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## Short Communication

# THERMODYNAMIC STUDIES WITH ACETYLTHIOCHOLINE ON NICOTINIC RECEPTORS OF MAMMALIAN SKELETAL MUSCLE *IN VITRO*

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**Abstract**—The temperature dependency of binding of acetylthiocholine, a specific nicotinic agonist, to the nicotinic receptor of mammalian skeletal muscle was studied using isotonic contractions of the rat denervated diaphragm preparation *in vitro*. The dissociation constants at different temperatures (22–39°) were determined by the Furchgott method using α-bungarotoxin as an irreversible antagonist. Both free energy of association ($\Delta G° = -22.93$ kJ/mol at 37°) and enthalpy of binding ($\Delta H° = -58.35$ kJ/mol) calculated from $K_d$ (dissociation constant) and slope of $\ln K_d$ versus $1/T$ (van't Hoff plot) respectively were found to be negative. The negative entropy value ($\Delta S° = -0.113$ kJ/mol/deg) obtained from the intercept of this van't Hoff plot differs from the large positive value obtained earlier employing radioligand binding studies of the nicotinic receptor of *Electrophorus electricus*.

*Key words:* nicotinic receptor; thermodynamics; acetylthiocholine; skeletal muscle; entropy; enthalpy; α-bungarotoxin

Thermodynamic studies are increasingly used to provide answers for various biophysical phenomena occurring at the receptor sites during drug receptor interactions. While extensive thermodynamic studies have been carried out on β-adrenergic, benzodiazepine and dopamine receptors [1], very little work has been done with nicotinic receptors in general and nicotinic receptors of the skeletal muscle in particular. From the radioligand binding studies of nicotinic receptors isolated from electric organ of eel fish, *Electrophorus electricus*, Maelicke *et al.* [2] could not find any thermodynamic differences between agonist and antagonist binding, both of which were found to be entropically favourable processes, as is the binding of choline to the nicotinic acetylcholine receptor from torpedo [3]. Since it is generally accepted that radioligand binding studies are not always sensitive to functional differences between the interactions of agonist and antagonist with the receptor [4], we have employed the *in vitro* denervated rat hemidiaphragm preparation which has the following advantages: (i) receptors are readily available for equilibrium binding with agonist, (ii) the time course of the desensitization process is slower [5] in denervated than at innervated end plate and, (iii) it is perhaps the only mammalian *in vitro* preparation which responds through a wide range of temperature permitting detailed analysis of the energetics underlying drug receptor binding. In the present study, acetylthiocholine has been used as the specific nicotinic agonist [6] instead of the commonly used acetylcholine which acts on both muscarinic and nicotinic receptors. The Furchgott receptor inactivation method [7] has been used for the determination of dissociation constants. The importance of the Furchgott method is that one can avoid the higher concentration range of the agonist which causes desensitization.

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† Abbreviation: α-BTX, α-bungarotoxin.

## *Materials and Methods*

*Denervated hemidiaphragm preparations.* Experiments were performed on denervated hemidiaphragm preparations of albino rats of the Sprague-Dawley strain (150–200 g) of either sex. Denervation of the left phrenic nerve was carried out according to the method of Mitchell and Silver [8] and the preparations were set up as described earlier [9]. The diaphragm was suspended in a 10 mL organ bath containing Kreb's solution (mmol: NaCl 118, KCl 4.7, $CaCl_2 6H_2O$ 2.5, $NaHCO_3$ 25, $KH_2PO_4$ 1.2, $MgSO_4 7H_2O$ 1.2 and dextrose 11.1) continuously aerated with 95% $O_2$ and 5% $CO_2$ and the pH was kept at 7.4. The muscle contractions were recorded isotonically using a simple lever having 8–10-fold magnification on a smoked kymograph.

*Experimental protocols.* The tissue preparations were kept in the bath solution for 2–2.5 hr for acclimatizing to the desired temperature before administration of any drug. Prior to partial inactivation of the receptors by α-BTX† ($1.28 \times 10^{-8}$ mol for 25 min) two dose–response curves were obtained in each experiment with acetylthiocholine iodide. Before obtaining the final dose–response curve sufficient time was allowed for washout of the free α-BTX from the tissue.

