and 20 μl of double-distilled deionized water (control) or 20 μl of an appropriate solution of (−)-nicotine, (+)-nicotine, or cotinine solution were added. The vials were gently shaken intermittently yet thoroughly for the next 2.5 h at room temperature. Then, the entire contents of the vials were transferred to ESR quartz cells and the ESR spectra were taken within 5 min.

HPLC detemination of the purity of nicotines and cotinine. The purity of the nicotines and cotinine were demonstrated through use of HPLC. A Perkin-Elmer series 2 HPLC with a 25 cm × 4.8 mm × 6.4 mm Partisil PXS 5/25 column was used. The materials were eluted with a mixture of ethyl acetate/methanol/ammonia in the ratio of 80 : 3 : 0.4 at a flow rate of 1 ml/min and a pressure of 800 lb/in^2. The compounds were monitored with an ultraviolet detector at 260 nm. A single compound was detected in each case. The nicotines had a retention time of 10 min, while cotinine had a retention time of 15 min.

Results and Discussion

ESR of oriented bilayers with 3-doxylcholestane and 5-doxylstearic acid

3-Doxylcholestane and 5-doxylstearic acid were incorporated into a dipalmitoylphosphatidylcholine (DPPC) bilayer and a mixed DPPC and dipalmitoylphosphatidylseine (DPPS) bilayer. The ESR spectra of the bilayers (Fig. 1A) show the normal orientation dependence of an immobilized spin label [5,6]. The spin-labeled bilayers were titrated with nicotine to determine the effect of nicotine on the bilayer structure or organization. At low concentrations of nicotine, there was no effect on the DPPC or the mixed DPPC/DPPS bilayer. At higher (greater than 10^{-2} M) nicotine concentrations, the ESR spectra of the spin-labeled mixed DPPC/DPPS bilayer showed the appearance of freely rotating spin label (Fig. 1B). In order to see mobile spin label in the ESR spectra of the DPPC bilayers, it was necessary to increase the concentration of nicotine by approx. 1000-fold

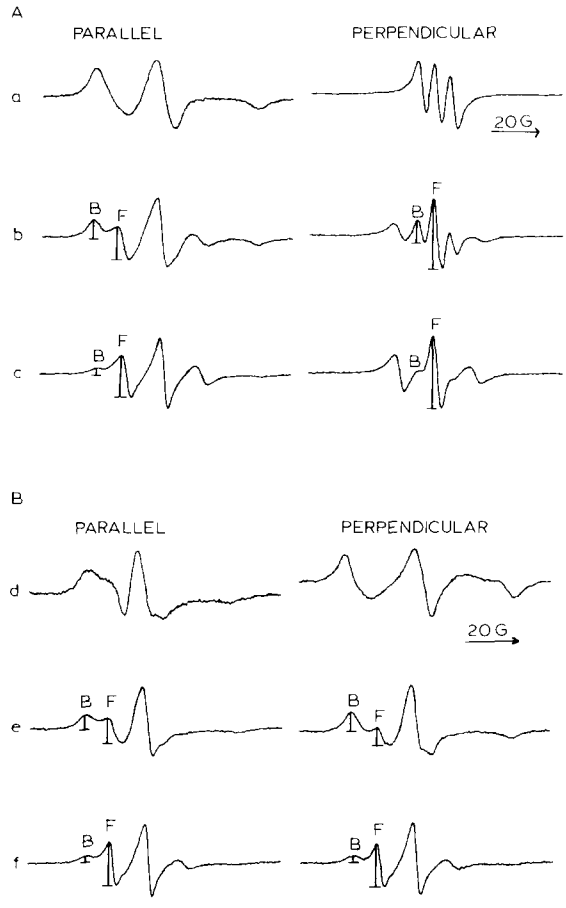

Fig. 1. A, ESR spectra of 3-doxylcholestane incorporated into DPPC/DPPS bilayers with the bilayers parallel and perpendicular to the applied magnetic field. a, control; b, and c, after treatment with 0.1 M and 0.175 M aqueous (−)-nicotine solutions, respectively. B, ESR spectra of 5-doxylstearic acid incorporated into DPPC/DPPS bilayers with the bilayers parallel and perpendicular to the applied magnetic field. d, control; e and f, after treatment with 0.1 M and 1 M aqueous (−)-nicotine solutions. Under these conditions, 5-doxylstearic acid incorporated into DPPC bilayers showed spectra as in d and were unperturbed with 1 M aqueous nicotine solution.

