Biochemical Pharmacology, Vol. 27, pp. 2519–2523.

0006-2952/78/1101-2519$02.00/0

EFFECTS OF NICOTINE ON BRAIN 1-PHOSPHATIDYLINOSITOL-4-PHOSPHATE AND 1-PHOSPHATIDYLINOSITOL-3,4-BISPHOSPHATE SYNTHESIS AND METABOLISM—POSSIBLE RELATIONSHIP TO NICOTINE-INDUCED BEHAVIORS

ROBERT J. HITZEMANN, REIKO NATSUKI and HORACE H. LOH
Langley Porter Neuropsychiatric Institute, and Departments of Pharmacology and Psychiatry, University of California, San Francisco, CA 94143, U.S.A.

(*Received* 18 *July* 1977; *accepted* 22 *December* 1977)

Abstract—The effects of nicotine and other cholinergic drugs on the initial incorporation of $^{32}P_i$ and [^{3}H]glycerol into 1-phosphatidylinositol-4-phosphate (DPI) and 1-phosphatidylinsitol-3,4-bisphosphate (TPI) in the rat brain microsomal fraction were studied. Nicotine and eserine significantly decreased and mecamylamine increased [^{32}P]- and [^{3}H]TPI levels. Atropine had no effect on labeled TPI levels. Dose–effect studies for nicotine revealed that as little as 0.1 mg/kg of nicotine, i.p., significantly decreased the accumulation of labeled TPI. It was concluded that pharmacologically relevant and non-convulsive doses of nicotine can markedly affect brain polyphosphoinositide synthesis and/or metabolism; the nature of the effects is consistent with theories suggesting that the conversion of TPI to DPI will release membrane lipid bound Ca^{2+} and, thus, increase membrane excitability.

The functions of 1-phosphatidylinositol-3,4-bisphosphate (TPI) and 1-phosphatidylinositol-4-phosphate (DPI) in the regulation of nervous system activity are unknown. Several theories of TPI and DPI function have stressed the binding of divalent cations to these phospholipids. Hawthorne and Kai [1] have suggested that Ca^{2+} bound on two molecules of DPI or TPI could bridge and obstruct an ion channel. The dephosphorylation of either phospholipid to phosphatidylinositol (PI) would release Ca^{2+} and consequently open the channel. Hendrickson and Reinersten [2, 3] have noted that there is a sufficient difference in Ca^{2+} affinity between TPI and DPI so that simply the dephosphorylation of TPI to DPI would lower membrane bound Ca^{2+} levels. Both the theories of Hawthorne and Kai [1] and Hendrickson and Reinersten [2, 3] are appealing, since it is known that Ca^{2+} is released from the nerve membrane during the development of the action potential [4].

It has also been suggested that a TPI to DPI conversion may be involved in the generation of action potentials at nicotinic cholinergic synapses in the central nervous system (CNS) [5]. This theory envisages acetylcholine (ACh) binding to an inhibitory subunit of TPI phosphomonoesterase, causing its dissociation from the catalytic subunit. The activated enzyme can then metabolize TPI to DPI. Michell [6] has raised some serious objections to this hypothesis on both theoretical and experimental grounds. Most obviously, this hypothesis is not compatible with the evidence indicating that the nicotinic receptor is an ACh-stimulated ionophoric membrane protein [7, 8].

In the present study, we have attempted to determine the role(s) TPI and DPI may play in central nicotinic cholinergic function by examining the effects of various doses of nicotine on the incorportion of $^{32}P_i$ and [^{3}H]glycerol into TPI and DPI. Initially, drug effects were examined in four brain regions (the diencephalon, the midbrain, the pons-medulla and the neostriatum) and in the microsomal and nerve ending-mitochondrial fractions. However, significant drug effects were found only in the pons-medulla and midbrain microsomal fractions and, thus, only these data, which have been combined, will be reported in this communication. In addition, the effect of mecamylamine, atropine and eserine on the incorporation of $^{32}P_i$ and [^{3}H]glycerol into TPI and DPI were examined.

MATERIALS AND METHODS

Materials. [^{32}P]phosphoric acid (carrier free) and [2-^{3}H]glycerol (sp. act. 10 Ci/m-mole) were obtained from New England Nuclear Corp. Nicotine, eserine, mecamylamine and atropine were purchased from Sigma Chemical Co., St. Louis, MO. All solvents were of analytical grade and were used without further purification.

