per h per mg protein) =0.0091 + 0.0064 X (1/mM pyrazinoic acid), $r = 0.99$], indicating the presence of only one enzyme in the XO fraction for both pyrazinamide and pyrazinoic acid. The conversion of pyrazinamide into 5-hydroxypyrazinamide or that of pyrazinoic acid into 5-hydroxypyrazinoic acid increased 1.30- and 1.37-fold, respectively, in the XO fraction when 0.7 mM NAD was added to the reaction mixture containing 10 mM pyrazinamide or 3 mM pyrazinoic acid respectively. It was thus demonstrated that some dehydrogenase(s) that metabolized both pyrazinamide and pyrazinoic acid existed in the XO fraction. As the staining pattern of the disc gels was not changed by the addition of NAD, this may suggest that the dehydrogenase(s) in this case was identical to xanthine dehydrogenase. The inhibitory studies with oxypurinol were done over a single period of 40 min, indicating that the greater the quantity of oxypurinol used, the more marked was the inhibition of conversion of either pyrazinamide or pyrazinoic acid in the XO fraction (data not shown).

As for pyrazinamide, however, there is evidence that xanthine oxidase may not be the main catalyst responsible for its oxidation. In one case, Weiner and Tinker found Compound II in the urine of a human subject pretreated with allopurinol; they did not find 5-hydroxypyrazinoic acid in this subject [2]. In another case, Auscher *et al.* [13] found Compound II in the urine of a xanthinuric subject who was supposedly without xanthine oxidase, though they did not find 5-hydroxypyrazinoic acid in this subject. These findings indicate that further study is warranted to identify in humans a pyrazinamide-oxidizing enzyme other than xanthine oxidase.

In summary, using disc gel electrophoresis and high performance liquid chromatography, it was shown that both pyrazinamide and pyrazinoic acid were oxidized to their 5-hydroxy forms by xanthine oxidase from human liver (K_m values of xanthine oxidase with pyrazinamide and pyrazinoic acid were 2.4 and 0.7 mM respectively).

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Mouse brain ATPase activities after chronic nicotine infusion

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Tolerance to the effects of nicotine develops with chronic exposure to the drug [1–5], and a portion of this tolerance may be related to the observation that chronic treatment with nicotine results in an increase in the number of putative nicotinic receptors [4–6]. The changes in receptor number do not seem to explain all of the tolerance, however [5].

Nicotinic receptors from *Torpedo* and *Electrophorus* contain ion channels that are activated after interaction with agonists [7]. The normal ionic conductance observed after the activation of these channels is the inward flux of Na^+ down its electrochemical gradient. Therefore, it may be that a persistent activation of nicotinic receptors arising from the constant presence of the nicotinic receptor agonist, nicotine, may increase intracellular Na^+ and result in a chronic depolarization of the nerve membrane.

Chronic membrane depolarization may indirectly affect the activity of the enzyme responsible for maintaining the membrane potential, Na^+, K^+-ATPase [8], by increasing the concentration of its intracellular ion substrate, Na^+. It has been reported that rats chronically exposed to nicotine in their drinking water appeared to have elevated levels of total Na^+, K^+-ATPase activity [9]. Na^+, K^+-ATPase in the brain is represented by two isozymes with different molecular weights [10], and different affinities for the cardiac glycosides [10, 11]. Both of these isozymes appear to function in ion transport [12]. The present study was undertaken to determine whether the two ouabain-sensitive Na^+, K^+-ATPase activities are equally affected by chronic nicotine treatment in four strains of mice that differ in their abilities to develop tolerance to the effects of nicotine [5].

Methods

Materials. ATP, phosphoenolpyruvate (PEP), ouabain octahydrate and pyruvate kinase (PK) were purchased from Boehringer–Mannheim (Indianapolis, IN) and bovine serum albumin, L-nicotine and imidazole were purchased from the Sigma Chemical Co. (St. Louis, MO).

Mice. Females of four inbred mouse strains were used: BALB/cByJ, C57BL/6J/Ibg, DBA/2J/Ibg and C3H/2Ibg. All mice were bred at the Institute for Behavioral Genetics at the University of Colorado, Boulder.

