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THE EFFECT OF NORADRENALINE ON THE INCORPORATION OF ^{32}P INTO BRAIN PHOSPHOLIPIDS

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Abstract—Rat brain homogenates incubated with noradrenaline for 5 min showed reduced incorporation of ^{32}P into phospholipids. Subcellular fractionation studies indicated that this effect of noradrenaline occurred only in the synaptosomal fraction.

Incubation with noradrenaline for 20–120 min increased the incorporation of ^{32}P into phospholipids. This effect occurred in both the synaptosomal and mitochondrial fractions and was inhibited by the α-blocking drug thymoxamine. Noradrenaline did not significantly increase ^{32}P incorporation into phosphoproteins.

The results are discussed in terms of a possible transmitter role for noradrenaline in the brain.

NORADRENALINE (NA) is present in discrete areas of the mammalian brain[1] where it may act as a neurotransmitter[2] and thus might be expected to act upon the neuronal membrane. Since phospholipids are a major component of neuronal membranes,[3] such an action could possibly be reflected in a change in the metabolism of membrane phospholipids. NA is actively transported into brain slices[4] and isolated nerve endings[5] by a mechanism which requires $[Na^+ + K^+]$ Mg ATPase. Hence transport of NA might entail increased turnover of a phosphorylated carrier in the membrane.

In the work reported here an attempt has been made to investigate the effect of NA on the incorporation of ^{32}P into phospholipids and phosphoproteins of rat brain homogenates and to determine whether such effects are a reflection of either a transmitter function of NA or of its transport by neuronal membranes. A preliminary report of part of this work has been made.[6]

MATERIALS AND METHODS

1. *Preparation of tissues*

All procedures were carried out at 4° in an MSE 40 refrigerated ultracentrifuge.

(a) *Preparation of brain homogenates.* Male Wistar rats (300–400 g) were killed by cervical dislocation, the brains removed and immediately placed in ice-cold 0·32 M sucrose. The cerebral hemispheres and cerebellum were removed and the remaining brain tissue homogenized in 10 vol. of sucrose–Tris–EDTA (0·32 M–10 mM–0·5 mM; pH 7·4) using a Teflon homogenizer with 0·25 mm clearance at 800 rpm. The homogenate was centrifuged at 1000 *g* for 10 min, the pellet discarded and the supernatant centrifuged at 100,000 *g* for 30 min. The final pellet was resuspended in an incubation medium containing (mM): Na^+, 141; K^+, 5·9; Ca^{2+}, 5·1; Mg^{2+}, 2·4; Cl^-, 120; SO_4^{2-}, 2·4; HCO_3^-, 25; glucose, 9·2; pargyline, 0·05; ascorbate, 1·14; EDTA, 0·124 to a final protein concentration of 3–5 mg/ml. Aliquots (3 ml) of this suspension were incubated

at 37° under O_2–CO_2 (95:5) with 25 μc ^{32}P-orthophosphate and NA where appropriate.

(b) *Preparation of subcellular fractions.* Homogenates were prepared from whole rat brain as described above except that sucrose solutions used during preparation contained 10 μg/ml (5×10^{-5} M) pargyline but no tris or EDTA. The homogenate was centrifuged at 1000 *g* for 10 min to remove nuclei and cell debris and the supernatant centrifuged at 10,000 *g* for 20 min. The supernatant from this second centrifugation (designated Fraction I) was saved. The pellet (corresponding to approximately 1 g original brain tissue) was resuspended in 3 ml 0·32 M sucrose and layered onto 13 ml 0·8 M sucrose. This was centrifuged at 100,000 *g* for 60 min in an 8 × 25 ml fixed-angle head.[7] The upper layer (Fraction II) was aspirated off and kept, the remainder of the supernatant discarded and the pellet resuspended in 3 ml 0·8 M sucrose. This was layered onto 13 ml 1·2 M sucrose and centrifuged at 100,000 *g* for 60 min. The upper layer (Fraction III) was aspirated off and saved, the remainder of the supernatant discarded and the pellet (Fraction IV) saved. Fractions II and III were diluted with an equal volume of 0·32 M sucrose and, together with Fraction I, centrifuged at 17,000 *g* for 30 min. These pellets and the Fraction IV pellet were then suspended in incubation medium (similar to that mentioned in (a) except that the glucose was replaced by 30 mM pyruvate and 3·3 mM fumarate) to a final protein concentration of 3–5 mg/ml. Aliquots (3 ml) of this suspension were incubated at 37° under O_2–CO_2 (95:5) with 25 μc ^{32}P-orthophosphate and NA where appropriate.

