The Binding of Noradrenaline to Monophosphoinositide: *In Vitro* Studies

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SUMMARY

Monophosphoinositide chromatographically purified from bovine brain extract shows in *in vitro* experiments a high capacity to bind noradrenaline. Using a simplified two-phase model consisting of an aqueous phase with noradrenaline and a lipophilic organic phase with monophosphoinositide, thermodynamic variables are calculated for the binding reaction. The binding capacity and the partition of lipid was strongly influenced by the presence of bivalent cations. From the results obtained a possible receptor function of monophosphoinositide for noradrenaline in synaptosomes is discussed.

INTRODUCTION

In previous work, phosphatidylserine (PS) was demonstrated to possess a capacity of binding uncharged noradrenaline (NA) in a simplified two-phase model (1). In continuation of these studies, the present work gives data for the ability of monophosphoinositide (PI) to bind NA.

The partition of NA is described as a function of concentration and of bivalent cations. The change in standard free energy, entropy, and enthalpy for the formation of the binding complex is estimated. The binding of NA to PI is discussed in relation to a possible function of this lipid in synaptic vesicles and synaptosomes as receptor for NA during storage and transmitter function of this catecholamine.

MATERIALS AND METHODS

Chemicals of highest commercially available purity from British Drug House were used for all studies. L-Noradrenaline was obtained from Sigma (USA).

Characterization of Lipid Receptor

Lipid material. Phosphatidylinositol was obtained from Koch-Light Laboratories, England (ex bovine brain, Folch fraction I, batch No. 23056) and further purified by column chromatography (see below).

Thin-layer chromatography (TLC). The purity of the lipids was determined by ascending thin-layer chromatography (2). An alkaline chloroform–methanol–25% NH_3 mixture (70:30:5 v/v) was used as the solvent system. The spots were identified by spraying the developed chromatogram with: (a) ammonium heptamolybdate in 0.1 *N* HCl, 70% $HClO_4$; (b) ninhydrin in acetone; (c) 2% resorcinol in 0.1 *M* $CuSO_4$ in concentrated HCl (3), followed by drying and heating to 100°.

Column chromatography. PI was isolated by chromatography on acetylated diethylaminoethyl (DEAE)-cellulose (4, 5). PI, 250 mg, was dissolved in 8 ml of chloroform and was applied to a 25 × 2.5 cm DEAE-cellulose column. Discontinuous elution with mixed solvents gave rise to the following fractions: 210 ml of glacial acetic acid eluted free fatty acids + PS; 200 ml of methanol washed out glacial acetic acid; and 200 ml of chloroform–methanol–25% NH_3 (4:10:0.5 v/v) eluted PI. The composition of the fractions was visualized by TLC as described above. The glacial acetic acid fraction revealed one spot when stained with ammonium heptamolybdate or

ninhydrin, detecting the presence of PS. Stained with ammonium heptamolybdate, the PI fraction similarly revealed only one spot, and no color appeared when it was sprayed with ninhydrin. Resorcinol did not reveal carbohydrate components in any fraction.

Determination of lipid phosphorus. This was performed according to the methods of Bartlett (6) and Parker (7). The purified PI showed a phosphate content of 2.7%, in agreement with the identity of the fraction with the monophosphate derivatives.

Determination of protein. The method of Lowry *et al.* (8) could not detect any protein in the lipid fractions used.

Infrared analysis. The PI fraction was analyzed in a Unicam SP-200 infrared spectrophotometer using a 100 mg KBr disk as reference. The spectrum obtained was analyzed in accordance with the law of group frequencies (9), and the result confirmed the material isolated to be PI.

Determination of NA. NA was fluorometrically determined by the slightly modified trihydroxyindole method as previously described (1, 10).