Experimental. Each male Sprague–Dawley rat (Simonsen Laboratories, Gilroy, CA) weighing 150–200 g had a permanent cannulae guide implanted in the skull over the lateral ventricle. Four to 5 days after surgery, the animals were given eserine, atropine, mecamylamine, nicotine or saline. In the initial series of experiments, 15 min after drug injection the animals were injected intraventricularly with 200 μCi $^{32}P_i$ and 50 μCi [^{3}H]glycerol dissolved in 20 μl of phosphate free Krebs–Ringer bicarbonate buffer. For the nicotine dose–effect studies, 5 min elapsed between drug and isotope administration. Twenty-

five or 60 min after isotope administration the animals were sacrificed and the brains were rapidly removed, cooled, dissected and homogenized in 19 vol. of 0.32 M sucrose + 10 mM Tris, pH 9.3. For the time course used, the incorporation of $^{32}P_i$ into TPI and DPI has been shown to increase linearly [9]. In some preliminary experiments we confirmed these results of Gonzalez-Sastre *et al.* [9] and also found that the incorporation of [^{3}H]glycerol increased linearly during the first hour after isotope administration. The conditions used to process the tissue have been shown to minimize post-mortem loss of TPI and DPI once the brain has been removed [10]. The levels of TPI and DPI obtained with this procedure are comparable to the levels found after rapidly freezing the brain in liquid N_2 [10].

Subcellular fractionation. The homogenate was centrifuged for 10 min × 1000 *g* to remove debris and nuclear material. The supernatant fraction was then centrifuged for 10 min × 17,000 *g*. After centrifugation, the crude mitochondrial pellet was washed three times using the original volume of sucrose–Tris medium. This procedure removes microsomal contamination [11] and yields a pellet containing approximately 40 per cent nerve endings [12]. The crude mitochondrial supernatant fraction was centrifuged for 1 hr × 100,000 *g* to yield the microsomal pellet which was used without any additional washing procedures.

Extraction of TPI and DPI. The method of Hauser and Eichberg [10] was used to extract TPI and DPI from the various subcellular fractions. Briefly, in this procedure the tissue is first extracted with chloroform–methanol (1 : 1, v/v) containing 1 mg/ml of $CaCl_2$. The tissue is then washed twice with chloroform–methanol (2 : 1, v/v) before extraction with chloroform–methanol–conc. HCl (2:1:0.01, v/v). Typically, 10-ml aliquots of the various solvents were used to extract the lipids from the brainstem microsomal or nerve ending-mitochondrial fractions. The acidic solvent extract was washed [13] and evaporated under reduced pressure at 30°. TPI and DPI were isolated from the extract by means of thin-layer chromatography [14]. The lipids were then extracted from the silica gel and their specific activities determined as described elsewhere [15].

Measurement of locomotor activity and rearing behavior. The effects of nicotine on locomotor activity and rearing behavior were measured in a simple open-field apparatus as described elsewhere [16]. Briefly, individual rats were habituated to the open-field for 60 min. After drug administration, the animals were immediately returned to the open-field apparatus and their behavior was observed. Data are expressed in terms of squares crossed and number of rears during the first 30-min post-drug interval.

RESULTS

Effects of nicotine and other cholinergic drugs on the accumulation of ^{32}P- and ^{3}H-labeled TPI and DPI. Since the various drug effects were examined at single time points (25 or 60 min) after isotope administration, it is appropriate to discuss the data in terms of labeled lipid accumulation rather than incorporation, even though at the time points used the specific activity of the phosphipids is increasing linearly. The effects of nicotine, eserine, mecamylamine and atropine on the accumulation of [^{32}P]- and [^{3}H]TPI and DPI into brainstem microsomal fractions are illustrated in Table 1. Eserine and nicotine significantly inhibited the accumulation of [^{3}H]- and [^{32}P]TPI without affecting the accumulation of labeled DPI. In contrast to the effects of nicotine and eserine, mecamylamine significantly increased the levels of [^{3}H]TPI (131 per cent) and [^{32}P]TPI (102 per cent). Atropine had no significant effects on [^{3}H]- or [^{32}P]TPI and DPI accumulation. None of the drugs tested had significant effects on the levels of unincorporated ^{32}P and ^{3}H in the brainstem.