(c) *Enzyme determinations.* Succinic dehydrogenase (SDH) was assayed by the succinate-dependent reduction of indophenol at 600 mμ.[8] Free lactic dehydrogenase (LDH) was assayed by the pyruvate-dependent oxidation of NADH at 340 mμ;[9] a figure for total LDH was obtained after the addition of 0·1 ml of 30% Triton X-100 to the cuvette and occluded LDH calculated from the difference.

2. *Chemical methods*

(a) *Extraction of phospholipids.* Incubation was stopped by the addition of 3 ml ice-cold 0·3 N perchloric acid. The contents of the incubation flasks were transferred to thick-walled glass centrifuge tubes in which the phospholipid extraction (based on the procedure of Mulé, 1967[10]) was carried out. The tubes were centrifuged at 2000 *g* for 20 min, the supernatant decanted and the pellet resuspended by homogenization in 5 ml 0·3 N perchloric acid. The acid-insoluble material was again separated by centrifugation and the perchloric wash repeated twice more. After the supernatant had been decanted from the final wash, the pellet was resuspended by gentle homogenization in 2 ml ethanol; 2 ml re-distilled chloroform were then added and the phospholipids extracted overnight at 4°. The following morning 5 ml 0·1 N HCl were added to each tube and the contents emulsified by homogenization. When centrifuged this emulsion separated into three phases. The upper water/ethanol layer was removed by aspiration and discarded; the interfacial disc of insoluble material was processed for phosphoproteins (see below) and the lower chloroform layer containing the lipids was sampled for chromatography and for determination of total radioactivity and phosphorus content. Total radioactivity was determined in a Packard Tricarb liquid scintillation spectrometer.

(b) *Extraction of phosphoprotein.* Phosphoproteins were extracted by a modification of the method of Heald.[11] The interfacial disc from 2(a) was washed with chloroform:

methanol (2:1 v/v) in a boiling tube. To the insoluble residue was added 1 ml 10 M urea followed by 2 ml of a saturated NaCl/ammonium sulphate solution. The tubes were placed in a boiling water-bath for 1 min and then centrifuged. The supernatant was decanted and the residue washed twice in 5 ml of the saturated NaCl/ammonium sulphate solution containing 15% urea. The insoluble material was digested overnight at 37° in 5 ml 1 N KOH. One ml 6 N HCl and 2·5 ml 10% TCA were then added. Any remaining insoluble material was centrifuged down and the supernatant sampled for determination of ^{32}P and total P.

(c) *Chromatography of phospholipids.* Aliquots (0·04 ml) of the chloroform layer from 2(a) were applied to silicic acid-impregnated paper (Whatman S.G. 81). Phospholipids were separated by ascending chromatography in the solvent system di-isobutylketone:acetic acid:water (40:25:5 v/v) as described by Marinetti.[12] After development the papers were dried in air. Direct chromatography of the chloroform extract yielded insufficient radioactivity for location by autoradiography. Hence an aliquot of the chloroform extract was concentrated on a rotary evaporator and chromatographed. The radioactivity was located by autoradiography using Kodak "No-Screen" medical X-ray film exposed for 7 days. The ^{32}P activity was seen as four discrete spots on the film, which was used as a template to cut individual chromatograms into four discs each of which was placed in 10 ml scintillation fluid and radioactivity determined by liquid scintillation counting. Recovery of applied ^{32}P was 80–90 per cent.

