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ALTERED SYNAPTOSOMAL ATPase ACTIVITY IN RAT BRAIN FOLLOWING PROLONGED *IN VIVO* TREATMENT WITH NICOTINE

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Abstract—Effects of prolonged *in vivo* treatment with nicotine on synaptosomal ATPase activity in rat brain were examined by employing doses of nicotine (0.02 and 0.04 mg/ml in the drinking water) which simulated intake by moderate and heavy smokers. Under these conditions the "low" dose of nicotine resulted in increased body weight whereas "high" dose of nicotine inhibited weight gain. Examination of synaptosomal ATPase activities revealed a dose and time dependent stimulatory/inhibitory effect. With "low" dose of nicotine, maximum stimulatory effect on ATPase activity was seen at the end of the 3rd week, while "high" dose stimulated the enzyme activities maximally by the 2nd week itself; further treatment up to the 4th week caused inhibition of the ATPase activities (10% to 43% decrease).

Studies in various mammalian species to assess the effects of nicotine on brain function and behaviour indicate that small doses of nicotine stimulate spontaneous motor activity while larger doses of the alkaloid depress this activity [1–6]. In tolerant animals, the depressant effects of nicotine decrease after repeated treatment with the alkaloid and marked stimulation of locomotor activity and rewarded responding are noted [7, 8]. Treatment of rats with nicotine during pregnancy was found to result in increased brain weight in the offspring [6]. Besides, higher doses of nicotine resulted in decrease in the maternal body weight [9, 10]. More recent studies by Sershen and Lajtha [11] indicate that nicotine treatment *in vivo* significantly influenced protein synthesis in the brain of adult males, mothers and newborn rats. Our own studies have shown that acute as well as chronic treatment with nicotine had a stimulatory effect on protein synthetic activity in rat brain, the enhancement in protein synthesis being more pronounced in the microsomal and mitochondrial–synaptosomal fractions [12]. There are, however, no reports in the literature which illustrate biochemical effects of prolonged nicotine treatment on brain function. Since changes in brain protein metabolism [11, 12] could be expected to influence brain function, the effects of prolonged exposure to nicotine on the synaptosomal enzyme system viz. ATPase have been examined in the present studies. Such studies become relevant since membrane transport ATPases play an important role in nerve transmission [13–17].

The results of our studies clearly indicate that treatment *in vivo* with nicotine for prolonged periods indeed results in alterations in the synaptosomal ATPase activities.

MATERIALS AND METHODS

Chemicals. Nicotine (free base) was obtained from British Drug Houses (Dorset, Poole, U.K.) and was distilled prior to use. Distilled nicotine was stored in a stoppered tube under refrigeration. Sodium salt of vanadium free ATP and ouabain were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical-reagent grade.

Animals. Male albino rats of Wistar strain weighing between 190 and 210 g (average weight 195.0 ± 3.10 g, S.E.M.) were used. The weights of the animals in various subgroups were matched closely at the initiation of the experiments; group to group variations in average weights of the animals did not exceed 5 g. Three animals were housed in a cage and were given nicotine in their drinking water at a concentration of either 0.02 mg/ml or 0.04 mg/ml. These doses simulate the intake of nicotine by moderate and heavy smokers [18] and are hereafter referred to as "low" and "high" dose respectively. Solutions of nicotine were made fresh daily in tap water. Control animals received only tap water. A daily record of weight gain and water intake by the animals was maintained. The animals had access to water and food *ad libitum*. Tap water or nicotine solutions were given in water-tight bottles so that there was practically no spillage of the fluids. After starting the nicotine regimen, the animals were killed at the end of 1, 2, 3 or 4 weeks for the isolation of synaptosomal membranes from their brains and study of ATPase activities.

Isolation of synaptosomal membranes. The animals were killed by decapitation and their brains were quickly removed and placed in beakers containing cold (0–4°) 0.32 M sucrose. The brain tissue was washed repeatedly to free it from adhering blood and was then used for the isolation of synaptosomal membranes essentially according to the procedure of Burgoyne and Rose [19] with some modifications. This procedure yields synaptosomal membranes free of mitochondrial and myelin contamination as revealed by electron microscopic examination [19].

