ACETYLCHOLINE-ATP BINDING BY DIRECT MEMBRANE ELECTRODE MEASUREMENT

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SUMMARY

Controversy concerning acetylcholine-ATP interaction and the possible role of such binding for acetylcholine storage in synaptic vesicles has been resolved by direct binding measurements using an acetylcholine selective membrane electrode. At pH 7.4, acetylcholine was found to bind ATP^{-4} with a 1:1 stoichiometry and a thermodynamic formation constant of $175M^{-1}$. The interaction of acetylcholine with $HATP^{-3}$ and $MgATP^{-2}$ was found to be much weaker with formation constants of approximately $20M^{-1}$ and $25M^{-1}$, respectively. The data indicate that ATP binding could not account for more than 20% of acetylcholine storage under the conditions known to exist in synaptic vesicles.

INTRODUCTION

It has recently been shown that ATP is a constituent of cholinergic synaptic vesicles (1,2), and there has been speculation as to the possible role of ATP in the storage of acetylcholine (ACh) in nerve terminals (2-4). The interaction of acetylcholine and ATP has been studied by several methods but with conflicting results (5-9). Investigations using Fourier transform ^{13}C and ^{14}N NMR (5) and microcalorimetry (6) obtained no evidence of interaction but more recent studies using NMR (7,8) and equilibrium dialysis (9) provided indirect evidence of complex formation but disagree about the stoichiometry of the complex. One study (7), using proton spin-lattice relaxation time (T_1) measurements, suggests a 4:1 acetylcholine-ATP complex, while another NMR study, using proton spin-spin relaxation time (T_2) measurements, indicates the formation of a

1:1 complex (8). None of the previous studies provide any quantitative information about the formation constant.

In this investigation, the interaction of acetylcholine and ATP is measured directly using a newly developed acetylcholine ion selective electrode (10). Ion selective electrodes have been proven to be useful in the determination of the thermodynamic formation constants and stoichiometries of metal ion complexes of ATP (11-13), ADP (14) and AMP (15). The electrode method permits the selective measurement of unbound acetylcholine and does not require the use of D_2O or alcohol as solvents in distinction to the NMR methods; as a result, interpretation of the data is straightforward and quantitative binding measurements become possible.

MATERIALS AND METHODS

Acetylcholine chloride and Na_2ATP were obtained from Sigma Chemical Company. After recrystallization from isopropanol, stock solution of acetylcholine were prepared in the following concentrations: 0.01, 0.02, 0.03, 0.04, 0.05 and 0.10M. ATP stock solutions were approximately 0.01 and 0.03M. Stock solutions of $MgCl_2$ (0.01M), NaCl (0.1M) and NaOH (0.05M) were prepared as previously described (15). All measurements were made at 25.0°C $\pm$.2°C.

Potentiometric measurements were made with an acetylcholine selective electrode in conjunction with a saturated calomel reference electrode. The method of potential comparison (16) was used to determine the formation constants. With this technique the potential of a standard solution of acetylcholine chloride is compared to that of a test solution containing a known amount of ATP. Increments of acetylcholine stock solution are added until the potentials of both solutions are the same, indicating that the activities of acetylcholine are equal. The sodium concentration of the standard solution was adjusted to be equal to that of the test solution by the addition of sodium chloride in order to cancel out any sodium ion interference on the electrode. The ionic strength was kept low by the use of low concentrations of reagents to allow for an accurate estimation of activity coefficients using the Davies equation (17). Measurements were made at various ratios of ACh to ATP at a pH of 7.4 $\pm$ .2 and of 6.0 $\pm$ .2. The pH was adjusted with NaOH. Measurements were also made in the presence of magnesium ion under identical conditions. An Orion Model 92-32 divalent cation electrode was used to measure the free magnesium ion activities.

