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Binding of narcotics and narcotic antagonists to triphosphoinositide

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Triphosphoinositide occurs mainly in the brain as a constituent of membrane. It is both water soluble and lipid soluble and has five negative charges concentrated in a molecule of about 1000 molecular weight. A role for triphosphoinositide in nerve transmission has been considered based on the observation that calcium ion can chelate with triphosphoinositide [1] and that phosphate turnover rate in triphosphoinositide is very rapid [2]. Apart from its physiologic functions, triphosphoinositide may also be involved in opiate action.

It has been reported that morphine administration can deplete brain calcium in animals [3,4] and, since the level of calcium fluctuates with the level of triphosphoinositide [5], there is a possibility that the action of morphine may involve triphosphoinositide. To find any fluctuation in the triphosphoinositide pool during the morphine action may be futile, as the majority of triphosphoinositide is merely a part of myelin structure, and only a small part of total pool size may fluctuate. Mulé [6] studied the turnover of triphosphoinositide and found it to be increased during morphine treatment.

It has been reported that acidic lipids, including triphosphoinositide, can bind with opiates with different affinities in different media. Thus, in the organic phase, morphine is bound more than naloxone, while in the water phase the reverse is true. The differences in binding properties have been related to biologic activity.* This communication reports a further study on the binding of three narcotic agonist–antagonist pairs to triphosphoinositide.

[^{3}H]Naloxone hydrochloride (23.6 Ci/m-mole) was obtained from New England Nuclear, and morphine sulfate from Mallinckrodt. The tartrate salts of levorphanol and [^{3}H]levorphanol (2.4 Ci/m-mole) were donated by Hoffmann-La Roche and naloxone hydrochloride by Endo Laboratories. Triphosphoinositide was extracted from rat brain and purified by the method of Michell *et al.* [7]. Based on the total and individual lipid phosphorus analyses after thin-layer chromatography with potassium oxalate impregnated Silica gel H plates and development with a solvent system of chloroform–methanol–4 N NH_4OH (9:7:2, v/v) [8], the preparation was established to contain no lipids other than phosphoinositide lipids which were present in the amount of 1% monophosphoinositide (MPI), 5% diphosphoinositide (DPI) and 70% triphosphoinositide (TPI). The stability of TPI was examined by the azure assay method [9]; TPI was stable over a year when stored at 25° in chloroform–methanol (19:1, v/v); under neutral pH in water, in heptane, or in octanol, TPI was stable over a month.

Two methods were used to study the binding of narcotic agonists or antagonists in various solvent systems. The binding was quantified by determining the concentration of drug needed to inhibit [^{3}H]levorphanol binding to triphosphoinositide by 50 per cent (ID_{50}).

Organic solvent–water partition. This method consisted of the addition of 1-ml of radioactive drug of varying concentrations in water at pH 6.0 to 1 ml of triphosphoinositide solution in heptane or octanol in a glass tube, 13 × 100 mm, and vortexing the mixture at medium speed for 1 min. After the mixture was centrifuged at 1500 *g* for 10 min, an 0.5-ml aliquot of both phases was removed and the radioactivity was determined by liquid scintillation spectrophotometry with 10 ml of Scinti Verse solution (Fisher Scientific Co.); the counting of an efficiency in this system was determined to about 40 per cent. The amount of drug bound to TPI in the organic phase was calculated by the simplified equation of Weber *et al.* [10]: $M_b = M_t - P \times M_W - M_w$, M_b being the amount of drug bound by TPI in the organic phase, M_t, the total amount of drug used, P, the partition coefficient of drug between the organic phase and the aqueous phase, and M_w, the amount of drug in aqueous phase. The dissociation constants of various TPI–drug complexes were obtained by Scatchard analysis [11]. $M_b/M_f = -M_b/K_d + M_m/K_d$.† M_b being the amount of drug bound, M_f, the amount of free drug, M_m, the maximum amount of drug which could be bound, and K_d, the dissociation constant of the TPI drug complex. The heptane–water partition was used to obtain ID_{50} values of various drugs using 5 μg/ml of triphosphoinositide and 5×10^{-8} *M*[^{3}H]levorphanol and varying concentrations of a test drug.

Equilibrium dialysis. A solution of triphosphoinositide in chloroform–methanol (19:1) was dried *in vacuo* with a rotatory evaporator and the residue was sonicated in water at pH 6.0 to make a 0.01% liposome solution. A coil of dialysis Visking membrane was boiled in 1 mM EDTA solution and then in distilled water and soaked in water at room temperature for several hr. A dialysis cell of 1 ml capacity was assembled with a piece of Visking membrane separating the inner from the outer chamber, each containing a small glass bead for the purpose of agitation. The

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†This equation is obtained by multiplying the original Scatchard equation by the amount of TPI added.