Assay of ATPase activities. $(Na^+ + K^+ + Mg^{2+})$-dependent ATPase activity was determined in a medium (total volume: 1.0 ml) containing 50 mM imidazole–HCl buffer pH 7.4, 120 mM NaCl, 10 mM

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KCl, 5 mM $MgCl_2$, and 4 mM ATP [19]. The reaction was started by adding 25–35 μg of synaptosomal membrane protein and was carried out at 37° for 20 min. At the end of the incubation period, the reaction was terminated by addition of 0.1 ml of 10% (w/v) SDS solution [20, 21]. Mg^{2+}–ATPase activity was determined with 5 mM $MgCl_2$ in the medium and by omitting both NaCl and KCl. $(Na^+ + K^+)$-dependent ATPase activity was calculated from the difference between the $(Na^+ + K^+ + Mg^{2+})$– and Mg^{2+}–ATPase activities [22].

For measurement of Ca^{2+}–ATPase activity, the medium employed was as given by Lin and Way and Sorensen and Mahler [23, 24] and contained in a 1.0 ml total volume: 50 mM imidazole–HCl buffer pH 7.4, 4 mM ATP and 1 mM $CaCl_2$.

Ouabain insensitive ATPase activity was determined by inclusion in the assay medium for $(Na^+ + K^+ + Mg^{2+})$–ATPase activity, 1.0 mM freshly prepared ouabain.

ATP solutions were made fresh prior to use by neutralizing ATP to pH 7.4 with Tris.

Protein estimation was according to Lowry *et al.* [25]. Determination of inorganic phosphate was as described by Fiske and Subba Row [26].

Results are given as mean ± S.E.M. Students *t*-test was used to determine statistical significance of differences between means.

RESULTS

The data on average daily water and nicotine intake by rats are summarized in Table 1. It is clear that the daily water intake was 6.9 ml/100 g body wt in the control rats and increased to 8.7 and 9.8 ml/100 g body wt respectively in the "low" and "high" nicotine groups (26 and 42% increase respectively in the two groups). The values for average daily intake of nicotine were 0.17 and 0.39 mg/100 g body wt in the two experimental groups. Based on these average daily intake values, the cumulative intake of nicotine in the "low" nicotine group for 1, 2, 3 and 4 weeks would amount to approximately 1.20, 2.41, 3.61 and 4.82 mg/100 g body wt; the corresponding values for the "high" nicotine groups would work out to about 2.74, 5.47, 8.21 and 10.95 mg/100 g body wt. The more than double intake of nicotine observed in the "high" nicotine group obviously corresponds to higher water intake (Table 1).

The increased water intake in general in the rats given nicotine in their drinking water is interesting to note. We do not, however, know as yet whether it is a genuine metabolic need as a consequence of inclusion of nicotine in the drinking water or whether these animals drink more of "nicotine" water because they "like" it. It may, however, be mentioned here that when nicotine is administered by substaneous route, water intake invariably decreases [27]. On the other hand there are reports in the literature which indicate that the rats begin to detect nicotine in solutions at 3 μg/ml [28] and actually prefer nicotine solution up to 16 μg/ml [29]. Interestingly, nicotine concentrations used in our studies [18] correspond to the latter values referred to above [29].

The data on body and relative brain weights as influenced by prolonged treatment with nicotine are summarized in Fig. 1. It can be seen that the untreated controls gained weight uniformly over the

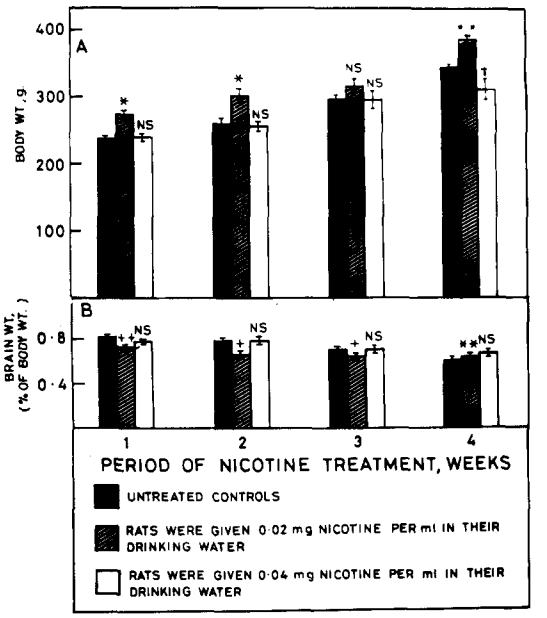

Fig. 1. Effect of prolonged treatment with nicotine on body and brain weights of the experimental rats. The animals were given either 0.02 mg/ml or 0.04 mg/ml nicotine in their drinking water as described in the "Materials and Methods" section. Control animals received only tap water. The results are given as mean ± S.E.M. of 4–12 independent observations: †$P < 0.05$ compared to untreated control; $^+P < 0.02$ compared to untreated control; $^{++}P < 0.01$ compared to untreated control; *$P < 0.002$ compared to untreated control; **$P < 0.001$ compared to untreated control; NS, not significant.