*Calculations and statistics.* Using α-BTX as irreversible nicotinic receptor blocker the dissociation constant of acetylthiocholine was determined by the Furchgott method [7]. Equieffective doses of the agonist before ([A]) and after ([A']) blockade by α-BTX were calculated by constructing horizontal lines on the dose–response curves and a reciprocal plot of these doses obeyed the straight line equation:

$$1/[A] = (1/q)/[A'] + (1/q - 1)/K_d \quad (1)$$

where $q$ is the fraction of the receptors remaining unoccupied after irreversible blockade. The dissociation constant ($K_d$) was obtained from the slope and intercept of Equation 1:

Table 1. Values of dissociation constant and thermodynamic parameters for the interaction between acetylthiocholine iodide and nicotinic receptor of the isolated preparation of rat hemidiaphragm (N = 6)

| | 22° | 30° | 33° | 37° | 39° |
|---|---|---|---|---|---|
| $K_d$ (mol) | $4.63 \times 10^{-5}$ | $7.33 \times 10^{-5}$ | $6.72 \times 10^{-5}$ | $1.39 \times 10^{-4}$ | $1.75 \times 10^{-4}$ |
| ±SEM (mol) | $1.50 \times 10^{-5}$ | $2.89 \times 10^{-5}$ | $0.78 \times 10^{-5}$ | $0.3 \times 10^{-4}$ | $0.35 \times 10^{-4}$ |
| $\Delta G°$(kJ/mol) | −24.98 ± 0.87 | −24.44 ± 1.06 | −24.48 ± 0.29 | −22.93 ± 0.84 | −22.52 ± 0.5 |
| $\Delta H°$(kJ/mol) | | | −58.35 ± 14.02* | | |
| $\Delta S°$(kJ/deg/mol) | | | −0.113 ± 0.046* | | |

* Calculated from van't Hoff plot: $\Delta H° = R$ (slope) and $\Delta S° = R$ (intercept).

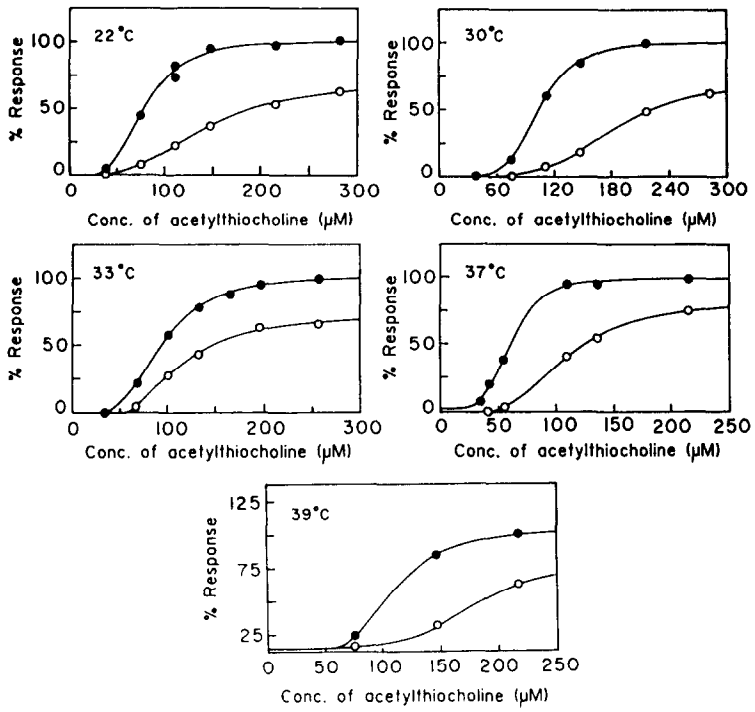


Fig. 1. Isotonic contraction of the rat denervated hemidiaphragm preparations in response to different concentrations of acetylthiocholine iodide measured at different temperatures in (●) the absence, and (○) presence of irreversible antagonist α-BTX ($1.28 \times 10^{-8}$ mol for 25 min). Single representative experiment out of six experiments is shown for each of the temperatures used.

$$K_d = (\text{slope} - 1)/\text{intercept} \quad (2)$$

This constant ($K_d$) was used to get the standard free energy

$$\Delta G° = -RT\ln(1/K_d) = RT\ln(K_d) \quad (3)$$

where $R$ is the universal gas constant (8.33 J/mol/deg) and $T$ is the absolute temperature. The enthalpy of binding ($\Delta H°$) can be evaluated from the integrated van't Hoff equation:

$$\ln K_d = (\Delta H°/R)(1/T) - \Delta S°/R \quad (4)$$

From the intercept of Equation 4 standard entropy change ($\Delta S°$) can be calculated. The slope of the plot of $\ln K_d$ versus $1/T$ is $\Delta H°/R$. Determination of $\Delta H°$ and $\Delta G°$ also allow calculation of entropy ($\Delta S°$) at each temperature from the following equation:

$$\Delta G° = \Delta H° - T\Delta S° \quad (5)$$

Means ± SE mean are given throughout.

*Compounds.* Acetylthiocholine and α-BTX were obtained from Sigma.