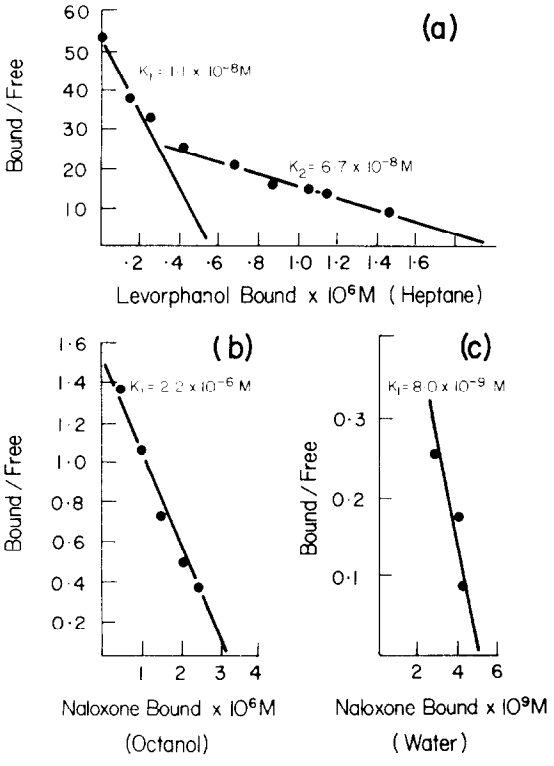

Fig. 1. Scatchard analysis of levorphanol and naloxone binding to triphosphoinositide. (a) [^{3}H]levorphanol binding to 5 μg triphosphoinositide in heptane by the partition method. (b) [^{3}H]naloxone binding to 50 μg triphosphoinositide in octanol by the partition method. (c) [^{3}H]naloxone binding to 100 μg triphosphoinositide in water by the equilibrium-dialysis method.

inner chamber was filled with 0.2 ml of 0.01% triphosphoinositide liposome solution, and the outer chamber was filled with 0.2 ml of [^{3}H]naloxone of varying concentrations in water at pH 6.0. The cells were shaken for 2 hr and the radioactivity of the solution from each chamber was determined by liquid scintillation spectrophotometry. The results were analyzed by the Scatchard method as described previously.

Binding of opiate to triphosphoinositide in the various solvent systems was studied over a wide range of concentrations from 1×10^{-9} to 1×10^{-5} M. A plot of the data according to Scatchard [11] is shown in Fig. 1: the ordinate indicates the ratio of bound to free drug, and the abscissa, the concentration of the drug bound. The reciprocal of the slope of the curve gives the dissociation constant for the drug–triphosphoinositide complex in that particular solvent. The affinity of the drug for triphosphoinositide is the reciprocal of the dissociation constant of the drug triphosphoinositide complex. Thus, a steep slope yields a low dissociation constant, and this reflects a high affinity site that becomes saturated at a low drug concentration. As the drug concentration increases, other sites with lower affinities become gradually saturated. As shown in Fig. 1a, the levorphanol binding to triphosphoinositide in heptane yielded dissociation constants of 1.1×10^{-8} M and 6.7×10^{-8} M.

Figure 1 (panels b and c) shows the Scatchard plot of the [^{3}H]naloxone binding in octanol and in water respectively. The dissociation constant of naloxone binding in octanol was 2.2×10^{-6} M, while that in water was 8×10^{-9} M.

In order to compare the binding of three agonist antagonist pairs to triphosphoinositide, the ID_{50} to inhibit the binding of 5×10^{-8} M [^{3}H]levorphanol to 5 μg triphosphoinositide was determined using the heptane–water partition method. The results, as summarized in Table 1, have been corrected for the partition of drugs in heptane and water [16]. The partition coefficients, $P_{h,w}$ of the drugs were as follows: levorphanol 0.011 levallorphan 0.04, morphine 0.0005, nalorphine 0.0035, oxymorphone 0.002, and naloxone 0.008. Since it had been shown in similar experiments that analgetic potency of narcotic drugs by intraventricular administration correlated with their ability to inhibit levorphanol binding to cerebroside sulfate, it is clear that the drug concentration in heptane is more crucial than that in water for determining the ID_{50} of each drug [16]. Invariably, the affinity of TPI for agonist was higher than that for its corresponding antagonist. Thus, with each pair, the ID_{50} of agonist was less than that of its antagonist analog, i.e. levorphanol < levallorphan; morphine < nalorphine; oxymorphone < naloxone.

Table 1. The ID_{50} of the three opiate agonists and their corresponding antagonists determined with the levorphanol heptane system*

Agonist/Antagonist	ID_{50}
Levorphanol	4.8×10^{-8} M
Levallorphan	1.1×10^{-7} M
Morphine	4.9×10^{-8} M
Nalorphine	2.5×10^{-7} M
Oxymorphone	2.4×10^{-7} M
Naloxone	4.7×10^{-7} M

* ID_{50} is defined as the concentration of drug in heptane required to inhibit the binding of 5×10^{-8} M [^{3}M]levorphanol to 5 μg triphosphoinositide by 50 per cent.

