MONITORING THE STEREOSPECIFICITY OF MORPHINE ACTION *IN VIVO* AND *IN VITRO* THROUGH BRAIN MEMBRANE LIPID FLUIDITY

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SUMMARY

Differential scanning calorimetry of crude brain mitochondrial lipids obtained from control and morphine treated rats was carried out and the lipid phase transition measured. Morphine treatment resulted in a significant decrease in the temperature range and enthalpy of the phase transition. This effect was found to be dose dependent and reversible both *in vivo* and *in vitro* by naloxone. Studies with levorphanol and dextrorphan demonstrated stereospecificity. Furthermore, the ether precipitable fraction of total lipid extracts was shown to mediate the opiate response.

INTRODUCTION

Recently, many investigators (1,2,3) have shown that *in vitro* addition of various anesthetic drugs to lipid bilayers induces melting or increased fluidity of the system. It has been suggested (4) that such a membrane response may be fundamental to the molecular action of these drugs *in vivo*. Indirect evidence of morphine induced changes in the physical state of the membrane lipids has been obtained in our laboratory (5) by the use of Arrhenius plots of membrane bound enzymes.

The present investigation is an attempt to provide information on the induction of changes in brain mitochondrial membrane lipids subsequent to the *in vivo* administration of morphine and its congeners.

Several investigators have shown (6,7) that the technique of differential scanning calorimetry (DSC) is a useful method to directly measure changes in membrane fluidity. It has been suggested that the insertion of certain foreign materials into the predominantly hydrophobic milieu of the membrane could induce changes in membrane fluidity as demonstrated by the changes in

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the enthalpy of the lipid phase transition.

Pert *et al* (8) have shown that 50% of the opiate receptors in brain homogenate are present in the crude mitochondrial fraction. In the experiments herein described we have studied the influence of opiate administration both *in vivo* and *in vitro* on the DSC profiles of lipid extracts obtained from crude brain mitochondrial preparations. This lipid extract was used as a model system to study the molecular mode of action of opiates on bilayers.

METHODS

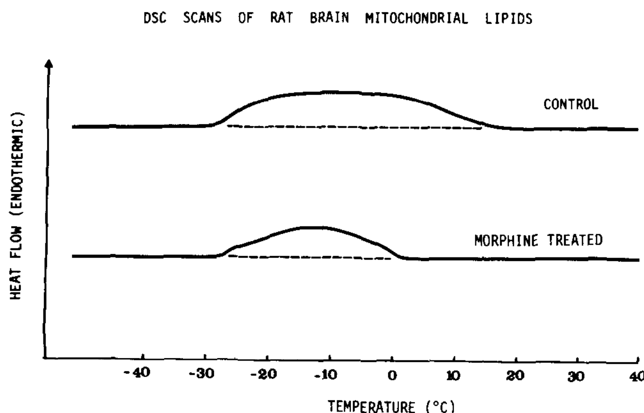
Male Sprague-Dawley rats weighing between 100-150 gm were used throughout the experiments. Control animals were injected with 200 μl of distilled water, the medium used to dissolve the drugs, while the experimental animals received either 5, 10, 15 or 25 mg/kg morphine, 5 mg/kg levorphanol, or 5 mg/kg dextrorphan, all intraperitoneally. In some experiments, naloxone (1 mg/kg i.p.) was injected 15 min. after opiate administration. The total volume of any one injection did not exceed 200 μl.

The animals were sacrificed 0.5 hr after opiate treatment by decapitation, the brain excised and the cerebellum dissected away. The tissue thus obtained was homogenized in ice cold medium (0.25 M sucrose, 5 mM EDTA, 5 mM tris-HCl, pH 7.4). A crude mitochondrial pellet was obtained according to the method of French (9). The lipids were extracted by the method of Kates (10). The total lipid extract was then dried under nitrogen and used for calorimetry. In some cases the ether precipitate was prepared from the total lipid extract by the method of Soto *et al* (11) and both supernatant and extract subsequently dried under nitrogen and used for the crossover experiments.