(greater than 1 M). The appearance of the freely rotating spin label in the case of the DPPC/DPPS bilayer can be accounted for by either release of the label from the bilayer structure into the hydration phase or by disruption of regions of the bilayer structure to allow free rotation of the spin label. Plots of the percent increase of the mobile spin label versus nicotine concentration are shown

in Fig. 2. The effect of nicotine is unique to the mixed DPPC/DPPS bilayer, indicating an interaction between DPPS and nicotine. This effect may be explained by an ionic interaction between the net negative charge on the DPPS headgroup and the positively charged nicotine molecule.

ESR of granulocytes spin labeled with 5-doxylstearic acid

Polymorphonuclear cells prepared from human blood by discontinuous Ficoll density gradient centrifugation were routinely 98% granulocytes and 1% each of basophils and eosinophils with a 95% viability. We were able to incorporate 5-doxylstearic acid but not 3-doxylcholestane into these intact cells. The ESR spectrum shown in Fig. 3a demonstrates that the spin label is largely immobilized upon incorporation into the cell. To our knowledge, this is the first report of a spin-labeled granulocyte. The amount of 5-doxylstearic acid taken up by the cells was somewhat variable, depending on the individual preparation of the cells, which were all from different donors.

When the spin-labeled cells were titrated with (−)-nicotine, the ESR spectra (Fig. 3b–c) showed a biphasic dependence of the concentration of mobile spin label on nicotine concentration. The first increase in the concentration of mobile spin label occurred in the micromolar region of nicotine concentration with a second increase in the millimolar region of nicotine concentration (Fig. 4). Thus, there is an effect of nicotine on cells that is not observed with either DPPC or mixed DPPC/DPPS bilayers. The binding of (−)-nicotine to the nicotine receptor of these cells occurs at a similar concentration with an IC_{50} value of $8 \cdot 10^{-7}$ M [3]. The concentration of nicotine in the blood after smoking is also comparable, approx. $2 \cdot 10^{-7}$ M [4]. These data suggest that the observed effects of (−)-nicotine at micromolar concentrations on polymorphonuclear cell membranes

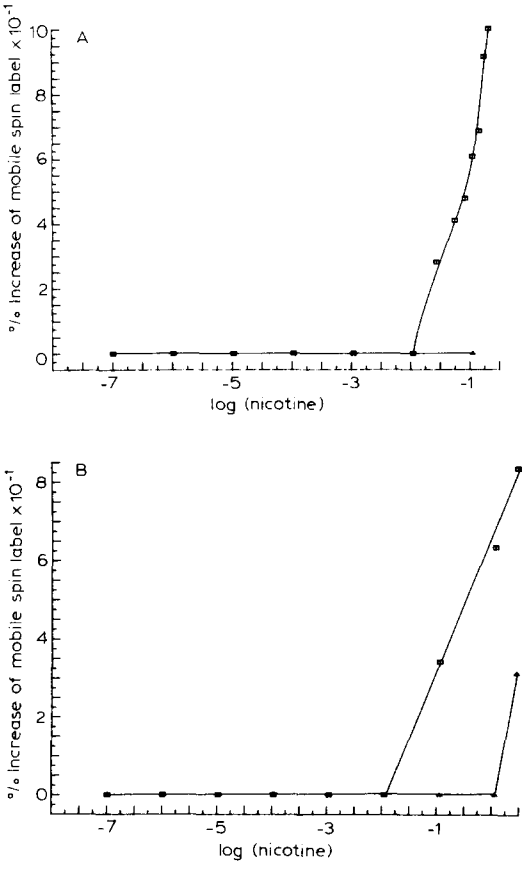

Fig. 2. A, plot of percentage of increase of mobile spin label versus nicotine concentration for DPPC (▲) and mixed DPPC/DPPS (□) bilayers labeled with 3-doxylcholestane. B, plot of percentages of increase of mobile spin label versus nicotine concentration for DPPC (▲) and mixed DPPC/DPPS (□) bilayers labeled with 5-doxylstearic acid. The data represent two experiments in which the values agreed within 3%.