Table 1. Average daily water and nicotine intake by rats

Treatment	Water intake (ml/100 g body wt)	Nicotine intake (mg/100 g body wt)
Control	6.93 ± 0.22	—
Nicotine (0.02 mg/ml)	8.72 ± 0.16*	0.172 ± 0.003
Nicotine (0.04 mg/ml)	9.79 ± 0.18*	0.391 ± 0.007*

The animals were given either tap water or nicotine solutions in tap water as indicated. Solutions of nicotine were made fresh daily. The values of water and nicotine intake are given as mean of 28 days ± S.E.M.

* $P < 0.001$.

Table 2. Synaptosomal ATPase activities in normal rat brain

	ATPase activity (μmoles of Pi/mg protein/hr)				
	1 week (6)	2 week (6)	3 week (16)	4 week (6)	Pooled (34)
$(Na^+ + K^+ + Mg^{2+})$–ATPase	11.76 ± 0.95	12.28 ± 0.42	12.78 ± 0.41	12.05 ± 0.44	12.28 ± 0.39
$(Na^+ + K^+)$–ATPase	5.56 ± 0.43	6.22 ± 0.52	6.68 ± 0.23	4.99 ± 0.32	6.06 ± 0.21
Mg^{2+}–ATPase	6.20 ± 0.60	6.07 ± 0.18	6.53 ± 0.49	7.31 ± 0.39	6.35 ± 0.28
Ca^{2+}–ATPase	4.11 ± 0.47	3.76 ± 0.21	4.08 ± 0.43	3.80 ± 0.45	3.85 ± 0.24
Ouabain insensitive	6.81 ± 0.77	5.66 ± 0.14	6.40 ± 0.51	7.81 ± 0.39	6.31 ± 0.31

Results are given as mean ± S.E.M. of the number of independent observations indicated in parentheses. Concentration of ouabain used was 1.0 mM.

entire experimental period. There was only a small increase in the brain weight during this period (data not given). Consequently the relative brain weight decreased. The animals in the "low" nicotine group gained more in their body weight (15–30% increase) than the controls and thus were heavier. Their brain weights were generally comparable to those of corresponding controls (data not given) but because of the increased body weight, the relative brain weights were decreased (Fig. 1). The animals in "high" nicotine group, by contrast, did not gain as much weight as those in the "low" nicotine group and their weights were almost comparable to the untreated controls except for the 4th week where a 10% decrease in the body weight was evident. No changes in the brain weight were, however, discernible.

Results on decreased body weights in the animals receiving "high" doses of nicotine are in agreement with the observations of others [9, 10, 30]. The increased body weights in the rats receiving "low" doses of nicotine, however, seem to be interesting. Of note in this connection is the reported observation that nicotine withdrawal resulted in significant weight gain in rats [30].

The data on ATPase activities for the synaptosomal membrane fractions isolated from normal rat brain at the periods of 1–4 weeks corresponding to the start of nicotine treatment are given in Table 2. It can be noted that throughout this period, all of the ATPase activities were practically unaltered, which is also reflected when these values for the entire period were pooled (last column of Table 2). It may also be pointed out that $(Na^+ + K^+)$– and Mg^{2+}–ATPase activities make up almost about 50%,