With respect to the sodium ion effect on opiate binding to triphosphoinositide, the binding of [^{3}H]levorphanol was inhibited completely by 100 mM NaCl, while that of [^{3}H]naloxone was not affected, as shown in Table 2.

Binding of opiate to triphosphoinositide had a different set of optimum experimental conditions for each particular drug. For levorphanol, it was in the heptane water partition system at 5 μg/ml of concentration; for naloxone, it was in an octanol water system at 50 μg/ml of concentration. As shown in Fig. 1 (panels a and b), the dissociation constants for levorphanol binding by the heptane water partition method were $K_1 = 1.1 \times 10^{-8}$ M and $K_2 = 6.7 \times 10^{-8}$ M and that for naloxone binding by the octanol–water partition method was 2.2×10^{-6} M. From our experiences with opiate binding to acidic lipids,* it is obvious that levorphanol binding to acidic lipids

Table 2. Effect of sodium ion on levorphanol and naloxone binding*

Opiate	Amount bound (pmoles) Without NaCl	Amount bound (pmoles) With NaCl	% Inhibition by NaCl
[^{3}H]levorphanol	22.06	0.56	97
[^{3}H]naloxone	2.87	3.08	0

* Binding in water phase in the absence or presence of 100 mM NaCl was performed with 1 ml of liposome solution containing 20 μg triphosphoinositide and 5×10^{-8} M [^{3}H]levorphanol or 5×10^{-7} M [^{3}H]naloxone by the partition method with 1 ml octanol.

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occurred at the heptane–water interface, where the lipids existed in at least two conformations yielding two dissociation constants, while naloxone binding to acidic lipids in the octanol–water system occurred in the octanol phase, in which the lipids existed as one molecular state producing one dissociation constant.

It was evident that octanol inhibited protonation of naloxone and, if protonated naloxone was the binding species, binding could be inhibited; consequently, the true constant should be much lower than the observed dissociation constant, 2.2×10^{-6} M. In order to remove this factor of inhibited protonation, we resorted to the equilibrium dialysis method using 100 μg/ml of concentration which nearly simulates the triphosphoinositide level in the rat brain. As shown in Fig. 1c, the dissociation constant of naloxone binding in water by the equilibrium dialysis method was 8×10^{-9} M which was about 270 times lower than that in octanol, providing evidence that protonated naloxone was the important species in naloxone binding. High affinity to bind opiate was evidenced by the ID_{50} values of levorphanol, levallorphan, morphine, nalorphine, oxymorphone and naloxone (shown in Table 1) ranging from 5×10^{-7} to 5×10^{-8} M. While these values approximate their pharmacologically active concentrations in the brain, this does not necessarily mean that triphosphoinositide is an opiate receptor. However, the binding studies in the presence of sodium are compatible with this possiblity. As shown in Table 2, triphosphoinositide binding of levorphanol is virtually completely inhibited in the presence of 100 mM NaCl, while that of naloxone is not. The significance of this sodium effect on the agonist and antagonist binding can be linked with the observations made by Simon *et al.* [12] and Pert and Snyder [13] who reported that sodium ion decreases the binding of agonist but not that of antagonist to opiate receptors. Although the sodium ion effect on agonist–receptor binding can be mimicked with the triphosphoinositide liposome system, it is not certain that the binding noted in brain homogenates involves triphosphoinositide *per se*, since *post mortem* hydrolysis of triphosphoinositide would continue to occur [14].

Lipid solubility and high affinity for opiate are common properties shared by cerebroside sulfate [15, 16] and triphosphoinositide. However, cerebroside sulfate is ten times more abundant than triphosphoinositide in the brain and, under such conditions, triphosphoinositide would not compete favorably with cerebroside sulfate for agonist binding. On the other hand, as our results suggest, if the action of antagonist is facilitated in aqueous solution rather than in the membrane lipid,* then the antagonist binding property of triphosphoinositide would be more in evidence, since agonist but not antagonist binding would be inhibited by tissue sodium.

There are no available studies concerning the allosteric effect of triphosphoinositide on the opiate binding to cerebroside sulfate except our preliminary experimental results which indicate that triphosphoinositide itself can antagonize the levorphanol binding to cerebroside sulfate. Based on our present and previous findings,* one might expect that an agonist might bind preferentially to cerebroside sulfate to establish a stable "dehydrated" state in the nerve membrane. However, this stable agonistic state can be easily perturbed or even destroyed allosterically by the introduction of an antagonist which might bind first with triphosphoinositide and then facilitate a change of conformation in cerebroside sulfate from the dehydrated to the hydrated state. This view is consistent with the fact that, where there is cerebroside sulfate, there is also always triphosphoinositide, the highest concentration of both lipids being found in the brain stem [17]. Based on the evidence we have obtained to date, it appears that triphosphoinositide as well as cerebroside sulfate might both be useful as models for studying the mechanisms involved in opiate agonist and antagonist action. Studies on the effect of other cations on drug binding to TPI and agonist–antagonist binding to other acidic phospholipids are in progress and will be reported in the near future.

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