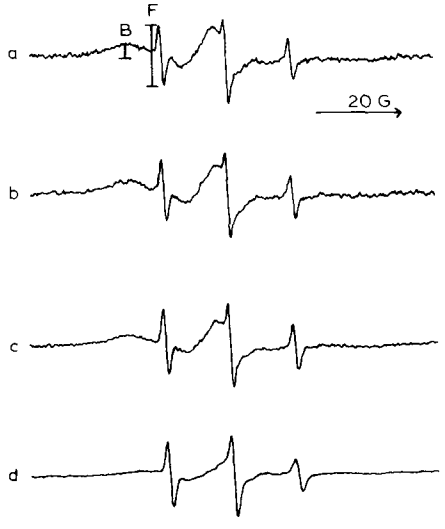

Fig. 3. ESR spectra of 5-doxylstearic acid incorporated into granulocytes. a, control; b, c and d, after treatment with 10^{-6}, 10^{-1} and 0.5 M aqueous nicotine solutions, respectively.

may occur in smokers. The results also raise the possibility that the action of nicotine is receptor mediated. ESR experiments were also conducted with cotinine, which is a major metabolite of nicotine. Titration of the cells with cotinine showed that there was no increase in the concentration of mobile radical in the micromolar region (Fig. 4). Likewise, cotinine has virtually no affinity for the nicotine receptor on granulocytes [3] and has no effect on granulocyte chemotaxis.

Fig. 4 also shows that the effect of nicotine on polymorphonuclear cell membranes is stereoselective for the (−)-isomer, since a comparable increase in the fraction of mobile spin label begins with a concentration of about 10^{-5} M (+)-nicotine. The stereoselectivity for binding to the nicotine receptor site is reversed, as evidenced by (+)-nicotine having a 30-fold greater affinity than the (−)-isomer [3]. The finding that (+)-nicotine has a higher affinity than the (−)-isomer {3]. may simply mean that (+)-nicotine better mimics the structure of the presumed native for the receptor. The binding of (+)-nicotine to the receptor would not necessarily have functional consequences, as apparently observed in both the ESR and chemotaxis experiments.

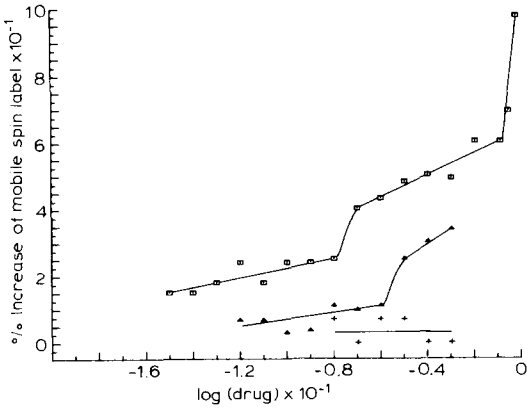

Fig. 4. The effects of (−)-nicotine (□), (+)-nicotine (▲) and cotinine (+), on the percentage of increase of mobile spin label with 5-doxylstearic acid incorporated into whole granulocytes suspended in phosphate-buffered saline (pH 7.4). The abscissa represents the log of the molar concentration of drug. The data represent three or four independent experients in which the values agreed within 5%.

Granulocyte chemotaxis

In order to compare the stereoselectivity of the effect of nicotine on polymorphonuclear cell membranes, using a functional parameter, the effects (+)- and (−)-nicotine on granulocyte chemotaxis were measured. As shown in Table I, (−)-nicotine was not a chemoattractant for granulocytes (10^{-7}–10^{-4} M) under conditions for which *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe) produced a robust stimulation. These data are in contrast to the report by Totti et al. [12] in which (−)-nicotine apparently acted as a chemotaxin. Although the methods were different – Boyden chambers compared with agarose plates – qualitative differences were unexpected. Lack of chemotactic activity was also demonstrated for (+)-nicotine, and the metabolites cotinine and nicotine *N* − oxide (data not shown). Fig. 5 shows that (−)-nicotine modulates the chemotaxis of granulocytes stimulated by fMet-Leu-Phe in a dose-dependent manner in the micromolar range. These concentrations of (−)-nicotine are comparable not only to that required for the membrane perturbation as monitored by ESR, but also that for binding to receptors.