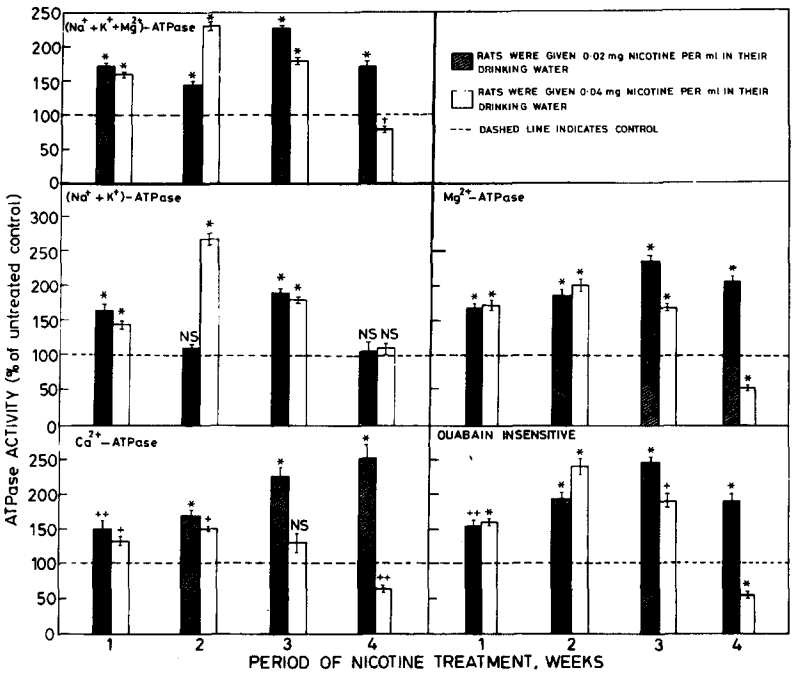

Fig. 2. Changes (% of control) in ATPase activities following prolonged treatment with nicotine. The animals were given nicotine in their drinking water as described in the "Materials and Methods" and in legend to Fig. 1. Various ATPase activities were determined as described in the text. Results are given as mean ± S.E.M. of 12–22 independent determinations: †$P < 0.05$ compared to untreated control; ††$P < 0.01$ compared to untreated control; *$P < 0.001$ compared to untreated control; NS, not significant.

each of the total, i.e. $(Na^+ + K^+ + Mg^{2+})$–ATPase activity. Also the ouabain insensitive ATPase activities compare fairly well with the Mg^{2+}–ATPase indicating the efficacy of ouabain treatment.

The synaptosomal ATPase activities in animals receiving "low" and "high" doses of nicotine are given in Fig. 2. It can be observed that with "low" doses of nicotine there was stimulation of the various ATPase activities as early as the 1st week ranging from 52 to 70%; maximum stimulation was seen at the end of 3rd week where the various ATPase activities increased by 106% to 143%. The stimulatory effect decreased slightly by the 4th week with extent of stimulation ranging from 69 to 107% for the various ATPase activities, except in the case of Ca^{2+}–ATPase where 207% stimulation was noted.

In case of the "high" dose nicotine group (Fig. 2) there was early stimulation of ATPase activities followed by inhibition. Thus the maximum stimulation was observable by the second week of treatment; the extent of stimulation ranged from 79% to 174%. By the end of the 3rd week, stimulation decreased slightly compared with the second week values, but by the end of the 4th week there was a significant decrease in the activity of all the ATPases (10% to 48% decrease compared to untreated controls).

DISCUSSION

The results summarized in the present communication have clearly shown biochemical evidence for the first time for altered synaptosomal function following treatment with nicotine for a prolonged period in terms of altered synaptosomal ATPase activities. Thus, it was observed that nicotine first stimulated the ATPase activities which declined or were inhibited depending on the time and dose of the treatment (Fig. 2). However, the effects were not synergistic, i.e. although the 2 week "low" dose and 1 week "high" dose rats received practically similar cumulative doses of nicotine, as was also the case for 4 week "low" dose and 2 week "high" dose groups, the effects on the various ATPase activities were not exactly identical. The time of treatment and the dose therefore seem to be two important but independent factors in nicotine effects. Nevertheless the results on early stimulation followed by decrease in the stimulation or inhibition of the ATPase activities would seem to be compatible with stimulatory and inhibitory effects of nicotine on CNS and locomotor and rewarded learning activities referred to above [1–8]. This becomes more relevant in view of the fact that the various ATPases play a significant role in the process of nerve transmission [13–17].

Since early stimulation followed by decrease in the ATPase activities were noted following *in vivo* treatment with nicotine (Fig. 2), attempts were made in separate experiments to examine effects of *in vitro* addition of nicotine at concentations of 1, 10, 100 μM and 1 mM on various ATPase activities with or without pre-incubation of the synaptosomal membranes with nicotine for up to 60 min. Such treatments did not significantly influence any of the ATPase activities (J. M. Shallom and S. S. Katyare, unpublished work). It would therefore seem that the observed *in vivo* effects are more specific and do not result due to mere membrane association of the alkaloid with the enzyme.