In a number of preliminary experiments, the effects of the various drugs were examined in four brain regions: the diencephalon, the neostriatum, the midbrain, and the pons-medulla. Additionally, two sub-cellular fractions, the microsomal and the nerve ending-mitochondrial, were examined in each brain region. The results from these preliminary experi-

Table 1. Effects of cholinergic drugs on the accumulation of ^{32}P- and ^{3}H-labeled TPI in the brainstem microsomal fraction* †

Group	[^{3}H]TPI	[^{3}H]DPI	[^{3}H]TPI / [^{3}H]DPI	[^{32}P]TPI	[^{32}P]DPI	[^{32}P]TPI / [^{32}P]DPI
			(per cent of control ± S. E.)			
Control	100 ± 13	100 ± 9	100 ± 11	100 ± 10	100 ± 8	100 ± 9
Eserine	60 ± 13‡	105 ± 15	57 ± 22†	52 ± 7	85 ± 7	61 ± 10‡
Mecamylamine	231 ± 19‡	111 ± 10	208 ± 6‡	202 ± 9	110 ± 6	184 ± 13
Atropine	103 ± 10	126 ± 14	82 ± 10	123 ± 9	112 ± 11	111 ± 8
Nicotine	55 ± 6‡	94 ± 8	59 ± 7‡	51 ± 4	94 ± 7	54 ± 6‡

* Rats were given 0.5 mg/kg of eserine, 2 mg/kg of mecamylamine, 30 mg/kg of atropine, 1 mg/kg of nicotine or saline, i.p., 15 min prior to the intraventricular injection of 200 μCi $^{32}P_i$ and 50 μCi [^{3}H]glycerol. The animals were sacrificed 1 hr later, brains were removed, dissected and subcellular fractions were prepared under alkaline conditions (see Materials and Methods). TPI and DPI were extracted from the microsomes and the specific activities of the phospholipids were determined. Data are the mean ± S. E. of at least ten experiments.

† The brainstem refers to the midbrain–pons-medulla.

‡ Significantly different from control, $P < 0.05$.

Table 2. Effects of nicotine on open-field behaviour*

Dose of nicotine (i.p.)	Rears/30 min	Squares crossed/ 30 min
0	13.8 ± 2.0	4.2 ± 1.1
0.1	22.7 ± 3.9†	6.2 ± 1.7
0.3	46.3 ± 2.1†	26.5 ± 1.9†
1.0	10.0 ± 1.3	31.7 ± 2.0†
3.0	0.3 ± 0.1	4.0 ± 1.3

* Animals were habituated to the open-field for 1 hr prior to drug administration. After drug or saline administration, the animals were immediately returned to the open-field apparatus and their behavior was observed for 30 min. In the present experiments the animals were monitored for rearing activity and square-crossing behavior. Details of the method are presented elsewhere [16]. Data are the mean ± S. E. obtained from six animals/drug dose.

† Significantly different from control, $P < 0.05$.

ments (N = 6) indicated that significant drug effects on the accumulation of labeled TPI and DPI were found only in the microsomal fractions of the midbrain and the pons-medulla. Since the drug effects in these two brain regions were virtually identical, in our subsequent studies we examined the drug effects in the midbrain–pons-medulla (the brainstem).

Effects of nicotine on exploratory and rearing behaviors. Marked effects of nicotine on exploratory and rearing behaviors were observed during the first 5–30 min after drug administration. The data in Table 2 indicate the bell-shaped nature of the nicotine dose–response curve. Maximal increases in square-crossing and rearing activities occurred after the administration of 1.0 and 0.3 mg/kg of nicotine respectively. Three mg/kg of nicotine produced a significant decrease in square-crossing activity from the increased levels of activity induced by the lower drug doses. The level of rearing activity in animals given 1.0 mg/kg of nicotine was no different than the control level. In animals given 3.0 mg/kg of nicotine, the levels of rearing activity were less than control levels.