Determination of Thermodynamic Constants

Binding assay. As mentioned earlier (1) this consisted of an organic phase (phase II) containing 400 μl of chloroform–methanol (2:1 v/v) with purified PI (75 μg/ml) and 400 μl aqueous phase (phase I) with NA in 2.5 m*M* Tris-HCl buffer pH 7.2. The total amount of NA in the two phases varied from 10 μg to 70 μg. In each run two tubes without lipid were used as controls, while two others containing lipid served as the experimental system. Each mixture was shaken 1 min in N_2 atmosphere to avoid oxidation of PI and NA; after 5 min to clear the phases, these were further separated by centrifugation in a Sorvall RC-2 superspeed centrifuge at 10,000 *g* for 5 min. Two aliquot portions from phase I were removed from each tube for NA determination. All values were thus measured four times with a standard deviation of $\pm$2.4%. The binding experiments were performed at three different temperatures: 305°K, 293°K, and 281°K ($\pm$0.1°).

Influence of Inorganic Ions on Binding

In the two-phase system with PI as receptor lipid, the binding of NA was studied in the presence of Ca^{2+} and Mg^{2+} in varying concentrations and at constant temperature (293°K). The two-phase system was brought to equilibrium with the total amount of NA to be bound, and 10 μl of aqueous phase I was replaced by 10 μl of buffered solution of $CaCl_2$ or $MgCl_2$, yielding final concentrations as indicated in the text. The two-phase system was again mixed to equilibrium, and 100 μl phase (I) was removed for NA determination.

RESULTS

The two phases used here, with PI as the receptor lipid in the nonpolar phase II and NA in the aqueous phase I, have been described in detail (1). Thus a certain amount of nonionized NA ($=NA_{II}$) is dissolved in phase II, and in this nonpolar state the binding to PI takes place:

$$NA_{II} + PI \rightleftarrows NA_{II}PI$$

The corresponding equilibrium constant is

$$K = \frac{(NA_{II}PI)}{(NA_{II})(PI)}$$

According to this equilibrium and assuming a monomolecular binding, there exits at a given pH value and PI concentration a certain maximum number of NA molecules that can be bound. If the equivalent number of PI molecules is called n_0 and the number of PI molecules bound to NA_{II} at a given total NA concentration is called n, then the Langmuir isotherm $\alpha = c/(c + K)$ applies, where c is the concentration of NA in phase I, K is equilibrium constant and α the degree of saturation (n/n_0).

Figure 1 shows a typical plot of α against c at pH 7.2 and 293°K. Volumes of phase I and II were kept constant, while concentrations of NA were varied. The curve follows the Langmuir isotherm.

With molecular weight 850 for PI, the molar binding ratio between total NA and total PI in the assay system used at satura-

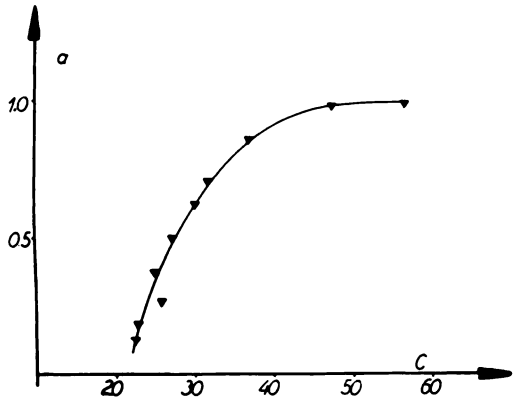

FIG. 1. *Saturation degree* $\alpha = n/n_0$ *against c at pH 7.2*

Here n is equivalent to NA_{II} bound to PI at a given total NA concentration, and n_0 the maximum equivalent of NA_{II} that can be bound; c is concentration of NA in phase I at a given total NA concentration.

tion was calculated to be 0.588 (Table 2) suggesting some PI molecules dispersed in the aqueous phase I.

From Fig. 1 the concentration of NA required to half-saturate PI can be calculated. This is 27.5 μg/0.4 ml and a K value of 2458 (293°K) is indicated

$$\begin{aligned}\Delta F &= -RT \ln K \\ &= -1.98 \times 293 \times 2.3 \times \log 2458 \\ &= -4.52 \text{ kcal/mole}\end{aligned}$$

This value agrees with that found for the electrostatic bond between NA and phosphatidylserine (Table 1).

Temperature Dependence of Free Energy

When binding experiments were performed at the same constant pressure, but with varying temperatures ($T = 281$°K, 305°K) the values for ΔF were slightly affected, as shown in Table 1. Stating the approximate linearity between ΔF and reciprocal temperature as observed, the change in enthalpy ΔH and entropy ΔS can be calculated by using the Gibbs-Helmholz equation.

