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MORPHINE AND THE INCORPORATION OF ^{32}ORTHOPHOSPHATE *IN VIVO* INTO PHOSPHOLIPIDS OF THE GUINEA PIG CEREBRAL CORTEX, LIVER AND SUBCELLULAR FRACTIONS

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Abstract—This investigation was initiated to ascertain whether morphine *in vivo* would alter the incorporation of ^{32}orthophosphate into phospholipids of guinea pig cerebral cortex, liver and subcellular organelles of these tissues. Morphine stimulated the incorporation of ^{32}orthophosphate into whole cerebral cortex phospholipids 119 per cent at 16 hr and 87 per cent at 24 hr after ^{32}orthophosphate as determined by total radioactivity measurements. A significant stimulation of the incorporation of ^{32}orthophosphate ranging from 41 to 207 per cent occurred at 16 and 24 hr with individual phospholipids. No statistically significant effect was observed with total radioactivity data obtained from whole liver, although significance was observed with phosphatidylinositol and phosphatidic acid at later time intervals. A predominantly stimulatory effect on phospholipid metabolism was obtained with total radioactivity determinations on the subcellular fractions of the cerebral cortex. The major stimulatory effect occurred with the individual phospholipids at 48 hr in the soluble supernatant fraction (248–403 per cent). Total radioactivity studies with liver subcellular fractions provided a statistically significant effect at 48 hr (79–134 per cent). The effect on individual phospholipids from liver subcellular fractions was similar to the total radioactivity data. Morphine had both an inhibitory and stimulatory effect on the total radioactivity determinations of mitochondrial subfractions from the cerebral cortex at 16, 24 and 48 hr. A similar effect was observed with mitochondrial fractions subjected to osmotic shock. Total radioactivity determinations on phospholipids from microsomal subcellular fractions of liver were both stimulatory and inhibitory at the various time intervals. A statistically significant stimulatory effect was observed with individual phospholipids at 48 hr from the smooth and rough microsomal liver subcellular fractions. It is concluded that morphine alters phospholipid metabolism *in vivo* and thus may directly affect cellular function in liver and neuronal activity in the central nervous system.

RECENT investigations[1–3] have demonstrated the predominantly stimulatory effect of morphine and nalorphine on phospholipid metabolism in cerebral cortex slices *in vitro* as well as with *in vivo* levels of morphine (1·1 nmoles/g). However, those studies did not demonstrate whether morphine *in vivo* could alter the incorporation of ^{32}orthophosphate ($^{32}P_i$) *in vivo* into phospholipids, thus providing evidence for a direct effect on phospholipid metabolism with pharmacological levels of drug. It was therefore most important to conduct these experiments in the intact animal utilizing both

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cerebral cortex and liver phospholipids. Studies were also initiated to ascertain whether the morphine phospholipid effect might be localized at a specific intracellular site. The results of these investigations are reported in this communication.

METHODS

Biological procedures

Guinea pigs weighing 300–700 g were injected intraperitoneally with 150 μc/100 g of $Na_2H^{32}PO_4$ and sacrificed at intervals of 4, 16, 24 and 48 hr. All guinea pigs were deprived of food, but had full access to water after the $^{32}P_i$ injection. Morphine (40 mg/kg as free base) was administered subcutaneously 1 hr prior to sacrifice. The control animals received 0·9% saline in a volume equivalent to the morphine injection 1 hr prior to sacrifice. The guinea pigs were killed by decapitation and the cerebral cortex or whole brain as well as a portion of the liver was removed and placed on ice immediately.

Chemical procedures

The brain and liver tissue were either homogenized in 5 ml of 0·3 N $HClO_4$ or in a 10% homogenate, which consisted of 0·32 M sucrose plus 10 μM Ca^{2+} for brain and 0·25 M sucrose for liver. The $HClO_4$ homogenates were directly extracted and the 10% homogenates were subjected to differential centrifugation to obtain the subcellular fractions as described by Mulé *et al.*[4] prior to extraction.

Extraction of phospholipids. The brain and liver perchloric acid homogenates were centrifuged at a maximum speed of 3000 *g* for 15 min and the sediment was washed twice by resuspending and centrifuging. The final sediment was resuspended in 4 ml chloroform–ethanol (1:1) and phospholipids were extracted as described by Hokin and Hokin.[5]

For the subcellular fractions, aliquots of brain and liver in sucrose were mixed with an equal volume of cold 10% trichloroacetic acid (TCA) and centrifuged at a maximum speed of 12,000 *g* for 10 min. The pellets were washed three times with 5% TCA by resuspending and centrifuging. The phospholipids were extracted from the final pellets as described above.

Chromatography and radioactive assay of the phospholipids. Aliquots of $CHCl_3$ extract were applied as spots on silicic acid-impregnated Whatman no. 1 paper.[6] The phospholipids were separated by ascending chromatography using the solvent system diisobutyl ketone–acetic acid–water (40:25:5, v/v). The phosphoinositides were separated with the solvent phenol–concentrated NH_3 (99:1, v/v) as reported by Santiago-Calvo *et al.*[7] After development, the chromatograms were dried at room temperature, stapled to Kodak No-Screen medical X-ray film, and placed in film holders. The films were then exposed for 10 days. With the staple marks as guides, the autoradiograms were superimposed over the chromatograms and the individual labeled phospholipids circled using a standardized template. The individual discs were cut out and transferred to liquid scintillation counting vials. Ten ml of phosphor solution was added and the radioactivity determined in the Packard Tri-Carb liquid scintillation spectrometer (model no. 3003). Quenching was corrected by the channels ratio method.[8] Radioactivity from duplicate or triplicate spots usually agreed within 10 per cent. All $^{32}P_i$ radioactivity was corrected for decay. Total radioactivity was determined

by evaporating aliquots of the phospholipid–$CHCl_3$ extracts to dryness in scintillation counting vials and adding 10 ml of phosphor solution to each vial.

Method of expressing radioactivity. In order to determine the specific activity of phosphorus (cpm/μmole of P) in the total $CHCl_3$ extract of the tissues and in the individual phospholipids, an identical amount of $^{32}P_i$ with the same specific activity was injected intraperitoneally into paired control and morphine-treated guinea pigs at the various time intervals. All chemical and biological manipulations were handled in an identical manner for both control and morphine-treated animals. The data were calculated to provide a specific activity per gram of the brain (cpm/μmole of P/g). The quantity of radioactivity determined in the sample was in the range of 10–100 cpm per μg of phospholipid-P for the brain and liver tissue respectively.