Chronic drug treatment. Mice were chronically treated with nicotine by constant intravenous infusion [4, 5]. The final treatment dose was 3.0 $mg \cdot kg^{-1} \cdot hr^{-1}$, and animals were treated with this dose for 10 days.

Tissue preparation. At the completion of the treatment period, mice were killed by cervical dislocation, and their brains were dissected into six regions: cerebral cortex,

hindbrain (pons-medulla), hippocampus, striatum, hypothalamus and midbrain (primarily thalamus). A whole particulate fraction was prepared [4, 5] and the final pellet was resuspended to a concentration of 1 mg protein/ml in 50 mM imidazole buffer, pH 7.2.

ATPase assay. ATPase activity was assayed as described previously [11, 12]. Buffer composition was: NaCl, 100 mM; KCl, 20 mM; $MgCl_2$, 3 mM; EDTA, 0.5 mM; ATP, 3 mM; PEP, 2 mM; and PK, 4 μg/ml. Reaction was begun by addition of the ATP, PEP, PK mixture. Incubation temperature was 37°. The reaction was terminated by the addition of ice-cold $HClO_4$ to a final concentration of 0.5 M. When the effect of ouabain on enzyme activity was determined, the cardiac glycoside (3×10^{-8} to 3×10^{-3} M) was added to the mixture containing all components, except ATP, PEP and PK. The different components of ATPase activity were routinely measured from results obtained at ouabain concentration of 0 M, 5×10^{-6} M and 5×10^{-3} M as described previously.

Protein. Protein was measured by the method of Lowry *et al.* [13] using bovine serum albumin as the standard.

Results

The results displayed in Fig. 1 are the profiles of ouabain inhibition of ATPase activity for saline- and nicotine-treated mice of the four inbred strains. The inhibition curves are shallow (range of Hill coefficients: 0.47–0.54), indicating deviation from simple mass action. The curves can be resolved into three components: (1) a ouabain-resistant component, (2) a ouabain-sensitive component with a K_i of approximately 1×10^{-4} M (high K_i), and (3) a ouabain-sensitive component with a K_i of approximately 5×10^{-7} M (low K_i). Values estimated for the inhibition constants are indicated on the figure, and the curves displayed are the theoretical best fit of the data. Chronic nicotine infusion had no effect on either the K_i values or the activity represented by each ATPase component in any of the four mouse strains.

Measurement of the three ATPase components was extended to five other brain regions to determine if a region-selective effect of nicotine treatment occurred. The results, summarized in Table 1, are the values for the two ouabain-sensitive components of the ATPase activity as well as for the ouabain-insensitive component as calculated from the differential ouabain-inhibition assay [12]. Some differences in the regional distribution of the activities were noted: the level of high K_i $Na,^+$ K^+-ATPase activity in cortex was higher than that in the other five regions, whereas the level of the low K_i $Na,^+$ K^+-ATPase activity was higher in hypothalamus than in the other five regions. Nicotine treatment had no effect on any ATPase component in any brain region of any mouse strain.

Discussion

In contrast to the results reported by Shallom and Katyare [9] for rats chronically exposed to nicotine in their drinking water, the results presented here indicate that the three ATPase activities measured (ouabain-insensitive, low K_i $Na,^+$ K^+-ATPase) were unaffected by chronic nicotine infusion in any of four mouse strains which display different capabilities for tolerance development.

The failure to observe any effects of nicotine treatment on $Na,^+$ K^+-ATPase activities in the fairly large brain areas assayed is not surprising. Comparison of the binding site densities for nicotinic receptors in brain (concentration of nicotine and α-bungarotoxin binding sites are about 100 fmol/mg protein [4, 5]) to that for $Na,^+$ K^+-ATPases (concentrations of ouabain binding sites are about