Effects of various doses of nicotine on the accumulation of ^{32}P- and ^{3}H-labeled TPI and DPI. The data in Table 3 illustrate that nicotine has marked effects on the accumulation of labeled TPI. Within the range of 0.1 to 1.0 mg/kg, nicotine induces a dose-related decrease in the accumulation of both ^{3}H- and ^{32}P-labeled TPI. However, 3.0 mg/kg of nicotine significantly increases the [^{3}H]TPI level above the level in the animals given 1.0 mg/kg. In contrast, 3 mg/kg produced no significantly different effect than 1 mg/kg on the [^{32}P]TPI level. Nicotine had no significant effect on the [^{32}P]DPI level, but did decrease the [^{3}H]DPI levels at doses of 0.3 and 1.0 mg/kg. Another sensitive and less variable index of the effects of nicotine on labeled TPI and DPI accumulation was the ratio of labeled TPI to DPI. The effects of 0.1 mg/kg of nicotine illustrate the sensitivity of this index. Although this dose did not significantly affect either the [^{32}P]TPI or [^{32}P]DPI level, the ratio of [^{32}P]TPI to [^{32}P]DPI was significantly ($P < 0.01$) decreased. These data suggest that nicotine can alter the pattern of incorporation without changing the net levels of labeled TPI + DPI.

Table 4. Effects of various doses of nicotine on the accumulation of ^{32}P- and ^{3}H-labeled PI in the microsomal fraction of the rat midbrain*

Nicotine (mg/kg, i.p.)	[^{32}P]PI (cpm/μmole lipid P) × 10^{-3}	[^{3}H]PI (cpm/μmole lipid P) × 10^{-3}
0	23.2 ± 1.4	15.4 ± 1.3
0.1	24.6 ± 4.7	20.7 ± 2.5
0.3	30.6 ± 1.1†	27.0 ± 2.0†
1.0	40.5 ± 1.4†	28.7 ± 2.2†
3.0	24.0 ± 4.9	25.6 ± 4.0†

* Details of drug and isotope administration are given in the legend to Tables 1 and 3.

† Significantly different from control, $P < 0.05$.

Effects of various doses of nicotine on the accumulation of ^{32}P- and ^{3}H-labeled phosphatidylinositol (PI). The data in Table 4 show that in a dose-related fashion nicotine increased the accumulation of both ^{32}P- and ^{3}H-labeled PI. One mg/kg induced the maximum effect, increasing the [^{32}P]PI level 75 per cent and the [^{3}H]PI level 86 per cent. Three mg/kg of nicotine significantly decreased the accumulation of [^{32}P]PI from the level obtained with 1 mg/kg; there was no significant difference between the levels of [^{32}P]PI in control and 3 mg/kg treated animals. A similar effect was not observed for [^{3}H]PI levels.

Table 3. Effects of various doses of nicotine on the accumulation of ^{32}P- and ^{3}H-labeled DPI and TPI in the microsomal fraction of the rat midbrain*

Dose of nicotine (mg/kg)	(cpm/μmole lipid P ± S. E.) × 10^{-2}		[^{3}H]TPI / [^{3}H]DPI	(cpm/μmole lipid P ± S. E.) × 10^{-3}		[^{32}P]TPI / [^{32}P]DPI
	[^{3}H]TPI	[^{3}H]DPI		[^{32}P]TPI	[^{32}P]DPI	
0	24.3 ± 3.5	11.2 ± 1.3	2.18 ± 0.26	15.2 ± 2.0	9.2 ± 0.9	1.66 ± 0.05
0.1	14.3 ± 2.0†	9.5 ± 1.4	1.51 ± 0.07†	13.2 ± 1.6	10.9 ± 1.8	1.21 ± 0.04†
0.3	10.4 ± 1.7†	8.4 ± 0.6†	1.23 ± 0.13†	11.6 ± 0.8†	9.9 ± 0.8	1.17 ± 0.06†
1.0	5.6 ± 0.8†	7.1 ± 0.8†	0.79 ± 0.10†	8.0 ± 1.6†	9.0 ± 0.8	0.89 ± 0.02†
3.0	11.9 ± 1.3†‡	9.3 ± 1.4	1.28 ± 0.15†‡	9.2 ± 2.0†	9.4 ± 1.0	0.98 ± 0.12†

* Details are similar to those described in the legend to Table 1 except that the isotopes were administered 5 min after nicotine and the animals were sacrificed 30 min after nicotine admiistration.

† Significantly different from control, $P < 0.05$.

‡ Significantly different from 1.0 mg/kg values, $P < 0.05$.