Total phospholipid phosphorous. Aliquots of suitably diluted $CHCl_3$ extracts from brain and liver were digested in 10 N H_2SO_4 and total phosphorus was determined by the method of Bartlett.[9]

Statistical analysis. The calculation of the standard errors embodies both the biological as well as the chemical variability of the experiments. The data were statistically analyzed as group comparisons for the calculation of t according to methods described by Snedecor.[10]

RESULTS

Morphine and the incorporation of $^{32}P_i$ in vivo into phospholipids of the whole cerebral cortex. Morphine markedly increased the incorporation of $^{32}P_i$ into phospholipids (Fig. 1) at 16 and 24 hr (87–119 per cent). No statistically significant effect was obtained at either 4 or 48 hr. The incorporation of $^{32}P_i$ into individual phospholipids of the cerebral cortex appears in Fig. 2. The effect of morphine on triphosphoinositide

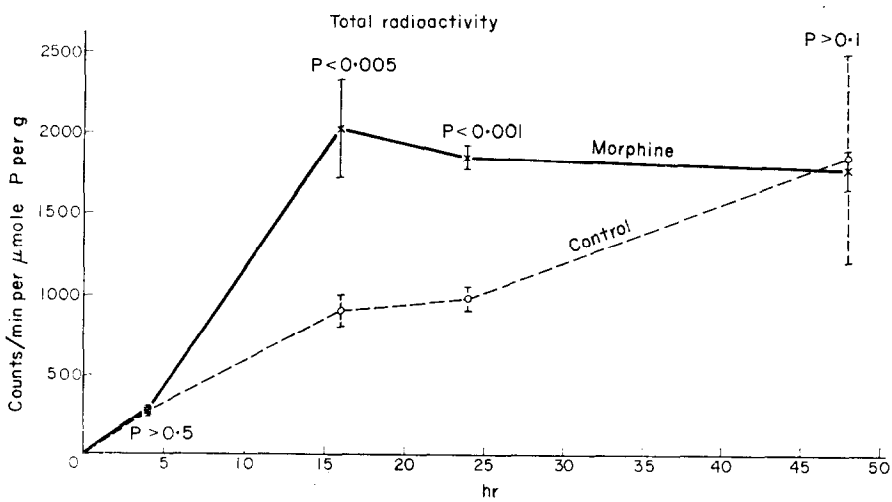

FIG. 1. Total radioactivities in the phospholipids extracted from the cerebral cortex after a $^{32}P_i$ (150 μc/100 g) i.p. injection to two to four guinea pigs. The time intervals of 4, 16, 24 and 48 hr represent time after $^{32}P_i$ administration. Morphine was administered s.c. (40 mg/kg, as free base) 1 hr prior to sacrifice. The control guinea pigs received a comparable volume of 0·9% saline s.c. 1 hr prior to sacrifice. Each value represents the mean obtained from four to eight determinations ± S.E.

TABLE 1. MORPHINE AND THE INCORPORATION OF $^{32}P_i$ INTO

Phospholipid	Time (hr)	Homogenate (cpm/μmole P/g)			Crude nuclear (cpm/μmole P/g)		
		C†	M‡	Δ % §	C	M	Δ %
Total radioactivity	16	109 ± 11	167 ± 12	+ 53‖	263 ± 10	269 ± 11	+ 2
	24	169 ± 12	174 ± 16	+ 3	269 ± 11	251 ± 12	− 7
	48	184 ± 9	377 ± 10	+ 105‖	209 ± 12	399 ± 8	+ 91‖
Phosphatidylinositol	16	10 ± 3	16 ± 5	+ 60	17 ± 2	17 ± 4	0
	24	11 ± 4	11 ± 3	0	14 ± 3	17 ± 5	+ 14
	48	10 ± 4	22 ± 3	+ 120‖	17 ± 1	20 ± 4	+ 18
Sphingomyelin	16	17 ± 4	22 ± 5	+ 29	22 ± 2	32 ± 6	+ 45‖
	24	13 ± 2	18 ± 6	+ 38	25 ± 4	26 ± 6	+ 4
	48	21 ± 1	41 ± 2	+ 95‖	25 ± 3	39 ± 5	+ 56‖
Phosphatidylcholine	16	32 ± 4	49 ± 3	+ 53‖	76 ± 8	94 ± 7	+ 24‖
	24	31 ± 6	53 ± 5	+ 71‖	45 ± 4	38 ± 6	− 16
	48	59 ± 4	157 ± 5	+ 166‖	47 ± 3	121 ± 10	+ 157‖
Phosphatidylserine + phosphatidylethanolamine	16	13 ± 6	17 ± 5	+ 31	21 ± 2	19 ± 3	− 10
	24	11 ± 4	12 ± 3	+ 9	10 ± 4	10 ± 3	0
	48	15 ± 2	36 ± 5	+ 140‖	13 ± 3	33 ± 5	+ 154‖
Phosphatidic acid	16	9 ± 6	11 ± 2	+ 22	11 ± 1	11 ± 4	0
	24	7 ± 4	8 ± 2	+ 14	5 ± 3	5 ± 2	0
	48	9 ± 1	281 ± 7	+ 211‖	5 ± 2	11 ± 2	+ 120‖

*$^{32}P_i$ (150 μc/100 g) was injected i.p. and the animals were sacrificed at the various times after $^{32}P_i$. The cerebral cortex was removed, subcellular fractions were prepared and the radioactivity was determined as described under Methods.

†C, control. From three to nine guinea pigs received 0·9% saline s.c. 1 hr prior to sacrifice. Each value represents the mean ± S.E. obtained from six to eighteen determinations.

(TPI) at 24 hr was stimulatory (207 per cent). Increased incorporation of $^{32}P_i$ occurred at 16 and 24 hr, ranging from 41 to 120 per cent for phosphatidic acid (PA), phosphatidylcholine (PC), and phosphatidylserine plus phosphotidylethanolamine (PS + PE). $^{32}P_i$ was increased at 16 and 48 hr for phosphatidylinositol (PI). No statistically significant stimulatory effect was observed with diphosphoinositide (DPI) or sphingomyelin (S) at each time interval.

Effect of morphine on the incorporation of $^{32}P_i$ in vivo *into phospholipids from cerebral cortex subcellular fractions.* Table 1 summarizes the data obtained with the cerebral cortex subcellular phospholipids. Statistically significant increased turnover of $^{32}P_i$ was observed at 48 hr for each phospholipid in the homogenate and soluble supernatant fraction. A significant effect was obtained with some of the phospholipids from the crude nuclear, crude mitochondrial, and microsomal fractions at 16, 24 and 48 hr after i.p. $^{32}P_i$. Phosphatidylcholine appeared to be most affected by morphine in each intracellular fraction and at the various time intervals. This effect may be quite important, since phosphatidylcholine represents from 25 to 40 per cent of the total lipid phosphorus in guinea pig brain.[11] The data on the total radioactivity indicated a statistically significant stimulation (59–109 per cent) primarily at 48 hr for the phospholipids in each subcellular fraction.