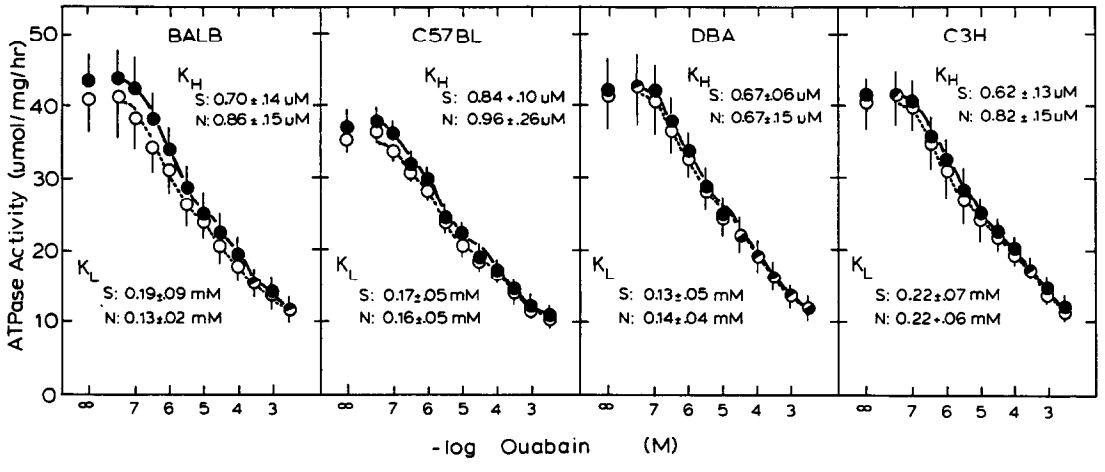

Fig. 1. Inhibition of cortical ATPase activity by ouabain. ATPase activities of saline-treated (○) and nicotine-treated (●) mice of the four strains were measured in the presence of ouabain at the concentrations indicated. Each point represents the mean ± SEM of six individual experiments. Each curve was fitted to the following model:

$$\text{Activity} = \text{ATPase}_{OI} + \frac{\text{ATPase}_{HOS}}{1 + ([\text{ouabain}]/K_H)} + \frac{\text{ATPase}_{LOS}}{1 + ([\text{ouabain}]/K_L)}$$

where activity is the total ATPase measured in the presence of any concentration of ouabain, ATPase_{OI} is ouabain-insensitive ATPase activity, ATPase_{HOS} is the ATPase activity highly sensitive to ouabain inhibition, ATPase_{LOS} is the ATPase activity less sensitive to ouabain inhibition, K_H is the inhibition constant observed at lower ouabain concentrations (higher affinity) and K_L is the inhibition constant observed at higher ouabain concentrations (lower affinity). The mean ± SEM for the two inhibition constants are indicated on the figure for saline (S) and nicotine (N) treated mice. Values calculated for each of the three activities are summarized in Table 1. The curves are those of best fit calculated from the model.