DISCUSSION

The purpose of the present study was (1) to determine if nicotine has any significant effects on the synthesis and metabolism of TPI and DPI and (2) to determine if there is any relationship between the effects of nicotine on these phospholipids and on behavior. The results shown in Table 1 illustrate that a variety of cholinergic drugs, including nicotine, can affect the initial accumulation of ^{32}P- and ^{3}H-labeled TPI and DPI in the microsomal fraction of the rat brainstem (pons-medulla-midbrain). There are several interesting aspects to these data. First, although the number of drugs examined was small, it appears that nicotinic rather than muscarinic cholinergic activity controls the pattern of label accumulation. Nicotine and eserine markedly inhibited and mecamylamine markedly stimulated the accumulation of [^{32}P]- and [^{3}H]TPI. Atropine, even a very high dose, had no effect on labeled TPI accumulation. Second, the drug effects were found only in the microsomal fraction, which is known to be enriched in putative nicotinic cholinergic receptors [17]. No effect was seen in the nerve ending-mitochondrial fraction, suggesting that the drug effects were specific for a peculiar pool of TPI and DPI. Third, the reproducibility of the drug effects in a large number of experiments indicates that, with the exercise of appropriate precautionary measures to prevent degradation, it is possible to measure drug effects on synthesis and metabolism of TPI and DPI in discrete subcellular fractions. The work of Hauser and Eichberg [10] suggested this possibility and the present study provides confirming evidence of their findings. That some portion of the microsomal TPI and DPI was lost during the preparation of the tissue cannot be dismissed, since it is known that these compounds are highly labile [10]. However, the rapid cooling and homogenization in alkaline sucrose apparently preserves a drug-responsive pool of TPI and DPI.

The effects of behaviorally relevant doses of nicotine on the accumulation of labeled TPI were investigated (Table 3). The dose range used (0.1 to 3.0 mg/kg) includes doses (0.1 and 0.3 mg/kg) of nicotine which maximally facilitate conditioned behaviors [18], and doses (0.3 and 1.0 mg/kg) which maximally increase locomotor activities. Interestingly, significant changes in the accumulation of labeled TPI were observed with the administration of as little as 0.1 mg/kg of nicotine. This dose, which significantly increased rearing activity (Table 2), also has been shown in rats to increase the rates of responding on fixed interval and fixed ratio schedules of reinforcement [18]. Higher doses of nicotine, e.g. 1 mg/kg, decrease fixed interval and fixed ratio response behavior but induce the maximal increase in some locomotor behaviors, e.g. square-crossing activity (Table 2). Similar to this effect in square-crossing activity, the maximal nicotine effect on label incorporation into TPI (and PI) was seen at 1 mg/kg. These data suggest that the changes in phospholipid accumulation may be functionally associated with certain kinds of nicotine-induced behaviors. The question remains as to whether the changes in behavior lead to the chemical change or vice versa. From the evidence accumulated to date, it is not possible to determine which sequence is the most likely; there is also the possibility that no relationship exists. However, the data do indicate that the accumulation of ^{32}P- and ^{3}H-labeled TPI is exquisitely sensitive to the administration of nicotine and that marked changes in phospholipid synthesis and metabolism are occurring at pharmacologically relevant doses.

The data suggest that nicotine has two effects on the accumulation of ^{32}P- and ^{3}H-labeled TPI and DPI. The most notable effect is the preferential decrease of ^{32}P- and ^{3}H-labeled TPI as compared to labeled DPI. The second effect is the change in the pattern of labeled TPI and DPI accumulation independently of what changes occur in the net accumulation of TPI plus DPI. For example, 0.1 mg/kg of nicotine had no significant effects on either [^{32}P]TPI or [^{32}P]DPI levels but did significantly decrease the [^{32}P]TPI to [^{32}P]DPI ratio. Similarly, we have observed that morphine or β-endophin can increase the labeled TPI to DPI ratio without affecting the net accumulation of labeled lipid.* Furthermore, none of the doses of nicotine tested significantly affected [^{32}P]DPI levels, while all but one significantly decreased [^{32}P]TPI levels. Given our present understanding of the mechanisms involved, the most plausible interpretation of these data is that nicotine either enhances TPI metabolism and/or blocks the synthesis of TPI from DPI. The effects of nicotine on ^{3}H-labeled TPI and DPI accumulation, which presumably is a measure of the drug effects on *de novo* phospholipid synthesis, are generally similar to the effects seen on [^{32}P]lipid accumulation except that (1) the decrease in [^{3}H]TPI is more pronounced than the comparable decrease in [^{32}P]TPI, and (2) [^{3}H]DPI levels were significantly decreased in the 0.3 and 1.0 mg/kg nicotine groups while no change was observed in [^{32}P]DPI levels. Thus, the newly synthesized TPI and DPI are most sensitive to the effects of nicotine. The mechanism(s) by which nicotine decreases [^{3}H]DPI + [^{3}H]TPI levels is not the result of a decrease in the level of [^{3}H]PI. In fact, [^{3}H]PI as well as [^{32}P]PI levels were significantly increased after nicotine administration. If there is only one pool of PI available for synthesis to TPI and DPI, these data then suggest that nicotine either directly or indirectly blocks the synthesis of DPI from PI.