(d) *Phosphorus determination.* Phosphorus was determined by the method of Itaya and Ui[13] as modified by Ferguson and Strauch.[14]

(e) *Materials.* ^{32}P-orthophosphate (30 c/mg, Radiochemical Centre); *d-l* noradrenaline HCl (Sigma); propranolol [1-isopropylamino-3-(1-naphthyloxy)-2-propanol HCl, 'Inderal', I.C.I.]; thymoxamine [4-(2-dimethylaminoethoxy)-5-isopropyl-2-methylphenyl acetate, 'Opilon', Warner]; pargyline (*N*-benzyl-*N*-methyl-2-propynylamine HCl, Abbot). All other chemicals used were of reagent grade. Chloroform was re-distilled once; chloroform and ethanol were gassed with N_2 before use.

RESULTS

1. *Effect of noradrenaline on ^{32}P incorporation into whole brain homogenates*

(a) *Total phospholipids.* Table 1 shows ^{32}P incorporation into total phospholipids (PIP) over a 2-hr period and the effect of NA. NA appeared to have a dual effect on PIP, initially decreasing incorporation and later increasing it. Analysis of variance was applied to the data for each time point separately so as to remove the between-experiment variation. (A significant treatment × time interaction for the combined data justified this.) The level of significance obtained at each time point is shown in Table 1. There was a significant decrease in PIP at 5 min ($P < 0·05$) and a significant increase at 30, 60 and 120 min ($P < 0·001$).

(b) *Chromatography of phospholipids.* Chromatography separated the phospholipid extract into four fractions identified by co-chromatography of standards and on the basis of their R_f values (in parentheses) as phosphatidylcholine (0·33), phosphatidylinositol (0·43), phosphatidylethanolamine + phosphatidyl serine (0·50), and phosphatidic acid (0·70).

Table 2 shows the effect of NA on incorporation of ^{32}P into these four fractions. In control homogenates the greater part of the radioactivity was incorporated into

TABLE 1. EFFECT OF NORADRENALINE ON INCORPORATION OF ^{32}P INTO PHOSPHOLIPIDS OF RAT BRAIN HOMOGENATES

Experiment	Incubation time (min)																	
	5			10			20			30			60			120		
	C*	NA†	PC‡	C	NA	PC	C	NA	PC	C	NA	PC	C	NA	PC	C	NA	PC
1	901	528	59	713	627	88	963	898	93	972	1262	130	250	2052	164	806	2531	140
2	1017	600	59	1083	850	79	1300	1292	99	1475	1996	135	2292	3196	139	2821	3404	121
3	476	268	56	494	679	137	1071	905	84	1310	1500	115	1970	2732	139	1536	2500	163
4	269	287	107	542	477	88	1056	—	—	569	991	174	1958	2630	134	2352	4394	187
5	535	318	59	449	621	138	1141	1020	89	1141	1601	140	2152	2702	126	2308	3763	163
	$0{\cdot}05 > P > 0{\cdot}01$			—			$0{\cdot}1 > P > 0{\cdot}05$			$0{\cdot}001 < P < 0{\cdot}01$			$P < 0{\cdot}001$			$P < 0{\cdot}001$		

* C = Control; † NA = $1{\cdot}57 \times 10^{-5}$ M l-noradrenaline bitartrate; ‡ PC = noradrenaline as % of control.
Values expressed as counts/min ^{32}P incorporated into total phospholipids per mg homogenate protein.

phosphatidic acid and NA caused a large increase in incorporation into this fraction. However, when the incorporation in the presence of NA was related to that in the controls, NA caused a similar percentage increase into each fraction.

(c) *Incorporation of ^{32}P into phosphoprotein.* Figure 1 summarises the experiments on incorporation of ^{32}P into total phosphoproteins of whole brain homogenates. NA had no significant effect on phosphoprotein turnover.

TABLE 2. INCORPORATION OF ^{32}P INTO PHOSPHOLIPIDS OF RAT BRAIN HOMOGENATES AFTER 120 min INCUBATION WITH ^{32}P-ORTHOPHOSPHATE

	Incorporation of ^{32}P (cpm/mg protein ± S.E.M.)		Increase in NA-treated (% of control)
	Controls	Plus NA*	
PC	40 ± 10	46 ± 8	15
PI	35 ± 7	47 ± 9	34
PE + PS	46 ± 13	53 ± 13	15
PA	137 ± 13	199 ± 23	45

* NA, 5 μg/ml noradrenaline;
PC = Phosphatidylcholine;
PI = Phosphatidyl-inositol;
PE + PS = phosphatidylethanolamine + phosphatidyl serine;
PA = phosphatidic acid.
Means of eight experiments.