RESULTS

Measurements were made at pH's of 7.4 and 6.0 to resolve the effects of $HATP^{-3}$ and ATP^{-4}, which exist in equilibrium. The pKa

value of 7.68 at zero ionic strength determined by Phillips et.al. (18) was used to calculate the concentrations of the two species. Calculations were done with the aid of a CDC 6400 computer using the procedure previously outlined (12,19).

The values obtained for the ACh-HATP constant were very small and were difficult to determine accurately by this method. A value of approximately 20 M^{-1} was obtained. The values obtained for the ACh-ATP complex under a range of conditions are summarized in TABLE I. Since the values remain constant over a wide range of ACh/ATP ratios, it is clear that a complex of 1:1 stoichiometry is formed. Measurements were also made at higher (4:1 to 6:1) ratios of ACh/ATP, and gave no evidence for any complex other than the 1:1 species.

The interaction of acetylcholine with $MgATP^{-2}$ was also studied. The previously determined formation constant for the $MgATP^{-2}$ complex (13) was used to calculate the concentration of the $MgATP^{-2}$ species. The formation constant for the ternary complex was low with an approximate value of 25 M^{-1}.

DISCUSSION

The numerical value of the formation constant for the ACh-ATP complex indicates a weak interaction. The magnitude of the constant is comparable to that of the sodium and potassium complexes of ATP, e.g. K = 228. M^{-1} (11). The even smaller value of the ACh-HATP formation constant indicates that essentially all the binding at pH = 7.4 is due to the ATP^{-4} species. As in the case of the ternary complex of ACh with MnATP (20), the ACh-MgATP complex also makes only a negligible contribution to acetylcholine binding.

In order to estimate the fraction of acetylcholine which could be bound to ATP under physiological conditions, it is necessary to convert the thermodynamic constants to concentration con-

TABLE I - Formation Constants for ACh-ATP Measured at 25°C[a]

pH	Ionic Strength x 10^2 M	[ACh-ATP] x 10^4 M	[ATP^{-4}] x 10^4 M	[ACh^+] x 10^3 M	K M^{-1}	T_{ACh}/T_{ATP}
7.469	3.27	1.12	16.7	1.56	165.	.42
7.415	3.25	1.24	16.2	1.56	189.	.42
7.438	1.32	.728	6.21	1.73	172.	1.22
7.414	1.32	.827	6.04	1.76	202.	1.22
7.440	3.38	2.99	15.0	4.72	164.	1.33
7.419	3.37	3.24	14.7	4.72	182.	1.34
7.384	2.11	1.58	9.13	3.34	161.	1.52
7.373	1.82	1.12	7.99	2.79	145.	1.45
7.341	1.35	1.39	4.87	3.44	211.	2.73
7.384	1.36	1.15	5.19	3.45	164.	2.71

[a] Mean K = 175. M^{-1}; Standard Deviation $\pm$ 28. (25 Determinations)

stants at an ionic strength of 0.15 M. This was done using the procedure of George et.al. (21) to approximate the activity coefficients. A calculation, assuming the vesicular acetylcholine concentration to be 0.1 M (22) and the ATP concentration to be 0.02 M, indicates that only about 14% of the acetylcholine would be bound to ATP. This represents a maximum estimate since it assumes the absence of any metal ions which would preferentially bind ATP. As has been shown, the interaction of acetylcholine with the metal complexed form of ATP is very weak. Furthermore, it can be seen that since the stoichiometry of the ACh-ATP complex is 1:1 and the ratio of acetylcholine to ATP in synaptic vesicles is approximately 5:1 (2), that even a much larger constant could account for no more than 20% of the total bound acetylcholine.

Thus, this study shows that ATP (by itself or in the presence of magnesium ions) cannot bind sufficient amounts of acetylcholine to account for a major fraction of the acetylcholine stored in synaptic vesicles. The results do not rule out the possibility, suggested by some investigators (1,3,4), that an ATP-protein complex is responsible for the acetylcholine storage. The protein, however, would have to provide the majority of binding sites.

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