Other studies from our laboratory (J. M. Shallom and S. S. Katyare, unpublished work) have in fact shown that prolonged treatment with nicotine results in decreased turnover and increased half-life of proteins in several sub-cellular components. Possibility of altered kinetic properties of this enzyme have also become apparent from the experiments currently in progress in our laboratory. Such studies can be expected to give further insights in the biochemical and molecular mechanisms underlying stimulatory/inhibitory effects of nicotine on CNS function and behavior.

REFERENCES

1. R. L. Volle and G. B. Koelle, in *The Pharmacological Basis of Therapeutics* (Eds. L. S. Goodman and A. Gilman), p. 585. Macmillan, New York (1970).
2. S. N. Pradhan and C. Bowling, *J. Pharmac. exp. Ther.* **176**, 229 (1971).
3. D. Bovet, F. Bovet-Nitti and A. Oliverio, *Science* **163**, 139 (1969).
4. J. M. Nelsen in *Drugs Addiction, Neurobiology and Influences on Behaviour*, Vol. 3 (Eds. J. M. Singh and M. Lal), p. 375. Stratton & Grune, New York (1974).
5. M. J. Marks, J. B. Burch and A. C. Collins, *J. Pharmac. exp. Ther.* **226**, 817 (1983).
6. D. B. Hudson, E. Meisami and P. S. Timiras, *Experentia* **29**, 286 (1973).
7. P. B. S. Clarke and R. Kumar, *Br. J. Pharmac.* **78**, 329 (1983).
8. P. B. S. Clarke and R. Kumar, *Psychopharmac.* **79**, 271 (1983).
9. A. A. Bartolini, M. Bernardi and S. Genedani, *Neurobehav. Toxicol. Teratol.* **4**, 545 (1982).
10. M. A. Peters and L. L. E. Ngan, *Arch. int. Pharmacodyn. Thér.* **257**, 155 (1982).
11. H. Sershen and A. Lajtha, *Neuropharmacol.* **18**, 763 (1979).
12. K. Sankaran, V. K. Asundi, S. S. Katyare and G. B. Nadkarni, *Baroda J. Nutr.* **8**, 67 (1981).
13. A. Schwartz, G. E. Lindenmayor and J. C. Allen, *Pharmac. Rev.* **27**, 3 (1975).
14. E. T. Wallick, L. K. Lane and A. Schwartz, *Ann. Rev. Physiol.* **41**, 397 (1979).
15. L. C. Cantley, *Curr. Topics Bioenerget.* **11**, 201 (1981).
16. K. E. O. Akerman and D. G. Nicholls, *TIBS* **8**, 63 (1983).
17. D. A. Powis and G. D. Wattus, *Fedn Eur. Biochem. Soc.* **126**, 285 (1981).
18. H. F. Krous, G. A. Campbell, M. W. Fowler, A. C. Catron and J. P. Farber, *Am. J. Obst. Gynacol.* **140**, 743 (1981).
19. R. D. Burgoyne and S. P. R. Rose, *J. Neurochem.* **345**, 510 (1980).
20. A. H. Sawas and J. C. Gilbert, *Biochem. Pharmac.* **30**, 1799 (1981).
21. Y. Tashima, *Biochem. biophys. Res. Commun.* **55**, 630 (1973).
22. M. Nimai-Gurgani and E. Meisami, *J. Neurochem.* **32**, 1027 (1979).
23. S. C. C. Lin and E. L. Way, *J. Neurochem.* **39**, 1641 (1982).
24. R. G. Sorensen and H. R. Mahler, *J. Neurochem.* **37**, 1407 (1981).

25. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
26. C. H. Fiske and V. Subba Row, *J. biol. Chem.* **66**, 375 (1925).
27. P. B. S. Clarke and R. Kumar, *Br. J. Pharmac.* **82**, 233 (1984).
28. D. Naguira, E. Zunino, L. Argueros and O. H. Viseras, *Eur. J. Pharmac.* **47**, 227 (1978).
29. M. S. G. Clark, *Br. J. Pharmac.* **35**, 367 (1969).
30. E. McNair and R. Brysen, *Pharmac. Biochem. Behav.* **18**, 341 (1982).