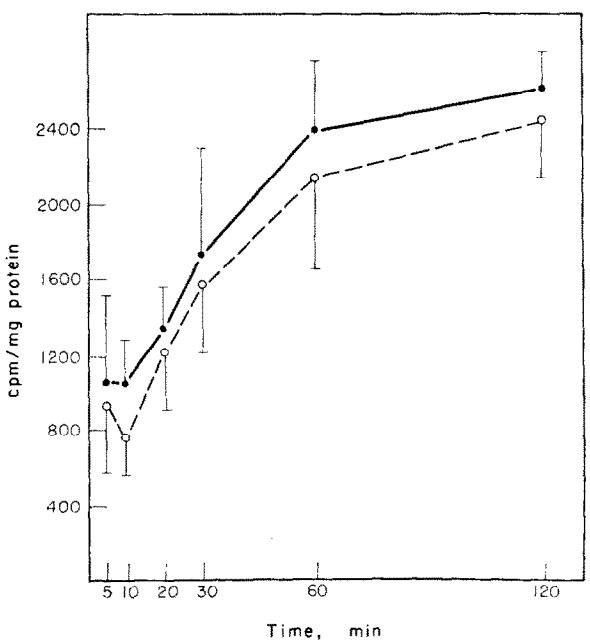

FIG. 1. Incorporation of ^{32}P into total phosphoproteins of rat brain homogenates. ○——○ Control; ●——● l-noradrenaline bitartrate 5 μg/ml ($1{\cdot}57 \times 10^{-5}$ M). Radioactivity expressed as ^{32}P incorporated into total phosphoprotein per mg protein in protein extract. Vertical bars represent standard errors. Each point the mean of four experiments.

2. *Effect of drugs known to interact with catecholamines*

To determine whether the observed stimulation of PIP by NA might be related either to NA transport or to an effect on membrane excitability we next studied the effect of drugs which interact with this system on PIP at 60 min. Cocaine and desmethylimipramine, which are potent inhibitors of NA uptake into brain,[4] did not prevent the effect of NA on PIP suggesting that this effect is not a reflection of NA transport. Drugs which are known to interact with catecholamine "receptors" however, did modify the action of NA. Preliminary experiments with blocking drugs suggested that control values might be raised by endogenous NA released during incubation of brain homogenates. To eliminate this possibility the experiments were performed on brains prepared from rats pre-treated with reserpine (5 mg/kg, i.p.; 42 hr and 18 hr before killing). The results are shown in Table 3. The stimulatory effect of

TABLE 3. EFFECT OF DRUGS ON THE INCORPORATION OF ^{32}P INTO TOTAL PHOSPHOLIPIDS OF RAT BRAIN HOMOGENATES AFTER 60 min INCUBATION WITH ^{32}P-ORTHOPHOSPHATE

Treatment	Incorporation of ^{32}P (cpm/mg protein ± S.E.M.)
Controls	2464 ± 128
Noradrenaline*	2825 ± 93‡
Noradrenaline* + Thymoxamine†	2314 ± 148 NS
Thymoxamine†	2259 ± 108 NS
Noradrenaline* + Propranolol†	3598 ± 219§
Propranolol†	2996 ± 118‡

* 5 μg/ml; † 50 μg/ml.
Values are means of six experiments.
Levels of significance of difference from controls by F-test:
‡ $P < 0.05$; §, $P < 0.01$; NS, not significant.