Various theories suggest that changes in the ratio of TPI to DPI affect the amount of membrane lipid bound Ca^{2+} [2, 3]. A decrease in the TPI to DPI ratio, causing a decrease in bound Ca^{2+}, would lead to increased membrane excitability. Conversely, an increase in the TPI to DPI ratio would decrease excitability. Given the results obtained in the present study with nicotine and mecamylamine, we conclude that there may be a relationship between the interconversion(s) of TPI and DPI and the level of nicotinic receptor excitability.

Acknowledgements—The authors acknowledge the technical assistance of Barbara Hitzemann and the editorial and typing assistance of Barbara Halperin and Kaye Welch. This study was supported in part by USPHS

* Unpublished observations.

Grants DA-00564, DA-01583 and MH-25487, and a grant from the Academic Senate, University of California, San Francisco. This is publication No. 77–29 from the Department of Pharmacology, University of California, San Francisco, CA.

REFERENCES

1. J. N. Hawthorne and M. Kai, in *Handbook of Neurochemistry* (Ed. A. Lajtha), Vol. 3, p. 491. Plenum Press, New York (1970).
2. H. S. Hendrickson and J. L. Reinersten, *Biochemistry* **8,** 4855 (1971).
3. H. S. Hendrickson and J. L. Reinersten, *Biochem. biophys. Res. Commun.* **44,** 1258 (1971).
4. A. L. Hodgkin and R. D. Keynes, *J. Physiol., Lond.* **138,** 253 (1957).
5. C. Torda, *A Depolarization-Hyperpolarization Cycle, A Molecular Model*, pp. 1–100. Torda, New York (1972).
6. R. H. Michell, *Biochim. biophys. Acta* **415,** 81 (1975).
7. G. Hazelbauer and J.-P. Changeux, *Proc. natn. Acad. Sci. U.S.A.* **71,** 1479 (1974).
8. D. M. Michaelson, J. R. Dugurd, D. L. Miller and M. A. Raftery, *J. Supramolec. Struct.* **4,** 419 (1976).
9. F. Gonzalez-Sastre, J. Eichberg and G. Hauser, *Biochim. biophys. Acta* **248,** 96 (1971).
10. G. Hauser and J. Eichberg, *Biochim. biophys. Acta* **326,** 201 (1973).
11. I. G. Morgan and L. S. Wolfe, *Biochim. biophys. Acta* **241,** 737 (1971).
12. C. W. Cotman, *Method. Enzym.* **31,** 445 (1974).
13. G. Hauser, J. Eichberg and F. Gonzalez-Sastre, *Biochim. biophys. Acta* **248,** 87 (1971).
14. F. Gonzalez-Sastre and J. Folch-Pi, *J. Lipid Res.* **9,** 532 (1968).
15. R. Natsuki, R. J. Hitzemann, B. A. Hitzemann and H. H. Loh, *Biochem. Pharmac.* **26,** 2095 (1977).
16. R. J. Hitzemann and H. H. Loh, *Eur. J. Pharmac.* **27,** 89 (1974).
17. A. DeBlas and H. R. Mahler, *Biochem. biophys. Res. Commun.* **72,** 24 (1976).
18. T. R. A. Davis, C. J. Kensler and D. B. Dews, *Psychopharmacologia* **32,** 51 (1973).