Dry lipid samples weighing 25 mg were dispersed in 25 μl of 50% aqueous ethylene glycol just prior to calorimetry. The choice of suspending medium was dictated by two considerations: a) an aqueous medium to promote bilayer formation, and b) ethylene glycol to prevent freezing. Scanning calorimetry was carried out in a Perkin-Elmer DSC-1, at a scanning rate of 5°C/min. The calorimeter records the differential power input necessary to heat the sample and reference at the same rate; when the sample undergoes a phase transition the difference in power input appears as a peak. The enthalpy of transition can be determined from the area under the peak. The calorimeter was calibrated with palmitic acid and tin.

RESULTS

The DSC scan of total lipids extracted from the crude brain mitochondrial preparations of control animals is shown in Fig. 1 along with that obtained after morphine treatment (25 mg/kg i.p.). The phase transition of control lipids commences at -26 ± 1°C and ends at 17 ± 1°C. The enthalpy of transition was found to be 2.2 ± 0.3 mcal/mg. As can be seen, morphine treatment resulted in a significant decrease in the transition temperature range, i.e., it began at -26 ± 1°C and terminated at -1 ± 1°C. The enthalpy of transition decreased to 1.1 ± 0.4 mcal/mg, as shown in Table I. This decrease in both temperature



	T_i(°C)	T_f(°C)	ENTHALPY (mcal/mg)
CONTROL	-26 ± 1	17 ± 1	2.2 ± 0.3
MORPHINE TREATED	-24 ± 1	-1 ± 1	1.0 ± 0.1
SIGNIFICANCE	N.S.	$p < .001$	$p < .001$

Fig. 1. Representative tracings of DSC scans of crude rat brain mitochondrial lipids obtained from control and morphine treated (25 mg/kg) animals. 25 mg lipid samples were suspended in 50% aqueous ethylene glycol and scanned at 5°C/min. T_i denotes the intial, T_f the final temperature of the phase transition.

range and enthalpy of transition exhibits a dose related effect (vid Table I).

To determine whether this was a specific opiate effect, naloxone (1 mg/kg i.p.) was administered 15 min. after morphine treatment (25 mg/kg i.p.). Naloxone completely reversed the opiate-induced changes in the lipid phase transition, i.e., both the range and enthalpy were found to be the same as those of the control. A dose of 1 mg/kg naloxone alone had no effect on the transition.

To test if these effects were stereoselective in nature, levorphanol (5 mg/kg i.p.) and its pharmacologically inactive enantiomer, dextrorphan, (5 mg/kg i.p.) were administered. Levorphanol treatment resulted in a lipid phase transition closely resembling that produced by 15 mg/kg morphine, i.e. the temperature range of the transition was found to be from -27 ± 6°C to 1 ± 3°C, while its enthalpy was 1.5 ± 0.1 mcal/mg. The administration of 5 mg/kg dextrorphan had no significant effect on the phase transition, the

Table I
Effect of various doses of *in vivo* morphine treatment on the lipid phase transition

DOSE (mg morphine/kg)	RANGE (°C)		ENTHALPY (cal/gm)
	$T_{initial}$	T_{final}	
0	-26 ± 1	17 ± 1	2.2 ± 0.3
5	-30 ± 1	9 ± 1	1.5 ± 0.4
10	-24 ± 4	5 ± 3	1.3 ± 0.2
15	-23 ± 3	-1 ± 2	1.3 ± 0.1
25	-24 ± 1	-1 ± 1	1.0 ± 0.1

values being -24 ± 4°C, 16 ± 4°C and 2.1 ± 0.4 mcal/mg.

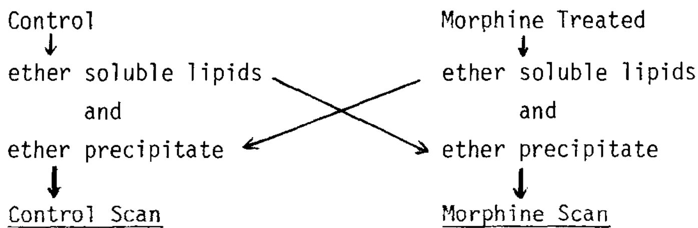
In an attempt to demonstrate that morphine's effects were not due to respiratory depression but rather to a direct interaction with the lipid extract, 50 nmol morphine/mg lipid or 100 nmol leucine enkephalin/mg lipid were mixed with samples obtained from control animals. The resultant DSC profiles were not significantly different from those obtained following *in vivo* administration of 25 mg/kg morphine. Moreover, addition of equimolar amounts of naloxone to each of the above systems reversed the opiate induced effect, as shown in Table II.