TABLE 4. EFFECT OF ADDITIVES ON INCORPORATION OF ^{32}P INTO PHOSPHOLIPIDS OF RAT BRAIN HOMOGENATES AFTER 60 min INCUBATION WITH ^{32}P-ORTHOPHOSPHATE

Additives		Incorporation of ^{32}P (cpm/mg protein)
A.	Glucose (10 mM)	4149
B.	Pyruvate (10 mM) + fumarate (3·3 mM)	8829
C.	B + cytochrome C (0·032 mM)	6018
D.	C + NAD (1 mM)	5328
E.	D + ADP (1 mM)	2008
F.	E + fluoride (19 mM)	1934

NA was blocked by thymoxamine which is known to antagonise the α effects of NA.[15] Propranolol, which blocks β effects,[16] itself stimulated PIP and the effect of NA was superimposed upon this. Isoprenaline (10 μg/ml), and 5-hydroxytryptamine (10 μg/ml) did not affect PIP.

3. *Effect of additives*

The effect on PIP of various additions to the medium is shown in Table 4. Substi-

tution of glucose by pyruvate and fumarate doubled ^{32}P uptake, but successive additions of cytochrome *c*, NAD, ADP and fluoride to the pyruvate–fumarate medium each lowered PIP. Hence in subsequent experiments an incubation medium was used which contained pyruvate and fumarate instead of glucose.

4. *Incorporation of ^{32}P into subcellular fractions*

Brain homogenates were split into four fractions by a combination of differential centrifugation and density separation as described under Methods.

(a) *Enzyme markers.* Fractions III and IV were assayed for succinic dehydrogenase (SDH, as a mitochondrial marker) and for lactic dehydrogenase (LDH, as a cytoplasmic marker) and the results are shown in Table 5. Pinched-off nerve endings

TABLE 5. THE DISTRIBUTION OF PROTEIN, OCCLUDED LACTIC DEHYDROGENASE (LDH) AND SUCCINIC DEHYDROGENASE (SDH) BETWEEN FRACTIONS III AND IV OF A BRAIN HOMOGENATE

Fraction	Protein (%)	Occluded LDH		SDH	
		%	RSA*	%	RSA
III	37	58	1·57	10	0·27
IV	63	42	0·67	90	1·43

* RSA = relative specific activity. Means of two experiments.

TABLE 6. EFFECT OF NORADRENALINE (NA) ON INCORPORATION OF ^{32}P INTO PHOSPHOLIPIDS OF SUBCELLULAR FRACTIONS OF RAT BRAIN HOMOGENATES INCUBATED FOR 5 min WITH ^{32}P-ORTHOPHOSPHATE

	NA (μg/ml)	cpm	μg P_i	cpm/μg P_i
Fraction III	—	6493 ± 1053	69 ± 10	103 ± 22
	5	4661 ± 151	74 ± 11	66 ± 7*
	50	4051 ± 273	74 ± 7	57 ± 6*
Fraction IV	—	6199 ± 1439	107 ± 12	58 ± 11
	5	5052 ± 196	113 ± 9	45 ± 2 NS
	50	5794 ± 351	110 ± 14	55 ± 5 NS

Values are means (±S.E.M.) of four experiments. Asterisks indicate levels of significance of difference from controls by F-test.
* $P < 0·05$; NS, not significant.

(synaptosomes) are likely to be the sole source of occluded LDH in these fractions and the distribution of LDH suggests that synaptosomes are distributed between fractions III and IV. However, the presence of the greater part of the SDH in Fraction IV suggests that the mitochondria are concentrated in this fraction.

(b) *Incubation for 5 min.* Each of these four fractions was incubated separately for 5 min in a ^{32}P-containing pyruvate–fumarate medium (see Methods). The PIP is shown in Table 6. NA depressed PIP at 5 min (as it did in whole brain homogenates) and this effect was confined to Fraction III.

TABLE 7. EFFECT OF NORADRENALINE (NA) ON INCORPORATION OF ^{32}P INTO PHOSPHOLIPIDS OF SUBCELLULAR FRACTIONS OF RAT BRAIN HOMOGENATES INCUBATED FOR 60 min WITH ^{32}P-ORTHOPHOSPHATE

	NA (μg/ml)	Incorporation of ^{32}P (cpm)	(cpm/μg P_i)
Fraction II	0	5264	25 ± 10
	5	7144	25 ± 4 NS
	50	6956	34 ± 2 NS
Fraction III	0	25,474	368 ± 28
	5	31,067	457 ± 7*
	50	35,438	448 ± 2*
Fraction IV	0	17,014	142 ± 2
	5	32,007	251 ± 15†
	50	35,532	271 ± 13†

Values are means (±S.E.M.) of three experiments. Levels of significance of difference from controls by F-test:
* $P < 0{\cdot}05$; †, $P < 0{\cdot}01$; NS, not significant.