The effect of morphine on the mitochondrial subfraction phospholipids appears in

PHOSPHOLIPIDS FROM CEREBRAL CORTEX SUBCELLULAR FRACTIONS*

Crude mitochondrial (cpm/μmole P/g)			Microsomal (cpm/μmole P/g)			Soluble supernatant (cpm/μmole P/g)		
C	M	Δ %	C	M	Δ %	C	M	Δ %
146 ± 12	162 ± 8	+ 11	128 ± 11	177 ± 12	+ 38‖	253 ± 8	311 ± 16	+ 23‖
172 ± 14	161 ± 7	− 7	194 ± 16	196 ± 8	+ 1	275 ± 12	266 ± 11	− 3
201 ± 12	318 ± 10	+ 59‖	222 ± 12	372 ± 8	+ 67‖	390 ± 16	1148 ± 12	+ 109‖
13 ± 4	9 ± 2	+ 31	7 ± 2	15 ± 3	+ 114‖	84 ± 3	119 ± 11	+ 42‖
8 ± 2	9 ± 3	+ 12	12 ± 2	15 ± 4	+ 25	70 ± 4	25 ± 2	− 64
9 ± 1	9 ± 3	0	8 ± 3	14 ± 3	+ 75‖	42 ± 4	147 ± 6	+ 250‖
13 ± 2	11 ± 2	− 15	7 ± 2	22 ± 4	+ 180‖	69 ± 7	117 ± 8	+ 69‖
27 ± 6	17 ± 3	− 37‖	18 ± 4	22 ± 5	+ 24			
22 ± 4	17 ± 5	− 23	16 ± 2	26 ± 5	+ 61‖	51 ± 6	253 ± 12	+ 396‖
42 ± 4	41 ± 5	− 2	19 ± 5	58 ± 10	+ 205‖	133 ± 10	158 ± 12	+ 19‖
41 ± 5	36 ± 3	− 12	57 ± 4	79 ± 8	+ 38‖	105 ± 8	36 ± 6	− 66‖
32 ± 6	106 ± 10	+ 231‖	65 ± 5	147 ± 6	+ 126‖	110 ± 7	495 ± 8	+ 350‖
14 ± 1	14 ± 2	0	8 ± 2	19 ± 5	+ 138‖	80 ± 4	19 ± 5	− 76‖
8 ± 3	7 ± 2	− 12	10 ± 2	13 ± 3	+ 30			
6 ± 1	12 ± 3	+ 100‖	21 ± 4	30 ± 5	+ 43	54 ± 6	188 ± 10	+ 248‖
6 ± 1	8 ± 4	+ 33	6 ± 2	10 ± 5	+ 67	70 ± 5	10 ± 4	− 86‖
5 ± 1	4 ± 2	− 20	5 ± 2	5 ± 3	0	44 ± 3	112 ± 11	+ 154‖
5 ± 2	4 ± 2	− 20	6 ± 1	9 ± 3	+ 50	31 ± 2	156 ± 8	+ 403‖

‡M, morphine. From three to nine guinea pigs which received 40 mg/kg (free base) of morphine s.c. prior to sacrifice. Each value represents the mean ± S.E. obtained from six to eighteen determinations.
§Percentage difference between the control and morphine-treated animals.
‖$P < 0.05$ as compared to the corresponding control values.

Table 2. In general, statistically significant effects were observed at 16 and 48 hr after $^{32}P_i$. The effect of morphine on the A subfraction (myelin fragments) phospholipids ranged from 67 to 233 per cent. The effect was substantially less for the B fraction (mixture of nerve endings, synaptic vesicles and membranes). The incorporation of $^{32}P_i$ into cholinergic nerve endings (C) was greater than that observed for the non-cholinergic nerve endings (D). In fact, morphine appeared to inhibit the incorporation of $^{32}P_i$ into most of the phospholipids of the D fraction at various time intervals. The most pronounced effect occurred with the phospholipids from the E fraction (free mitochondria). At 16 and 24 hr the turnover of $^{32}P_i$ was inhibited and at 48 hr the morphine effect was predominantly stimulatory (36–82 per cent) in the E fraction. The phospholipids in the fractions obtained after hypoosmotic shock (M_1, M_2, M_3) of the crude mitochondria (Table 2) were both stimulated and inhibited at the various time intervals.

It would appear that phosphatidylcholine was the most prominently affected phospholipid in the mitochondrial subfractions. It is interesting, however, that the effect on phosphatidylcholine in the various fractions was not stimulatory in all cases.

Morphine and the incorporation of $^{32}P_i$ into phospholipids from guinea pig liver. Figure 3 shows the effect of morphine on the incorporation of $^{32}P_i$ into total phospholipids (total radioactivity) and individual phospholipids of liver. The total radioactivity