A weak interaction between fMet-Leu-Phe and the nicotine receptor site was observed in a previous study [3]. Thus, fMet-Leu-Phe at relatively high concentrations was able to reduce the specific binding of nicotine to intact granulocytes. The IC_{50} value for fMet-Leu-Phe was $5 \cdot 10^{-5}$ M.

TABLE I

EVALUATION OF (−)-NICOTINE AS A CHEMOATTRACTANT FOR GRANULOCYTES

Chemotactic Index, CI. Results are expressed as means of two to four experiments each with six replications. The mean value of CI for 10^{-6} M fMet-Leu-Phe was 2.65 in this series of experiments.

Concentration (M)	CI
$1 \cdot 10^{-7}$	0.031
$5 \cdot 10^{-7}$	0.075
$1 \cdot 10^{-6}$	0.055
$5 \cdot 10^{-6}$	0.045
$1 \cdot 10^{-5}$	0.085
$5 \cdot 10^{-5}$	0.032
$1 \cdot 10^{-4}$	0.116

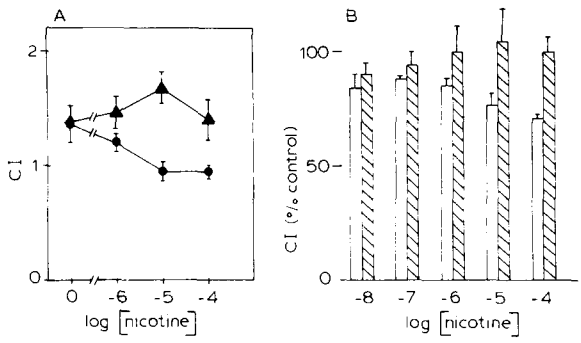

Fig. 5. Effects of (−)-nicotine (●, open bars) and (+)-nicotine (▲, hatched bars) on the chemotaxis of granulocytes. A, Representative experiments with points showing means ± S.D. of six determinations. The ordinate is the chemotactic index (CI). B, composite of several experiments with error bar representing the means ± S.E. of three to five independent experiments, each with six determinations. The data are expressed relative to the control value without nicotine for each experiment.

The potency of (−)-nicotine for producing a decrement in polymorphonuclear cell chemotaxis is considerably greater than that reported by Bridges et al. [13] who found that millimolar concentrations were required. This may be due to the fact that the chemoattractant in their study was endotoxin-activated serum, which is chemotactic for granulocytes because of the generation of complement factors. Further, there is evidence that complement components and *N*-formylated peptides induce chemotaxis by different mechanisms [14].

In contrast to results obtained with higher concentrations of (−)-nicotine [15], there was no decrement in chemokinesis (random migration) with the concentrations used in this study. For example, with 10^{-6} M (−)-nicotine, the value was 109 ± 7 expressed as the mean percent of control values without nicotine ± S.E. of five experiments each with six determinations.

Conclusion

The major findings reported in this paper are that (−)-nicotine causes a specific perturbation of granulocyte membranes and a modulation of chemotaxis. Whether the observed decrement in chemotaxis is directly related to the membrane perturbation is unknown. The possibility that these effects are receptor-mediated is supported by the following observations.

(i) The effects on membranes and chemotaxis occur at concentrations of (−)-nicotine comparable to that required for binding to noncholinergic receptors.

(ii) Cotinine, which affects neither membrane parameters as monitored by ESR measurements nor chemotaxis, does not bind to the receptor.

(iii) We have found that red blood cell ghosts do not show the nicotine-induced membrane perturbation at low concentrations (Gala, Kreilick and Hoss, unpublished observation). Likewise, these cells do not contain nicotine receptors.

In conclusion, the finding that (−)-nicotine produces effects on granulocyte membranes and chemotaxis at concentrations comparable to plasma nicotine levels after smoking supports the notion that some of the effects of smoking may be mediated by noncholinergic nicotine receptors in these cells.

Acknowledgements

The work described here was supported by grants from the Council for Tobacco Research, U.S.A., Inc. We thank Leo G. Abood for helpful discussions and Jan-Ping for technical assistance.

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