*Results and Discussion*

The present study has revealed that the binding of acetylthiocholine to the nicotinic receptor of the mammalian skeletal muscle is temperature dependent as is evident from the values of dissociation constant (Table 1) at different temperatures ranging from 22 to 39°. The dissociation constant was determined based on the shift of the dose–response curve by using α-BTX at each temperature. Such a procedure eliminates physiological influences like enzyme activity, muscle activity, etc. Affinity (inverse of dissociation constant) of the agonist increased with decrease in temperature. α-BTX ($1.28 \times 10^{-8}$ M for 25 min) produced typical rightward and downward shift (Fig. 1) of the dose–response curve (at each temperature) which is in agreement with the findings of Neubig *et al.*

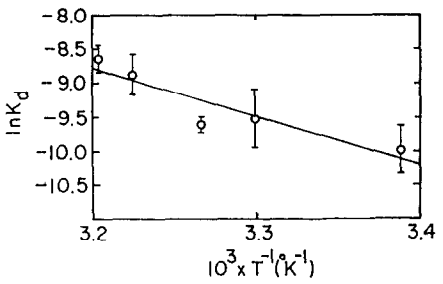


Fig. 2. Van't Hoff plot of ln $K_d$ versus $1/T$ has been shown with linear regression ($r = -0.92$). Dissociation constant was measured at five temperatures, viz. 22, 30, 33, 37 and 39°. Slope and intercept of the line yielded the enthalpy and entropy values, respectively. Each point shows mean ± SEM of six experiments.

[10]. Since it is not necessary to get the upper part of the dose–response curve for determining dissociation constant, higher doses have been avoided in our experiments. No sign of desensitization was observed in terms of contraction in the dose range employed in our experiments. The double reciprocal plot of equieffective doses which are obtained by constructing horizontal lines on the blocked and unblocked dose–response curve of acetylthiocholine yielded a straight line with linear regression ($r = 0.99$) according to Equation 1. The dissociation constant was calculated from the slope and intercept of this straight line. The values of the apparent dissociation constant (Table 1) obtained in the present study were found to be comparable with the previous work on nicotinic receptor isolated from postsynaptic membrane of Torpedo by Neubig and Cohen [10] who measured the efflux of $^{22}Na^+$ as response to each concentration of carbamylcholine and determined the dissociation constant by the method of partial inactivation of the receptors using $\alpha$-BTX.

Using a van't Hoff plot (Fig. 2), the thermodynamic parameter $\Delta H°$ of binding was calculated and found to be negative (Table 1). Free energy of association and entropy were also found to be negative. Negative $\Delta G°$ indicates that the agonist binding is spontaneous and the negative $\Delta H°$ value indicates its exothermic nature. The thermodynamically unfavourable decrease in entropy was compensated by the large negative enthalpy value. The negative entropy value obtained in the present study differs from the unusually large positive value obtained (on an average 0.5 kJ/mol/deg) by Maelicke *et al.* [2], who acknowledged the fact that the magnitude of the reaction entropy is quite unexpected in this type of ligand receptor interaction. However, the large positive $\Delta S°$ observed by Maelicke *et al.* may be due to hydrophobic association and/or ionic interaction [11].

The present finding of a negative enthalpy value in the interaction between acetylthiocholine and nicotinic receptor of the skeletal muscle may be due to van der Waals and hydrogen bond formation during drug receptor interaction [11]. This finding is consistent with the observations made with other drug receptor interactions, e.g. adrenoceptors, benzodiazepine receptors, etc. [12–14]. The energetically unfavourable decrease in entropy implies an increased orderliness due to drug receptor complex formation. Loss of translational and rotational freedom of the ligand by binding with the receptor has been used to explain such a decrease in entropy [15]. Similar or other factors were apparently sufficient in the present study to overcome increases in entropy due to, for example, (a) melting of water molecules surrounding the receptor and ligand during complex formation [13], and (b) vibrational freedom due to additional bond formation and internal motions [15]. Entropy changes in the drug receptor binding have been explained by assuming conformational change at the receptor [2, 13]. Conformational change during drug binding at the receptor may affect the rigidity at the receptor. Positive change in entropy may indicate flexibility whereas negative entropy change may indicate rigidity at the receptor. Our finding of negative entropy change suggests the latter phenomenon. Binding of one species to another normally results in the loss of flexibility of the combiner.

In conclusion, using the specific nicotinic receptor agonist, acetylthiocholine, an attempt has been made for the first time to determine the thermodynamic parameters of the nicotinic receptors at the skeletal muscle using *in vitro* isolated rat hemidiaphragm preparation. Although there are limitations inherent in the inability to measure thermodynamic quantities for the drug receptor reaction directly, it is still a useful way to examine the results of the above analysis to an approximately limited extent [12]. Under the conditions used, the interaction occurred with a decrease in free energy, enthalpy and entropy.

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