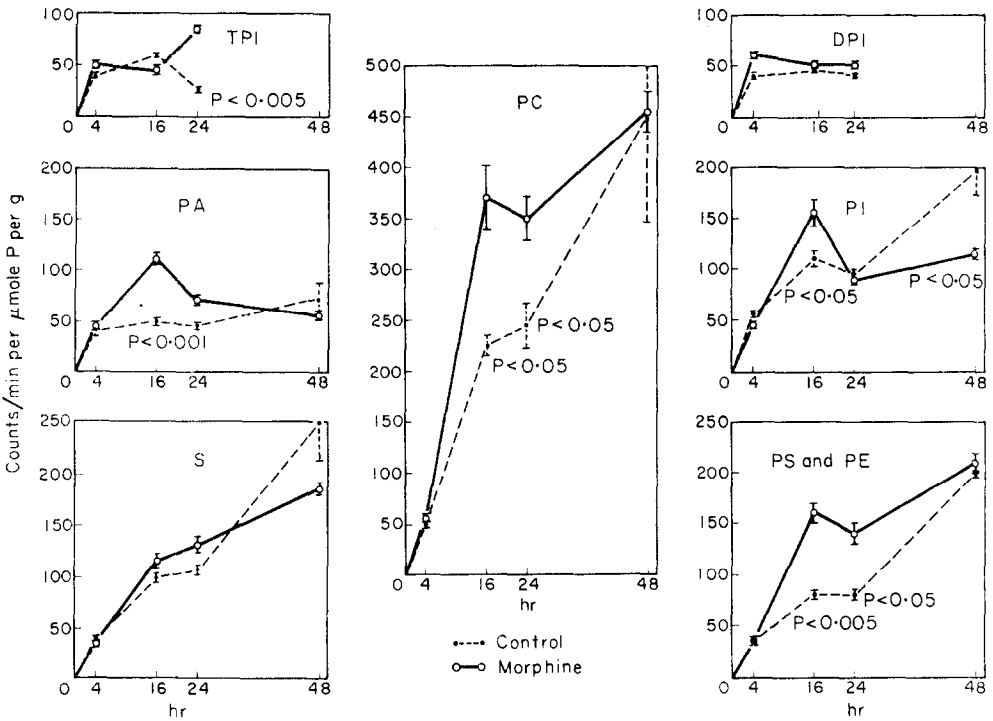

FIG. 2. Effect of morphine on the incorporation of $^{32}P_i$ into individual phospholipids of the cerebral cortex at various time intervals *in vivo*. $^{32}P_i$ (150 μc/100 g) was injected i.p. into two to four guinea pigs. The time intervals of 4, 16, 24 and 48 hr represent time after $^{32}P_i$ administration. A 40 mg/kg (free base) s.c. injection of morphine was administered 1 hr prior to sacrifice and a comparable volume of 0·9% saline was injected s.c. into the control guinea pigs. Each value represents the mean obtained from four to eight determinations ± S.E. TPI, triphosphoinositide; PA, phosphatidic acid S, sphingomyelin; PC, phosphatidylcholine; DPI, diphosphoinositide; PI, phosphatidylinositol; PS & PE, phosphatidylserine and phosphatidylethanolamine.

data show that no statistically significant effect occurred at 4, 16, 24 and 48 hr after $^{32}P_i$. However, with respect to the individual phospholipids, the incorporation of $^{32}P_i$ into phosphatidic acid was significantly stimulated at 24 (34 per cent) and 48 (78 per cent) hr. Phosphatidylinositol was significantly stimulated (59 per cent) at 24 hr and no statistically significant effect was obtained at any other time interval with the other phospholipids from the guinea pig liver extract.

Morphine and the incorporation of $^{32}P_i$ into phospholipids from liver subcellular fractions. The effect of morphine on the incorporation of $^{32}P_i$ into total phospholipids as well as into the individual phospholipids obtained from the liver subcellular fractions is presented in Table 3. The primary statistically significant effect with respect to total radioactivity occurred at 48 hr (79–134 per cent) with no statistically significant effect observed at 16 or 24 hr. The individual phospholipids from each subcellular fraction were predominantly stimulated at 48 hr after $^{32}P_i$. This stimulatory effect was most pronounced with the phospholipids from the soluble supernatant fraction. The effect at 16 and 24 hr was both stimulatory and inhibitory, and apparently

TABLE 2. INCORPORATION OF $^{32}P_i$ INTO PHOSPHOLIPIDS EXTRACTED FROM SUBFRACTIONS OF THE CRUDE MITOCHONDRIAL FRACTION OF GUINEA PIG CEREBRAL CORTEX*

		Crude mitochondrial subfractions (cpm/μmole P/g)																							
	Time	A			B			C			D			E			M_1			M_2			M_3		
Phospholipids	(hr)	C†	M‡	Δ %§	C	M	Δ %	C	M	Δ %	C	M	Δ %	C	M	Δ %	C	M	Δ %	C	M	Δ %	C	M	Δ %
Total radioactivity	16	89 ± 12	51 ± 11	−43‖	83 ± 10	72 ± 8	−13	88 ± 17	78 ± 14	−11	121 ± 21	116 ± 14	−4	89 ± 8	80 ± 12	+10	350 ± 12	128 ± 18	−63‖	341 ± 30	160 ± 10	−53‖	234 ± 28	211 ± 20	−17
	24	43 ± 8	87 ± 10	+102	102 ± 14	84 ± 11	−18	110 ± 12	76 ± 16	−31	151 ± 30	94 ± 10	−38‖	190 ± 14	77 ± 18	−54‖	164 ± 16	163 ± 20	+1	219 ± 19	168 ± 14	−23‖	240 ± 30	275 ± 10	+14
	48	112 ± 11	173 ± 14	+54‖	131 ± 12	254 ± 15	+94‖	144 ± 18	260 ± 19	+80‖	179 ± 31	294 ± 12	+64‖	198 ± 12	422 ± 12	+113‖	155 ± 20	156 ± 17	+1	214 ± 20	254 ± 29	+19	353 ± 26	358 ± 18	+1
Phosphatidyl-inositol	16	4 ± 1	12 ± 4	+200	9 ± 1	2 ± 1	−78‖	12 ± 2	16 ± 3	+33	19 ± 3	7 ± 2	−63‖	77 ± 5	24 ± 2	−69‖	21 ± 3	34 ± 8	+62‖	12 ± 3	8 ± 3	−33	28 ± 2	22 ± 4	−21
	24	5 ± 1	9 ± 2	+80	6 ± 1	6 ± 1	0	7 ± 2	9 ± 2	+28	9 ± 2	4 ± 1	−56‖	17 ± 3	5 ± 2	−70‖	16 ± 4	21 ± 2	+31	8 ± 2	5 ± 1	−38			
	48	8 ± 2	16 ± 3	+100‖	6 ± 1	12 ± 2	+71‖	7 ± 1	12 ± 1	+71‖	7 ± 1	12 ± 4	+71‖	11 ± 2	20 ± 4	+82‖	27 ± 1	28 ± 6	+4	6 ± 1	20 ± 2	+233‖	20 ± 3	34 ± 2	+70‖
Sphingomyelin	16																64 ± 2	9 ± 1	−86‖	43 ± 12	47 ± 8	+4	38 ± 3	13 ± 3	−66‖
	24	6 ± 1	20 ± 2	+233‖	12 ± 2	12 ± 2	0	13 ± 2	20 ± 3	+54‖	16 ± 3	7 ± 2	−56‖	21 ± 4	8 ± 2	−62‖	22 ± 4	27 ± 3	+23	13 ± 3	11 ± 2	−27	24 ± 2	26 ± 3	+7
	48	12 ± 4	16 ± 3	+33	18 ± 3	25 ± 4	+39‖	17 ± 2	24 ± 4	+39‖	18 ± 5	26 ± 4	+44‖	23 ± 3	32 ± 3	+39‖									
Phosphatidyl-choline	16	8 ± 2	17 ± 4	+112‖	17 ± 4	25 ± 3	+47‖	14 ± 5	15 ± 3	+7	36 ± 2	35 ± 10	−3	186 ± 11	46 ± 3	−75‖	21 ± 3	3 ± 1	−86‖	79 ± 14	15 ± 11	−84‖	46 ± 10	44 ± 9	−4
	24	5 ± 3	7 ± 2	+90	30 ± 5	23 ± 5	−17	32 ± 3	44 ± 5	+38‖	46 ± 4	27 ± 8	−41‖	55 ± 4	21 ± 2	−58‖	15 ± 3	20 ± 4	+33	39 ± 5	67 ± 12	+33‖	78 ± 12	51 ± 4	−35‖
	48	8 ± 1	25 ± 2	+212‖	39 ± 7	68 ± 4	+74‖	46 ± 6	75 ± 4	+63‖	12 ± 6	39 ± 7	+225‖	65 ± 6	96 ± 13	+48‖	14 ± 4	59 ± 12	+321‖	97 ± 16	30 ± 4	+347‖	82 ± 5	82 ± 6	0
Phosphatidyl-serine— phosphatidyl-ethanolamine	16				5 ± 1	3 ± 1	−40	3 ± 1	5 ± 1	+67	14 ± 3	10 ± 2	−28	69 ± 4	22 ± 8	−67‖	7 ± 3	1 ± 0·1	−86	13 ± 2	5 ± 1	−62‖	32 ± 8	18 ± 9	−44‖
	24	3 ± 1	4 ± 2	+33	11 ± 2	10 ± 2	−9	12 ± 3	10 ± 2	−17	17 ± 2	7 ± 1	−59‖	20 ± 6	5 ± 2	−75‖	4 ± 1	8 ± 2	+100‖	16 ± 3	12 ± 2	−25			
	48	4 ± 1	8 ± 2	+100	15 ± 3	28 ± 4	+87‖	12 ± 4	30 ± 5	+130‖	14 ± 5	26 ± 2	+86‖	24 ± 3	43 ± 3	+79‖	5 ± 2	6 ± 2	+20	8 ± 2	6 ± 1	−25	42 ± 2	42 ± 10	0
Phosphatidic acid	16	6 ± 1	5 ± 1	0	2 ± 0·2	1 ± 0	−50	2 ± 0·1	2 ± 0·3	0	8 ± 2	4 ± 2	−50	37 ± 6	11 ± 2	−70‖	5 ± 2	1 ± 0·1	−80	5 ± 2	2 ± 0·6	−60	35 ± 3	14 ± 4	−60‖
	24	2 ± 0·5	3 ± 1	+50	4 ± 1	4 ± 1	0	5 ± 1	5 ± 1	0	6 ± 2	3 ± 1	−50	10 ± 4	3 ± 1	−70‖	3 ± 1	5 ± 1	+67	5 ± 2	2 ± 1	−60			
	48	3 ± 1	5 ± 2	+67‖	4 ± 2	6 ± 2	+50‖	5 ± 2	7 ± 1	+40	7 ± 2	14 ± 2	+100	14 ± 2	19 ± 8	+136‖	2 ± 0·4	2 ± 0·8	0	2 ± 1	1 ± 0·4	−50	17 ± 7	19 ± 4	+12