Table 1. Effect of nicotine infusion on ATPase activities

Strain	Treatment	Brain region					
		Cortex	Midbrain	Hindbrain	Hippo-campus	Striatum	Hypo-thalamus
		Ouabain-insensitive ATPase					
BALB	Saline	10.9 ± 1.7	8.1 ± 1.5	6.7 ± 1.0	11.0 ± 1.5	11.9 ± 1.5	11.0 ± 2.5
	Nicotine	11.6 ± 1.8	8.4 ± 1.5	6.6 ± 0.9	12.3 ± 1.5	12.3 ± 1.5	9.7 ± 0.9
C57BL	Saline	9.9 ± 0.7	7.9 ± 1.1	6.7 ± 1.1	12.0 ± 2.0	12.2 ± 2.0	9.9 ± 2.2
	Nicotine	10.4 ± 0.9	8.6 ± 1.0	7.2 ± 0.9	12.4 ± 1.6	10.7 ± 1.8	10.7 ± 1.8
DBA	Saline	11.8 ± 2.1	7.9 ± 1.3	6.9 ± 1.0	11.8 ± 1.2	14.0 ± 1.9	12.0 ± 3.1
	Nicotine	11.1 ± 1.6	8.4 ± 0.9	6.6 ± 0.7	12.3 ± 1.7	11.9 ± 1.4	11.7 ± 2.2
C3H	Saline	11.1 ± 1.1	7.4 ± 0.8	6.8 ± 0.9	11.1 ± 1.2	13.6 ± 1.9	11.5 ± 1.6
	Nicotine	11.8 ± 1.1	8.4 ± 0.9	7.1 ± 0.6	12.2 ± 1.5	13.7 ± 1.6	11.0 ± 1.2
		Low K_i ouabain-sensitive ATPase					
BALB	Saline	17.7 ± 1.9	16.8 ± 1.5	14.2 ± 1.2	16.0 ± 1.7	16.8 ± 2.1	22.6 ± 2.6
	Nicotine	19.3 ± 1.5	15.5 ± 1.6	14.5 ± 0.9	16.1 ± 1.6	16.1 ± 1.2	22.6 ± 2.7
C57BL	Saline	14.0 ± 1.3	14.7 ± 1.1	14.1 ± 1.2	14.2 ± 1.7	15.0 ± 1.7	22.4 ± 3.4
	Nicotine	15.7 ± 1.5	15.6 ± 1.7	14.3 ± 1.3	15.2 ± 1.6	14.9 ± 1.1	25.6 ± 3.4
DBA	Saline	16.8 ± 2.0	14.5 ± 1.5	14.1 ± 0.8	15.2 ± 1.1	16.2 ± 1.3	25.0 ± 3.0
	Nicotine	17.3 ± 1.7	17.2 ± 1.8	13.5 ± 1.0	16.9 ± 1.5	15.8 ± 2.1	23.8 ± 2.3
C3H	Saline	16.5 ± 1.6	14.3 ± 1.7	14.2 ± 1.2	14.9 ± 1.3	17.2 ± 1.8	27.1 ± 2.9
	Nicotine	16.9 ± 1.3	14.6 ± 1.5	15.3 ± 1.8	14.3 ± 2.1	18.0 ± 3.0	25.1 ± 2.4
		High K_i ouabain-sensitive ATPase					
BALB	Saline	12.5 ± 1.5	7.3 ± 1.0	7.4 ± 1.3	8.4 ± 1.4	7.6 ± 1.4	7.4 ± 1.2
	Nicotine	13.1 ± 2.0	7.9 ± 1.2	7.3 ± 1.1	8.6 ± 1.4	8.7 ± 1.7	7.7 ± 1.2
C57BL	Saline	11.5 ± 0.5	7.8 ± 0.9	7.4 ± 0.9	7.5 ± 1.1	6.1 ± 1.3	8.3 ± 1.6
	Nicotine	11.3 ± 0.5	7.9 ± 1.3	7.5 ± 1.0	7.9 ± 1.3	8.3 ± 0.9	9.1 ± 1.6
DBA	Saline	14.0 ± 1.6	8.5 ± 1.2	7.3 ± 1.0	7.5 ± 1.2	7.0 ± 1.4	8.2 ± 1.5
	Nicotine	15.2 ± 1.3	7.6 ± 1.1	7.3 ± 1.0	8.0 ± 1.5	7.6 ± 1.3	6.9 ± 1.6
C3H	Saline	13.8 ± 1.7	7.6 ± 0.9	7.4 ± 0.9	8.0 ± 1.2	7.5 ± 1.3	9.0 ± 1.2
	Nicotine	13.1 ± 1.3	7.6 ± 1.0	6.9 ± 1.2	8.0 ± 1.6	8.4 ± 1.6	8.6 ± 1.3

ATPase activities were obtained from differential ouabain-inhibition assays. Units of enzyme activity are μmol of phosphate produced per mg protein per hr. Results represent mean ± SEM of six individual assays. No significant differences between saline- and nicotine-treated mice were found.

100 nmol/mg protein [11]) indicates that enzyme sites outnumber putative nicotinic receptor sites by about six orders of magnitude. Therefore, the perturbation of ion levels postulated as a result of stimulation of nicotinic receptors is likely to be quantitatively inconsequential compared to the overall capacity of brain tissue to actively transport monovalent cations. However, the possibility that local alterations of $Na,^+$ K^+-ATPase may occur on those brain cells rich in nicotinic receptors cannot be dismissed, particularly since ion movement through activated channels is fast relative to the enzymatic transport of these ions. In addition, activation of voltage-dependent Na^+ channels in these cells would further increase intracellular Na^+ content.

In summary, chronic nicotine treatment had no effect on the activity of any of three ATPase activities measured. The results indicate that chronic treatment with this agent, which may act in brain as it does in the periphery by increasing the flux of Na^+ across the membrane, does not cause sufficient disruption of normal ion metabolism to induce measurable changes in ion-transport enzymes. It seems unlikely, therefore, that overall changes in ion metabolism occur in the brain as a result of chronic nicotine treatment.

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