Calculation of ΔH

$$\begin{aligned}\Delta H &= \left(\frac{\partial(\Delta F/T)}{\partial(1/T)}\right)_p \\ &= \frac{T_2\Delta F_1 - T_1\Delta F_2}{T_2 - T_1} \\ &= \frac{-4.52 \times 281 + 293 \times 4.39}{-12} \\ &= -1346 \text{ cal/mole}\end{aligned}$$

Calculation of ΔS

$$\begin{aligned}-\Delta S &= \left(\frac{\partial \Delta F}{\partial T}\right)_p \\ &= \frac{\Delta F_1 - \Delta F_2}{T_1 - T_2} \\ &= \frac{-4.52 + 4.39}{12} \\ &= +10.8 \text{ e.s.u.}\end{aligned}$$

Effect of Ca^{2+} *and* Mg^{2+}

The distribution of PI, determined as orthophosphate (P_i) in the two phases in the presence of varying amounts of Ca^{2+} or Mg^{2+} is shown in Table 2. Both metals increased the solubility of PI in the organic phase II. The optimal effect is encountered at 2 mEq of Ca^{2+} in the system, which at the PI concentration used caused a complete solution of PI in phase II. The amount of NA bound in phase II was similarly increased as shown in Table 2. Na^+, K^+, or Zn^{2+} did not induce any change in distribution of NA or PI in the two phases.

TABLE 1

Calculation of thermodynamic variables for lipid binding of noradrenaline at varying temperatures and constant pressure (1 atm)

Complex	ΔF (293°K) (kcal/mole)	ΔF (281°K) (kcal/mole)	ΔF (305°K) (kcal/mole)	ΔS (e.s.u.)	ΔH (cal/mole)
PS-NA	−4.67[a]	—	—	—	—
PI-NA	−4.52	−4.39	−4.82	+10.8	−1346

[a] Data of Formby (1).

TABLE 2

Effect of Ca^{2+} and Mg^{2+} on binding capacity and partition of lipid in the two-phase system at constant temperature and pressure (293°K, 1 atm)

Added cation (mEq/l)	NA bound (μg)	$\frac{\mu\text{moles NA}}{\mu\text{moles } P_i}$	$\frac{\mu\text{moles } P_i \text{ phase I}}{\mu\text{moles } P_i \text{ phase II}}$
Ca^{2+} (0.5)	3.82 ± 0.12	0.638	0.3080
Ca^{2+} (1.0)	4.49 ± 0.09	0.750	0.2780
Ca^{2+} (1.5)	5.68 ± 0.11	0.949	0.0650
Ca^{2+} (2.0)	7.10 ± 0.16	1.187	0.000
Mg^{2+} (1.0)	3.90 ± 0.08	0.648	0.2981
Mg^{2+} (2.0)	4.70 ± 0.08	0.789	0.2214
None	3.50 ± 0.14	0.588	0.3352

Electron Spin Resonance[1]

Using a magnetic field of 8 kgauss the Ca^{2+}–lipid–NA complex in the organic phase II was studied. However, no unpaired electrons could be detected.

DISCUSSION

In previous work using a simplified two-phase model system a high capacity of phosphatidylserine to bind NA was shown. In the present studies using the same experimental conditions, evidence is provided for a strong binding of NA to PI.

Calculation of ΔF for the formation of the complex PI-NA at three different temperatures reveals the binding to be electrostatic. The experimental measurements show that ΔS accompanying the binding reaction is a positive number, indicating a total increase in the freedom of motion of the system and possibly explained by an interaction of molecular components with the solvents before mixing. The negative change in ΔH indicates the binding reaction to be an exergonic process at constant pressure.