*$^{32}P_i$ (150 μc/100 g) was injected i.p. and the animals were sacrificed at the various times after $^{32}P_i$. The cerebral cortex was removed and subfractions of the crude mitochondria were prepared (Mulé *et al.*[4]). The incorporation of $^{32}P_i$ was determined as described under Methods. See text for definition of the various subfractions.
†C, control. Three guinea pigs received 0·9% saline s.c. 1 hr prior to sacrifice. Each value represents the mean ± S.E. from six determinations.
‡M, morphine. Three guinea pigs received 40 mg/kg (free base) of morphine s.c. 1 hr prior to sacrifice. Each value represents the mean ± S.E. from six determinations.
§Percentage difference between the control and morphine-treated animals.
‖$P \leq 0.05$ as compared to the corresponding control values.

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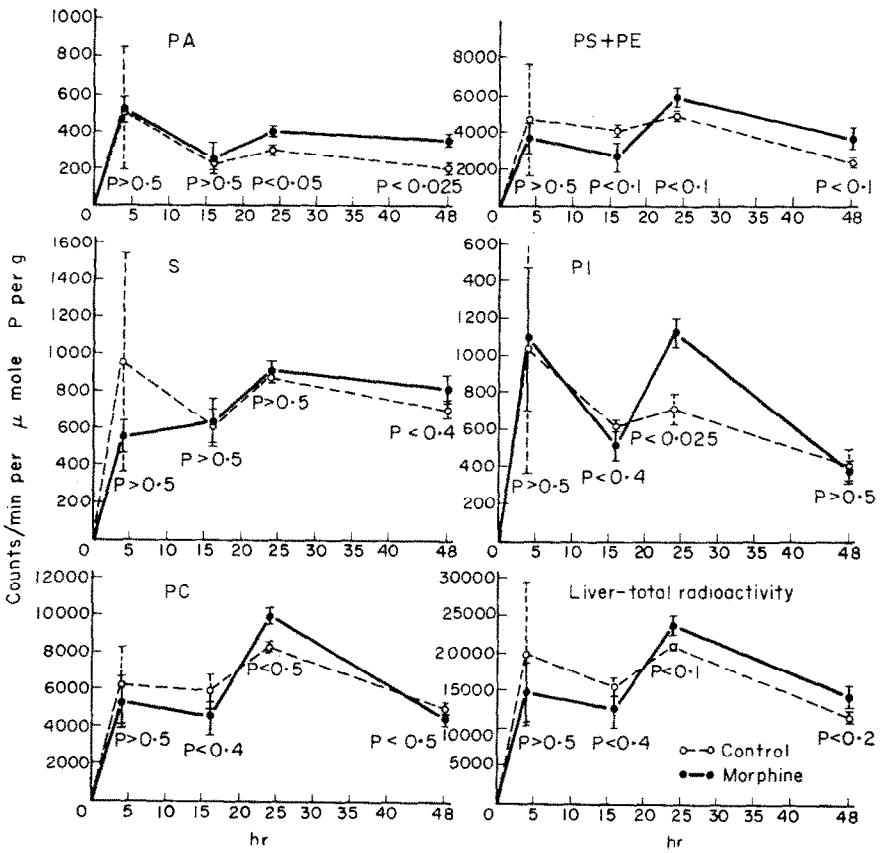

FIG. 3. Incorporation of $^{32}P_i$ into individual phospholipids and the total radioactivity obtained from liver after an i.p. injection of $^{32}P_i$ (150 μc/100 g) into three guinea pigs. The time intervals of 4, 16, 24 and 48 hr represent time after $^{32}P_i$ administration. A 40 mg/kg (free base) s.c. injection of morphine was administered 1 hr prior to sacrifice. The control guinea pigs received a comparable volume of 0·9% saline s.c. 1 hr prior to sacrifice. Each value represents the mean obtained from six determinations ± S. E. PA, phosphatidic acid; S, sphingomyelin; PC phosphatidylcholine; PS + PE, phosphatidylserine plus phosphatidylethanolamine; PI, phosphatidylinositol.

varied with the phospholipid, subcellular fraction, and time interval. The incorporation of $^{32}P_i$ into phosphatidylinositol, however, was stimulated from 51 to 160 per cent at the various time intervals in the soluble supernatant fraction.