Several investigators have shown that a stereospecific binding material, consisting primarily of proteins and cerebroside sulfate, can be isolated from the total brain lipid extract by ether precipitation (12,13). In order to determine if this material could be involved in the opiate-induced perturbation of mitochondrial lipids, samples were separated into ether precipitable and ether soluble fractions and a set of experiments were performed according to the following scheme (crossover experiment):

Table II

Effect of opiate agonists and antagonist *in vitro* on the lipid phase transition

TREATMENT (*IN VITRO*)	RANGE (°C)		ENTHALPY (cal/gm)
	$T_{initial}$	T_{final}	
Control	-26 ± 1	17 ± 1	2.2 ± 0.3
Morphine (50 η mol/mg)	-26 ± 1	-1 ± 1	1.1 ± 0.4
Morphine (50 η mol/mg) plus Naloxone (50 η mol/mg)	-28 ± 5	17 ± 2	2 ± 0.3
Enkephalin (100 η mol/mg)	-23 ± 2	-1 ± 1	1.1 ± 0.7
Enkephalin (100 η mol/mg) plus Naloxone (100 η mol/mg)	-24 ± 2	14 ± 4	2 ± 0.2



Ether soluble control lipids were mixed with the ether precipitate from morphine (25 mg/kg i.p.) treated animals. The resultant calorimetric scans of this mixture were similar to those of the total lipid extract obtained from morphine treated animals, as shown in Table III. When ether soluble mitochondrial lipids from morphine treated animals were mixed with control ether precipitate, the lipid phase transition was found to be normal.

Table III
Crossover Experiment

SAMPLE	RANGE (°C)		ENTHALPY (cal/gm)
	$T_{initial}$	T_{final}	
Control total Lipid extract	-26 ± 1	17 ± 1	2.2 ± 0.3
Morphine Lipid plus Control ppt.	-25 ± 3	17 ± 2	1.9 ± 0.2
Control Lipid plus Morphine ppt.	-26 ± 4	6 ± 3	0.9 ± 0.1
Morphine treated total Lipid extract	-24 ± 1	-1 ± 1	1.0 ± 0.1

DISCUSSION

The significant decrease observed in both the range and enthalpy of the lipid phase transition as measured by differential scanning calorimetry demonstrates that following morphine administration at pharmacological concentrations, both _in vivo_ and _in vitro_ there is a perturbation of brain mitochondrial lipids, indicative of a disruption of the lipid packing order; this perturbation is dependent on the presence of morphine treated ether precipitable material. Manifestation of this effect _in vitro_ precludes the possibility of an indirect mode of action.

The results described in this communication suggest that this direct action is a receptor mediated process rather than being a nonspecific membrane effect. It is reversible both _in vivo_ and _in vitro_ by naloxone and mimiced _in vitro_ by leu-enkephalin, a putative natural ligand for the opiate receptor (14). Further, levorphanol, a synthetic narcotic agonist also produced scans similar to those obtained following morphine treatment, while administration of dextrorphan (its pharmacologically inactive enantiomer) resulted in phase

transitions virtually identical to those of control lipid extracts.

Crude brain mitochondrial lipid extracts were used in these studies since they have been shown to contain a high concentration of opiate receptors (8) as well as being one of the few mammalian membrane preparations having a low cholesterol content (15). The latter is an important consideration in DSC, since high concentrations of cholesterol obscure the observed phase transition (16)

Preliminary work in our laboratory using ^{14}C-morphine has shown that morphine isolates with the lipid extract, the ether precipitate having a higher specific activity than the soluble fraction. This suggests that the presence of morphine is a prerequisite for the above described effects; it does not, however, eliminate the possibility that morphine induces a long term change in receptor conformation.

In the present study the ether precipitate has been shown to be responsible for the mediation of opiate action. We have analyzed this fraction and found it to contain both cerebroside sulfate and hydrophobic protein, the two substances identified in whole brain lipid extracts as possible components of the opiate receptor (12,13). Therefore, it appears that differential scanning calorimetry of crude brain mitochondrial lipids offers a unique opportunity to study the opiate receptor under a variety of conditions.

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