(c) *Incubation for 60 min.* Fractions were incubated for 60 min in the presence of ^{32}P and the results are shown in Table 7. NA increased PIP at 60 min as it did in whole brain homogenates, and this effect was seen in Fractions III and IV.

DISCUSSION

In these experiments NA had a dual effect on ^{32}P incorporation into total phospholipids (PIP) of brain homogenates during incubation *in vitro*: an initial depression and a subsequent stimulation (Table 1). The question arises whether either of these effects is related to a physiological function of NA in the brain.

The first possibility is that the increase in PIP caused by NA was associated with the NA uptake mechanism of neuronal membranes. This seems unlikely as cocaine and desmethylimipramine, both of which inhibit accumulation of NA by brain slices,[4] did not affect the stimulation of PIP by NA. The NA uptake system shows high substrate specificity and, since it is easier to visualise this specificity residing in a protein than in a lipid, we tested the effect of NA on incorporation of ^{32}P into phosphoprotein of whole homogenates but could find no significant effect (Fig. 1). It might be worthwhile to repeat this on a synaptosomal fraction which is the fraction which takes up NA.[5]

Acetylcholine, which almost certainly has a transmitter function in the brain, has been shown to stimulate PIP in brain slices[17] and to inhibit PIP in isolated nerve endings.[18] Thus it is possible that the effect on PIP reported here could similarly represent a transmitter effect of NA in the brain. Iontophoretically-applied NA excites some neurones and inhibits others[19] and it is just possible that the depression of PIP reported here at 5 min could be related to a rapid transmitter action in the brain and/or that the stimulation of PIP seen after 30 min incubation could be related to a more gradual "modifier" effect.

The depressant effect appeared to be confined to Fraction III (synaptosomes) and thus might represent an effect on the excitability of the synaptosomal membrane. The

sites of action of NA as a central transmitter are unknown but there are three possible interpretations of this effect upon synaptosomes. The membranes of synaptosomes are almost entirely pre-synaptic and so NA might act upon them as a presynaptic transmitter. Alternatively, NA could be acting as a postsynaptic transmitter on the subsynaptic web which is known to be associated with some synaptosomes at least.[20, 21] Lastly it is possible that all neuronal membranes react similarly to the different transmitters and that selectivity resides in the locus of release of the different transmitters.

The stimulant effect became apparent only 30 min after exposure to NA. It was seen in both Fractions III and IV and so could be synaptosomal and/or mitochondrial or could be occurring in some other contaminant of these fractions. The relationship between amine levels and mood[2] suggests that NA may have a "modifier" effect in the brain exerted over a longer time period. This might well consist of a slowly-developing adaptive change in the membrane which modifies its excitability and could be reflected in the slowly-developing increase in PIP reported here. The stimulant effect of NA on PIP could be classified as an α effect since (a) it was blocked by thymoxamine, which blocks the α effects of catecholamines in peripheral tissues[15] and (b) isoprenaline, which has only β actions peripherally, did not stimulate PIP. Several workers have postulated the existence of α-receptors in the mammalian brain. The α-blocker phenoxy benzamine antagonises the actions of NA on rat cerebral cortex *in vivo*[22] and Fiszer and de Robertis[23] have suggested that brain α-receptors may be lipid in nature and are extractable with a solvent system giving a total lipid extract similar to that obtained in the present experiments.

The stimulation of PIP by propranolol could be a reflection of its effect on brain function which may not be related to β-receptor blockade.[24]

The increase in PIP is specific for NA in that it is not caused by 5-hydroxytryptamine or by GABA or histamine (unpublished observations).

In a paper published while this report was in preparation Hokin[25] reported that NA increases PIP into slices of guinea-pig brain after 60 min incubation.

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