The effect of morphine on phospholipids from the microsomal subcellular fractions of liver appears in Table 4. The total phospholipid (total radioactivity) stimulatory effect appeared to occur at 16 hr (39–73 per cent) with both a stimulatory and inhibitory effect at 24 and 48 hr. The effect on the individual phospholipids from the smooth microsomal fraction was variable, except for the stimulated incorporation of $^{32}P_i$ into phosphatidic acid (2–42 per cent) at each time interval. A similar effect was observed for the rough microsomal fraction phospholipids where the stimulation obtained with phosphatidic acid ranged from 468 to 33 per cent over the time interval 16–48 hr. Microsomal-2 and microsomal supernatant fraction phospholipids were both stimulated and inhibited at the various time intervals.

TABLE 3. MORPHINE AND THE INCORPORATION OF $^{32}P_i$ INTO

Phospholipids	Time (hr)	Subcellular fractions: Homogenate C†	Homogenate M‡	Homogenate Δ %§	Crude nuclear C	Crude nuclear M	Crude nuclear Δ %
Total radioactivity	16	3734 ± 1074	3659 ± 1280	− 2	3235 ± 1105	2910 ± 685	− 10
	24	3302 ± 1450	3255 ± 1120	− 1	3135 ± 1402	3148 ± 382	0
	48	2796 ± 895	5002 ± 1202	+ 79‖	2680 ± 505	5204 ± 1025	+ 94‖
Phosphatidyl-inositol	16	118 ± 14	150 ± 18	+ 27‖	99 ± 11	127 ± 20	+ 28
	24	118 ± 10	107 ± 16	− 9	153 ± 8	108 ± 12	− 29‖
	48	75 ± 8	111 ± 11	+ 48‖	69 ± 7	101 ± 11	+ 46‖
Sphingomyelin	16	191 ± 10	212 ± 12	+ 11	178 ± 12	218 ± 41	+ 22
	24	160 ± 14	142 ± 16	− 11	198 ± 18	182 ± 30	− 8
	48	233 ± 8	368 ± 12	+ 58‖	308 ± 19	556 ± 19	+ 81‖
Phosphatidyl-choline	16	1688 ± 20	1789 ± 21	+ 6	1295 ± 21	1294 ± 82	0
	24	1466 ± 62	1398 ± 40	− 5	1253 ± 41	1291 ± 62	+ 3
	48	1343 ± 41	2212 ± 54	+ 65‖	1046 ± 24	1861 ± 38	+ 78‖
Phosphatidyl-serine + phosphatidyl-ethanolamine	16	796 ± 80	652 ± 24	− 18‖	835 ± 48	529 ± 105	− 37‖
	24	594 ± 16	581 ± 40	− 2	525 ± 20	515 ± 34	− 2
	48	491 ± 21	885 ± 28	+ 80‖	505 ± 65	949 ± 80	+ 88‖
Phosphatidic acid	16	46 ± 6	29 ± 8	− 37	88 ± 21	41 ± 16	− 53‖
	24	40 ± 8	24 ± 6	− 40‖	56 ± 12	38 ± 8	− 32
	48	41 ± 12	74 ± 4	+ 89‖	56 ± 11	131 ± 14	+ 134‖

*$^{32}P_i$ (150 μc/100 g) was injected i.p. and the animals were sacrificed at the various times after $^{32}P_i$. The liver was removed, subcellular fractions were prepared, and the total radioactivity as well as the incorporation of $^{32}P_i$ into individual phospholipids was determined as described under methods.

†C, control. Three guinea pigs at each time interval received 0·9% saline s.c. 1 hr prior to sacrifice. Each value represents the mean ± S.E. of six determinations.

DISCUSSON

The results of the present study clearly show that morphine alters phospholipid metabolism *in vivo*. This effect is present in phospholipids from the cerebral cortex, liver and subsequent subcellular organelles of these tissues.

The incorporation of $^{32}P_i$ into phospholipids from the cerebral cortex of control guinea pigs increased through 48 hr. Morphine, however, effectively stimulated total phosphatide turnover only at 16 and 24 hr. A similar effect was observed with the individual phospholipids obtained from the cerebral cortex. This phospholipid effect of morphine was also observed with cerebral cortex slices in previous studies *in vitro*.[1–3] The mechanism whereby morphine initiates this effect is unknown, but in all probability represents a direct effect on the biosynthesis of the phosphatides. As stated previously, the most likely candidate for this effect is the essential phospholipid biosynthetic intermediate D-1, 2-diglyceride. In support of a direct effect on biosynthesis is the fact that no significant difference in the total amount of radioactivity was found in the acid-soluble phosphate esters from the control and morphine-treated guinea pigs, thus eliminating a secondary effect on precursor phosphatide synthesis.[1]

Intracellular fractionation of the cerebral cortex showed quite clearly that the

PHOSPHOLIPIDS FROM GUINEA PIG LIVER SUBCELLULAR FRACTIONS*

(cpm/μmole P/g)