Using the two-phase system with the organic phase II saturated with NA, a further increase in the solubility of NA in the organic phase II was caused by Ca^{2+} and Mg^{2+}. These results can be interpreted by supposing that PI molecules dispersed in aqueous systems are oriented with their polar heads toward the aqueous phase. In the presence of Ca^{2+} or Mg^{2+}, coordination complexes are formed, causing the PI dispersion to assume a water-in-oil configuration and thus explaining the observed increase in solubility in the organic phase II of the PI–metal complex (11, 12). The net results are shown to be an increase in solubility of NA in the organic phase II where a ternary metal–PI–NA complex could be formed (13). The extra uptake of NA in the presence of Mg^{2+} or Ca^{2+} could also be due to inclusion of NA in water solution trapped in lipid micelles in the lipid phase. However, the amount of water solubilized in the micelles of phospholipids barely increases above 0.3 g per gram of lipid (14, 15). Thus, the amount of NA taken up in bulk water is only 1–3% of the total uptake.

PI is a constituent of cell membranes in general and has been reported to be present in the membranes of synaptosomes and synaptic vesicles (16, 17). In earlier investigations it was suggested that NA could be liberated from the diffusible PS–NA complex by some kind of ionic exchange (18, 19), after diffusion through the membrane. Thus a similar function of PI could be possible (20, 21) although the present data may also indicate a possible presence of metal–PI–NA complexes in granules in which the transmitter is synthesized and stored (22). The liberation of chemically bound NA into the cytoplasmic pool containing free NA could be connected to the metabolism of the membrane polar lipids in storage particles (23–25). Al-

[1] Kindly performed by Dr. S. O. Nielsen, Danish Atomic Energy Centre, Risø.

ternatively, it could proceed as suggested by Maas and Colburn (13); here the presence of ATP in an aqueous phase strongly reduced metal incorporation into an organic phase, so that a reversible mechanism could release complex-bound NA from membrane.

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REFERENCES

1. B. Formby, *Mol. Pharmacol.* **3**, 284 (1967).
2. E. Stahl, *Pharmazie* **11**, 633 (1956).
3. H. O. Christensen Lou and J. Clausen, *J. Neurochem.* **12**, 619 (1965).
4. G. Rouser, A. J. Bauman, G. Kritchevsky, D. Heller and J. O'Brien, *J. Am. Oil Chemists Soc.* **40**, 425 (1963).
5. G. Rouser, D. Heller and G. Kritchevsky, *J. Am. Chemists Soc.* **38**, 544 (1961).
6. G. R. Bartlett, *J. Biol. Chem.* **234**, 466 (1958).
7. F. Parker, *J. Lipid Res.* **6**, 455 (1965).
8. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
9. L. J. Bellamy, "The Infra-red Spectra of Complex Molecules." Methuen, London, 1958.
10. J. Häggendal, *Acta Physiol. Scand.* **59**, 242 (1963).
11. M. Walman and H. Wiener, *Nature* **200**, 486 (1963).
12. H. E. Carter and E. J. Weber, *Lipids* **1**, 16 (1966).
13. J. W. Maas and R. W. Colburn, *Nature* **208**, 41 (1965).
14. P. H. Elworthy and D. J. McIntosh, *J. Phys. Chem.* **68**, 3448 (1964).
15. A. W. Cuthbert, B. A. Callingham, S. Warren and E. Painter, *J. Pharm. Pharmacol.* **19**, 313 (1967).
16. J. Eichberg, V. P. Whittaker and R. M. C. Dawson, *Biochem. J.* **92**, 91 (1964).
17. V. P. Whittaker, *Ann. N.Y. Acad. Sci.* **137**, 982 (1966).
18. E. Rojas and J. M. Tobias, *Biophys. Soc. Abstr.* WE9 (1964).
19. E. Rojas and J. M. Tobias, *Biochim. Biophys. Acta* **94**, 394 (1965).
20. R. B. Ellis and J. N. Hawthorne, *Biochem. J.* **96**, 19P (1962).
21. T. Gilliard and J. N. Hawthorne, *Biochem. J.* **88**, 38P (1963).
22. E. Costa, D. J. Boullin, W. Hammer, W. Vogel and B. B. Brodie, *Pharmacol. Rev.* **18**, 577 (1966).
23. B. W. Agranoff, R. M. Bradley and R. D. Brady, *J. Biol. Chem.* **233**, 1077 (1958).
24. H. Paulus and E. P. Kennedy, *J. Biol. Chem.* **225**, 1203 (1960).
25. R. J. Rossiter and F. B. Palmer, *Proc. 2nd Meeting Federation European Biochem. Soc.* p. 250 (1966).