Crude mitochondrial			Microsomal			Soluble supernatant		
C	M	Δ %	C	M	Δ %	C	M	Δ %
3151 ± 1097	3597 ± 1124	+ 14	4403 ± 1102	4512 ± 1620	+ 2	2833 ± 890	4223 ± 1920	+ 49
2751 ± 635	2623 ± 820	− 5	3775 ± 985	3863 ± 1180	+ 2	3155 ± 680	3191 ± 1130	+ 1
2221 ± 620	4707 ± 940	+ 112‖	3013 ± 820	5653 ± 1130	+ 88‖	2070 ± 620	4852 ± 710	+ 134‖
93 ± 11	145 ± 6	+ 56‖	153 ± 16	200 ± 20	+ 31‖	109 ± 15	165 ± 18	+ 51‖
100 ± 18	78 ± 8	− 22	161 ± 14	120 ± 12	− 25	127 ± 9	203 ± 12	+ 60‖
56 ± 7	94 ± 10	+ 68‖	89 ± 7	151 ± 10	+ 72‖	73 ± 11	190 ± 16	+ 160‖
120 ± 41	143 ± 8	+ 19	202 ± 16	206 ± 8	+ 2	214 ± 12	321 ± 18	+ 50‖
122 ± 20	115 ± 31	− 6	243 ± 12	200 ± 18	− 18	173 ± 8	174 ± 20	+ 1
117 ± 8	267 ± 24	+ 128‖	200 ± 20	416 ± 12	+ 108‖	158 ± 20	459 ± 14	+ 191‖
1197 ± 60	1546 ± 72	+ 29‖	1952 ± 102	1949 ± 91	0	1455 ± 91	2439 ± 150	+ 68‖
1068 ± 34	1004 ± 62	− 6	1591 ± 40	1401 ± 62	− 12‖	1648 ± 102	1536 ± 48	− 7
1014 ± 42	1858 ± 81	+ 83‖	1335 ± 68	2503 ± 102	+ 87‖	1187 ± 60	2388 ± 84	+ 101‖
709 ± 40	631 ± 19	− 11	1049 ± 140	749 ± 90	− 29	687 ± 61	733 ± 41	+ 7
533 ± 36	477 ± 28	− 11	585 ± 68	621 ± 50	+ 6	456 ± 32	488 ± 19	+ 7
435 ± 40	900 ± 92	+ 107‖	432 ± 71	900 ± 40	+ 108‖	344 ± 38	764 ± 41	+ 122‖
138 ± 20	69 ± 8	− 50‖	13 ± 10	21 ± 12	+ 62	29 ± 4	22 ± 8	− 24
76 ± 16	59 ± 4	− 22	20 ± 6	27 ± 10	+ 35	25 ± 11	27 ± 6	+ 8
82 ± 12	228 ± 20	+ 178‖	27 ± 4	58 ± 9	+ 115‖	35 ± 8	54 ± 6	+ 54‖

‡M, morphine. Three guinea pigs at each time interval received 40 mg/kg (free base) s.c. of morphine 1 hr prior to sacrifice. Each value represents the mean ± S.E. of six determinations.
§Percentage difference between the control and morphine-treated animals.
‖$P < 0.05$ as compared to the corresponding control value.

phospholipid effect was not localized in a specific subcellular organelle. However, the major stimulatory effect appeared to occur with the phospholipids from the supernatant fraction. It is interesting to note that this fraction also contained the highest percentage of ^{3}H-morphine of the intracellular fractions analyzed for morphine from guinea pig brain.[4]

Subfractionation of the crude mitochondrial fraction provided data which were both stimulatory and inhibitory with respect to the effect of morphine on phosphatide metabolism. The major stimulatory effect appeared to occur in fraction A. This fraction represents predominantly myelin fragments, as reported by De Robertis *et al.*[12, 13] However, the predominant morphine effect was observed in fraction E, which contained free mitochondria.[12, 13] Since Green and Fleischer[14] have shown that phospholipids in mitochondria are essential for electron transfer, it would seem that the narcotic analgesics might interfere with cellular oxidative functions. However, investigators[15, 16] have reported that quite high concentrations of morphine (10^{-4} to 10^{-2}M) have no effect on the O_2 uptake or lactic acid production of the cerebral cortex.

The incorporating activity of $^{32}P_i$ after morphine in the phospholipids of the

TABLE 4. MORPHINE AND THE INCORPORATION OF $^{32}P_i$ INTO

		Microsomal fractions					
	Time	Smooth microsomes			Rough microsomes		
Phospholipid	(hr)	C†	M‡	Δ %§	C	M	Δ %
Total radioactivity	16	47,166 ± 2750	74,043 ± 3000	+ 57‖	47,287 ± 4050	67,310 ± 4120	+ 42‖
	24	95,503 ± 4,250	59,024 ±1980	− 38‖	76,345 ± 3050	63,657 ± 3680	− 17‖
	48	43,666 ± 3500	47,866 ± 4100	+ 10	42,804 ± 2800	42,965 ± 4100	0
Phosphatidyl-inositol	16	1878 ± 150	1738 ± 194	− 7	1609 ± 80	2243 ± 190	+ 39‖
	24	1953 ± 201	1151 ± 91	− 41‖	2005 ± 190	1733 ± 120	− 14
	48	1191 ± 180	1988 ± 115	+ 67‖	2088 ± 101	2195 ± 135	+ 5
Sphingomyelin	16	2984 ± 190	2976 ± 240	0	1873 ± 135	2550 ± 168	+ 36‖
	24	3981 ± 130	4103 ± 160	+ 3	1934 ± 208	4681 ± 314	+ 142‖
	48	3819 ± 185	2336 ± 168	− 39‖	2734 ± 162	1722 ± 193	− 37‖
Phosphatidyl-choline	16	29,214 ± 3080	27,336 ± 1980	− 6	14,712 ± 1835	27,511 ± 1145	+ 87‖
	24	46,955 ± 2150	24,328 ± 2206	− 48‖	40,556 ± 4160	28,672 ± 4060	− 29‖
	48	18,031 ± 1965	20,465 ± 2400	+ 13	18,588 ± 1902	23,026 ± 2190	+ 24‖
Phosphatidyl-serine + phosphatidyl-ethanolamine	16	10,603 ± 1090	14,833 ± 1682	+ 40‖	6509 ± 1051	13,729 ± 1253	+ 111‖
	24	17,505 ± 1051	13,841 ± 2000	− 21‖	13,536 ± 1102	15,391 ± 1959	+ 14
	48	9002 ± 985	8522 ± 674	− 5	9289 ± 900	9032 ± 632	− 3
Phosphatidic acid	16	541 ± 62	769 ± 34	+ 42‖	165 ± 30	938 ± 63	+ 468‖
	24	603 ± 91	617 ± 104	+ 2	101 ± 21	500 ± 48	+ 395‖
	48	289 ± 48	335 ± 29	+ 16	331 ± 41	439 ± 23	+ 33‖

*$^{32}P_i$ (150 μc/100 g) was injected i.p. and the animals were sacrificed at various times after $^{32}P_i$. The liver was removed and microsomal subcellular fractions were prepared (Mulé *et al.*[4]). Total radioactivity as well as the incorporation of $^{32}P_i$ into individual phospholipids was determined as described under Methods.

†C, control. Three guinea pigs received 0·9% saline s.c. 1 hr prior to sacrifice. Each value represents the mean + S.E. of six determinations.

subcellular fragments following hypoosmotic shock (M_1, M_2, M_3) was both stimulatory and inhibitory. The effect on the M_1 fraction (swollen mitochondria, myelin fragments, nerve ending membranes, intersynaptic filaments and subsynaptic web) was observed predominantly at 16 hr and was inhibitory. In the M_2 fraction, the major lipid affected was phosphatidylcholine at each time interval. This fraction represents the morphologic storage units for acetylcholine.[12] It would of course be quite interesting if a correlation between the morphine phospholipid effect and synaptic transmission existed. In this regard, investigators[17–21] have shown an increased incorporation of $^{32}P_i$ into phosphatidic acid and phosphatidylinositol after excitation of sympathetic ganglia with either acetylcholine or electrical impulses. It is thought that this effect is related to the action of the synaptic transmitter on the post-synaptic cell. In the case of morphine, the primary phospholipid affected in the M_2 fraction (acetylcholine storage fraction) was phosphatidylcholine, but the most significant percentage stimulation occurred at 48 hr with phosphatidylinositol.

No statistically significant effect was obtained between the control and morphine treated animals with respect to total radioactivity in the phospholipids obtained from the whole liver. Significant effects were obtained with two individual phospholipids

MICROSOMAL SUBCELLULAR FRACTIONS FROM GUINEA PIG LIVER*

(cpm/μmole P/g)

Microsomal-2			Microsomal supernatant		
C	M	Δ %	C	M	Δ %
7125 ± 1050	9872 ± 1280	+ 39‖	5781 ± 1202	9985 ± 980	+ 73‖
13,301 ± 2050	10,040 ± 3021	− 25	13,384 ± 2050	9140 ± 1680	− 33
6665 ± 980	6291 ± 1080	− 6	5224 ± 1100	5615 ± 850	+ 7
258 ± 40	388 ± 81	+ 50‖	148 ± 8	318 ± 31	+ 115‖
405 ± 80	420 ± 68	+ 4	535 ± 41	397 ± 28	− 26
350 ± 65	260 ± 34	− 26	250 ± 20	263 ± 42	+ 5
603 ± 40	336 ± 27	− 44‖	394 ± 41	370 ± 62	− 6
506 ± 38	443 ± 19	− 12	716 ± 18	476 ± 41	− 34‖
346 ± 61	272 ± 49	− 21	428 ± 91	410 ± 33	− 4
4159 ± 1090	3462 ± 1650	− 23	2031 ± 430	3685 ± 1200	+ 81‖
6302 ± 1902	3802 ± 1405	− 40‖	6943 ± 620	4523 ± 1290	− 35‖
2815 ± 982	2853 ± 699	+ 1	2516 ± 340	2803 ± 920	+ 11
1806 ± 450	1934 ± 320	+ 7	862 ± 192	1469 ± 111	+ 70‖
2483 ± 932	2290 ± 419	− 8	2391 ± 695	1803 ± 433	− 25
1298 ± 685	1233 ± 391	− 5	1202 ± 140	1152 ± 109	− 4
101 ± 28	62 ± 31	− 39	50 ± 12	89 ± 12	+ 74‖
64 ± 19	79 ± 24	+ 23	99 ±18	85 ± 19	− 14
43 ± 16	39 ± 14	− 9	60 ± 21	115 ± 16	+ 92‖

‡M, morphine. Three guinea pigs received 40 mg/kg (free base) s.c. of morphine 1 hr prior to sacrifice. Each value represents the mean ± S.E. of six determinations.
§Percentage difference between the control and morphine-treated animals.
‖$P < 0.05$ as compared to the corresponding control value.

(phosphatidylinositol and phosphatidic acid) at 24 and 48 hr after $^{32}P_i$ administration. After liver cell subfractionation, significant effects (as measured by total radioactivity) were observed in all fractions at 48 hr. Individual phospholipids were primarily affected at 48 hr and most significantly in the soluble supernatant fraction. Subfractionation of the liver microsomal fraction provided a predominantly stimulatory effect for both the total radioactivity and the individual phospholipids at 16 hr.

The specific activities of the various phospholipids in the liver were in the order of PC > PE > S > PI > PA. This relationship appeared to be true whether the phospholipids were extracted from whole liver or subcellular fractions, and morphine did not alter this relationship. The specific activity in the smooth and rough microsomal subfractions for phosphatidylcholine and phosphatidylserine plus phosphatidylethanolamine was greater than that observed for other phospholipids in the microsomal subfractions. This was not surprising, since these lipids represent the largest concentration in the liver.[22, 23] However, it appears that these phospholipids are predominantly associated with the rough and smooth endoplasmic reticulum. Morphine stimulated the incorporation of $^{32}P_i$ into these lipids only at 16 hr, and was both inhibitory and stimulatory at other time intervals. The data, unfortunately, do not provide any

direct information as to whether the effect of morphine is on the synthesis of phosphatidylcholine by the CDP-choline pathway[24] or methylation of phosphatidylethanolamine.[25] The latter pathway would be dependent upon the formation of radioactive CDP-ethanolamine. The same analogy would hold for the effect of morphine directly on phosphatidylethanolamine or on phosphatidylserine metabolism or on both.

The physiological significance of the phospholipid effect of morphine might be correlated with a functional alteration of phospholipid–ionic complexes at the cell membrane level of organization. Phospholipids do interact with ions,[26–29] constitute a major fraction of cell membranes,[11, 30, 31] and provide polar heads which may act as ion-exchange sites for the flow of ions through cell membranes.[32, 33] Thus, the pharmacological action of morphine may be interpreted as a successful competition with ions for the binding sites on the polar heads of the membrane phospholipids, which subsequently affects ion conductance, membrane permeability and neuronal excitability. Certainly, the following observations support a phospholipid–polyvalent cation–morphine interrelationship: (1) morphine and nalorphine alter phospholipid metabolism in the cerebral cortex;[1–3] (2) narcotic analgesics inhibit the binding of Ca^{2+} to phospholipids;[29] (3) analgesia induced by narcotic analgesics is antagonized by Ca^{2+} and enhanced by chelating agents;[34] (4) Ca^{2+} antagonizes the morphine inhibition of gut contractions produced by coaxial stimulation;[35] (5) a marked increase in the urinary excretion of Ca^{2+} occurs after the administration of morphine;[36] (6) morphine causes an increase in plasma Mg^{2+} levels and inhibits Ca^{2+} transport across the rat intestine;* (7) the inhibition of O_2 uptake in potassium-stimulated cortex slices apparently may only be shown in a Ca^{2+}-free medium.[37]

Although it appears that phospholipids acting as ion exchangers provide a reasonable model for narcotic drug action, it is difficult to explain the displacement of Ca^{2+} from phospholipids by local anesthetics[32, 38] and non-narcotic stimulants and depressants.[28, 29] Therefore, a definitive relationship or lack of such a relationship between the morphine phospholipid effect and the mechanism of action of narcotic drugs await further investigation.

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